Microparticles as novel biomarkers/effectors in severe sepsis

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A thesis submitted in partial fulfilment of the requirements of the University of London for the degree of Doctor of Philosophy

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I, Hazem Mohamed Shokry Lashin, confirm that the research presented in this thesis is my own original work or that where it has been performed in collaboration with others, this is duly acknowledged below and my contribution is indicated. Previously published material is also acknowledged.

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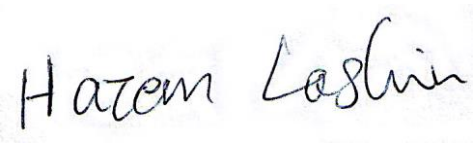
Details of collaboration and publications:

This work was supported by HCA international.

Patients' samples were obtained from the Genomic Advances in Sepsis (GAinS) biobank, Wellcome Trust for Human Genomics, Oxford, UK. The samples were obtained from the biobank and sent to me by Miss Emma Davenport or Miss Katie Burnham. Otherwise, all samples were generated by myself. I collected and analysed the data presented herein, except for imaging flow-cytometry analyses kindly performed by Mr Hefin Jones.

Signed:  Date: April 18th, 2015

Abstract

Microparticles (MP) are submicron structures produced by all cells upon activation or apoptosis that act as a non-soluble means of communication between cells. They ferry proteins, bioactive lipids, RNA and receptors, as well as ridding cells of redundant organelles and toxins. They have been recently investigated for their pathophysiological role and as potential biomarkers/effectors in many diseases. In severe sepsis, studies of MP so far have produced inconsistent and even conflicting results. In this project, it was demonstrated that cell derived MP subsets vary according to the cause of severe sepsis (community acquired pneumonia (CAP) or faecal peritonitis (FP)), where CAP patients had higher levels of circulating MP. Surprisingly, FP patients MP levels were comparable to healthy volunteers. Further stratification of MP subsets according to their expression of the protein alpha-2-macroglobulin (A2M) has yielded better differentiation between the two diseases. The A2M expressing MP were significantly higher in survivors of community acquired pneumonia sepsis, but there was no similar association in patient with FP.

Granulocyte macrophage colony-stimulating factor (GM-CSF) and interferon γ (IFN-γ) are being studied as possible adjuvant therapies in sepsis. They seem to reverse the immune-paresis that ensues after the initial insult. MP produced from whole blood stimulated with GM-CSF and IFN- γ were studied in this project. Both GM-CSF and IFN- γ increased MP expressing A2M over control. These MP elicited a pro-inflammatory phenotype when incubated with neutrophils or endothelial cells which may contribute to the potential benefits of GM-CSF and IFN- γ in severe sepsis.

Acknowledgments

First, I would like to thank Professor Mauro Perretti my supervisor for his enthusiasm and motivation towards this project that was inspiring to me. Also, I would like to thank him for giving me the space to develop my project and for his invaluable advice at the times when I needed them.

I would like to thank Dr Suchita Nadkarni for teaching me the basic laboratory skills and guiding me through the initial steps of this project and for her continued support during the latter stages.

I would also like to thank Professor Charles Hinds my second supervisor for allowing me the opportunity to do this project and his continued support during it especially at the difficult times. Professor Hinds also provided me with invaluable career development advice.

Dr Lucy Norling has provided me with great guidance and support during those latter stages of this project and during writing of this thesis that would otherwise have been immensely difficult.

I would like to thank my colleagues Sarah Headland, Rachel Wright, Patricia De-Souza, Hefin Jones and Dianne Cooper for their help and support during this project. I would like to thank them and everyone else at the department of Biochemical pharmacology for all the good times we spent together.

Finally, I would like to thank my wife Diala Lashin and my daughter Yara Lashin for their continued unconditional love, support and patience during the course of this project. I also am grateful for my mother Dr Zeinab El-Shafey for her encouragement and support.

Table of contents

List of figures

List of tables

List of abbreviations:

AP-1:	Activator protein 1
aPC:	Activated Protein C
AKI:	Acute Kidney Injury
ARDS:	Adult Respiratory Distress Syndrome
APC:	Allophycocyanin
A2M:	Alpha-2-Macroglobulin
AnxA7:	Annexin A7
AV:	Annexin V
AMP:	Annexin V positive Microparticles
BUN:	Blood Urea Nitrogen
CLP:	Caecal Ligation and Perforation
CCL:	CC chemokine ligand
CXCL:	CXC chemokine Ligand
CXCR:	CXC chemokine Receptor
COPD:	Chronic Obstructive Pulmonary Disease
CAP:	Community-Acquire Pneumonia
CARS:	Compensatory Anti-inflammatory Response Syndrome
CHF:	Congestive Heart Failure
COX:	Cyclooxygenase
cMP:	control Microparticles
DAMP:	Danger-associated molecular patterns
DVT:	Deep venous thrombosis
DNA:	Deoxyribonucleic Acid
DM:	Diabetes Mellitus
DIC:	Disseminated Intravascular Coagulopathy
eCRF:	electronic Case Report Form
EC:	Endothelial Cells
EMP:	Endothelial Microparticles
EPCR:	Endothelial Protein C Receptor
ECAM:	Epithelial adhesion molecule
ECM:	Extra-cellular matrix
FITC:	Fluoroescein Isothocyanate
Fl-:	Fluoroescence channel
FMLP:	Formyl-Methionyl-Leucyl-Phenylalanine
FSC:	Forward Scatter Channel
eNOS:	Endothelial Nitric Oxide Synthase
EryMP:	Erythrocyte Microparticles
GM-CSF:	Granulocyte-macrophage colony stimulating factor
GM-CSFR:	Granulocyte-macrophage colony stimulating factor receptor
GPCR:	G-protein-coupled receptor
GAinS:	Genomic Advances in Sepsis study
HLA:	Human leucocyte antigen
HV:	Healthy Volunteers
HO-1:	Hemoxygenase-1

HDU:	High Dependency Unit
HUVEC:	Human Umbilical Vein Endothelial Cells
IgG:	Immunoglobulin G
IgM:	Immunoglobulin M
iNOS:	inducible Nitric Oxide Synthase
IBD:	Inflammatory Bowel Disease
ITGAV:	Integrin alpha v
IL:	Interleukin
ICAM-1:	Intracellular Adhesion Molecule 1
ICU:	Intensive Care Unit
JAM:	Junctional adhesion molecule
LAIR-1:	Leucocyte Associated Immunoglobulin like Receptor 1
LPS:	Lippopolysaccharide
LRP:	Lipoprotein receptor-related protein
MAS:	Macrophage activation syndrome
MFI:	Mean Fluorescence Intensity
Mel-CAM:	Melanoma cell adhesion molecule
MLS:	Myosin light chain
MHC:	Major histocompatibility complex
mRNA:	messenger Ribonucleic Acid
MSC:	Mesenchymal Stem Cells
MMP:	Matrix metaloprotinases
miRNA:	micro Ribonucleic Acid
MP:	Microparticles
MARS:	Mixed Anti-inflammatory Response Syndrome
mAB:	monoclonal Antibody
MODS:	Multi-Organ Dysfunction Syndrome
NM:	Nano-medicine
NLR:	Nucleotide-binding oligomerization-domain protein-like receptors
NADPH:	Nicotinamide Adenine Dinucleotide Phosphate
NO:	Nitric Oxide
NFκB:	Nuclear Factor κB
NK:	Natural killer cells
NKT:	Natural killer T cells
NET:	Neutrophil Extra-Cellular Trap
NMP:	Neutrophil Microparticles
OSA:	Obstructive Sleep Apnea
PAK:	p21- activated protein kinase (PAK)
PAF:	Platelet-activating factor
PBMC:	Peripheral blood mononuclear cells
PRR:	Pattern recognition receptors
PVD:	Peripheral Vascular Disease
PBS:	Phosphate Buffered Solution
PSGL-1:	P-selectin glycoprotein ligand 1
PS:	Phosphatidylserine
PMT:	Photomultiplier tubes

PE:	Phycoerythrin
PE-CY5:	Phycoerythrin-Cascade Yellow 5
PAI-1:	Plasminogen Activator Inhibitor 1
PAMP:	Pathogen Associated Molecular Pattern
PFP:	Platelet Free Plasma
PMP:	Platelet MP
PPP:	Platelet Poor Plasma
PECAM-1:	Platelet Endothelial Cell Adhesion Molecule 1
PMNs:	Polymorph Nuclear Leucocytes
PAC-1:	Procaspase Activating Compound 1
PC:	Protein C
PtnS:	Protein S
PRR:	Pattern recognition receptor
ROS:	Reactive Oxygen Species
RA:	Rheumatoid Arthritis
rhGM-CSF:	recombinant human granulocyte-macrophage colony stimulating factor
RNA:	Ribonucleic Acid
sMP:	sepsis Microparticles
SOFA:	Sequential Organ Failure Assessment
SS:	Severe Sepsis
SSC:	Side Scatter Channel
shMP:	sham Microparticles
SD:	Standard Deviation
SEM:	Standard Error of the Mean
SIRS:	Systemic Inflammatory Response Syndrome
SLE:	Systemic Lupus Erythematosus
TB:	Tuberculosis
Th:	Helper T cells
TM:	Thrombomodulin
TF:	Tissue Factor
TFPI:	Tissue Factor Pathway Inhibitor
TIMP:	Tissue inhibitors of metalloproteinases
tPA:	tissue Plasminogen Activator
TLR:	Toll Like Receptor
TNF:	Tumour Necrosis Factor
TNFR:	Tumor necrosis factor receptor
uPA:	urokinase type Plasminogen Activator
uPAR:	urokinase type Plasminogen Activator receptor
VEGF:	Vascular endothelial growth factor
VCAM-1:	Vascular cell adhesion molecule - 1

Chapter 1: Introduction

1.1 Inflammation:

1.1.1 Overview:

Inflammation is an adaptive response triggered by noxious stimuli such as infection and tissue injury. A controlled inflammatory response is crucial for host protection, but when dysregulated it could be harmful as is seen in severe sepsis and septic shock.

In microbial triggered inflammation, toll like receptors (TLR) and nucleotide-binding oligomerisation-domain protein-like receptors (NLR) of the tissue resident macrophages and mast cells are activated leading to the production of chemokines, cytokines, eicosanoids and products of proteolytic cascades. This response leads to plasma proteins and leucocytes (particularly neutrophils) gaining access to the site of inflammation via the activated endothelium. These neutrophils then attempt to kill the invading organism by producing reactive oxygen species (ROS) and other toxic products, which can also result in local tissue damage[1].

When the acute inflammatory response is successful, it results in the elimination of the invading pathogen and the processes of resolution and repair are instigated. These processes are mediated by resident and recruited macrophages *via* the production of bioactive lipid mediators (e.g. lipoxins, resolvins) and pro-resolving peptides. Lipoxins inhibit the recruitment of neutrophils and instead promote the recruitment of monocytes, which remove cellular debris and promote local tissue

remodelling. Resolvins and protectins along with growth factors (e.g. transforming growth factor – β) play a role in resolution of inflammation and initiating tissue repair[1].

When the acute inflammatory response fails to eliminate the offending agents, the inflammatory process persists and acquires new characteristics. The neutrophil infiltrate is replaced by macrophages and T cells. If the combined effect of these cells is still not enough to overcome the pathogen a chronic inflammatory process ensues involving granuloma and tertiary lymphoid tissue formation [1].

1.1.2 Neutrophils in inflammation

The neutrophils play a major role in acute inflammation and are a key component of the rapid response of the host to danger and injury. These cells are the first leucocytes recruited to the site of inflammation and are capable of eliminating the invading pathogen *via* multiple mechanisms that entail release of toxic agents, remodelling proteolytic activities and a variety of autacoids and other inflammatory mediators. Neutrophils are crucial for clearance of infection.

1.1.2.1 Neutrophil recruitment

Neutrophils are recruited to the site of inflammation *via* a well-recognised cascade involving the following steps: capture, rolling, adhesion, crawling and finally transmigration. This process is initiated by changes on the surface of endothelial cells that have been activated by inflammatory mediators released by tissue resident

macrophages that came in contact with pathogens. Endothelial cells can also be activated directly through the activation of their pattern recognition receptors (PRR)[2].

Neutrophil capture. Following activation, the endothelial cells expression of the pre-stored P-selectin from Weibel-Palade bodies is increased within minutes and is followed by the *de novo* production of E-selectin within 90 minutes. Once present on the surface of endothelial cells, the selectins bind to their neutrophil ligands (e.g. P-selectin glycoprotein ligand 1 (PSGL1)) facilitating the tethering (capturing) of free flowing neutrophils to the surface of endothelial cells and their rolling in the direction of blood flow[2].

Neutrophils roll on the endothelial surface at a shear stress of 1 to 10 dyne/cm^2 which requires the rapid formation and breaking of bonds. P-selectin-PSGL-1 dissociation at the rear of the neutrophil is balanced by formation of new bonds at the front of the cell to facilitate a smooth process. Also, long membrane tethers are formed at the rear end of the neutrophil that catapult to the front of the rolling cell to form a "sling". The sling has PSGL-1 patches distributed along its length which attach to the endothelial selectins and as the cell roll forwards these bonds are broken in step like fashion[2, 3].

Neutrophil adhesion. Full activation of neutrophils is mediated *via* specific priming by pro-inflammatory cytokines (e.g. tumour necrosis factor – α (TNF-α) and IL-1β) and also *via* contact with activated endothelial cells followed by exposure to pathogen-associated molecular patterns (PAMP), chemoattractants and growth factors. One

of these chemokines is IL-8, which plays a crucial role in neutrophil activation and adhesion to endothelial cells *via* the neutrophil receptor CXCR2 resulting in conformational change of the neutrophil surface integrins (e.g. CD11b). This results in the integrins having higher affinity to their ligands (e.g. intra-cellular adhesion molecule 1 (ICAM-1)), which is important for firm adhesion. This binding also activates signalling pathways inside the neutrophil, thereby stabilising adhesion and initiating cell motility[2].

Neutrophil crawling. Transmigration of neutrophils occurs at the endothelial cell-to-cell junction. The neutrophils actively crawl towards these sites. This crawling under shear conditions occurs perpendicular to the direction of blood flow while maintaining adhesion to endothelial surface at all times. This process relies upon the interaction between ICAM-1 expressed on the endothelial surface and integrin αMβ2 (CD11b/CD18) expressed on neutrophils[4].

Crawling does not require an active chemokine gradient. Under shear conditions in vitro, neutrophils still crawl perpendicular to the flow and when the flow is stopped the neutrophils crawl in random directions[2].

Neutrophil transmigration. In order to reach the site of inflammation or infection, the neutrophils have to cross the endothelium in a process called “transmigration”. This process requires integrins, CAMs (ICAM-1, ICAM-2 and vascular cell adhesion protein 1 (VCAM-1) as well as different junctional proteins such as CD31, CD99, junctional adhesion molecules (JAM) and epithelial cell adhesion molecule (ECAM).

Neutrophil transmigration across the endothelium occurs either para-cellularly (between cells) or trans-cellularly (through cells). Para-cellular transmigration occurs at junctions between cells where there are less junctional proteins and the alignment between cells is less organised[5]. This process requires the breakdown of junctional intercellular protein bonds such those formed by vascular-endothelial (VE)-cadherin[2]. In trans-cellular transmigration, endothelial cells form microvilli that extend around the adherent neutrophils to form “domes”. These domes shield the neutrophils from the blood stream and allow them to pass through the endothelial cells in an orderly fashion[4, 6].

The neutrophils then cross the basement membrane through areas with low density of extra-cellular matrix molecules (<60% of otherwise dense regions). These regions had less collagen IV, laminin 10 and nidogen 2 representing the path of least resistance for neutrophils[7].

1.1.2.2 Neutrophil antibacterial activities

At the site of infection, neutrophils employ multiple methods to eliminate the invading pathogen. When a pathogen is encountered, it is phagocytosed by the neutrophil and once it is inside a phagosome, the neutrophil releases reactive oxygen species (ROS), cathepsins, defensins, lactoferrin and lysozymes stored in granules into the phagosome to kill the invading organism. This process of degranulation can also occur in the extracellular vicinity of the cell in order to attack the offending pathogen.

The neutrophils can also eliminate extracellular pathogens by releasing neutrophil extracellular traps (NETs). NETs are formed of core DNA elements with histones, proteins (cathepsins, lactoferrin) and enzymes (metalloproteinase, neutrophil elastase). The NETs trap and directly kill pathogens through its own histones, proteins and enzymes as well as facilitating phagocytosis of the trapped pathogen[8].

1.1.3 Endothelial response to inflammation

1.1.3.1 Endothelium at rest

The endothelium is a single layer of cells lining all blood vessels. It is considered the largest organ of the body covering an area of 4000 – 7000 m^2 and weighing 1/5 kg. The endothelium contributes to each step of the process of haemostasis as it maintains and regulates blood flow, controls vessel permeability and quiesces circulating leucocytes[9, 10].Blood flow is maintained by the endothelium through inhibition of the coagulation system *via* the expression of tissue factor pathway inhibitor (TFPI) which inhibits the initiation of coagulation, the expression of heparin sulphate proteoglycan that inactivates thrombin, the expression of thrombomodulin which indirectly activates protein C and the production of nitric oxide (NO) and prostacyclin that inhibit platelet aggregation[10, 11].

The endothelium regulates blood flow through maintaining tight junctions between endothelial cells thereby inhibiting the passage of plasma proteins and circulating cells to the tissues while maintaining active mechanisms of transport of certain plasma proteins through the endothelial barrier[12].

The endothelial-leucocyte interaction is inhibited at rest as the endothelial cells maintain adhesive proteins such as P-selectin and chemokines such as IL-8 within secretory vesicles called Weibel-Palade bodies[13, 14] and the transcription of E-selectin, ICAM-1 and VCAM-1 is inhibited. Also the basal production of NO by endothelial cells contributes to endothelial and leucocytes quiescence *via* the inhibition of pro-inflammatory gene expression in endothelial cells, inhibition of Weibel-Palade bodies' fusion with the cell surface[15] and inhibition of leucocyte activation[10].

1.1.3.2 Endothelial activation

Activation of endothelial cells is defined as the acquisition of new capabilities by the resting cells. This process is divided into rapid responses that are not dependent on new gene expression (Type I activation) and slower responses that depend on up-regulation of gene expression (Type II activation). Both kinds of activation result in increased local blood flow, localised leakage of plasma proteins into the tissues and localised recruitment and activation of leucocytes which then enter the site of inflammation[10].

Type I endothelial activation is typically mediated by ligands that bind the extra-cellular domains of the G-protein-coupled receptors (GPCR) resulting in the intracellular release of Ca^{+} from the endoplasmic reticulum and RHO activation.

Increased cytosolic Ca^+ results in enhanced production of prostacyclin and nitric oxide (NO) causing increased blood flow locally. Increased vascular leak occurs as a results of the interaction between the Ca^+ and RHO pathway, this interaction increases myosin light chain (MLC) phosphorylation, causing contraction of actin filaments that are attached to junctional proteins, with opening of the gaps between endothelial cells[16]. The increase in cytosolic Ca^+ also has a role in leucocyte recruitment as it initiates the exocytosis of Weibel-Palade bodies brining P-selectin to the endothelial cell surface, as well as generating platelet-activating factor (PAF). The expression of both P-selectin and PAF on the endothelial surface initiates leucocyte tethering (capture) followed by integrin activation that initiates the process of neutrophil transmigration[10, 17].

Type II endothelial activation. Signalling by GPCR lasts for 10 to 20 minutes and is followed by receptor desensitisation, thereby limiting the inflammation and neutrophil recruitment induced by this process. A more sustained inflammatory response is required to maintain the process of pathogen elimination which is provided by type II activation.

The mediators of this type of activation are tumour necrosis factor – α (TNF-α) and IL-1 principally derived from activated leucocytes. TNF-α and IL-1 bind to tumour necrosis factor receptor -1 (TNFR-1) and IL-1 receptor 1 on the endothelial cell surface resulting in activation of the transcription factors nuclear factor-κB (NF-κB) and activator protein 1 (AP-1) leading to new gene transcription and protein

translation[18]. This response requires hours rather than minutes making it a slower process in comparison to type I activation.

Type II activation causes increased localised blood flow via increased production of prostacyclin at a much higher concentration in comparison to type I but still depending on the increase in cytosolic Ca^{+}. Vascular leakage induced by type II activation is mediated via TNF-α and IL-1 stimulation of endothelial cells to reorganise their actin and tubulin cytoskeletons resulting in opening of inter-cellular gaps[19]. The leakage seen with type II activation is of larger plasma proteins in comparison to type I and results in the formation of a firm provisional matrix often described as “induration” in comparison to the soft oedema produced by type I. The synthesis and expression of IL-8 and E-selectin on the surface of endothelial cells render type II activation more effective in terms leucocyte recruitment. E-selectin (functionally similar to P-selectin and IL-8) triggers firm attachment of neutrophils to endothelial cells and induces *diapedesis* into the tissues. This process can only act effectively if the leucocytes are tethered to the endothelial surface, a phenomenon which is reliant on type I activation preceding type II[10].

Type II activation evolves over time with ICAM-1 and VCAM-1 expression increasing while E-selectin expression decreases. Also, there is an increase in chemokine ligand 2 (CCL2) production which favours transition to monocyte rich infiltrate over the 6 to 24 hours following the initial activation. By 24 hours, leucocyte mediated endothelial cell injury contributes to inflammation; TNF-α and IL-1 in conjunction with

other mediators such as interferon-γ can induce endothelial cell death through the activation of procaspase-8 and the release of cathepsin B[20]. This injury is also pro-coagulant through shedding of anticoagulants, exteriorisation of MP expressing phosphatidylserine which favours coagulation[21], exposure of basement membrane collagen which supports the adhesion and activation of platelets and tissue factor synthesis and exteriorisation[22]. This pro-coagulant activity predisposes to thrombus formation which helps to sequester the pathogen and at the same time causes further damage to the vessel wall with a risk of haemorrhage[10].

1.1.3.3 Resolution of endothelial activation

Type I activation undergoes spontaneous resolution as a result of receptor desensitisation but type II activation can persist as long as the stimulating agents (e.g. cytokines) are present. Type II activation resolves when cytokines are eliminated following successful removal of the pathogen, as well as *via* several negative feedback mechanisms resulting in down-regulation of the genes that perpetuate inflammation[10].

The process of endothelial activation results in the acquisition of new capabilities by endothelial cells. This process starts with the more rapid Type I activation which is followed by the slower but more potent Type II activation. The activation process results in increased blood flow to the site of inflammation accompanied by increased leakage of plasma proteins and leucocyte recruitment to the site of inflammation. This inflammation resolves once the pathogen has been eliminated.

1.1.4 Resolution of inflammation

Resolution of inflammation is an active process aimed at restoring tissue integrity and function following the achievement of inflammatory goals (e.g. bacterial clearance). This is achieved through abolition of chemokine signalling in order to block further neutrophil tissue infiltration, as well as the attraction of monocytes and macrophages to clear apoptotic neutrophils which results in re-programming of macrophages towards a pro-resolution phenotype[23].

Chemokine depletion. Chemokines are chemoattractants that orchestrate the migration of leucocytes towards the site of inflammation. Abolishing this migration is a crucial step to reduce further inflammation and initiation of resolution via chemokine cleavage and sequestration. Matrix metalloproteinase (MMP) 12 secreted by macrophages cleaves CXC-chemokines and also depletes CC-chemokines, rendering them unable to attract neutrophils and monocytes respectively[24]. In an environment where there are pro- (LPS) and anti-inflammatory (IL-10) signals chemokine receptors can be uncoupled from signalling G-proteins to form “decoy” receptors[25]. Chemokine sequestration is a process by which chemokines bind to chemokine “decoy” receptors; this binding does not translate into leucocyte trafficking but rather causes chemokine trapping[25]. IL-10 also inhibits down regulation of (CC-chemokine receptor) CCR1, CCR2 and CCR5 resulting in further chemokine sequestration[26].

Neutrophil apoptosis. Neutrophil clearance is another important step in resolution of inflammation. Once migrated, neutrophil's lifespan is modified by multiple factors. For example, macrophages secret ligands of death receptor such as Fas-ligand and TNF-α which promote neutrophil survival in low concentrations and neutrophil apoptosis in high concentrations[27]. These pathways manipulate ROS production in neutrophils which is a key event in initiating neutrophil apoptosis. Neutrophils undergoing apoptosis produces protein such as annexin A1 which inhibits further neutrophil recruitment through binding formyl peptide receptor (FPR) – 2. Annexin A1 also promotes neutrophil apoptosis and clearance by macrophages[28]. Lactoferrin is another protein secreted by neutrophils with similar effects to annexin A1[29].

Macrophage reprogramming. Macrophages ingest apoptotic neutrophils via *efferocytosis* which switches off pro-inflammatory mediator production and initiates production of anti-inflammatory mediators (e.g. IL-10 and transforming growth factor (TFG) – β)[30]. These "resolution-phase" macrophages possess high levels of antigen processing and presentation capabilities, as well as producing lymphocyte chemoattractants such as XCL1, CCL5 and CXCL13[31, 32]. Certain subtypes of lymphocytes (B1, NK, γδT, $CD4^+CD25^+$, B2) migrate to the site of resolving inflammation and exert protective effects[33]. These resolution-phase macrophages express lower levels of CD11b, have reduced responses to toll like receptor (TLR)-4 ligands and have enhanced capacity to ingest apoptotic neutrophils[34].

Restoration of tissue function. Tissue recovery following inflammation is a complex process that involves coordination between macrophages, stem cells, progenitor cells and stromal cells to prevent fibrosis and restore function.

In this phase, macrophages produce anti-inflammatory and pro-repair mediators such as IL-1 receptor antagonist, IL-10, transforming growth factor (TGF)-β and vascular endothelial growth factor (VEGF)[35]. TGF-β promotes tissue repair and wound healing via enhanced fibroblast differentiation into myofibroblasts, expression of tissue inhibitors of metalloproteinases (TIMP) that regulate remodelling of extra-cellular matrix (ECM) and collagen synthesis by myofibroblasts[36]. VEGF promotes angiogenesis and restoration of oxygen supply in healing wounds[37]. Macrophages also produce metalloproteinases and TIMPs to control ECM turnover.

Furthermore, macrophages engage in cross-talk with mesenchymal stem cells (MSC) where macrophages promote MSC growth and motility. In turn MSC promote macrophage differentiation into a pro-resolution, pro-repair phenotype[35, 38].

Once pathogen elimination has been achieved, the process of resolution of inflammation ensues. This is an active process aiming to restore tissue integrity and function that is mediated through blocking of further neutrophil infiltration and attracting monocytes and macrophages to clear the apoptotic neutrophils. These macrophages now undergo a process of reprogramming to attain a pro-resolution phenotype.

1.1.5 Mediators of resolution

Resolution of inflammation is controlled by several classes of mediators such as autacoids, bioactive lipids and proteins. Below is a succinct focus on a few of the pro-resolution mediators.

Lipid mediators of resolution. Lipid mediators play a role in balancing inflammation and resolution. Prostaglandin (PG) – E2 and D2 play a pro-inflammatory role, while in the resolution phase the pro-resolution lipoxins, resolvins and protectins become predominant. Lipoxins inhibit the migration of neutrophils into sites of resolving inflammation and promote monocyte translocation into these same sites. They also reduce neutrophil expression of CD11b/CD18, ROS production, NFκB activity and production of pro-inflammatory soluble mediators. In monocytes, lipoxins promote neutrophil phagocytosis. Resolvins are another class of pro-resolution lipid mediators that reduce neutrophil infiltration and reduce monocyte, macrophage and dendritic cell NFκB activation in response to TNF-α[23, 39].

Peptide mediators of resolution. IL-10 (produced mainly by resident macrophages) is a mediator of inflammation resolution as it suppresses the production of pro-inflammatory mediators such as TNF-α and IL-1, as well as preventing maturation of antigen presenting cells (e.g. dendritic cells) resulting in suppressed T-helper cell proliferation and differentiation[40]. This effect protects the tissues from the harmful effects of unchecked inflammation and promotes resolution[41]. TGF-β produced by macrophages on phagocytosis of apoptotic neutrophils[30] is another important

mediator of resolution. TGF-β was found to suppress the production of pro-inflammatory cytokines by macrophages and other cells, as well as promoting tissue repair and wound healing[36]. VEGF also promotes resolution of inflammation and restoration of tissue function by stimulating angiogenesis[37].

1.2 Severe sepsis

1.2.1 Overview

Severe sepsis (SS) is a systemic inflammatory response to infection associated with coagulopathy and acute multi-organ failure. The incidence of SS is increasing worldwide with stubbornly high morbidity and mortality despite recent advances in understanding and management of the disease. In the USA it is estimated that 750,000 patients develop SS annually of whom 210,000 die[42]. The incidence of SS in the UK was around 66 per 100,000 population in 2003 up from 46 per 100,000 in 1996 with mortality approaching 30 per 100,000 up from 23 in the same period [43]. SS accounted for 23.5% of admissions to Intensive Care Units (ICU) across the UK in 1996 increasing to 28% in 2004 [43]. Non-surgical cases accounted for more than 70% of all SS admissions to ICU [43].

Sepsis as defined by the Surviving Sepsis Campaign International Guidelines 2012 refers to infection with systemic manifestations of inflammation and is considered severe when associated with sepsis-induced organ dysfunction or tissue hypo-perfusion. The campaign has devised criteria for the diagnosis of sepsis as follows[44]:

1. **Infection, documented or suspected, and some of the following**:
    1. General variables
        1. Fever (>38.3°C)
        2. Hypothermia (core temperature <36°C)
        3. Heart rate > 90/min or >2 SD above the normal value for age
        4. Tachypnoea
        5. Altered mental status
        6. Significant oedema or positive fluid balance (>20 mL/kg over 24 hrs)
        7. Hyperglycaemia (plasma glucose >140 mg/dL or 7.7 mmol/L) in the absence of diabetes mellitus
    2. Inflammatory variables
        1. Leucocytosis (WBC count >12,000/μL)
        2. Leucopenia (WBC count <4000 /μL)
        3. Normal WBC count with >10% immature forms
        4. Plasma C-reactive protein >2 SD above the normal value
        5. Plasma procalcitonin >2 SD above the normal value
    3. Hemodynamic variables
        1. Arterial hypotension (SBP <90 mm Hg; MAP <70 mm Hg; or an SBP decrease >40 mm Hg in adults or <2 SD below normal for age)
    4. Organ dysfunction variables
        1. Arterial hypoxaemia (PaO^2/FIO^2<300)
        2. Acute oliguria (urine output <0.5 mL/Kg hr or 45 mmol/L for at least 2 hrs, despite adequate fluid resuscitation)
        3. Creatinine increase >0.5 mg/dL or 44.2 μmol/L
        4. Coagulation abnormalities (INR >1.5 or aPTT >60 seconds)
        5. Ileus (absent bowel sounds)
        6. Thrombocytopaenia (platelet count, <100,000/μL)
        7. Hyperbilirubinaemia (plasma total bilirubin >4 mg/dL or 70 μmol/L)
    5. Tissue perfusion variables
        1. Hyperlactataemia (>upper limit of lab normal)
        2. Decreased capillary refill or mottling

The same guidelines devised a goal-orientated approach to the initial management of SS, which is called resuscitation. The aim of this approach is to correct the SS induced tissue hypo-perfusion. The tissue hypo-perfusion is defined, as “hypotension after initial fluid challenge” or a blood lactate level of more than or equal to 4 mmol/L. Within the first six hours the aim of resuscitation is to achieve:

1. Central venous pressure 8 -12 mm Hg
2. Mean arterial pressure ≥ 65 mm Hg
3. Urine output ≥ 0.5 ml/kg/hr
4. Central venous or mixed venous oxygen saturation of 70% or 65% respectively
5. Normalising lactate levels

The next step is to obtain at least two sets of blood cultures; at least one of them is drawn percutaneously before commencing antimicrobial therapy. Also performing any necessary imaging to confirm a potential source of infection.

The guidelines then recommend commencing empirical antibiotic therapy of one or more agents effective against all likely pathogens. This has to be modified later if and when the causative organism is identified. The antibiotic therapy should continue typically for 7 to 10 days. Anti-viral agents should be used when viral infections are suspected.

Source control is the next step in which anatomical sources of infection are identified and dealt with promptly in the least physiologically insulting fashion.

Another important recommendation is initiating selective oral and digestive decontamination strategies to reduce the incidence of ventilator-associated pneumonia.

The surviving sepsis campaign has formulated a “bundle” to help implement these recommendations. The first 4 steps have to be completed within 3 hours of diagnosis and the rest within 6 hours. This bundle consists of:

1. Measure lactate level
2. Obtain blood cultures prior to administration of antibiotics
3. Administer broad spectrum antibiotics
4. Administer 30ml/kg crystalloid for hypo-perfusion or lactate ≥ 4 mmol/L
5. Apply vasopressors (for hypotension not responding to initial fluid resuscitation) to maintain mean arterial pressure ≥ 65 mm Hg
6. If hypo-perfusion persists despite initial fluid resuscitation measure central venous pressure and central venous oxygen saturation
7. Re-measure lactate if it was initially elevated

The guideline also offers recommendations on haemodynamic support and adjunctive therapy in SS. These are mainly:

1. Fluid resuscitation of at least 30ml/kg using crystalloids and albumin
2. Vasopressors use to maintain mean arterial pressure ≥ 65 mm Hg, the main vasopressor they advocate is Norepinephrine
3. Inotropes could be used if myocardial dysfunction is suspected
4. Hydrocortisone could be used to supplement the treatments above, if the required blood pressure could not be reached

Other supportive measures which have been advised by the campaign are:

1. Blood products: Red blood cell transfusion to maintain haemoglobin level between 7 and 9 gm/dL. Platelets transfusion when count below 10,000x 10^9/L or in special situations.
2. Mechanical ventilation strategies for SS associated acute respiratory distress syndrome (ARDS)
3. Sedation: To be minimised in SS patients
4. Glucose control: Maintain blood glucose levels between 6 and 10 mmol/L
5. Renal replacement therapy: Continuous renal replacement strategies are advocated in SS patients with acute renal failure
6. Deep Venous Thrombosis (DVT) prophylaxis: Low molecular weight heparin is the first line
7. Stress ulcer prophylaxis: H2 blockers or proton pump inhibitors could be used
8. Nutrition: Enteral or parenteral nutrition to be commenced as soon as possible

Expect for antibiotics the main management strategy for SS as outlined above remains supportive. Many novel therapeutic agents (e.g. anti-TNF and anti-IL1) have

shown promise in vitro and in animal studies but did not show benefit when tested in clinical trials[42]. Other agents have shown benefits in initial clinical trials but have subsequently been found to be ineffective or even harmful (e.g. recombinant human Activated Protein C)[45]. The repeated failure to discover new therapeutic agents in SS at least in part reflects our limited understanding of the underlying mechanisms but could also be due to the complexity of the physiological response and the heterogeneity of the patient population in terms of age, gender, co-morbidities, medications and causative pathogen. Also the timing of therapy varies significantly between patients and genetic polymorphisms may be associated with individual differences in response to the original insult and therapies [46].

1.2.2 Pathophysiology of severe sepsis

Sepsis develops when the initial host response to the invading organism is amplified and subsequently dysregulated. This response is driven by multiple factors including microbial virulence, the extent of bacterial invasion, the host's premorbid condition as well as their immune phenotype. The host response can cause cell and tissue damage as in multiple organ dysfunction syndrome (MODS) and even multi-organ failure. In severe sepsis, the invading organism triggers both the innate and the adaptive immune systems. Innate immunity is the initial defence against the pathogen, while the adaptive immune system provides more sophisticated targeted defence later in the disease course. The adaptive immune system recognises specific pathogens and mounts a stronger response each time the host encounters the same organism [47].

Following stimulation by an invading organism, innate immune cells produce large amounts of cytokines, chemokines, complement-activation mediators and intra-cellular danger signals (alarmins). Also innate immune cells act directly on the invading organism through phagocytosis and killing [48].

Adaptive immunity is activated later through interaction with antigen-presenting cells and soluble mediators. The cells of the adaptive system then proliferate to generate effector cells that produce a specific repertoire of cytokines [48] . The “cytokine storm” is thought to be the trigger for the dysregulated systemic immune response that characterises severe sepsis.

1.2.2.1 Pathogen recognition and initiation of immune response

Cells initially recognise a potential threat through their pattern recognition receptors (PRR) which are stimulated with pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs). The PAMPs include bacterial cell wall components (e.g. Lipopolysaccharide – LPS and peptidoglycan) and microbial nucleic acids, while the DAMPs are host related and derived from damaged tissue such as DNA, ROS, heat shock proteins and fibrinogen[49].

TLRs are a group of PRRs that play an important role in the development of severe sepsis. They are expressed mainly by innate immune cells including neutrophils, monocytes and macrophages as well as endothelial cells. When they are stimulated by their ligands, TLRs induce the production of cytokines via nuclear factor κB (NFκB) pathway activation[49].

1.2.2.2 Pro-inflammatory response and cytokine storm

Upon activation of PRRs a number of pro-inflammatory cytokines and chemokines, includes IL-1β, IL-6, IL-8, interferon (IFN)-γ and tumour necrosis factor (TNF)-α are secreted initially mainly by macrophages. IL-1β (one of the initial cytokines produced in response to TLR stimulation) has the capacity to enhance the production of most other pro-inflammatory cytokines, chemokines and nitric oxide as well as increasing the expression of adhesion molecules. IL-1β also stimulates the bone marrow to produce inflammatory effector cells such as neutrophils and platelets. The complement system also acts to re-inforce and exaggerate the innate immune response by opsonising pathogens in order to facilitate their phagocytosis and lysis[49, 50].

Neutrophils are the first cells recruited to the site of inflammation in order to participate in bacterial clearance but subsequently these leucocytes can cause tissue damage through cytotoxicity, degranulation, increased vascular permeability and release of several pro-inflammatory mediators and proteases. Monocytes follow neutrophils to the site of inflammation where they participate in pathogen clearance and cytokine production to perpetuate inflammation. They also differentiate locally into macrophages and dendritic cells which are professional antigen presenting cells driving inflammation further by recruiting cells of the adaptive immune system[49, 50].

1.2.2.3 Hypo-inflammatory response and immune-paresis

On the other hand macrophages and neutrophils also release anti-inflammatory cytokines such as IL-10 and TGF–β, which play an important regulatory function in sepsis. The prolonged release of these mediators may produce the immune-paresis seen particularly in the later stages of the disease. This is mirrored by cells of the adaptive immune system which tend to release pro-inflammatory mediators at the onset of sepsis and anti-inflammatory mediators later on, thereby contributing to host susceptibility to nosocomial infections [51]. In addition, immune cells may become exhausted in sepsis and chemoattractant receptors can be de-sensitised (through phosphorylation of the intracellular tail and/or blockade of recycling), contributing to immune-paresis and an inadequate response to xenobiotic agents (see section 1.2.2).

Achieving a balance between pro- and anti-inflammatory mechanisms is an important determinant of outcome in sepsis. The early hyper-immune phase could be rapidly fatal whilst the immuno-paresis that develops later in the course of the disease predisposes to recurrent infection, persistent organ failure and late deaths. The initial surge in pro-inflammatory mediators and the hyper-immune state is termed the systemic inflammatory response syndrome (SIRS). This phase is important for successful elimination of the invading pathogen but also could cause injury (“collateral damage”) to the host. The anti-inflammatory mechanisms that are triggered almost simultaneously and as a result of SIRS have been termed the compensatory anti-inflammatory response syndrome (CARS) [51, 52].

The CARS can limit the damage mediated by SIRS but plays no role in pathogen elimination. Thus CARS could be harmful to the patient if poorly regulated or miss-timed rendering the host susceptible to acquired infections and causing further morbidity and mortality[51, 53] (**Figure 1.1**).

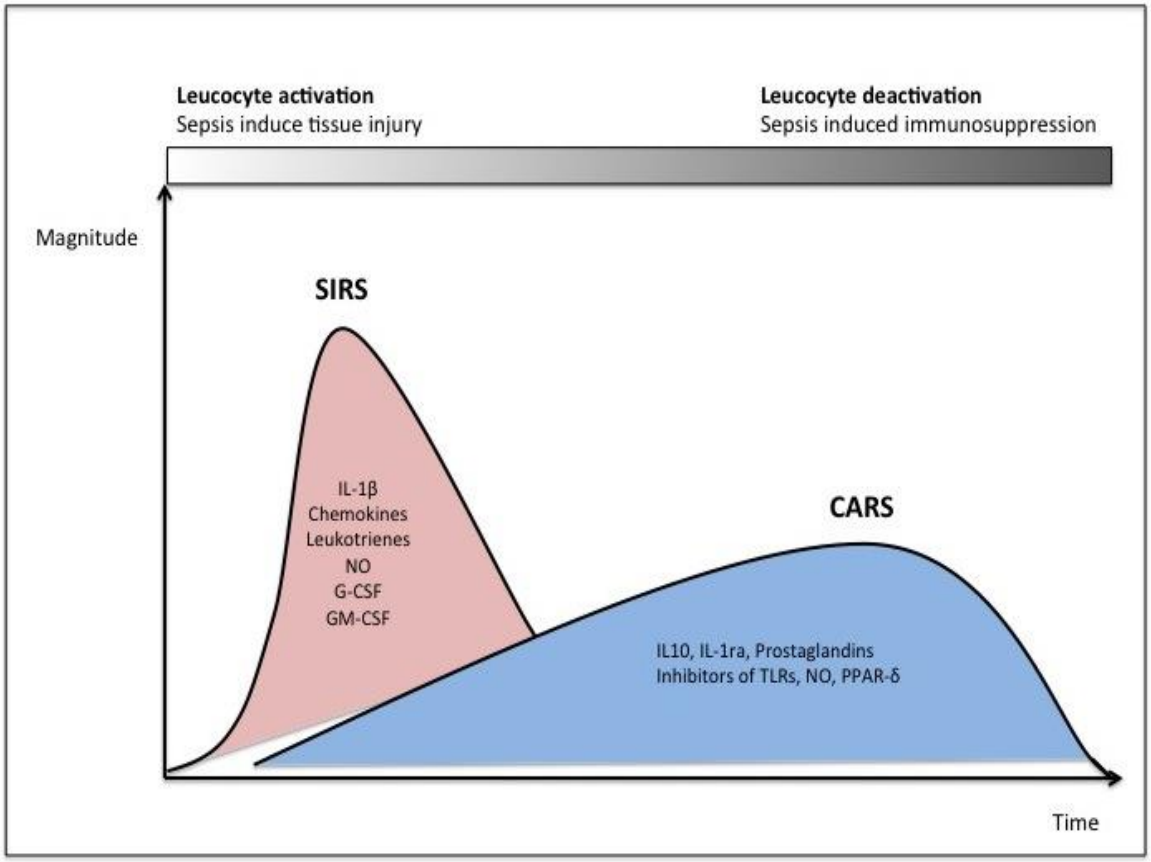


Figure 1.1. Schematic depicting pathophysiological phases in sepsis, including mediators involved, biological effects and clinical manifestations. CARS: Compensatory anti-inflammatory response syndrome, G-CSF: granulocyte colony stimulating factor, GM-CSF: granulocyte-macrophage colony stimulating factor, IL: interleukin, NO: nitric oxide, PPAR-δ: peroxisome proliferator-activated receptor δ, SIRS: Systemic inflammatory response syndrome, TLR: toll like receptor [54].

1.2.2.4 Mitochondrial dysfunction

Organ injury in sepsis is multifactorial. Increased tissue oxygen demand in the presence of inflammation, diminished oxygen delivery due to microcirculatory redistribution, hypovolaemia and myocardial suppression as well as endothelial failure and micro-thrombi formation are some of the factors resulting in organ dysfunction and failure.

Mitochondrial dysfunction is another mechanism that may contribute to organ failure. In sepsis, oxidative stress and the production of nitric oxide, carbon monoxide and hydrogen sulphide are implicated in the inhibition of mitochondrial respiration and mitochondrial damage[55]. As a consequence the mitochondria fail to replace depleted adenosine triphosphate (ATP) which leads to activation of cell death pathways and ultimately organ failure.

1.2.3 Neutrophils in sepsis

One of the main pathological features of sepsis is neutrophil reprogramming associated with dysregulated trafficking and function. This manifests as impaired neutrophil recruitment to sites of infection, neutrophil sequestration in remote sites causing local damage (e.g. Acute Respiratory Distress Syndrome (ARDS) secondary to sequestration in the lungs) and dysregulation of neutrophil effector responses. This impairment of function can cause defective innate immune responses.

Early in the course of sepsis, the rigidity of the neutrophil cell membrane increases and deformability is reduced due to the effect of bacterial endotoxins and pro-inflammatory cytokines[56]. This effect is positively correlated with the severity of infection and leads to neutrophil sequestration in the capillary beds, particularly in the lungs and the liver[56]. This sequestration results in occlusion of micro-vessels contributing to local ischaemia and subsequently organ dysfunction. The increase in rigidity may also affect margination and rolling of neutrophils[57].

Another effect of the pro-inflammatory cytokines and endotoxins is the up-regulation of inducible nitric oxide synthase (iNOS) resulting in increased nitric oxide (NO) production. This mediator inhibits adhesion molecule expression on both leucocytes (integrins and selectins) and the endothelial cells (selectins and immunoglobulin-like) resulting in the inhibition of neutrophil migration[57, 58]. Furthermore, NO enhances the expression of hemoxygenase-1 (HO-1), which can interfere with the rolling and adhesion of neutrophils[59].

In sepsis there is also release of chemoattractants that act at the G-protein-coupled receptors (GPCR) expressed by neutrophils. The high concentration of these chemoattractants leads to functional desensitisation due to several mechanisms including GPCR down-regulation, phosphorylation and inactivation. One of the GPCRs that is down regulated is CXCR2, in part due to the internalisation of occupied receptors[60]. This in turn results in decreased binding to CXCL2 and failure of neutrophil migration to the site of infection. Sepsis also causes changes in other

relevant chemokine receptors such as C-C chemokine receptor 2 (CCR2). This receptor is up-regulated in sepsis in response to TLR stimulation resulting in increased un-regulated migration and accumulation of neutrophils in distant organs, rather than targeted migration towards sites of infection[61].

Neutrophils express a number of TLRs, which when stimulated by PAMPs and endogenous inflammatory mediators results in the release of pro-inflammatory cytokines, chemokines and antimicrobial peptides. In early sepsis TLRs are persistently activated leading to excessive production of inflammatory mediators, contributing to tissue injury and the overall clinical picture[62]. The excessive and/or prolonged stimulation of the TLRs in sepsis causes receptor down-regulation and inhibition of their associated intracellular signalling pathways[63]. Moreover, the anti-inflammatory mediators released by other cells such as IL-10 produced by macrophages also inhibit TLR mediated neutrophil activation[64]. These observations suggest that overall sepsis is associated with a state of neutrophil hypo-responsiveness.

Neutrophils also produce extracellular traps (NETs) in response to exposure to microbial pathogens. NETs physically trap the invading pathogens, facilitating the antimicrobial effector function of leucocytes, as well as having intrinsic antimicrobial properties[65]. There is also evidence that NETs contribute to excessive inflammation and tissue injury[66].

Sepsis seems to result in neutrophil reprogramming by the suppression of several gene clusters and the induction of others, leading to substantial changes in neutrophil effector function. This process manifests in the suppression of genes responsible for the inflammatory response, immune modulation, oxidant production and phagocytosis and the induction of genes regulating the production of antimicrobials (e.g. lipocalin and cathelicidins) [67].

In conclusion, neutrophil function is greatly altered by sepsis resulting in dysregulated trafficking and anti-microbial effects. These alterations contribute to immune dysregulation and the clinical picture of sepsis.

1.2.4 Endothelium in sepsis

The endothelium is a single layer of cells lining all blood vessels and is considered the largest organ of the body covering an area of 4000 – 7000 m^2 and weighing 1/5 kg. The endothelium contributes to each step of the process of haemostasis[9]. The endothelium also defends the body against invading organisms by recruiting immune cells to the site of infection, releasing inflammatory mediators and encouraging local activation of the coagulation system to limit the spread of the causative organism[9]. “Endothelial dysfunction” can cause tissue damage when exaggerated and widespread.

Endothelial denudation is a feature of sepsis induced endothelial dysfunction and is a result of sub-endothelial oedema and detachment of endothelial cells (EC)[68, 69].

This detachment leads to an increase in the number of circulating EC; the extent of the increase is related to illness severity. The EC also undergo changes manifested as nuclear vacuolation, protrusion and cytoplasmic fragmentation[69]. Inflammatory mediators released in response to sepsis and hypoxia can trigger EC apoptosis[70] and the apoptotic EC in turn express adhesion molecules[71] which play a role in attracting more white cells. They also express phosphatidylserine (PS), which acts as a pro-coagulant surface[21]. This denudation impairs the endothelial natural barrier facilitating the passage of cells, inflammatory mediators and plasma into the interstitial compartment. This leakage phenotype causes damage to vital organs such as the lungs[72].

In sepsis there is enhanced expression of endothelial adhesion molecules either on the EC or in the plasma. The leucocyte derived inflammatory mediators such as TNF-α and IL-1β enhance the expression of endothelial adhesion molecules such as endothelial selectin (E-selectin) and intracellular adhesion molecule (ICAM)-1[73]. These adhesion molecules promote neutrophil rolling and adherence at the site of infection. Furthermore, some studies have shown a positive correlation between the levels of circulating E-selectin and ICAM-1 and the severity of sepsis[74, 75]. This overexpression of adhesion molecules in sepsis is another factor leading to neutrophil accumulation in vital organs causing tissue damage through the production of cytokines, proteases and oxygen free radicals[76].

The endothelium normally expresses thrombomodulin (TM) which enhances the activation of protein C, a natural anticoagulant. In sepsis, the neutrophils recruited

to the site of infection, release elastases and other proteinases that breakdown endothelial TM[77]. Also the inflammatory mediators released by the neutrophils(TNF-α and IL-1β) and the surrounding inflamed tissue can decrease the endothelial expression of TM [78]and increase expression of tissue plasminogen activator (tPA). The increased expression of tPA results in heterogeneous fibrin deposition and contributes to local ischaemia[9]. These mechanisms contribute to the hyper-coagulable state seen in sepsis.

Endothelial dysfunction and disordered nitiric oxide (NO) production are the main causes of circulatory failure in sepsis. Early in the septic process there is down-regulation of endothelial nitric oxide synthase (eNOS) induced by the neutrophil inflammatory mediators[79]. Decreased NO production is associated with arteriolar vasoconstriction in some organs, such as the digestive tract. As the disease progresses there is a vast increase in the production of NO by inducible Nitric Oxide Synthase (iNOS) leading to vasodilatation and poor perfusion contributing to organ failure[80].

In sepsis there is a dramatic increase in the production of reactive oxygen species (ROS) by neutrophils, other immune cells and to a lesser extent in the endothelium[81]. Accumulation of ROS in endothelial cells results in increased production of toxic compounds that damage proteins and DNA and cause injury to the EC[82]. ROS also reduce NO availability through various mechanisms including inhibition of eNOS[83].

This process affects vascular tone, platelet adhesion[84] and permeability[85] leading to vascular obstruction and further reductions in organ perfusion.

The endothelium is key player in the pathogenesis of severe sepsis. EC activation and dysfunction are important determinants of the host response and help to explain the complex clinical picture of sepsis. The inflammatory insult to the endothelium leads to loss of cell-cell junctional integrity and vascular leakage. The breakdown of this natural barrier facilitates tissue invasion by microorganisms, leakage of fluid into the extravascular compartment leading to hypotension and tissue oedema (tissue oedema in vital organs contributes to multi organ failure), impairment of oxygen and nutrient transport to the tissues and disruption of the anticoagulant actions of the endothelium.

1.2.5 Coagulation in sepsis

Coagulopathy is the third major component of the pathophysiological changes in sepsis. The initial over production of pro-inflammatory mediators stimulates the production of tissue factor (TF) from several sources including monocytes and endothelial cells, triggering the coagulation cascade[86-88]. The activated coagulation enzymes can affect specific inflammatory and endothelial cell receptors, thereby modulating the immune response[89]. The activation of coagulation and down-regulation of antithrombotic mechanisms on the endothelium conspire to produce coagulopathy. This coagulopathy can vary in severity from a minor fall in the platelet count and a subclinical prolongation of the clotting times to the full picture of a

consumption coagulopathy (i.e. Disseminated Intravascular Coagulopathy – DIC)[90, 91].

In sepsis the causative agent and the ensuing immune response affect fibrin formation and deposition by up-regulating the pro-inflammatory pathways, down-regulating the physiological anticoagulants[92] and suppressing fibrinolysis[93].

Endothelial cells and monocytes are known to express TF in response to a variety of stimuli. These cells also exhibit other pro-coagulant properties thus providing a surface for initiation and propagation of clotting with fibrin formation. Neutrophils[94], eosinophils[95] and platelets[96] also express TF when stimulated. There is some evidence to suggest that these cells acquire TF from microparticles (MP)[97]. However the activated monocyte seems to be the main trigger for the clotting cascade in sepsis[98]. In animal models of DIC secondary to sepsis, fibrin deposits in vital organs, in these organs TF expression is enhanced[99, 100]. TF is detected mainly in the circulating monocytes and the macrophages infiltrating these organs and in the same animals the circulating monocytes and macrophages exhibit strong TF activity[100, 101]. Furthermore monocyte MPs strongly expressed TF and are increased in sepsis[100].

In sepsis, suppression of fibrinolysis is mediated by increased levels of plasminogen activator inhibitor 1 (PAI-1)[102]. There is also an increase in tPA in plasma, but the balance is towards the PAI-1 and the anti-fibrinolytic activity[103]. Also the enhanced thrombin production might influence fibrin production by producing denser fibrin that

resists fibrinolysis. Activated platelets also contribute to this process by altering fibrin stability, making it less susceptible to lysis[104] as well as reducing the ability of the tPA and plasminogen to bind to the fibrin[105].

1.2.6 Immuno-modulatory therapies in severe sepsis

Sepsis morbidity and mortality remain stubbornly high and the management of these patients remains mainly supportive. Initially as the harmful effects of sepsis were thought to be due to a hyper-immune response, many therapeutic interventions aimed at suppressing immunity were tested, but they were all found to be ineffective or even harmful. As our understanding of sepsis advanced and the concept of immune dysregulation followed by hypo-immunity become more accepted, a new approach was adopted where the aim is to reverse the immune-paresis seen in the later stages of sepsis. Novel therapeutic agents are being tested to that effect including granulocyte macrophage colony stimulating factor (GM-CSF), interferon-γ (IFN-γ), IL-7, IL-15, anti-programmed cell-death receptor 1 and anti-B and T lymphocyte attenuator[106].

1.2.6.1 Granulocyte-macrophage colony stimulating factor (GM-CSF)

GM-CSF is one of three known colony stimulating factors, namely macrophage-colony stimulating factor (M-CSF), granulocyte-colony stimulating factor (G-CSF) and GM-CSF. GM-CSF is produced from a variety of cells including macrophages, dendritic cells, smooth muscle cells, fibroblasts, chondrocytes and T-cells. GM-CSF induces dendritic cell maturation, granulocyte activation and microglia proliferation, as well as differentiation of natural killer T cells and alveolar macrophage. In addition,

both lymphocytes and endothelial cells have GM-CSF receptors but their precise function is still unknown. GM-CSF knockout mice can develop pulmonary alveolar proteinosis suggesting that GM-CSF may be necessary for maintaining normal pulmonary physiology[107].

The GM-CSF receptor (GM-CSFR) is a heterodimer consisting of α subunit which contains the binding site for the agonist and a β subunit which it shares with IL-3 and IL-5 receptors. This receptor is expressed on haematopoietic cell surfaces in low concentrations and it is also expressed on the surface of granulocytes, monocytes, macrophage progenitor cells, macrophages, dendritic cells, megakaryocytes, plasma cells, T lymphocytes, endothelial cells, uterine cells, gastrointestinal epithelial cells, astrocytes, oligodendrocytes and microglia[108].

Binding of GM-CSF to its receptor results in the activation of the Janus kinases (JAK) resulting in the phosphorylation of signal transducers and activators (STAT) which migrate to the nucleus in order to direct the transcription of genes related to cell differentiation[109]. GM-CSF also activates protein kinase C to induce cell proliferation and prevents cell death by phosphatidylinositol 3 kinase and JAK/STAT5-Bcl-2 signalling[110]. It induces inflammation via NFκB activation[111] and regulates toll like receptor (TLR)-2 and TLR-4 expression in neutrophils[112] and TLR-2 in monocytes[112] as well as inducing IL-12 and TNF-α release by monocytes and monocyte chemoattractant protein (MCP)-1 expression[113].

Function of GM-CSF. GM-CSF plays a role in inflammation, as it enhances the survival of monocytes, macrophages and neutrophils and induces production of pro-inflammatory cytokines by these cells, thereby contributing to pathogen and tumour elimination[114]. GM-CSF increases neutrophil expression of adhesion molecules[115], and enhances their response to chemotactic factors, phagocytosis and production of ROS[116]. GM-CSF also increases neutrophil survival and enhances its major histocompatibility complex (MHC)-II expression, enabling T cell activation[117]. Monocytes and monocyte derived dendritic cells exhibit increased expression of MHC-II, along with other antigens in response to GM-CSF thereby enhancing their immune response[118]. Also, M1 macrophages are activated by GM-CSF resulting in increased production of TNF-α and interferon γ and suppressed production of IL-10[119].

GM-CSF has been implicated in a variety of diseases such as Felty's syndrome, rheumatoid arthritis and various other autoimmune, metabolic and cardiovascular diseases. As an example, local over expression of GM-CSF in the stomach leads to autoimmune gastritis and GM-CSF knockout mice are less prone to develop encephalitis, myocarditis and arthritis. These knockout mice are on the other hand obese, hyperphagic, have a decreased number of macrophages and lower expression of pro-inflammatory cytokines[107].

GM-CSF as an adjuvant therapeutic agent. GM-CSF is used widely as a haemopoietin. The human recombinant GM-CSF (hrGM-CSF) is produced in

Saccharomyces cerevisiae[120], Escherichia coli[121] and Chinese hamster ovary cells[122]. It is used for shortening the time to neutrophil recovery following chemotherapy in acute myeloid leukaemia[123] and for myeloid reconstitution following bone marrow transplant[124]. hrGM-CSF is also used as a second line therapy for neutropenia as it induces a small increase in neutrophil count as well as in the number eosinophils and basophils. It is thought be inferior to G-CSF in terms of myeloproliferative function[107].

GM-CSF was found to have a protective role in the gastrointestinal tract where it maintains the integrity of local innate immunity. Indeed, the lack of GM-CSF activity may be related to the immune deficiency typical of Crohn's disease. Patients with this disease have lower levels of GM-CSF[125] and clinical trials have shown that the subcutaneous injection of hrGM-CSF can induce remission of Crohn's disease [126, 127]. GM-CSF also has been found to enhance the in vivo and in vitro microbicidal activity of neutrophils and macrophages against organisms such as *Aspergillus fumigatus hyphae*, *Candida glabrata* and *Histoplasma capsulatum*[128, 129]. Nevertheless, there have been no clinical trials of GM-CSF as an adjuvant treatment against invasive fungal infections. This effect was also observed in children with HIV where their neutrophils were stimulated in vitro with GM-CSF resulting in an enhanced neutrophil bactericidal activity against Staphylococcus aureus[130].

GM-CSF in sepsis. Following the initial septic insult in, a state of immune-paresis ensues rendering the patient vulnerable to hospital acquired infections and causing further morbidity and mortality.

Novel therapeutic approaches have been adopted in an attempt to “re-ignite” the immune system. GM-CSF has been studied in this context to test its effect as an immune-modulatory agent in severe sepsis. In a study examining the effects of GM-CSF on reversing the immune-paresis observed in trauma, cardiac surgery and severe sepsis (impaired immune response was defined as impaired TNF-α production following in vitro incubation of ex-vivo whole blood with endotoxins and decreased monocyte HLA-DR expression). In this study, ex-vivo blood was incubated with GM-CSF, which resulted in an improvement of both parameters in the cardiac surgery cohort comparable and the same trend was observed in the sepsis but to a lesser extent. In vivo, GM-CSF was found to also increase monocyte HLA-DR expression in septic patients accompanied by a significant rise in white cell count[131, 132]. Moreover, in patients with sepsis GM-CSF was found to increase adhesion molecule expression on neutrophils and monocytes and significantly protect against nosocomial infections without, reducing illness severity or overall mortality.

A clinical trial was undertaken in adults with severe sepsis to further examine the effects of GM-CSF. Patients who received the treatment had higher neutrophil, monocyte and T lymphocyte counts as well as higher circulating TNF-α levels.

Moreover, monocytes had higher HLA-DR expression and they were more responsive to endotoxins in vitro in terms of pro-inflammatory cytokine release (TNF-α, IL6 and IL8). The GM-CSF group also had shorter mechanical ventilation times and a tendency towards shorter ICU and hospital stay, but there was no difference in severity scores or mortality[133]. Similar observations were noted in children[134]. GM-CSF also seems to improve pulmonary dysfunction related to sepsis, with a reduction in alveolar neutrophil count[135].

The picture that emerges from these studies suggests that GM-CSF improves immune responses in patients with severe sepsis, particularly those who have immune-paresis as evidenced by biomarkers, and that this improvement is associated with a positive effect on respiratory function, although overall there is no alteration in disease severity or mortality.

1.2.6.2 Interferon-gamma

Interferons were initially described as mediators that interfere with viral replication, but now they are understood to play a wider role in innate and adaptive immune responses. Interferons are classified as type I and type II; type I includes interferon-α, -β, -ω and -τ while type II is interferon-γ (IFN-γ). Type I interferons are secreted in low levels by all cells including haematopoietic cells, fibroblasts and macrophages and they are expressed in response to viral infections, while type II interferon is mainly secreted by $CD4^+$ and $CD8^+$ T lymphocytes and natural killer (NK) cells.

Recently however, IFN-γ was found to be produced by B cells, natural killer T (NKT) cells and antigen presenting cells in response to inflammatory insults[136].

Secretion of IFN-γ is induced by IL-12 and IL-18 from antigen presenting cells[137]. On encountering a pathogen macrophages produce IL-12 and macrophage inflammatory protein (MIP) - 1α which attracts NK cells to the site of inflammation and IL-12 promotes the production of IFN-γ in these cells[138]. Continued production of IL-12 and IL-18 by macrophages, NK and T cells further enhances the expression of IFN-γ[139]. Increased production of IFN-γ has been associated with enhanced resistance to pathogens. On the other hand, IL-4, IL-10, transforming growth factor (TGF)-β and glucocorticoids suppress the production of IFN-γ[139-141].

Function of interferon-gamma. IFN-γ up-regulates the MHC I[142] enhancing the ability of cytotoxic T cells to recognise foreign proteins, which is important for host ability defences against intra-cellular pathogens and the initiation of cell mediated immunity. IFN-γ also up-regulates the expression of class II MHC on the cell surface of $CD4^+$ T cells, B cells, dendritic cells, monocytes and macrophages[143].

Specific cytotoxic immunity induced by IFN-γ is promoted via direct and indirect mechanisms. Directly, IFN-γ induces the cytotoxic mechanisms in helper T cells (Th) – 1 which promote innate cell mediated immunity (via NK cells), specific cytotoxic immunity (via T cells) and macrophage activation[144]. Indirectly, IFN-γ up-regulates antigen processing, presentation and co-stimulation molecules on antigen

presenting cells to increase $CD4^+$ differentiation. Also, IFN-γ promotes differentiation of naive CD4+ into Th-1 phenotype through IL12 production[145, 146]. IFN-γ and its inducer IL-12 coordinate the link between pathogen recognition by the innate immune cells and the induction of cell specific adaptive immunity by forming a positive feedback loop perpetuating and magnifying the Th-1 response.

Another function of IFN-γ is activation of the bactericidal effector functions of macrophages namely pinocytosis, receptor mediated phagocytosis and bactericidal abilities[147]. These bactericidal actions in macrophages include the production of reactive oxygen species, nitric oxide and lysosomal enzymes release with a similar effect on neutrophils[148]. IFN-γ also provides a survival signal for macrophages[136].

Leucocyte trafficking to the site of inflammation is also induced by IFN-γ through the up-regulation of adhesion molecules (e.g. ICAM-1)[149]or indirectly through enhanced cytokine production (e.g. monocyte chemoattractant protein (MCP)-1, CXCL-9, MIP-1)[150-152].

IFN-γ enhances the innate immune response to inflammation as well as providing a link between innate immunity and the more specific adaptive immune response.

Interferon-gamma as a therapeutic agent. IFN-γ has been tested as a therapeutic agent in many diseases. In most of these trials a genetically engineered form of human IFN-γ has been used (IFN-1b)[153].

In cancer, studies have shown that IFN-γ is crucial to tumour surveillance, tumour regression, inhibition of tumour angiogenesis and proliferation and sensitisation of tumour cells to apoptosis. IFN-γ also stimulates anti-tumour immune activity via the up-regulation of MHC class I and II. In recurrent bladder cancer, intravesical injection of IFN-γ resulted in a reduction in tumour recurrence[154]. In ovarian malignancy, the combination of IFN-γ and standard treatment may be more effective than standard treatment alone, although other studies have shown the reverse[155, 156]. IFN-γ is also an approved treatment for adult T-cell leukaemia in Japan where it is reported to induce remission when injected locally into the lesion[156].

Tuberculosis (TB) is another disease where IFN-γ is being tested. IFN-γ activates alveolar macrophages, which constitutes an important defence mechanism against TB[157]. In multidrug resistant TB, IFN-γ was found to stabilise patients' body weight, render sputum smears negative and decrease mycobacterium burden, as well as reducing the size of pulmonary lesions[158].

In hepatitis B (HBV), IFN-γ was found not to have a significant impact, despite immune system modulation[159]. On the other hand, in hepatitis C (HCV) infection when used as a pre-treatment to the standard regime, IFN-γ enhanced immune activity and seemed to improve viral clearance[160]. IFN-γ also reduced hepatic fibrosis caused by HBV[161] and HCV in a selected group of patients[162].

In immune-compromised patients, opportunistic infections are a major cause of morbidity and mortality. IFN-γ was found to reduce *cryptococcal neoformans* burden in the cerebral spinal fluid of HIV positive patients following 2 weeks of treatment[163]. In conjunction with standard treatment, IFN- γ was reduced all opportunistic infections in HIV patients as well as improving their survival, although these effects did not reach statistical significance[164]. *Candida*, herpes and cytomegalovirus infections seem to be especially vulnerable to IFN-γ treatment[164].

IFN-γ has a potential role as an adjuvant therapeutic agent in many diseases, but further research is required to better target patients that would benefit from this immune-modulatory cytokine.

Interferon-gamma in sepsis. Trials of IFN-γ in sepsis have yielded conflicting results[50]. Some studies have shown that production of IFN- γ and TNF-α by in-vitro stimulated whole blood obtained from septic patients was markedly suppressed in comparison to control patients and healthy volunteers[165]. Furthermore, in a murine model of severe sepsis, serum levels of IFN-γ and IL-12 were reduced in comparison to control[166]. Also, peripheral blood mononuclear cells (PBMC) obtained from septic patients produced markedly reduced levels of IFN-γ in comparison to control. Interestingly, this effect was reversed when the cells was rested in fresh medium prior to stimulation. Furthermore, PBMCs obtained from septic patients who developed a second infection and those who died produced less IFN-γ when stimulated in-vitro in comparison to rest of the cohort. These findings suggest that

impaired IFN-γ production by PBMCs in sepsis may be reversible, the degree of this impairment may influence the effectiveness of immune system and those with the most severe impairment are more likely to die[167].

Therapeutically, IFN-γ was found to enhance in-vitro TNF-α production in ex-vivo monocytes obtained from septic patients. Subsequently, IFN-γ was tested in a small group of septic patients with reduced monocyte HLA-DR expression. Treatment with IFN-γ enhanced HLA-DR expression, increased serum TNF-α and improved outcome[168]. These observations were confirmed in another small study in which IL-6 serum levels were also increased confirming the immune-stimulatory effects of IFN-γ in severe sepsis[169].

On the other hand a phase III multicentre placebo controlled trial did not find any benefit of IFN- γ in terms of reducing nosocomial infections acquired by severe burns patients[170]. In another multicentre placebo controlled trial examining the effect of IFN- γ on infections associated with severe injuries showed a statistically significant decrease in the number of infections in the treatment arm and a trend towards lower deaths related to infection, although there was no effect on overall mortality[171].

These results are suggest that IFN-γ may useful as adjuvant therapy in severe sepsis, particularly in patients with immune-paresis evidenced by biomarkers such as decreased monocyte expression of HLA-DR. Ongoing clinical trials are exploring further the potential of IFN-γ in this subset of sepsis patients[172].

1.3 Microparticles

1.3.1 Introduction

Cell-to-cell communication is a vital aspect of an effective host response to insults such as bacterial invasion. Thus, cell-to-cell communication is crucial to activate alerting and protective mechanisms both in the affected site and systemically. A novel means of non-soluble communication has recently emerged focused on plasma membrane-shed vesicles, called microparticles (MP) or ectosomes. MPs were first described by Wolf in 1967[173]. At the time MP were thought to be simple, non-specific cell debris produced during cell death and were called “platelet dust”. However, it is now evident that MP play a role in a variety of biological functions; including coagulation, inflammation, cellular homeostasis and cell survival, intercellular communication, and transport. There is increasing evidence that MP are “multipurpose carriers” facilitating the intercellular exchange of trans-membrane receptors, mRNA, microRNA, and signalling molecules. Furthermore they promote cellular survival by removing dangerous or redundant intracellular compounds [174-176](Figure 1.2).

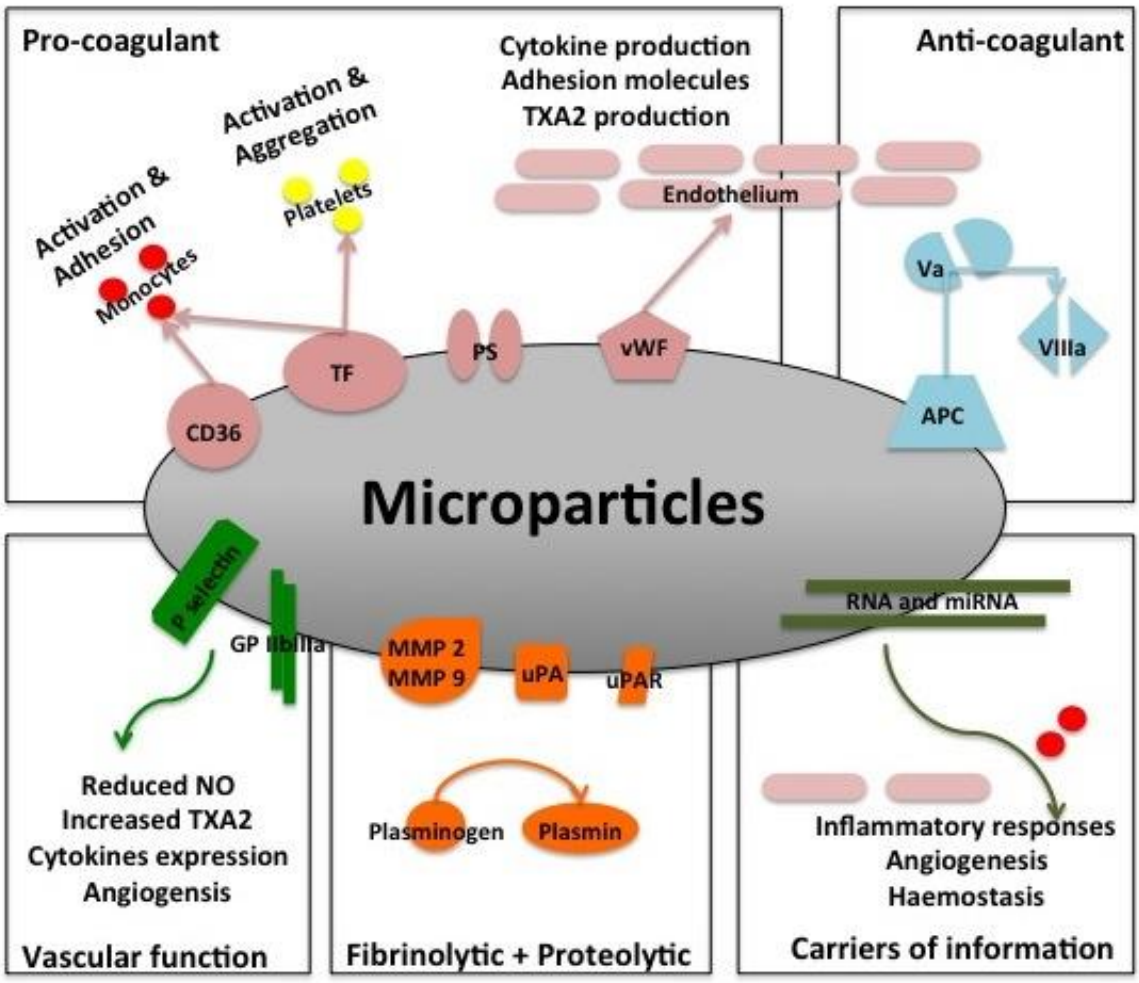


Figure 1.2. Biological effects of microparticles. APC: activated protein C. MMP: metalloproteinase. miRNA: micro ribonucleic acid. NO: nitiric oxide. PS: phosphatidylserine. RNA: ribonucleic acid. TXA2: thromboxane A2. TF: tissue factor. uPA: urokinase-like plasminogen activator. uPAR: urokinase-like plasminogen activator receptor. vWF: von-Willibrand factor.

1.3.2 Microparticle formation

Upon cell activation the endoplasmic reticulum and the plasmatic calcium channels are open, leading to calcium influx into the cytosol with activation of scramblase and inhibition of flippase enzymes. These enzymes act on the cell wall resulting in the loss of normal lipid symmetry and the exteriorization of Phosphatidylserine (PS). During the translocation of the membrane phospholipids, the bonds that stabilise the intracellular skeletal proteins are disturbed promoting plasma membrane budding.

This in turn triggers the activation of proteolytic calpains and lipid-binding gelsoins that result in cytoskeleton remodelling, cell surface vesiculation and membrane phospholipid hydrolysis [177] culminating in MP formation and shedding (Figure 1.3).

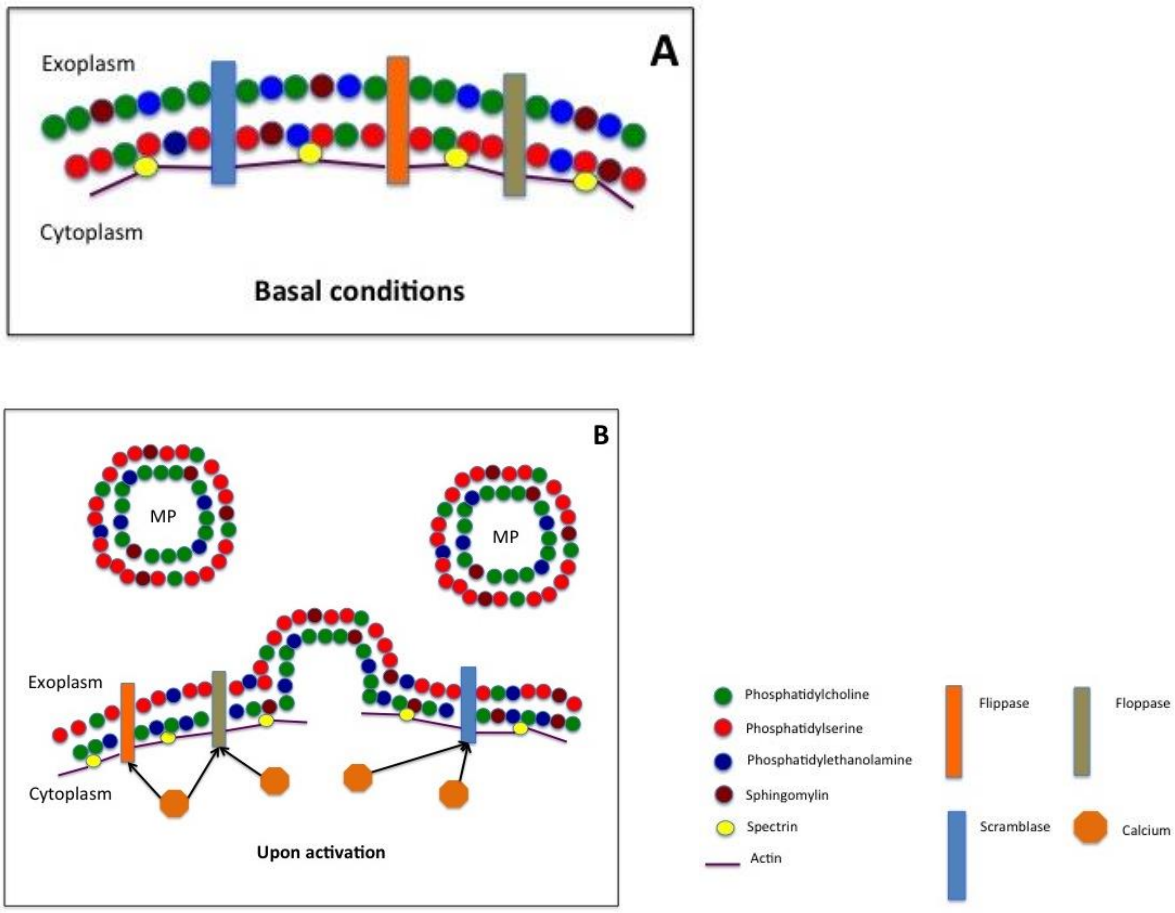


Figure 1.3. **Microparticle formation**. A: Basal conditions. B: Upon activation. MP: microparticles

1.3.2.1 Lipid membrane remodelling

Cell membrane is the first structure to be involved in MP formation. Under resting conditions phosphatidylserine and phosphatidylethanolamine are the main components of the outer layer while phosphatidylcholine and sphingomyelin compose the inner leaflet of the cell membrane bi-layer. This distribution is not constant, but it is an active process governed by ATP-dependent enzymes to reach

equilibrium [178]. Phosphatidylserine and phosphatidylethanolamine are kept on the inner layer of the membrane by the enzyme amino-phospholipid translocase (or flippase) while phosphatidylcholine and sphingomyelin are kept on the outer layer by the enzyme floppase[179]. Another enzyme (scramblase) acts to disturb this distribution. Upon activation, calcium membrane channels and cytoplasmic reticulum are open allowing calcium influx into the cytosol; resulting in the inhibition of flippase enzyme and activation of scramblase. This process results in disturbance of the "normal" distribution of lipids on the cell surface and the expression of phosphatidylserine on the outer layer of the membrane surface[180].

1.3.2.2 Cytoskeletal disruption

The cytoskeleton is formed of a protein network which maintains stability through protein-protein and protein-lipid interactions. Cell membrane lipids are connected to actin *via* the protein spectrin. When lipid translocation commences following calcium influx, the links anchoring membrane lipids to spectrin are disrupted resulting in membrane budding. This process results in actin regulatory proteins activation which further enhances calcium influx to perpetuate the process[181]. Calpins are another group of enzymes activated by cytosolic calcium influx. Calpins cleave cytoskeletal filaments (talin and α-actin) aiding membrane vesiculation leading to MP formation. When this process is inhibited by calpeptin, MP production is reduced[182]. Other mechanisms are involved in MP formation as calpins inhibition does not completely inhibit MP production. One of these mechanisms is dependent on p21-activated protein kinases (PAK) reorganisation of actin facilitating MP formation[183]. This

pathway is triggered by cell membrane lipid changes[184]. Another calpins independent pathway is mediated via mitogen-activated protein kinases (MAPK). The p38 MAPK is involved in inflammatory conditions such as sepsis and rheumatoid arthritis associated with increased MP production acting as downstream messenger for TNF-α. Inhibition of p38 MAPK results in reduction of MP production[185]. Another mechanism is triggered during apoptosis as the enzyme caspase-3 cleaving the Rho-kinase ROCK-1 resulting in phosphorylation of myosin light chain leading to cell membrane contraction and MP release[186]. In response to thrombin and in the absence of apoptosis, another Rho-kinase (ROCK-2) activated by caspase 2 induces cytoskeletal remodelling and MP shedding[187]. This pathway was inhibited by fluvastatin which was found to reduce MP production from endothelial cells[188].

The variety in the pathways leading to cytoskeletal changes leading to MP release probably is determined by the stimulus for their release and the parent cell[189].

1.3.2.3 Microparticles content

MP express a variety of bioactive substances, transmembrane receptors and adhesion molecules on their surface reflecting the parent cell membrane. During MP formation, the membrane engulfs a wide array of cytokines, chemokines, enzymes, growth factors and signalling proteins[190, 191] as well as functional genetic material such as microRNA[192].

Although MP transmembrane proteins and cargo resemble the parent cell, some components are selectively enriched depending on the parent cell and the stimulus

for MP release[191, 193, 194]. As an example MP produced by activated neutrophils express 10 times more integrin $\alpha_M\beta_2$ compared to MP derived from resting neutrophils[195]. Also endothelial cells when activated produced MP enriched with E-selectin and ICAM-1 while apoptotic cells produced MP enriched with CD105 and PECAM-1[196].

In chemotherapy resistant cancer, the malignant cells produce MP rich in the chemotherapeutic agent doxorubicin and these cells were found to have higher profile of genes related to MP production compared to chemotherapy sensitive cells[197]. A similar observation was found in ovarian cancer cells resistant to cisplatin[198]. These observation suggest a genetic mechanism behind the preferential loading of MP with chemotherapeutic agents in chemotherapy resistant cancer cells.

Confocal microscopy analysis of cells stimulated with complement C5b-9 demonstrated an orchestrated mechanism of MP formation and shedding that produced MP enriched in C5b-9[199] which suggests that MP content packaging is not a random process.

Lipid rafts are plasma membrane micro domains rich in certain proteins and lipids. Raft domains and lateral plasma membrane organisation segregate proteins and lipids resulting in inclusive or exclusive sorting[200]. Studies have shown that endothelial cells express lipid rafts rich in PECAM-1 and this response varies according to the stimulus. Also this raft expression correlated with subsequent MP

release rich in PECAM-1 surface expression[201]. Stimulated monocytes also developed lipid rafts rich in tissue factor and PSGL-1 which was subsequently found to be preferentially expressed on the produced MP[202]. This suggests a role for lipid rafts in MP enrichment.

These observations taken together suggest that MP content packing is probably an active process with multiple underlying mechanisms that depend on the cell of origin and its state as well as the stimulus for MP release.

1.3.3 Microparticle function

MP are important contributors to, but play a paradoxical role in coagulation. They are generally pro-coagulant due to the expression of PS on their surface, which triggers the intravascular coagulation cascade. This property is estimated to be 50 to 100 fold more powerful than the effect of activated platelets. Moreover, some MP express tissue factor (TF) on their surface rendering them even more pro-coagulant. MP originating from platelets can transfer membrane proteins such as integrins, P-selectin and von Willibrand factor as well as thromboxane A2 to distant cells triggering coagulation signals [177]. Conversely, a recent study has shown that Activated Protein C can induce the release of anticoagulant MP from the endothelium that express the protein C receptor[203]. Also recent research has shown that endothelial MPs can be pro-fibrinolytic as they provide a catalytic surface for the cleavage of plasminogen into plasmin by expressing urokinase-type plasminogen activator (uPA) and its receptor (uPAR). uPA and uPAR can also activate matrix

metaloprotinases (MMP) which in turn promote cell migration and angiogenesis. The activation of MMP by MP encourages extracellular matrix degradation and turn over in clinical situations such as tumour progression[177].

Several studies have shown that MP contain a variety of nucleic acids which could be transferred to other cell lines. Some MP contain up to 4730 human gene transcripts associated with cell-to-cell signalling, cellular growth, regulation of gene expression and lipid metabolism including CASP8AP2, EIF2C2(AGO2), CREG2, and VASP. One of the main nucleic acids present in MP is micro RNA (miRNA) that enables communication between cells and organs to modify gene expression in the target tissues[177, 204].

MP may also play a role in tissue repair. MP derived from mesenchymal stem cells (MSC) were as effective as MSC themselves in the stimulation of renal tubular epithelial cell proliferation and the inhibition of apoptosis in vitro. Moreover, they were similarly effective in accelerating the functional and morphological recovery from acute kidney injury (AKI) in immune-deficient mice. This effect was abolished when MP RNA was destroyed; suggesting that this regenerative effect is mediated via RNA transfer[205]. In another study by the same group, it was found that MP derived from MSC produce the same protective effect in immuno-competent rats, in which AKI was induced by ischaemia reperfusion injury[206]. MP derived from human liver stem cells enhance the proliferation, with concomitant inhibition of apoptosis of human and rat hepatocytes in vitro. In the same study it was found that these MP

when injected in vivo induced morphological and functional recovery of rats that had undergone 70% hepatectomy. These effects were again found to be related to RNA transfer and were abolished when MP RNA was destroyed[204]. These studies suggest that MP play a role in tissue repair and regeneration, probably through RNA transfer between the cells of origin and target cells. These findings indicate that MP may have therapeutic potential.

Altogether the studies discussed above, which are a small representative sample of the many investigations conducted on MP, are indicative of the considerable potential of MP both for therapy and as biomarkers in a variety of diseases from rheumatic to cardiovascular disease, from diabetes mellitus to renal disease, from malignancy to malaria and also in sepsis which is the focus of the investigations described herein.

1.3.4 Microparticles in disease

1.3.4.1 Microparticles in rheumatic diseases

Several studies in patients with rheumatic disease have found elevated circulating levels of MP and in particular platelet derived MP (PMP) and endothelial MP (EMP). MP levels are most raised in conditions with a strong vascular component such as rheumatoid arthritis (RA)[207].

In RA and other arthritides (psoriatic arthropathy, gout and juvenile idiopathic arthritis) high concentrations of MP are found in the synovial fluid (SF) of the affected

joints. PMP were the most abundant MP population in the SF (much higher than in plasma from the same patients) and MP from immune cells were also present, but at much lower concentrations. These MP were found to be pro-inflammatory and in mice induced cytokine production from synovial fibroblasts via IL-1. These investigators found that collagen receptor glycoprotein IV was a key trigger for PMP release in arthritis[208].

Along with their pro-inflammatory and pro-thrombotic roles, MP may contribute to the pathogenesis of rheumatic diseases through the formation of immune complexes. In one study MP bound to complement components (C1q, C3, C4) were abundant in the SF with lower levels of the same MP population in the same patients' plasma. Also the MP expressing complement activator molecules (IgG and IgM) were more abundant in the SF of the RA patients than in plasma and when compared to controls. These results suggest that MP contribute to the RA disease process by activating complement system in the synovial compartment[209]. A recent study has shown increased numbers of T lymphocyte derived MP in the RA patients' synovial fluid of RA patients when compared to those with osteoarthritis[210].

Studies also have shown that MP can have a paradoxical relationship with disease severity. In systemic sclerosis the total number of circulating MP was significantly higher than in healthy controls, whereas MP numbers were inversely correlated to skin thickness (a measure of disease severity) and patients with skin ulcers (severe

disease) had particularly low MP levels [211]. In SLE the total number of MP was found to be lower in patients in comparison to healthy volunteers [212].

These studies suggest that MP play a significant role in rheumatic diseases and that in the future MP have potential to act as biomarkers for these diseases and perhaps as therapeutic target.

1.3.4.2 Microparticles in cardiovascular diseases

Circulating microparticles are considered to be useful biomarkers for vascular injury and have been shown to correlate with disease severity in many cardiovascular diseases including acute myocardial infarction, atherosclerosis, pre-eclampsia, metabolic syndrome and hypertension. For example PMP, EMP and MP derived from erythrocytes (EryMP) were found to be raised in individuals with metabolic syndrome in comparison to healthy volunteers[213]. Other studies have suggested that EMP may have potential as predictive biomarkers in ischaemic stroke complicating diabetes[214].

MP can operate as effectors in many cardiovascular diseases. Circulating plasma MP isolated from patients with myocardial infarction, diabetes mellitus, pre-eclampsia, metabolic syndrome and obstructive sleep apnoea (OSA) have been shown to cause endothelial dysfunction via decreased NO signalling, enhanced protein nitration on endothelial cells and increased plasmatic oxidative stress[215]. In a rat model of pulmonary hypertension induced by hypoxia, MP decreased eNOS

activity and increased oxidative stress in pulmonary endothelial cells resulting in decreased NO bioavailability[216].

It has been suggested that MP isolated from the vitreous fluid of patients with diabetic retinopathy may impact on disease progression. These MP enhanced endothelial cell proliferation and new vessel formation when injected into mice. MP isolated from atherosclerotic plaques in humans may also contribute to the disease as they increase the initial endothelial dysfunction, promote intra-plaque new vessel formation and increase plaque thrombogenicty resulting in increased plaque progression[217].

Various medications have been investigated as means of reducing the deleterious effects of MP in cardiovascular diseases. A combination of pitavastatin and eicosapentaneoic acid (an omega-3 fatty acid from fish oil) significantly lowered the PMP numbers in the plasma in comparison to eicosapentaneoic acid alone in diabetic patients with hyperlipidaemia[218]. Atorvastatin was found to lower the expression of pro-coagulant proteins and adhesion molecules on PMP in patients with peripheral vascular disease[219]. Diabetic patients who are receiving pioglitazone were shown to have lower plasma levels of EMP [220].

Other interventions also could lower circulating MP in cardiovascular disease. Weight reduction by reducing food intake was shown to reverse PMP overproduction in obese patients[221]. Also continuous positive airway pressure treatment for patients

with OSA has improved endothelial dysfunction and lowered plasma levels of EMP, in particular those expressing E-selectin[222].

Therefore, it can be concluded that MP seem to play a key role in the pathogenesis, course and outcome of cardiovascular disease.

1.3.4.3 Microparticles in cancer

There is accumulating evidence that MP play a role in the survival of malignant cells, tumour invasiveness and metastatic disease. MP promote cancer cell survival through ridding the cells of stress chemicals such as caspase 3, which promote apoptosis[223]. MP also contribute to multi-drug resistance in cancer as these tumour cells secrete MP with high concentration of chemotherapeutics in comparison to cancer cells that are not multi-drug resistant[198]. Another mechanism is through MP suppression of immune cells as they induce T cell apoptosis and inhibit immune cell proliferation[224-226]. Tumour cells could also escape the immune system by receiving proteins from MP derived from cells other than their own and thereby imitating normal host cells (e.g. transfer of CD41 from platelet MP to cancer cells)[227]. It is also proposed that the pro-coagulant properties of some MP could help cancer cells evade the immune system by forming a thrombotic layer which coats and protects the tumour from detection[228, 229].

MP may enhance tumour invasiveness by participating in extracellular matrix degradation. MP carry proteases such as MMP-2, MMP-9 and uPA that may assist

in tumour cell invasion, metastatic disease and angiogenesis[229, 230]. In a study of ascites-derived MP in ovarian cancer, the activity of the enzymes MMP-2, MMP-9 and uPA was concentrated in the MP and the late stage MP showed higher activity of these enzymes than those from early stage tumours[231]. MP play a role in tumour metastasis, as the tumour MP transfer oncogenes or other genetic information from the primary tumour site to distant sites, causing malignant transformation of these cells[232].

In the future MP may have a role as biomarkers or as therapeutic targets in cancer. In one study, higher PMP levels were associated with shorter survival in patients with refractory prostate cancer[233]. Other studies investigating gastric, pancreatic and breast cancer found high PMP levels to be associated with more advanced disease and shorter survival times. On the therapeutic front, inhibition of MP release could be a potential treatment target in cancer[229].

1.3.5 Microparticles in sepsis

Attempts have been made to characterise microparticles in septic plasma but the results of these studies were varied and sometimes even contradictory. Others have attempted to investigate the pathophysiological effects of MP in relation to sepsis.

1.3.5.1 Characterisation of microparticles

Many studies have focused on platelet derived MP (PMP) as it is the most abundant MP population in plasma. These papers reported conflicting results when

the PMP count in septic patients was compared to healthy volunteers. Some reported that PMP increase in septic patients[234-236], whereas others reported that there were no differences[237, 238]. In one study it was reported that PMP counts might be reduced in sepsis[239]. Results were also inconsistent when MP populations derived from leucocytes, granulocytes, monocytes and endothelium were studied. Finally, some investigators were unable to detect these MP populations (Table 1.1).

Number of patients, origin of sepsis	Platelet MP	Endothelium MP	Leucocyte MP	Lymphocyte MP	Monocyte MP	Granulocyte MP	Erythrocyte MP	
n=7. Meningococcal sepsis	↑	=		=	=	↑	=	Nieuwland et al – 2000[234]
	AV/CD61	AV/CD62E		AV/CD4 AV/CD8 AV/CD20	AV/CD14	AV/CD66b	AV/CD235a	
n=9. Post operative, pancreatitis, E.Coli meningitis, Trauma	↓	↓				↑	=	Joop et al – 2001 [239]
	AV/CD61	AV/CD62E AV/CD144				AV/CD66b	AV/CD235a	
n=26. Trauma, Penumonia, Intra abdominal, Nec Fasc, Enterocoloitis	↑							Ogura et al – 2001[235]
	CD42a							
n=21. Necrotising fasciitis, Pneumonia, Peritonitis, Wound infection, Meningitis, Mediastinitis			=					Fujimi el al – 2002[240]
			CD11b					
n=16. Not clear.	=	ND		ND	ND	ND		Janiszewski et al – 2004[237]
	AV/CD61 AV/CD42b	AV/CD31		AV/CD3	AV/CD14	AV/CD15		

Number of patients, origin of sepsis	Platelet MP	Endothelium MP	Leucocyte MP	Lymphocyte MP	Monocyte MP	Granulocyte MP	Erythrocyte MP	
n=35. Pneumonia, Urosepsis, Soft tissue infection, Other	=	↑	**ND**					Soriano et al – 2005[238]
	CD31+/CD42+	CD31+/CD42+	CD45					
n=36. Pneumonia, Abdominal, Soft tissue, Urosepsis, Other.	↑	↑	↓		=	=	=	Mostefai et al – 2008[236]
	AV/41	AV/CD146	AV/CD45		AV/CD11b	AV/CD66b	AV/235a	
n=35. Gram-negative, Gram-positive, Fungal	↑							Woth et al – 2012[241]
	AV/CD41 AV/CD42a AV/CD61 AV/PAC1							
n=37. Gram-negative, Gram-positive, Fungal	↑							Tokes-Fuzesi et al – 2012[242]
	AV/CD41 AV/CD42a AV/PAC1							
n=30, Pneumonia, Intra-abdominal, Soft tissue, Urosepsis, Other.		=						Van Lerssel et al – 2013[243]
		CD31+/CD42b-						

Table 1.1. Studies describing microparticle population in sepsis. AV: Annexin V, MP: microparticles, ND: not detected, ↑: increased, ↓: decreased, =: no difference.

Many factors may be contributing to these conflicting findings, including the heterogeneity of the septic population and differences in the methods employed to separate microparticles from whole blood. Even though all these investigators used flow-cytometry to detect MP, they used flow-cytometers from different manufacturers, different size definitions and surface markers to identify MP populations (Table 1.1).

Renal impairment is one of the commonest complications of sepsis. An association was found between renal impairment and a high PMP count compared to septic patients without renal impairment[242]. Although, this observation was non-specific and could have been attributed to other factors, a conclusion reached by the authors themselves in a subsequent study[241]. In this latter publication an association was found between higher PMP counts and sepsis due to fungal infection.

DIC is another common and serious complication of sepsis. In one study, MP derived from endothelial cells and leucocytes were increased in patients with this complication and increases in EMP were strongly associated with early DIC[244].

MP were also examined in one study for a possible correlation with disease outcome but none could be demonstrated[238].

In all the above investigations circulating plasma MP were the focus of investigation, however MP can also be detected in fluid obtained from infected foci. Broncho-alveolar lavage (BAL) fluid and abdominal washings collected from patients with pneumonia and intra-abdominal infection respectively were found to be rich in neutrophil derived microparticles whilst other MP subtypes could not be detected[245].

1.3.5.2 Pathophysiological role of microparticles in sepsis

Effects on coagulation. MP may play a role in sepsis-induced coagulopathy. This phenomenon may be related in part to the expression of tissue factor (TF) on the surface of microparticles; an effect that has been demonstrated in a murine model

of endotoxaemia. The MP produced in response to intra-abdominal injection of lipopolysaccharide (LPS) had more pronounced pro-coagulant activity, which was abolished with TF antibody. In vivo, the activation of coagulation in endotoxaemic correlated with MP TF activity[246].

In a model of endotoxaemia in man, in which small doses LPS was administered to healthy volunteers, there was an 8-fold increase in MP dependent TF activity peaking at 3 hours post exposure to LPS, which returned to baseline at 8 hours; these changes were concomitant to a 25-fold increase in TF mRNA at 3 hours which returned to baseline at 24 hours[247]. The peak activity coincided with peak levels of the cytokines IL-6, IL-8 (pro-inflammatory) and IL-10 (anti-inflammatory). The peak also coincided with increases in TAT and prothrombin fragments indicating activation of the coagulation system[246]. Consistently in studies, a high inter-individual variability in MP-TF activity was noted which might be due to the “high” and “low” LPS responder phenomenon.

MP-TF activity is thought to be mainly attributed to monocyte derived MP. Monocytes stimulated with LPS produced MP that can shorten clot formation time. This effect can be abolished with the use of anti-TF antibodies suggesting that the shortened clotting time is due to TF expression on these MP and that monocyte derived microparticles have a role to play in sepsis induced coagulopathy[248].

In patients with septic shock secondary to meningococcal disease, the thrombin generation capacity of MP was greater than in patients with meningitis[249]. In these patients the plasma concentration of LPS, correlated positively with thrombin generation and the pro-coagulant activity of MP was significantly higher than in patients with meningitis only. In vitro, a shorter clot formation time was observed in the septic shock cohort. Both the thrombin generation capacity and the clot formation time were reduced when anti TF antibody was used, suggesting that these effects are probably due to TF expression on the MP surface[249]. This study also found large inter-individual variation in the pro-coagulant capacity of MP. This variability was also noted in an earlier study investigating the thrombin generation capacity of MP from patient with multi-organ failure[239].

Effects on inflammation. MP from septic rats (sMP) elicit a pro-inflammatory reaction when injected into healthy rats. The mean arterial pressure of the rats injected with sMP was significantly lower compared to controls and many of them died while all the controls survived. This could be explained by increased iNOS expression and the consequent vasodilation and hypotension[250].

In mice injected with human sMP there is differential expression of molecules associated with inflammation. Enhanced expression of eNOS, iNOS, COX-1, COX-2, NFκB and extra-cellular superoxide dismutase (EC-SOD) was observed in the hearts and lungs of these mice, while this effect was not detected in the livers and kidneys. In contrast to the previous study there was no increase in NO production in

response to sMP but there was increased O_2^-production preferentially in the heart and liver[251].

Human platelet MPs seem to express two subunits of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. This expression was two-fold higher in septic patients compared to healthy volunteers. Also MP from septic patients generated more superoxide when added to NADPH, which might contribute to MP induced vascular cell apoptosis[237].

Further exploration of the function of MP from septic patients function has revealed that when injected in mice they can potentiate the effect of vasoconstrictor agents such as serotonin while they did not alter the function of vasodilators such as acetylcholine. This increased responsiveness could be mediated through the vasoconstrictor metabolites of COX-1 such as thromboxane. Also sMP induce the production of thromboxane A2, which seemed to protect against vascular hypo-reactivity. These effects seemed to be evoked by MP of non-platelet origin[236]. In another study the same group found that sMP significantly increased IL-10 mRNA expression in tissue engineered vascular media (TEVM). When tested IL-10 significantly enhanced histamine induced contraction in LPS treated TEVM and improved the survival of mice injected with LPS by protecting them against septic shock and preventing LPS induced vascular hypo-reactivity[236].

Activated protein C (aPC) possesses cytoprotective functions in sepsis and other inflammatory conditions. In an interesting recent study, administration of aPC to septic rats generated a distinct MP profile[252]. These MP from septic rats given aPC when injected into healthy rats did not induce the hypotensive picture produced by MP from septic rats without aPC. This protective effect was found to be due the high thromboxane A2 content MP induced in response to aPC. Further investigation revealed that MP induced in reponse to aPC reduced the expression of the pro-inflammatory NFκB and iNOS. This suggests that aPC has modulates the MP production in septic rats and these MP might have a protective role in sepsis[252].

1.3.6 Alpha-2-macroglobulin positive microparticles

Investigations conducted by this research group, before the start of the project described in this report, have shown that neutrophils stimulated in suspension (fluid phase) or when adherent to an endothelial monolayer produced MP with different protein contents. About 30% of the protein content was uniquely expressed in each of the MP populations[253]. The adherent MP contained significant amounts of alpha-2-macroglobulin (A2M) and ceruloplasmin (CERU) while those in suspension contained more of the heat shock 70 kDa protein (Figure 1.4). Both subsets contained the same quantity of annexin A1, lactoferrin and β-actin. These proteins were also expressed on the surface of the MP as determined by flow-cytometry[253].

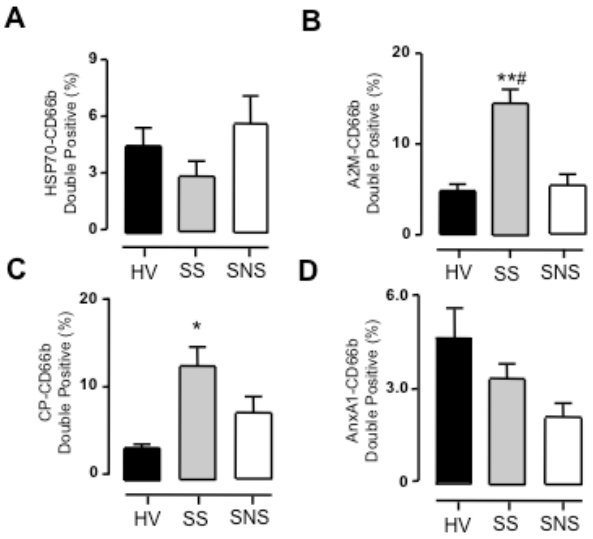


Figure 1.4. Neutrophil MP A2M levels within 24hr of admission to intensive care are elevated in patients that survive a septic event. Surface expression of HSP70, A2M, CP and AnxA1 was assessed on neutrophil (CD66b+) MP in plasma samples from healthy volunteer (HV), patients that survived the septic event (SS) and septic non-survivors (SNS). The percentage of double positive microparticles was assessed using flow cytometry. Results are mean ± SEM 10 individuals per group. (*P<0.05; **P<0.01 vs. HV plasma or SNS plasma, #P<0.05 vs. SNS as appropriate by one-way ANOVA).

To assess the translational value of these in vivo observations, the MP expressing the proteins mentioned above were quantified in the plasma of community acquired pneumonia (CAP) patients. In this cohort of patients, plasma levels of neutrophil derived MP expressing A2M and CERU were higher than in healthy controls. When incubated with HUVEC, these MP modified gene expression producing a longer lasting. The adherence of MP up-regulated the expression of pro-inflammatory genes such IL-1β and STAT3, while the fluid phase neutrophil MP down-regulated other pro-inflammatory genes such as STAT1 and CXCL6.

Taken together these results provide compelling evidence that MP derived from the same cell type under different conditions vary in their protein content, surface expression and effect on target cells. The A2M containing and expressing NMP appear to be endowed with protective actions in sepsis[253].

In a follow up study, A2M enriched MP (A2M-E) were generated as a form of nanomedicine and tested in a murine model of sepsis, in comparison to the free A2M protein and vehicle. The A2M-E MP were found to reduce bacterial load both locally and systemically, reduced neutrophil count locally, increased pro-resolving and reduced pro-inflammatory lipid mediators, reduced neutrophil infiltration of the lungs and reduced overall mortality. These actions were lost when A2M was blocked. The effect of A2M-E on human neutrophils was also tested. They increased the exposure and activation of CD11b on the surface of human neutrophils. Neutrophil recruitment and adhesion to HUVEC was also enhanced[254].

1.3.5 Other extra-cellular vesicles

Cells produce vesicles other than MP including exosome and apoptotic bodies. These vesicles play a similar role to MP in intercellular communication as well as waste management and coagulation[255].

1.3.5.1 Exosomes

Exosomes are smaller than MP with a reported diameter of 0.1 micron produced by most cells and could be detected in body fluids including blood, urine and cerebrospinal fluid. In contrast to MP they are produced via the inward budding of endosomal membranes resulting in the accumulation of vesicles within large multi-vesicular bodies (MVB) inside the cells. MVB are either degraded within the cell by fusing with lysosomes or they fuse with the cell membrane releasing the vesicles into the cellular microenvironment. Once released, the vesicles are termed

exosomes[256]. The exosomes express parent cell membrane components on their surface and include genetic material within, acting as inter-cellular messengers[256].

Exosomes are also surrounded by a phospholipid membrane that has a same orientation to the parent cells in comparison to MP which tend to have a phospholipid membrane with a reverse orientation of phospholipids. The exosomes membrane is also rich in cholesterol, sphingomyelin and ceramide[189]. Exsosomes contain characteristic set of proteins including proteins involved in membrane transport and fusion (e.g. GTPase, annexins and flotillin), components of the endoscomal sorting complex required for transport complex (e.g. Alix), tumour susceptibility gene 101, heat shock proteins, integrins and tetraspanins (e.g. CD63, CD81 and CD82)[257].

1.3.5.2. Apoptotic bodies

When cells undergo apoptosis they produce vesicles expressing phosphatidylserine on their surface with a diameter between 1 and 5 microns rendering them larger than MP and are called apoptotic bodies[189]. Differentiating between apoptotic bodies and platelets is rather difficult as they have a similar size ranges and both express phosphatidylserine[189].

Cells produce two distinct types of apoptotic bodies derived from either plasma membranes or from endoplasmic reticulum. The apoptotic bodies derived from plasma membrane contain DNA and histones, while those derived from endoplasmic reticulum do not possess DNA and histones but express immature glycoepitopes[258].

Apototic bodies are thought to play a role in the immune process where the defective clearance of these vesicles could lead to the development of auto-immune diseases such as systemic lupus erythematosus (SLE)[259].

Similar to MP, apoptotic bodies act as a multi-purpose carrier. Proteomic analysis of apoptotic bodies revealed that they share a significant number of proteins with MP[260]. Also, similar to MP they contain microRNA that have an effect on target cells[261].

1.4 Detection of microparticles

Many modalities can be employed to characterise MP and each of these detection tools have its strengths and weaknesses. This choice of a tool or a group of tools will depend on the aims of each study. Other factors have to be taken into consideration when choosing a tool such as availability, local expertise and cost. These tools are usually categorised into optical and non-optical detection methods.

1.4.1 Optical methods

Most modalities employ light based methods in order to detect MP including the tool most commonly used to detect these vesicles which is flow-cytometry.

Optical microscopy. The sample is illuminated by visible light in a bright-field microscope. The light scattered on MP is collected by a lens on a charge-coupled device camera. This method could detect MP down to 0.2 micron in diameter.

Measuring a concentration of MP in a specific volume is possible but would be very time consuming and would offer no information regarding proteins expression or biochemical composition of MP.

Some optical microscopes are able to detect fluorescence using a spectral filter prior to detection by charge-coupled device camera and thus allowing the detection of MP surface protein expression using a fluorescent probe[262]. This also could be used for calculating the concentration of a certain MP population that have been labelled with fluorescent probe in a sample. This method would not be suitable for high throughput studies as it is time consuming.

Dynamic light scattering. This method could potentially detect particles ranging in size between 1 nm and 6 microns[263]. Dynamic light scattering relies on Brownian motion of particles in a fluid and the smaller particles have higher Brownian motion. The Brownian motion will cause intensity fluctuation of the scattered light that could be detected. This modality performs well in detecting MP of the same size. When the sample contains MP with different sizes dynamic light scattering tool tends towards detection of larger particles. This tool risks missing the smaller size MP and it does not provide any information about cellular origin or composition[264].

Scattering and fluorescence flow-cytometry. This is the most commonly used modality for MP characterisation. It will be discussed in details later in this section.

Nanoparticle tracking analysis. Particles in a fluid are illuminated by laser beam and the scattered light is collected by an optical microscope. It can detect particles as small as 50 nm. The particles show as moving bright spots and this movement is detected over several minutes using special software[265]. This method allows accurate detection of size distribution of MP in a sample, but it is time consuming and does not offer any characterisation information.

Combining this method with fluorescence detection allows for further characterisation of MP.

Raman spectroscopy. The sample is illuminated with a laser light. The vibration of a molecule in the sample will result in a change in the wave length of the scattered light, which can be detected by spectrometers. The pattern of vibration is molecule specific. Also the signal strength correlates with the number of molecules. This method allows the detection of molecular composition of MP without labelling and it can estimate the size of MP. Raman spectroscopy can determine the concentration of MP in a known volume[174]. This modality requires several hours for each sample.

Stimulated emission depletion microscopy. It is a high resolution fluorescence microscopy with high resolution. This method can determine the size and fluorescence labels of MP but also potentially could offer information on the morphology and the distribution of labelled epitopes on MP surface. Currently this method has been employed to characterise organelles such the endoplasmic

reticulum inside a living cell[266]. This method is still under development and is estimated to require several hours for MP detection and characterisation.

Fluorescence correlation spectroscopy. This method was originally introduced to measure molecular diffusion parameters. It can determine the absolute size distribution and fluorescence signal of particles in a fluid through detecting the fluorescence intensity fluctuation caused by the Brownian motion of particles. It can detect vesicles smaller the 50 nm[174]. This modality could detect size distribution and concentration as well as phenotypes of MP.

1.4.2 Non-optical methods

Transmission electron microscopy. This tool uses electrons instead of photons. As the wavelength of electrons is much smaller than photons, the electron microscope has a much higher resolution rendering it capable of determining the size and morphology of microvesicles. This modality requires the sample to be fixed and dehydrated which will affect the size and morphology of the particles. Also it requires the concentration of particles, which will have an effect on the results making determining the concentration of particles in the original sample not possible. Immuno-gold labelling could afford some information regarding biochemical composition of particles[267].

Atomic force microscopy. This microscope scans a sample surface to create a three-dimensional image with a submicron resolution. It can be used to measure the size

of microvesicles in their physiological state. Antibodies could be used to characterise the vesicles phenotype. For the microscope to detect the vesicles they have to be firmly bound to a flat surface which could affect the morphology of the vesicles and as the efficiency of the binding process is not known, the concentration of the vesicles cannot be accurately measured[268].

1.4.3 Flow-cytometry

When a sample is injected into a flow-cytometer the particles are dispersed into a 3 dimensional space called the *central channel*, which is enclosed within an outer sheath that contains a faster moving fluid called the *sheath fluid*. The fast moving sheath fluid creates a massive drag on the narrower central channel which alters its fluid velocity, hereby creating a single file of particles; this process is called *hydrodynamic focusing*. Under optimal conditions (laminar flow) the two fluids will not mix (Figure 1.5)[269].

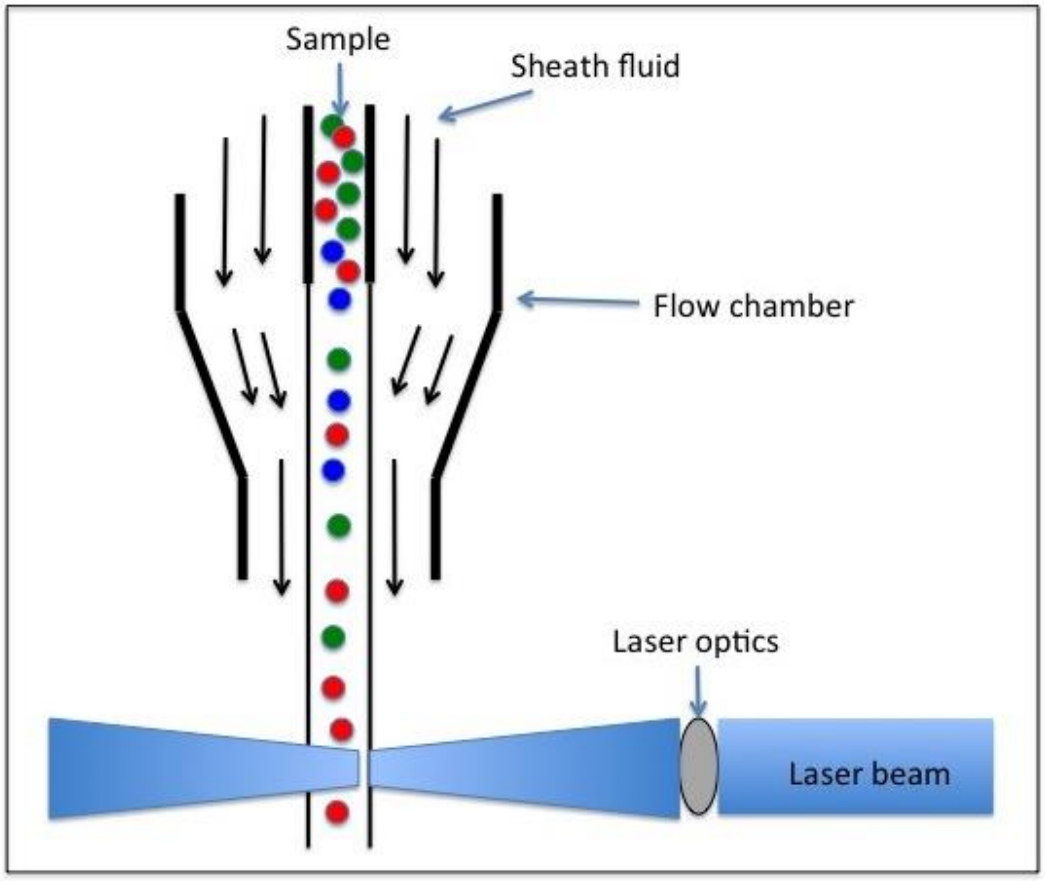


Figure 1.5. Schematic demonstrating the process of hydrodynamic focusing inside a flow-cytometer cell.

Following hydrodynamic focusing, each particle passes through one or more beams of light. The flow-cytometer then gathers information about each particle based on the scattering of light or emission of fluorescence (if the particles are fluorochrome labelled). The most commonly used sources of light are either a laser or an arc lamp. The focus here will be on laser as a light source, as this is used in the BD LSRFortessa. The light scattered in the forward direction is collected by a lens known as the *forward scatter channel* (FSC) while the light scattered at a 90 degree angle is collected through another lens called the *side scatter channel* (SSC). The FSC intensity equates to the particle size and the SSC one provides information about

particle granularity. Both FSC and SSC are unique to each particle and can be used to differentiate between various particles in a heterogeneous sample.

Fluorescence measurements taken at different wavelengths also provide data about surface receptors and protein expression of particles and cells as well. Flow-cytometry uses separate fluorescence channels to detect the light emitted using either silicon photodiodes or photomultiplier tubes (PMT). The specificity of detection is controlled through optical filters, which block certain wavelengths and transmit others (Figure 1.6)[269].

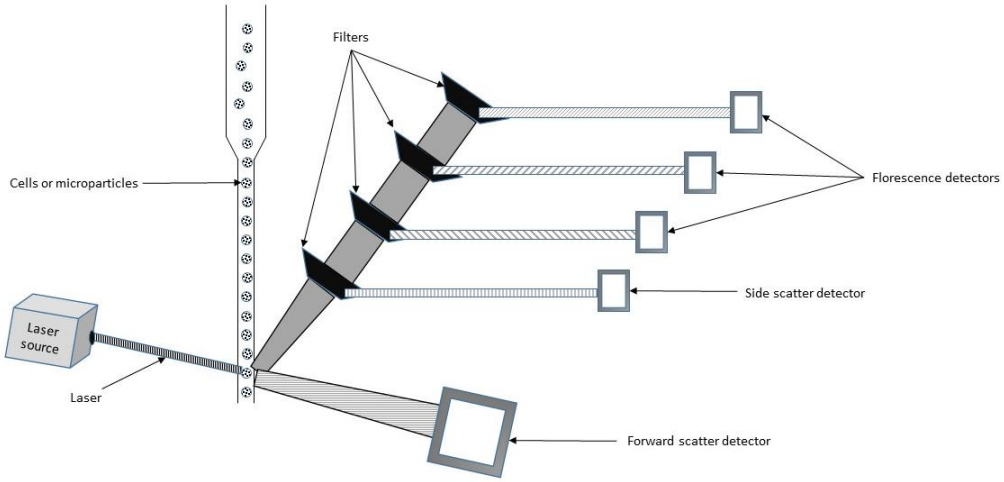


Figure 1.6. Schematic demonstrating the light system inside a flow cytometer

When a photo-detector receives light, a small current is generated with amplitude that is proportional to the amount of light detected. This signal is then amplified to allow graphical representation. Log amplification is usually used for fluorescence as it harmonises the signals, resulting in a distribution that can be represented as a histogram. The output from each detector is called the *parameter* (e.g. forward

scatter, side scatter or fluorescence) while the data acquired within each parameter is referred to as *events,* i.e. the number of particles or markers of interest.

Fluorochromes: are dyes that accept light (*excitation*) and re-emit the light at a higher wavelength (*emission*). Emission follows excitation in a matter of a few nanoseconds and this process is called *fluorescence*. The excitation wavelength is critical to the amount of light a fluorochrome will absorb. A fluorochrome will have a range of wavelengths at which it will absorb light and within this range there is a peak at which the maximum amount of light is absorbed. The excitation at this peak will produce more intense fluorescence emission. These optimal conditions are called *maximal absorption* and *maximal emission* wavelengths (Figure 1.7)[269].

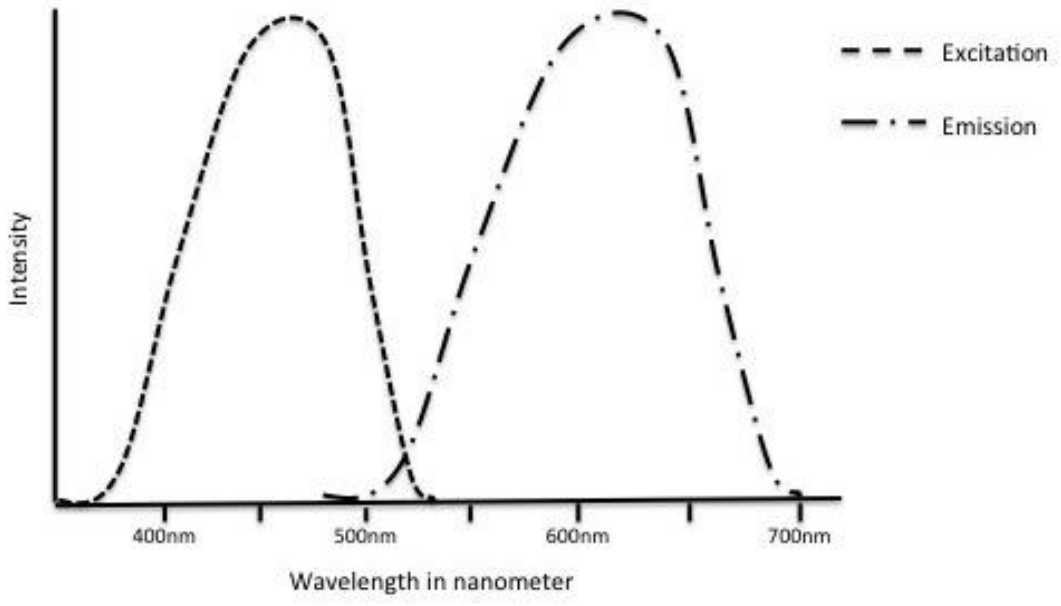


Figure 1.7.Schematic representing the excitation and emission curves of a fluorochrome.

Fluorescent probes such as fluorochrome-conjugated antibodies are useful to directly target a specific epitope to facilitate the measurement of its biochemical and biological properties using flow-cytometry. They are useful in many applications such as:

- identifying and quantifying distinct populations of cells or MP, surface receptors and proteins or intra-cellular organelles
- immuno-phenotyping
- measuring enzyme activity and apoptosis studies.

Changing the excitation light and the use of more than one fluorochrome facilitates the simultaneous analysis of several parameters in the same sample, which forms the basis for multi-colour fluorescence studies. There are a large and expanding number of fluorochromes that can be used in flow-cytometry[269].

The use of more than one fluorochrome in the same study carries the risk of emission profile overlap, rendering the measurement of each distinct fluorochrome emission more difficult. Using fluorochromes with very different emission spectra could rectify the problem, but this approach is not always practical. Another solution is to apply fluorescence *compensation*. Compensation calculation is based on measuring how much of each fluorochrome "spills" into the other fluorochrome channels through control measurements using single fluorochrome experiments. This mathematical compensation is then applied to the multi-colour studies[269].

Gating is an important flow-cytometry technique in which the groups of cells or MP of interest are selectively visualised while eliminating results from unwanted particles or noise. Gating was traditionally achieved according to physical characteristics on a typical graph of SSC versus FSC. Newer gating strategies use florescence combined with scatter parameters[269].

One of the most commonly used ways to present flow-cytometry data is as a *histogram*. These histograms display a single measurement parameter (fluorescence or light scatter intensity) on the x-axis and the number of events on the y-axis. Histograms are useful in enumerating the number of cells or particles that possess a physical property or which express the marker of interest. The cells or particles expressing this physical property are called the *positive dataset*. Under optimal conditions a flow-cytometer would produce a histogram with a distinct peak that would represent the positive dataset, but frequently a multi-peak histogram is produced making identifying the desired positive data set difficult. This problem can be overcome through the use of negative isotype controls [269] which are subtracted from the positive dataset to reveal the “true positive” events.

1.4 The project

1.4.1 Proteins

Microparticles express proteins on their surface that reflect their parent cell and other proteins that may have a role in their function. Detection of these proteins will facilitate microparticles characterisation. The proteins used in this project are:

A2M: is a member of the alpha-macroglobulin superfamily and is present in the body fluids of humans and other species. A2M is a high molecular weight (720 kDa) homotetrameric glycoprotein formed of 4 identical subunits (180 kDa each). This protein has a variety of complex functions, but most important is the inhibition of a wide range of proteases, which does not involve blocking the active site of these enzymes. A2M is also involved in the transport and regulation of many proteins such as transferrin, defensins and myelin. In addition A2M binds and modifies the activity of many important cytokines such as platelet-derived growth factor, nerve growth factor, IL-1 and IL-6. A2M also binds and regulates the activity of hormones. Moreover, this glycoprotein said to be protective against various infections. Binding of A2M to these mediators (e.g. IL-1 and IL-6) may alter its conformation to enable binding to a specific receptor, termed LRP1, a member of the family of low-density lipoprotein receptors. A2M may also have diagnostic and prognostic utility in many conditions including cardiac diseases, pancreatitis and gastric cancer. Finally, A2M may be used as a vehicle for vaccine and medications. This protein could be damaged by reactive species produced by the host and/or the invading organism resulting in pathological effects [270].

CD66b: is a trans-membrane protein with a cytoplasmic tail and is considered to be part of the immunoglobulin superfamily and the carcinoembryonic antigen like subfamily. The molecular weight pf CD66 is ~95-100 kDa. It is expressed on the surface of neutrophils and other granulocytes. CD66b is known to be involved in neutrophil adhesion, aggregation and activation[271].

CD14: is a 55kDa glycoprotein expressed on the surface of macrophages and monocytes. It acts as an receptor for LPS, facilitating its interaction with TLR, thus resulting in macrophage activation [272].

CD3: consists of 4 distinct chains (CD3γ, CD3δ and two CD3ε) and is associated with T-cell receptors (TCR). CD3 is found on all mature T cells, natural killer T cells and some thymocytes. CD3 is also known as T3 and is a member of the immunoglobulin super family that plays a role in antigen recognition, signal transduction and T cell activation.

CD235a: is also known as glycophorin A and is a member of the glycophorin family. It is a type I sialoglycoprotein present in the cell membrane as a homodimer. CD235a is expressed by erythroid precursors and erythrocytes. It bears the antigenic determinants of the MNS blood groups and is thought to be an inhibitor of haemagglutination and haemolysis. This protein is a binding site for *plasmodium falciprum*, influenza virus and hepatitis A virus.

CD41: is 125/25 kDa alpha subunit of the gpII/IIIa (CD41/61) complex. CD41 is a member of the integrin family and is a heterodimer of a heavy chain and a light chain linked by a disulphide bond. It is primarily expressed on platelets and megakaryocytes and it is involved in platelet aggregation and attachment. CD41/61 is a receptor for fibrinogen, fibronectin, von Willebrand factor and thrombin.

CD31: is also known as Platelet Endothelial Cell Adhesion Molecule 1 (PECAM-1). PECAM-1 is a trans-membrane glycoprotein with a molecular weight of 130-140 kDa and is a member of the immunoglobulin superfamily of adhesion molecules. It is highly expressed on the surface of endothelial and haematopoietic cells. CD31 plays an important role in the regulation of the immune response as it has both pro- and anti-inflammatory functions. Its pro-inflammatory function is mainly to facilitate the trans-endothelial migration of leucocytes while its anti-inflammatory properties include dampening of leucocyte activation, suppression of pro-inflammatory mediators production and maintaining the integrity of the endothelial barrier [273].

CD51: is also known as integrin alpha v (ITGAV) and vitronectin receptor alpha chain. ITGAV is a type I integral membrane glycoprotein which forms a heterodimer with integrin β1, β3, β5, β6 and β8. Thus, CD51 contains two disulphide-linked sub units of 125 kDa and 24 kDa and is expressed on endothelial cells, fibroblasts, macrophages, platelets and osteoclasts among others. CD51 binds vitronectin, von Willebrand factor, fibronectin, thrombospondin, osteopontin, fibrinogen and laminin. As an adhesion molecule it plays important roles in leukocytes homing and rolling. CD51 also mediates bone absorption and angiogenesis.

CD146: is also known as melanoma cell adhesion molecule (Mel-CAM), MUC18, S-Endo-1, MCAM. Mel-CAM is a membrane glycoprotein and is considered to be part of the immunoglobulin superfamily. CD146 is expressed on endothelial cells, smooth muscle cells and myofibroblasts among others. It is also expressed on many

malignant cells including melanoma cells. Mel-CAM mediates heterophilic cell-cell adhesion and participates in signal transduction. It is involved in outside-in signalling and may contribute to local adhesion, re-organisation of cytoskeletal structures, maintaining cell form as well as control of cell migration and proliferation[274].

1.4.2 Overview

The objective of the work described in this thesis was to better understand the role of MP in the pathogenesis, evolution and outcome of sepsis. I planned to quantify and characterise circulating MP in the plasma of septic patients, with particular attention to neutrophil-derived MP and their surface protein expression. I then investigated putative associations between the number and characteristics of the MP and clinical outcome. The investigations build on the findings of recent studies performed by our group in which my supervisors have shown that the protein content and surface expression of neutrophil-derived MP vary according to the environment and the nature of the stimulus. Moreover, in a small group of patients with septic shock, the number of neutrophil derived MP expressing alpha-2-macroglobulin (A2M) was significantly higher in survivors than in non-survivors. Members of our group have also demonstrated that A2M/CD66b positive MP can activate endothelial cells and promote the adhesion of freshly prepared leucocytes. These findings, coupled with the observation that administration of MP enriched with A2M, packaged as a nanomedicine, improved outcome and enhanced bacterial clearance in a murine model of sepsis, indicate the potential of certain subsets of MP to ameliorate the immune paresis characteristic of sepsis, and perhaps thereby improve outcome.

Intriguingly, MP prepared from the plasma of septic shock survivors (which expressed higher levels of A2M on the CD66b positive MP subset), but not those from non-survivors, could activate endothelial monolayers and increase leucocyte adhesion.

My goal, was to extend these observations and further explore the potential of MP to impact on the clinical management of patients with sepsis. This thesis will focus on two well-defined cohorts of septic patients with sepsis due to CAP and faecal peritonitis.

Besides neutrophil derived MP I characterised and quantified MP derived from other cells that have a role in sepsis. Specifically, I examined MP derived from monocytes, lymphocytes, platelets, erythrocytes and endothelium. I also investigated the changes in these MP over time as well the relation between the levels of these MP and the severity of sepsis and outcome.

The main population of MP I have studied were neutrophil MP ($CD66b^+$) and their surface expression of A2M. I also investigated monocyte ($CD14^+$), lymphocyte ($CD3^+$), erythrocyte ($CD235^+$) and platelet ($CD41^+$) derived MP and their A2M surface expression. I also quantified endothelium derived MP utilising more than one marker (CD146, CD31 and CD51) as these markers have been shown to correlate to acute and chronic conditions, respectively[275]. Also CD31 positive MP have been

shown to correlate with illness severity and outcome in many diseases, particularly conditions affecting the cardiovascular system[276].

1.4.3 Hypotheses:

1. Circulating cell derived microparticle concentrations are increased in severe sepsis.
2. The alpha-2-macroglobulin positive neutrophil microparticles are related to disease severity and outcome in severe sepsis.
3. Microparticles expressing alpha-2-macrogloulin derived from other cells are also related to disease severity and outcome.
4. Microparticle plasma concentration and alpha-2-macroglobulin expression change as sepsis evolves.
5. Microparticle profiles in severe sepsis will differ according to the source of infection.
6. Microparticles derived from stimulated whole blood possess immune-modulatory effects.

1.4.4 Objectives:

1. To quantify and characterise circulating cell derived microparticles in the plasma of two well defined cohorts of severe sepsis patients (community acquired pneumonia and faecal peritonitis).

2. To quantify neutrophil derived microparticles positive for alpha-2-macroglobulin in the same cohorts and confirm or refute their relationship to outcome.
3. To quantify and characterise (alpha-2-macroglobulin surface expression) microparticles derived from cells other than neutrophils (monocytes, lymphocytes, erythrocytes, and platelets) in these patients.
4. To relate the number and protein expression of microparticles derived from cells other than neutrophils (monocytes, lymphocytes, erythrocytes, platelets and endothelial cells) to outcome in the same groups of patients.
5. To document changes over time in microparticle numbers and alpha-2-macroglobulin expression in these patients.
6. To explore the effect of novel therapeutic adjuvant agents on whole blood production of alpha-2-macroglobulin expressing microparticles and their cells of origin.
7. To examine the effects of microparticles obtained from stimulated whole blood on aspects of neutrophil and endothelial function.

Chapter 2: Materials and methods

2.1 Materials

2.1.1 Cell Culture

Dulbecco's phosphate buffered saline (DPBS) with calcium and magnesium, foetal bovine serum (FBS), fungizone, human Serum, medium 199 with Earle's salts with L-glutamine (M199), Penicillin/Streptomycin and Roswell park memorial institute medium 1640 (RPMI) with L-glutamine were purchased from GE healthcare, Buckinghamshire, UK. Trypsin/EDTA (0.025%/0.01%) was purchased from Invitrogen, Paisley, UK. Dextran (molecular weight 450,000-650,000), gelatine Type B from bovine Skin, Hanks balanced salt Solution 10X, Histopaque 1077, phosphate buffered saline (PBS) and sodium citrate were purchased from Sigma-Aldrich, Poole, UK. Lipopolysaccharide from Escherichia Coli strain 0111:B4 purchased from Sigma-Aldrich, Poole, UK. Granulocyte macrophage colony stimulating factor and interferon gamma were purchased for Peprotech, London, UK.

2.1.2 Flow-cytometry antibodies for microparticles characterisation

Antigen	Isotype	Fluorochrome	Working Concentration	Clone	Source
CD66b	Mouse IgM, κ	PE	0.125 µg/ml	G10F5	Biolegend
CD146	Mouse IgG2a, κ	APC	0.50 µg/ml	SHM-57	Biolegend
CD14	Mouse IgG1, κ	APC	0.50 µg/ml	61D3	eBioscience
CD31	Mouse IgG1, κ	PE	0.25 µg/ml	WM59	Biolegend
CD51	Mouse IgG2, κ	FITC	0.1 µg/ml	NKI-M9	Biolegend
CD3	Mouse IgG2a, κ	PE	0.375 µg/ml	HIT3a	eBioscience
CD235	Mouse IgG2b, κ	PE	0.75 µg/ml	HIR2	eBioscience
LAIR1	Mouse IgG1	PE	0.12 µg/ml	NKTA255	eBioscience
Annexin A7	Rabbit IgG	PE-CY5	1 µg/ml	Polyclonal	Bioss
CD41	Mouse IgG1, κ	FITC or PE	0.25 µg/ml	HIP8	Biolegend
A2M	Mouse IgG1	FITC	1 µg/ml	IA5	BioMac

Table 2.1. Monoclonal antibodies used for microparticle detection. APC: allophycocyanin, A2M: alpha-2-macroglobulin, FITC: fluoroesceinisothiocyanate, LAIR1: leucocyte associated immunoglobulin like receptor 1, PE: phycoerythrin, PE-CY5: phycoerythrin-cascade yellow 5.

2.1.3 Flow-cytometry antibodies for cell characterisation

Antigen	Isotype	Fluorochrome	Working Concentration	Clone	Source
CD11b	Mouse IgG1, κ	APC	0.1 µg/ml	ICRF44	eBioscience
CD62L	Mouse IgG1, κ	PE-Cy5	0.1 µg/ml	DREG-56	eBioscience
ICAM-1	Mouse IgG1, κ	PE	0.1 µg/ml	HA58	eBioscience
VCAM-1	Mouse IgG1	FITC	0.1 µg/ml	1.G11B1	AbdSerotec
E-Selectin	Mouse IgG1	FITC	0.1 µg/ml	1.2B6	AbdSerotec
Tissue factor	Mouse IgG1, κ	APC	0.1 µg/ml	HTF-1	eBioscience

Table 2.2. Monoclonal antibodies used for cell antigen detection. APC: allophycocyanin, FITC: fluoroesceinisothiocyanate, ICAM-1: intra cellular adhesion molecule-1, PE: phycoerythrin, PE-CY5: phycoerythrin-cascade yellow 5, VACM-1: vascular cell adhesion molecule-1.

2.1.4 Flow-cytometry proteins for microparticles characterisation

Protein	Fluorochrome	Working concentration	Source
Annexin V	Pacific Blue	0.2 µg/ml	Biolegend

Table 2.3. Annexin V used for microparticle detection.

2.1.5 Other media

Annexin V binding buffer 10X was purchased from BD Pharmingen, San Diego, USA. Fluorescein isothiocyanate (FITC)-Dextran MW 40,000 was purchased from Sigma-Aldrich, Poole, UK.

2.1.6 Flow-cytometer

The flow-cytometer used in this project is BD LSRFortessa manufactured by BD Bioscience, San Diego, Ca, USA. This device contains 4 lasers with wavelengths 355nm, 405nm, 488nm, 640nm. The flow rates for this flow-cytometer are low: 12 µl/min, medium: 35 µl/min and hi: 60 µl/min. The flow-cytometer is calibrated daily by the specialist flow-cytometry laboratory staff.

2.2 Patients and samples

Patients admitted to intensive care units with severe sepsis or septic shock secondary to community acquired pneumonia or faecal peritonitis were recruited into the Genomic Advances in Sepsis (GAinS) study. Plasma obtained from the recruited patients was stored in a bio-bank at the Wellcome Trust Centre for Human Genetics. Colleagues from the Wellcome Trust Centre, with whom I am collaborating, provided the plasma at the time points required for each patient. They provided clinical data recorded using a comprehensive electronic web-based Case Report Form (eCRF), including demographics, comorbidities, differential white cell count, platelet count and illness severity scores.

2.2.1 GAinS study inclusion criteria

Patients recruited to the study were more than 18 years of age admitted to the high dependency units (HDU) or intensive care units (ICU) in the participating centres, with community acquired pneumonia (CAP) [defined as in reference [277] – febrile illness associated with cough, sputum production, breathlessness, leucocytosis and radiological features of pneumonia acquired in the community or within less than 2 days of hospital admission] or faecal peritonitis (FP) defined as inflammation of the serosal membrane lining the abdominal cavity secondary to contamination by faeces as diagnosed at laparotomy.

The use of standard diagnostic criteria for CAP and an unambiguous definition of FP, together with adjudication by two experienced clinicians ensured accurate

diagnosis. The causative organism of the CAP (where known) was documented. These patients may be admitted with, or may later develop, and progress through the spectrum of responses to infection, sepsis/severe sepsis/septic shock defined according to the extensively validated and widely accepted “Society of Critical Care Medicine/American College of Chest Physicians” consensus criteria definitions [278] and may be admitted with, or later develop specific organ failures (e.g. Acute lung injury/Acute respiratory distress syndrome as defined in reference [279]) which will be scored using the extensively validated and widely accepted “Sepsis-related Organ Failure Assessment” (SOFA) methodology [280].

2.2.2 GAinS study exclusion criteria

Patients were excluded when: patient, next-of-kin or legal representative unwilling or unable to give informed consent, the patient was less than 18 years of age, the patient was already enrolled in an interventional research study of a novel / unlicensed drug / therapy (patients enrolled in interventional studies examining the clinical application or therapeutic effects of widely accepted, “standard” treatments” are not excluded), the patient was pregnant, had an advanced directive to withhold or withdraw life sustaining treatment or was admitted for palliative care only, the patient was immune-compromised (known regular systemic corticosteroid therapy, exceeding 7mg/kg/day of hydrocortisone or equivalent, within three months of admission and prior to acute episode, known regular therapy with other immunosuppressive agents, e.g. azathioprine, known to be HIV positive or have acquired immunodeficiency syndrome as defined by the Centre for Disease Control,

patient had a neutrophil count less than 1 x 10^9/L due to any cause including metastatic disease and haematological malignancies or chemotherapy, but excluding severe sepsis and organ or bone marrow transplant receiving immuno-suppressive therapy).

2.2.3 Sample handling and storage

Following consent or assent, 10 ml whole blood was collected into ethylenediaminetetraacetic acid (EDTA) tubes on days 1, 3, and 5 of ICU admission. Samples were cold-centrifuged at 2,500 g for ten minutes. The resulting platelet poor plasma (PPP) was immediately (within 15 minutes) frozen and stored at -80 C as eight (500μL each) aliquots of which 100μL was sent to me for MP analysis. I then re-centrifuged the PPP at 13000 g for two minutes to generate platelet free plasma (PFP) suitable for microparticle detection by flow-cytometry.

2.3 Flow-cytometry

Flow-cytometry was the method used to identify and characterise microparticles (MP) in plasma.

2.3.1 Sample staining for flow-cytometry

Fluorescent monoclonal antibodies were incubated with 5 μL of platelet free plasma (PFP) in the dark for thirty minutes at room temperature. The antibodies and the PFP were diluted in medium up to a volume of 10 μL for accuracy of pipetting. Annexin V was added to the reaction as a general marker for MP. The staining combinations

are detailed in Table 2.4. The antibody combinations are formulated so as to minimise interference between various fluorochromes on flow-cytometry and to reduce the need for compensation.

Staining combinations	Cell of origin
Annexin V (Pacific Blue) + anti CD66b (PE) + anti A2M (FITC)	Neutrophils
Annexin V + anti CD14 (APC) + Anti A2M (FITC)	Monocytes
Annexin V + anti CD41 (PE) + Anti A2M (FITC)	Platelets
Annexin V + Anti CD3 (PE) + Anti A2M (FITC)	Lymphocytes
Annexin V+ Anti CD235 (PE) + Anti A2M (FITC)	Erythrocytes
Annexin V + Anti CD31 (PE)	Endothelial
Annexin V + Anti CD146 (APC)	Endothelial
Annexin V+ Anti CD51 (FITC)	Endothelial

Table 2.4. Staining combination for microparticles detection by flow-cytometry and conjugated fluorochromes. A2M: alpha-2-macroglobulin, APC: Allophycocyanin, FITC: FluoroesceinIsothocyanate, PE: Phycoerythrin

2.3.2 Reducing noise

As explained in the introduction, the sheath fluid plays an important role in the process of hydrodynamic focusing and also carries the cells or particles through the light source; contaminants in sheath fluid could, therefore, be counted as positive events. The standard sheath fluid that was used in our flow-cytometry laboratory was distilled water or phosphate buffered solution (PBS). By literature review it was noted that some researchers have used filtered sheath fluid and others have used degassed double filtered sheath fluid in order to minimise any possible false positive results due to contaminants in the sheath fluid. In preliminary experiments standard distilled water sheath fluid and double filtered degassed sheath fluid were compared.

Distilled water was passed through a 0.4 µm filter into a glass container and then applied negative pressure to the container in order to extract as many of air bubbles as possible. The filtered, degassed sheath fluid was poured into the flow-cytometer sheath fluid container and another filter of 0.2 µm pore size is installed in the tubing between the sheath fluid container and the machine. Then 10,000 events were acquired and the time taken to acquire this number of events between the standard sheath fluid and the double filtered degassed fluid was compared. Using the filtered sheath fluid 185 seconds were required to acquire 10000 events while with the standard sheath fluid the same number of events were acquired over a 15 seconds period. It was concluded that more accurate results would be obtained by using the double filtered degassed sheath fluid (Figure 2.1).

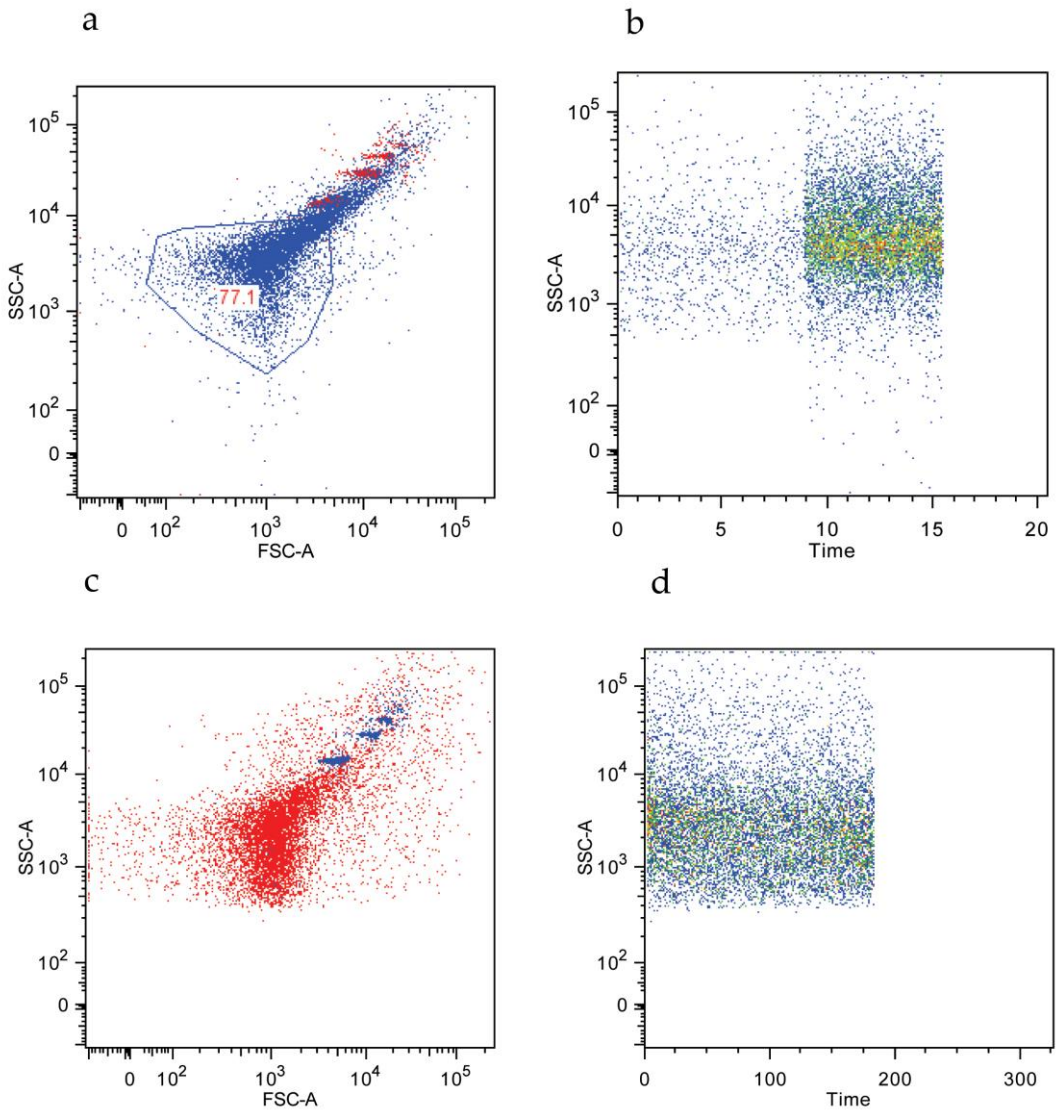


Figure 2.1.Flow-cytometry SSC/FSC plots comparing acquisition of 10,000 with standard and double filtered degassed sheath fluids. Sterile double filtered phosphate buffered saline (PBS) was subjected to flow-cytometry using either standard sheath fluid or double filtered, degassed fluid. a. The SSC/FSC plot for the 10,000 events acquired with the standard sheath fluid. b. The SSC/Time plot showing the time taken to acquire 10,000 events (15 seconds). c. The SSC/FSC plot for 10,000 events from the same sample acquired with the double filtered degassed sheath fluid. d. The SSC/Time plot showing the time taken to acquire 10,000 events (185 seconds). FSC: forward scatter, SSC: side scatter

2.3.3 Further noise reduction

To further reduce the noise that could interfere with microparticle detection, various cleaning methods of the flow-cytometer were employed.

Cleaning the flow-cytometer to achieve a low noise level in preliminary experiments was proving rather difficult even with running flow-cytometer rinse for up to an hour before every analysis. This method produced variable degrees of cleanliness. Then a deep cleaning method was employed where the sheath fluid was replaced with rinse as well as using it in through the sample probe and again that produced variable results in terms of noise reduction.

Then I tried double filtering the rinse fluid through 0.22 µm filters and that seemed to consistently reduce the noise to an acceptable level (Figure 2.2).

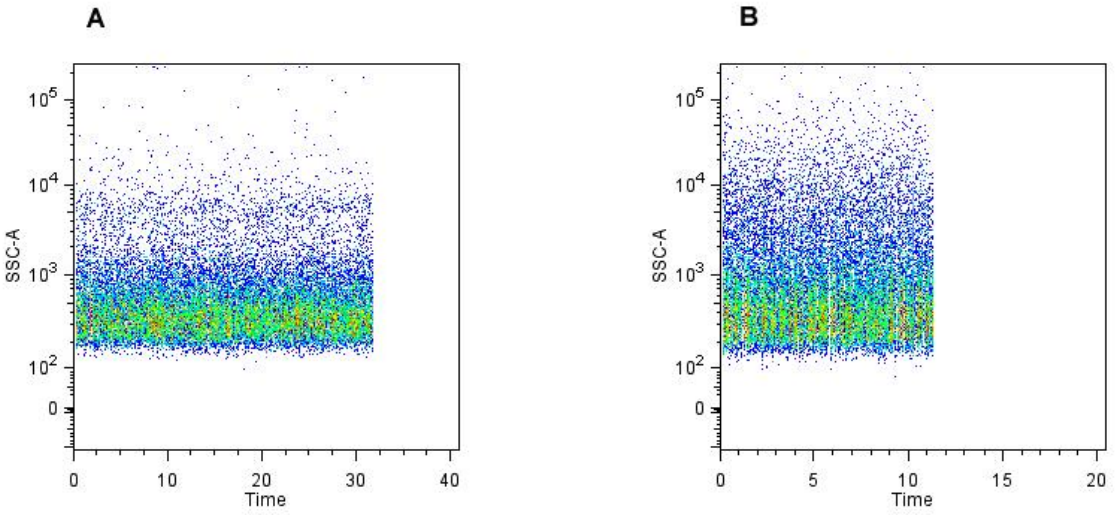


Figure 2.2. Comparison of double filtered flow-cytometer rinse fluid to standard rinse fluid. Both kinds of rinse fluid were processed through the flow-cytometer to acquire 10000 events. Double filtered rinse fluid took 3 times longer to acquire 10,000 events in comparison to standard rinse fluid. A: SSC/Time plot of the double filtered rinse fluid demonstrating that 10,000 events were acquired in over 30 seconds. B: SSC/Time plot of the standard rinse fluid demonstrating that 10,000 events were acquired in just over 10 seconds. SSC: side scatter.

2.3.4 Flow-cytometer threshold

An electronic *threshold* in the flow-cytometer can be set to limit the number of events acquired; this threshold is usually set to one parameter (e.g. FSC, SSC). The function of the electronic threshold is to eliminate events that are thought of as noise such as cell debris and electronic noise. For the acquisition of cells on the flow-cytometer I used a threshold of 5,000 set to both FSC and SSC, but for microparticles (MP) the standard threshold was 500 for both parameters. Therefore running the experiments on a threshold of 500 risked missing the smaller MP. In preliminary experiments the same sample was acquired using the standard threshold and the lowest possible electronic threshold (200) to compare the differences in MP count. The sample was double stained with annexin V (AV) and anti-CD66b PE antibody. Lowering the threshold to 200 significantly increased the events detected (Table 2.5).

	Threshold 500	Threshold 200	Fold increase
Annexin V positive	177	1875	11
Anti-CD66b positive	251	1543	6
Annexin V/Anti-CD66b positive	5	74	15

Table 2.5. The effects of reducing the flow-cytometer threshold on the number of microparticles detected. Healthy volunteers' plasma was stained using Annexin V – Pacific blue and anti-CD66b - PE fluorescent monoclonal antibodies and processed using flow-cytometry. The table shows events acquired at flow-cytometer thresholds of 500 and 200 and the increase expressed as folds. The data here is expressed as microparticles/μL (n=1). PE: phycoerythrin.

2.3.5 Size calibration in flow-cytometry

When a sample is acquired through a flow-cytometer, an SSC/FSC dot plot is produced representing all the acquired events. This dot plot does not directly indicate the size of the events acquired. Fluorescent polystyrene beads of a known size are usually used as a comparator to map the sizes on the FSC plot so that a gate can

be constructed around these beads that will include all particles of the desired size. This strategy is used to minimise noise and to exclude small size platelets and even smaller particles such as exosomes.

MP range in size between 0.1 to 1 μm. In order to formulate a population gate that would include these MP, I used 0.1 and 1 μm FITC fluorescent polystyrene beads. Also a batch of 0.5 μm was used to demonstrate that the flow-cytometer could differentiate between all three sizes of beads, so making it likely that it would be able to differentiate between MP of various sizes.

The beads come in highly concentrated solutions requiring dilution. In preliminary experiments I titrated the three beads sizes that I would use later for size calibration. The concentrations used and the optimum concentrations (Table 2.6) were selected based on as tight a population of beads as possible either on SSC/FSC or SSC/FITC plots (Figures 2.3, 2.4, 2.5).

Bead concentration	Bead size
1 x 10^4 / ml	
5 x 10^4 / ml	
1 x 10^5 / ml	
5 x 10^5 / ml	**1 μm**
1 x 10^6/ ml	**0.5 μm, 0.1 μm**
5 x 10^6/ ml	
10 x 10^6 / ml	

Table 2.6. The concentrations used for polystyrene bead titration and the optimum concentration for each bead size. Bead size is denoted next to the optimum concentration for its use.

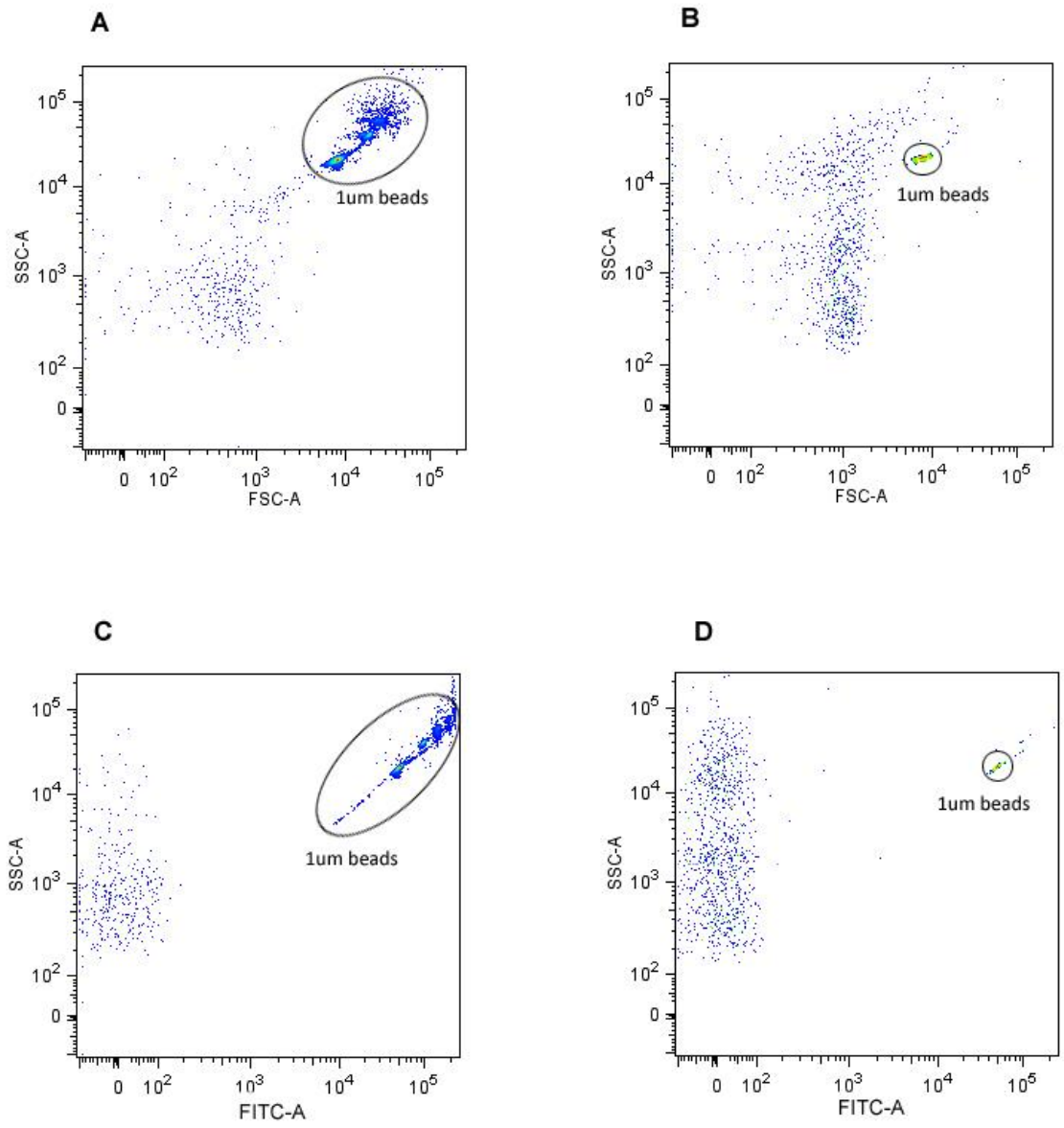


Figure 2.3. Titration of 1 µm polystyrene FITC fluorescent beads. Various concentrations of the 1µm beads acquired through flow-cytometry to find the tightest population of beads using both SSC/FSC and SSC/FITC. Beads are enclosed by an ellipse. A: SSC/FSC plot of 1 x 10^7/ml where the bead population was not so tight. B: SSC/FSC plot of 5 x 10^5/ml where the beads population was tightest. C: SSC/FITC plot of 1 x 10^7/ml where the bead population was no so tight. D: SSC/FITC plot of 5 x 10^5/ml where the bead population was tightest. FITC: fluorescein isothiocyanate, FSC: forward scatter channel, SSC: side scatter channel.

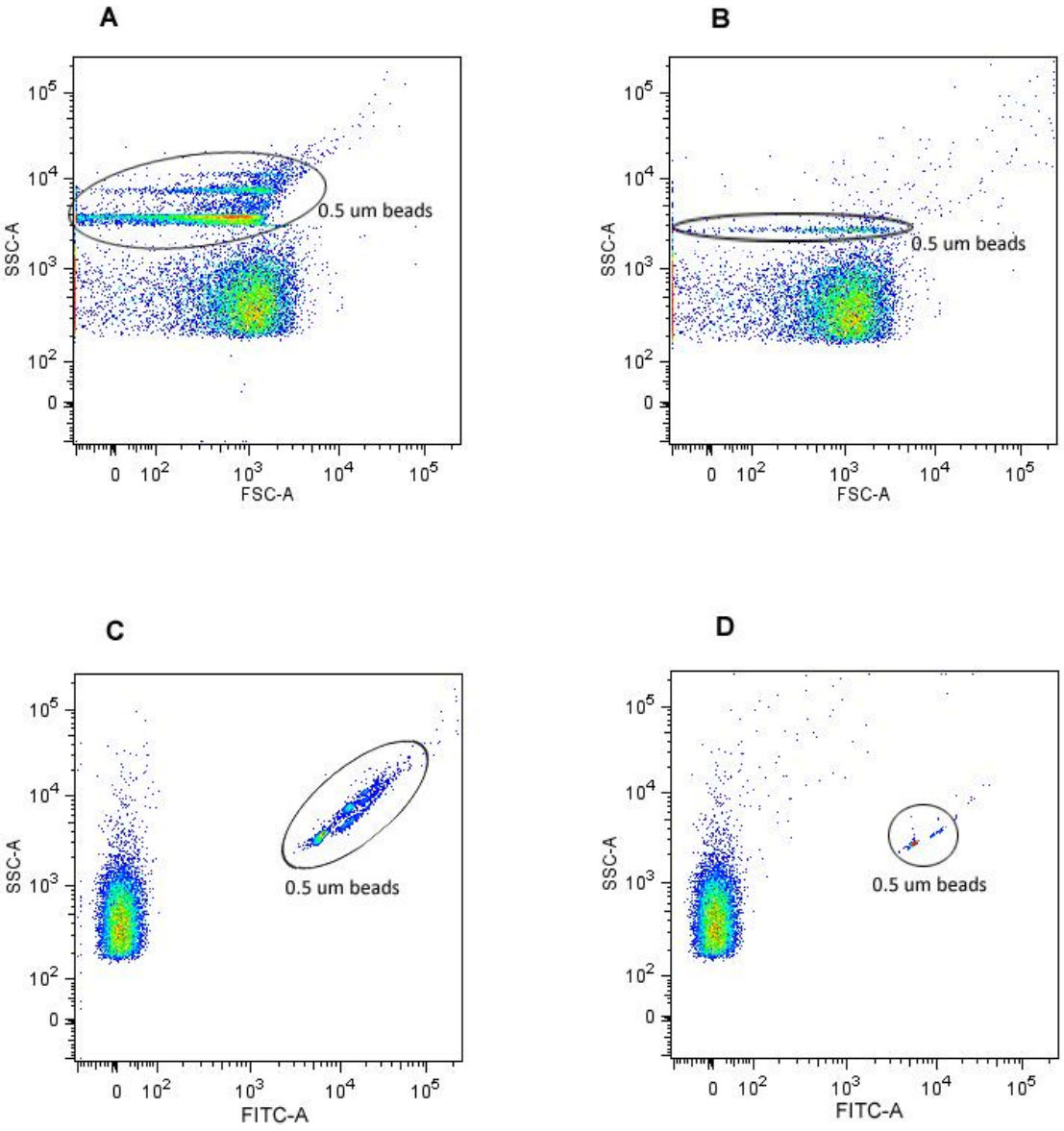


Figure 2.4. Titration of 0.5 µm polystyrene FITC fluorescent beads. Various concentrations of the 0.5µm beads acquired through flow-cytometry to find the tightest population of beads using both SSC/FSC and SSC/FITC. SSC/FSC could not resolve the bead population adequately, but the SSC/FITC could. Beads are enclosed by an ellipse. A: SSC/FSC plot of 1 x 10^7/ml where the bead population was not so tight. B: SSC/FSC plot of 1 x 10^6/ml where the bead population was tighter, but was not tight enough. C: SSC/FITC plot of 10 x 10^6/ml where the bead population was not so tight. D: SSC/FITC plot of 1 x 10^6/ml where the bead population was tightest. FITC: fluorescein isothiocyanate, FSC: forward scatter channel, SSC: side scatter channel.

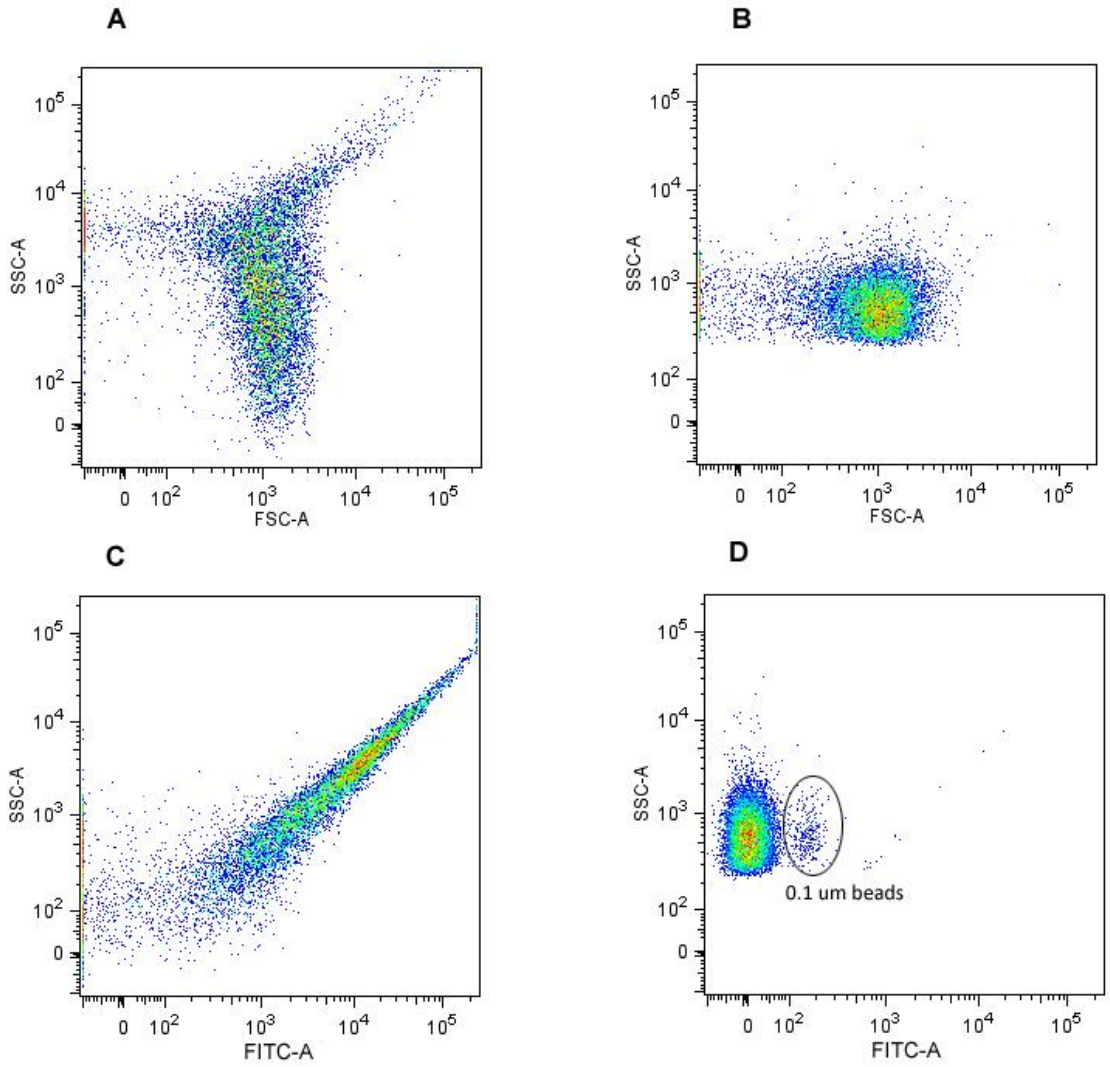


Figure 2.5. Titration of 0.1 µm polystyrene FITC fluorescent beads. Various concentrations of the 0.1µm beads acquired through flow-cytometry to find the tightest population of beads using both SSC/FSC and SSC/FITC. SSC/FSC could not resolve the bead population adequately, but the SSC/FITC could. A: SSC/FSC plot of 1 x 10^7/ml where the bead population was widely scattered. B: SSC/FSC plot of 1 x 10^6/ml where the bead population was hidden inside the noise cloud. C: SSC/FITC plot of 1 x 10^7/ml where the bead population was no so tight. D: SSC/FITC plot of 1 x 10^6/ml where the bead population was tightest. FITC: fluorescein isothiocyanate, FSC: forward scatter channel, SSC: side scatter channel.

2.3.6 Particle size resolution in flow-cytometry

The forward scatter channel (FSC) is usually used for size detection. Preliminary experiments demonstrated that the flow-cytometer I am using could not adequately

differentiate between the various sizes of beads in FSC histogram and that they overlap (Figure 2.6).

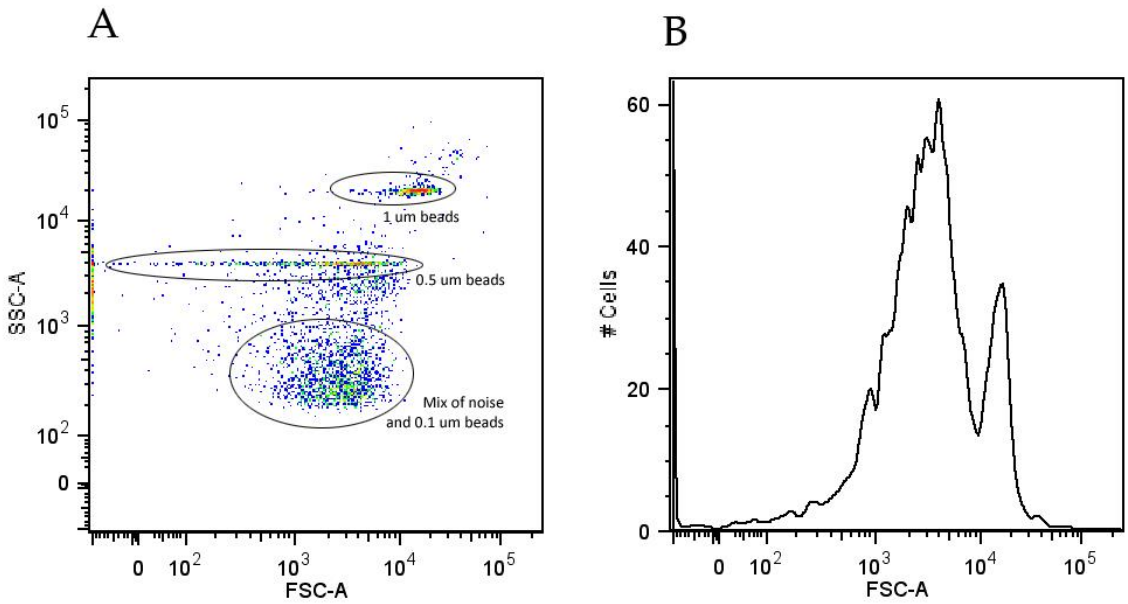


Figure 2.6. SSC/FSC dot plot and FSC histogram of 0.1, 0.5 and 1 µm beads acquired on flow-cytometry showing that it is difficult to differentiate between these beads using FSC. A**.** SSC/FSC dot plot showing a distinct population of the 1µm beads in the upper right corner of the dot plot, 0.5µm beads as a more widely dispersed population in the middle and 0.1µm population is difficult to distinguish from the background noise. B**.** FSC histogram of the 0.1, 0.5 and 1µm beads showing considerable overlap. FSC: forward scatter channel, SSC: side scatter channel.

Therefore, other channels that might be able to differentiate adequately between the various sizes of beads were tested. The side scatter channel (SSC) and the FITC fluorescence channel were able to differentiate between all three populations of beads (Figure 2.7).

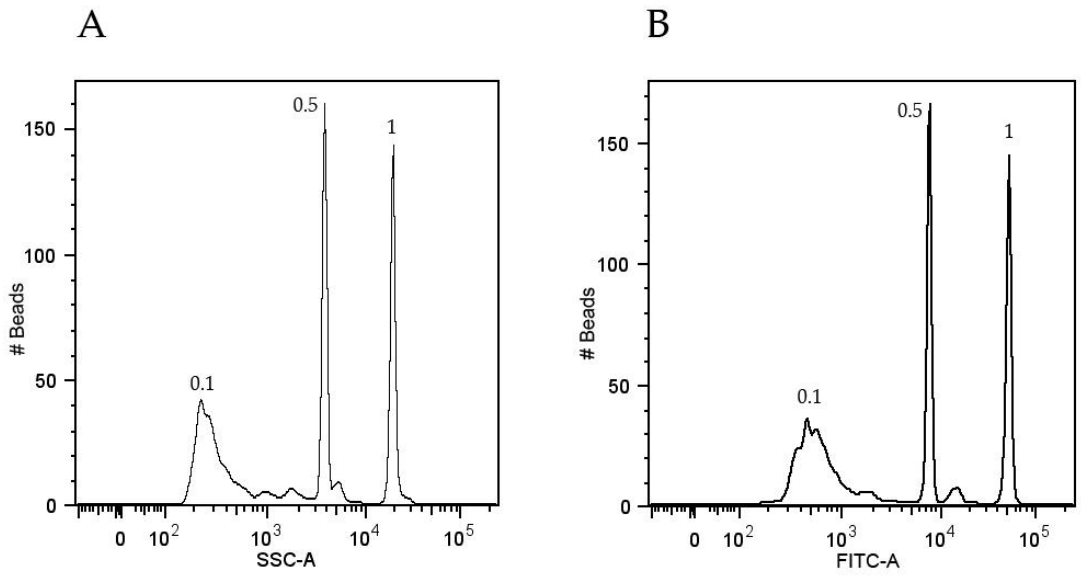


Figure 2.7. Two histograms for 0.1, 0.5 and 1 µm beads of the SSC and FITC channels showing that these two channels could adequately differentiate between all three populations of bead. A**.** SSC histogram showing three distinct peaks for each of the three bead populations. B**.** FITC histogram showing three distinct peaks for each of the three bead populations. FITC: fluorescein isothiocyanate, SSC: side scatter channel.

From these preliminary experiments it was concluded that the best way to formulate a gate to include the desired population is to use the SSC/FITC dot plot, which is then back-gated to SSC/FSC and a gate is drawn to include the resultant population. The gate is subsequently used to identify the MP population (Figure 2.8). This approach is known as *back gating*[281, 282].

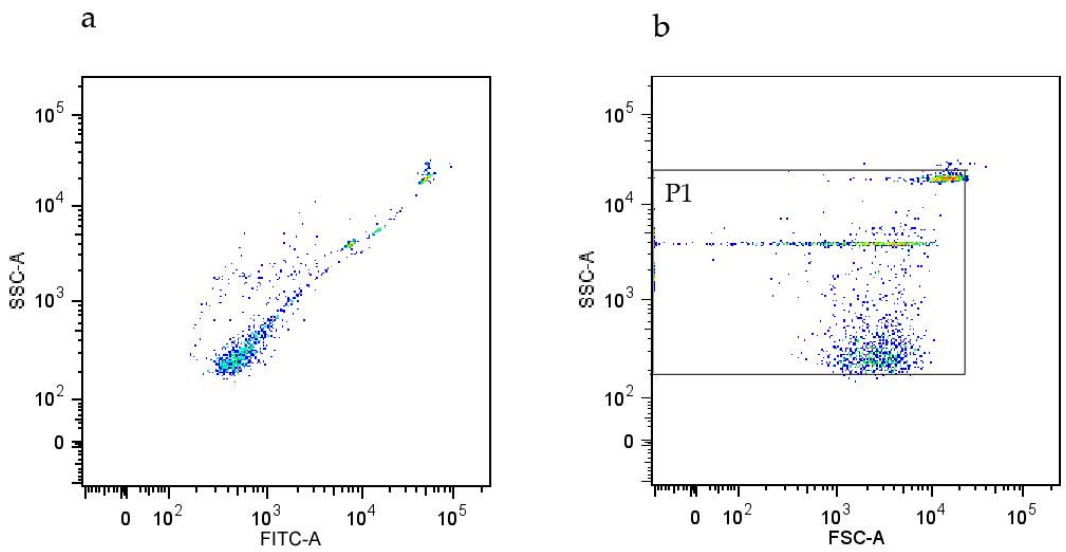


Figure 2.8. SSC/FITC and SSC/FSC of the 0.1, 0.5 and 1 µm bead populations and the gate drawn around them using "back gating" to identify the MP population. The three bead sizes (0.1, 0.5 and 1 µm) at the optimum concentrations were acquired using flow-cytometry. **a.** SSC/FITC dot plot of the three bead populations. **b.** SSC/FSC dot plot of the same populations identified through back gating of the previous dot plot population. **P1.** The gate drawn around the populations that would be later used for MP identification. FITC: fluorescein isothiocyanate, FSC: forward scatter channel, SSC: side scatter channel.

2.3.7 Sample acquisition and microparticle counting formula

Each sample is acquired for 30 seconds on the "Hi" acquisition option of the flow-cytometer. The high acquisition flow rate may encourage "swarm effect" which may risk under-estimating the number of MP. This was mitigated by ensuring good mixing prior to acquisition. The Hi acquisition processes 60 µL per minute.

For calculation of MP count / µL of PFP: $N * \frac{V}{D} =$ MP/ µL.

N is the total number of MP per sample. V is the volume acquired (usually 30 µL). D is the dilution (usually 1 in 40).

Other researchers have used counting beads for microparticles enumeration[283].

2.3.8 Gating strategy

In the protocol optimisation experiments, pacific blue conjugated Annexin V (AV) was used as a general marker for MP. As there is no isotype control for AV, control values were obtained by omitting calcium in the incubation buffer (phosphate buffered saline with no calcium that contains the same final concentration of AV was used). In this way the pacific blue background noise was determined and then subtracted from the total events in stained plasma samples. The AV positive population identified from the previous step was further characterised using one or two monoclonal antibodies. The isotype controls for these monoclonal antibodies were used to identify the non-specific binding and this was subtracted from the positive population (Figure 2.9).

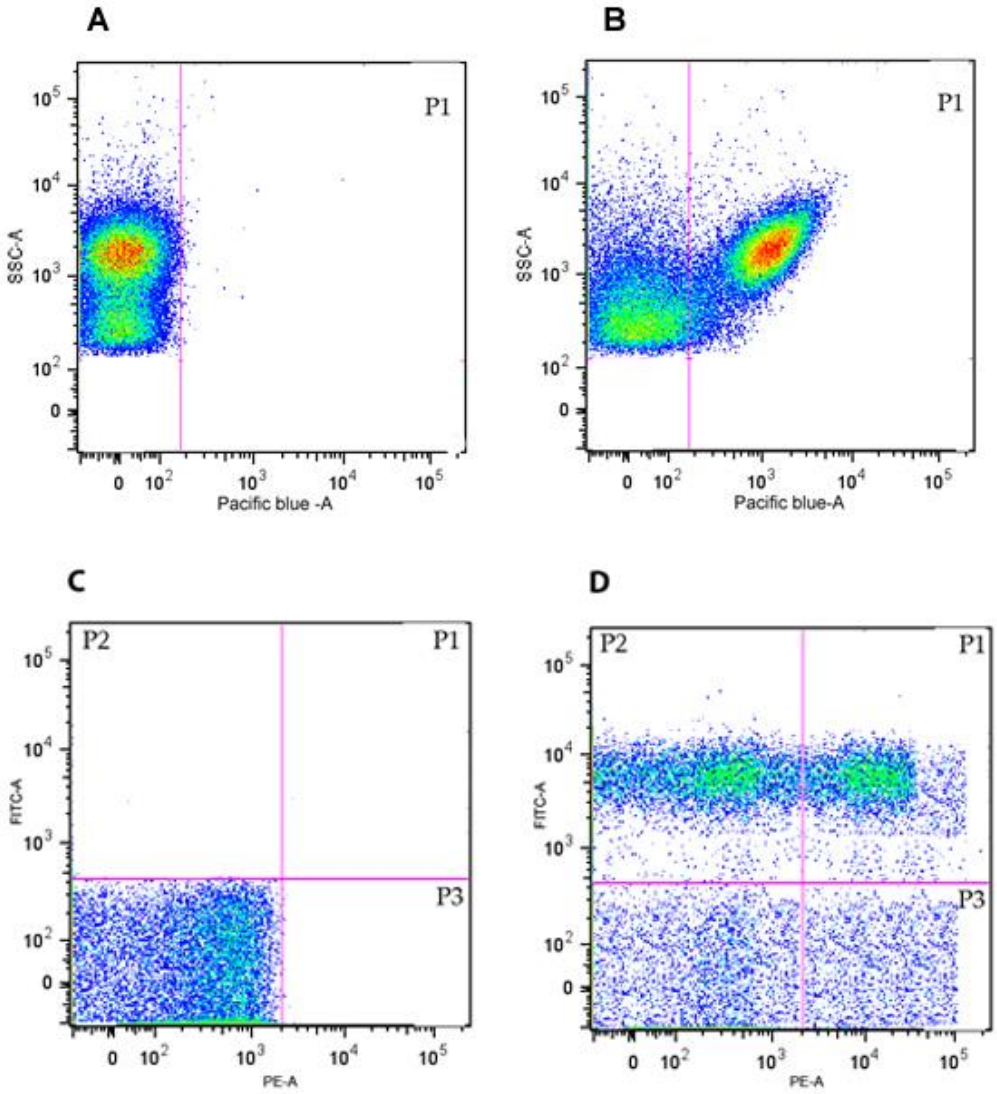


Figure 2.9.Gating strategy. Plasma obtained from healthy volunteers was stained with Pacific Blue fluorescent AV as well as PE and FITC conjugated monoclonal antibodies (anti-66b and A2M respectively) and processed using flow-cytometry. A**.** SSC/Pacific blue dot plot of a sample not stained with AV showing the pacific blue background noise and the quadrants drawn to subtract the noise from positive events. B**.** A sample stained with AV with the quadrants from the unstained sample applied, showing the positive population. P1**.** The AV positive population. SSC: side scatter channel. Healthy volunteer plasma was stained with A**.** Isotype control background noise gated out. B**.** Double stained sample using the quadrants formulated from the isotype controls. P1: Double positive population. P2: FITC positive population. P3: PE positive population. A2M: alpha-2-macroglobulin. FITC: Fluorescein isothiocyanate, PE: phycoerythrin

2.4 Microparticle preparation

2.4.1 Whole blood stimulation for microparticle production

Venous blood was collected from healthy volunteers using a 21 gauge needle into commercially available glass bottles containing 0.068 ml of 7.5% ethylenediaminetetraacetic acid (EDTA) solution acting as an anticoagulant. The blood was incubated with and without compounds as indicated in a 37^{o}C shaking water bath for the specified period.

2.4.2 Isolation of microparticles from whole blood

Whole blood was centrifuged at 1500 x *g* for 15 minutes to pellet the cells. The platelet poor plasma (PPP) was then aspirated and re-centrifuged at 13000 x *g* for 2 minutes to pellet the platelets. The platelet free plasma (PFP) was then aspirated and used for MP detection.

2.4.3 Microparticle concentration

In order to test the effect of MP on various cells, the MP had to be concentrated. This was achieved by centrifuging platelet free plasma at 19000 x *g* for 30 minutes at 4^{o}C. The supernatant was subsequently discarded and the MP pellet was re-suspended in the same original volume in sterile double filtered phosphate buffered saline solution. The suspension was then re-centrifuged using the same protocol as above. The supernatant was again discarded and the MP pellet was then re-suspended in 1/10 of the original plasma volume (Figure 2.10).

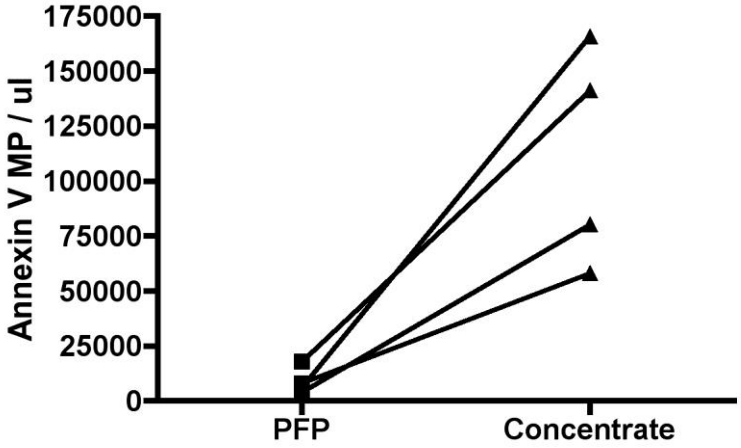


Figure 2.10.The effect of microparticle concentration on the number of microparticles per microliter. Platelet free plasma of 4 healthy volunteers was centrifuged twice at 19000 x *g* for 30 minutes at 4^{o}C and the microparticles pellet was re-suspended in 1/10 of the original plasma volume. The microparticles concentrate was stained with fluorescent Annexin V and the samples were analysed using flow-cytometry. MP: microparticles, PFP: platelet free plasma.

2.4.4 Microparticles sorting

In order to study the characteristics of a MP subset expressing a certain protein, the MP population has to be sorted to separate the desired subset from the whole population.

In an attempted to separate MP subsets using florescence sorting, 10 ml of whole blood was stimulated with granulocyte macrophage colony stimulating factor (GM-CSF) in a concentration of 100 ng/ml for half an hour. Next, platelet free plasma was prepared in the manner described elsewhere and then the MP were concentrated in 1/10th of the original volume. The resultant MP concentrate was then stained for A2M using FITC monoclonal antibody.

The sample was processed using the BD FACSARIA II cell sorter. The machine detected only a small number of events (about 1 million events).

The A2M MP were sorted in a total volume of 8 ml, which was then re-concentrated into 1/10th of the volume. The sample was stained for A2M using the same monoclonal antibody and quantified A2M MP in the sample on the more sensitive BD LSRFortessa. This has yielded an A2M positive population of MP that represented 73.6% of the all events including noise (Figure 2.11) with more than 64,000 positive events per microliter.

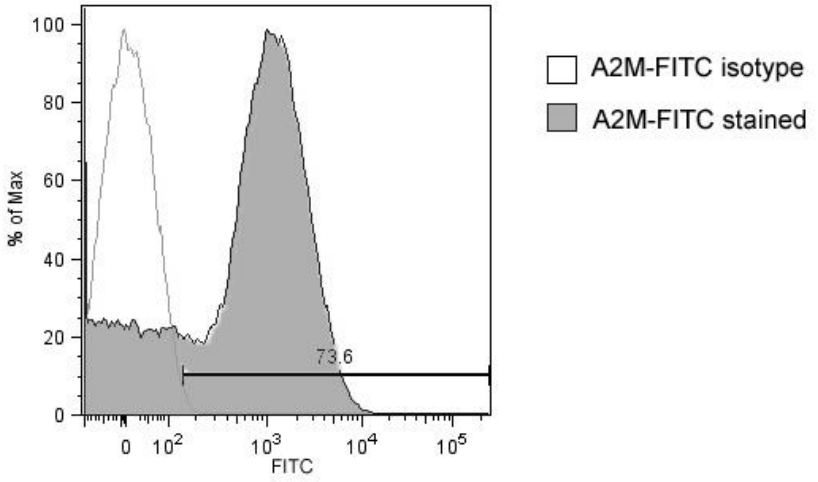


Figure 2.11. Histograms of the alpha-2-macroglobulin (A2M) sorted microparticles and thier isotype control. Whole blood from healthy volunteers was stimulated with granulocyte macrophage colony stimulating factor (GMCSF) for 30 minutes at 37^{o}C and microparticles were concentrated. The A2M positive microparticles were labelled with A2M-FITC fluorescent monoclonal antibody and sorted to separate A2M using FACS. Following the sorting process, microparticles were stained again with A2M-FITC antibody and processd using flow cytometry. 73.6% of the microparticles in the sample positive for A2M. A2M: Alpha-2-macroglobulin, FITC: Fluorescein isothiocyanate.

2.5 Human umbilical vein endothelial cells

2.5.1 Isolation and culture of primary human umbilical vein endothelial cells (HUVEC)

The Royal London Hospital midwifery unit was the source of the umbilical cords. Phosphate buffered saline (PBS) solution containing penicillin (100U), streptomycin

(10µg/ml) and fungizone (2.5µg/ml) was used as incubation medium for the umbilical cords. The cords were stored at 4ºC until processing.

The interior umbilical vein was digested used collagenase to isolate the HUVECs as described previously[284]. Briefly, at one end of the umbilical vein a needle was introduced and secured using a clamp. A sterile syringe was used to infuse 30mls of sterile PBS into the vein to remove any blood remaining in the lumen and to detect any perforations. The other end of the vein was then clamped and 20 to 25 ml of serum free M199 medium containing 0.1% type II collagenase, penicillin (100U), streptomycin (10µg/ml) and fungizone (2.5µg/ml) was introduced. The vein was incubated in at 37ºC in a humidified incubator containing 5% CO_2 for 15 minutes.

The solution contained in the vein was then transferred to a 50ml falcon tube. PBS solution (30mls) was then used to wash the vein and added to the same tube. The solution was centrifuged at 300g for 10 minutes, the supernatant was discarded and the pelleted cells were re-suspended in 10mls of complete medium (M199 containing 20% human serum, penicillin (100U), streptomycin (10µg/ml) and fungizone (2.5µg/ml)). The solution was then transferred to a 0.5% gelatine pre-coated T75 flask (75cm^2).

The cells were incubated at 37ºC in a humidified incubator containing 5% CO_2. The medium was changed at 24 hours and every 48 hours thereafter until the cells

reached 95% confluence. This procedure yielded approximately 0.5 – 1.5 x 10^6 cells per umbilical cord.

When the desired level of confluence was achieved, the cells were washed with PBS prior to the addition of 0.025% Trypsin/0.01% EDTA solution to detach the cells. Once the morphology of the cells started to change from spindle shape to oval and they began to detach the flask was firmly tapped to aid the detachment of the cells; complete medium was then added to render the trypsin inactive. The cells were split between T75 flasks, which were incubated at 37^{o}C with 0.5% gelatine for 20 minutes prior to cell addition. Passage 3 was the maximum stage of cell culture used for experiments.

2.5.2 Stimulation of human umbilical vein endothelial cells with microparticles

HUVECs prepared as above were plated as indicated at least overnight to achieve confluence. The supernatant was aspirated and replaced with HUVEC incomplete medium (M199 containing penicillin (100U), streptomycin (10µg/ml) and fungizone (2.5µg/ml)). The MP were added at a concentration of 1 – 2 x 10^6/ml to the cells with or without lipopolysaccharide 100ng/ml and placed in an incubator containing 5% CO_2 at 37^{o}C for 4 hours.

2.5.3 Assessment of adhesion molecule and tissue factor expression on human umbilical cord endothelial cells by flow-cytometry

The cells were then seeded in 12 well plates that were pre-incubated at 37^{o}C with 0.5% gelatine for 20 minutes prior to seeding of cells. The plates were then incubated in an incubator containing 5% CO_2 at 37^{o}C for 24 to 48hrs to reach confluence. Each well contained approximately 5 x 10^5 HUVEC.

The HUVEC cells were stimulated with MP (1 – 2 x 10^6/ml) with or without lipopolysaccharide (LPS – 100 ng/ml) and placed in incubator containing 5% CO_2 at 37^{o}C for 4 hours. The supernatant was then discarded and the cells were trypsinised and washed in complete medium as above and transferred to a 15ml falcon tube. The tube was then centrifuged at 300 x *g* for 10 minutes to pellet the cells and the supernatant was discarded. The cells were then plated in a 96 well plate and each well contained approximately 2 x 10^5 HUVEC cells suspended in phosphate buffered saline (PBS). The cells were then incubated with intra-cellular adhesion molecule - 1 (ICAM-1) phycoerythrin (PE), vascular cell adhesion molecule – 1 (VCAM-1) – fluorescein isothiocyanate (FITC), E/P selectin – FITC or tissue factor – allophycocyanin (APC) conjugated monoclonal antibodies for 30 minutes on ice in the dark. The cells were subsequently washed twice with PBS and re-suspended in 200µl of 1% Paraformaldehyde fixative (PFA) solution ready for analysis using flow-cytometry. Isotype controls (IgG1) were used for accurate calibration of the flow-cytometer. Adhesion molecules were expressed as median florescence intensity (MFI) units in the respective channel.

2.5.4 In-vitro flow chamber assay

This assay was used to examine the effect of MP on neutrophil-endothelial interaction under flow conditions. Human umbilical vein endothelial cells (HUVEC) were grown to confluence in T75 flasks as described above. The cells were then trypsinised and washed and pelleted as described above. The HUVECs were then re-suspended in 700µl of complete medium (M199 containing 20% human serum, penicillin (100U), streptomycin (10µg/ml) and fungizone (2.5µg/ml)). The cells (100µl per chamber) were subsequently plated in 6 chamber µ-Slide VI0.4 (Ibidi, Munich, Germany) (Figure 2.12), which were pre-incubated at 37^{o}C with 0.5% gelatine for 20 minutes prior to seeding of cells. The µ-Slides were then incubated in a 37^{o}C incubator containing 5% CO_2 over night to reach confluence. The medium was then aspirated and discarded. Serum free medium, with or without lipopolysaccharides or MP was used (150µl per chamber) for HUVEC stimulation. The slides were incubated in the same conditions as above for a further 4 hours.

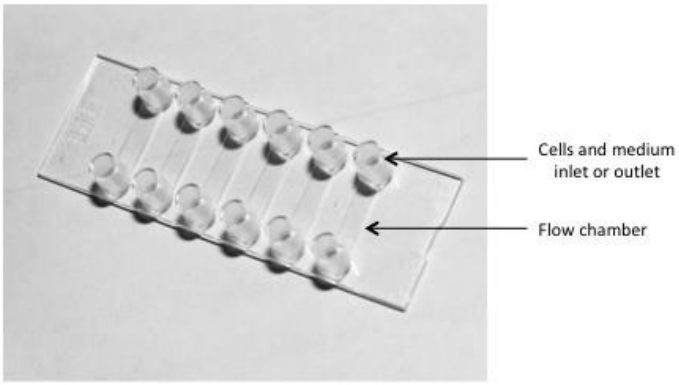


Figure 2.12. Ibidi µ-Slides containing 6 individual chambers were used for the flow chamber assay.

Neutrophils were isolated as detailed below, diluted in phosphate buffered saline (PBS) supplemented with Ca^{2+} and Mg^{2+} containing 1% bovine serum albumin (BSA) to a concentration of 1 x 10^6/ml and incubated in a 37^{o}C water bath for 10 minutes prior to infusion over the HUVEC monolayer.

An automated syringe pump system (Harvard Apparatus) was used to produce a shear stress of 1 dyne/cm^2, which was calculated using an adaptation of Poiseuille's law:

Wall shear stress (dyne/cm^2) = Mean flow velocity (mm/s) x [8/tube diameter (mm)] x viscosity (Poise)

This takes into consideration the cylindrical vessel flow rate; the wall shear stress to volumetric flow rate through the chamber equation[285]:

$$\tau_w = 6\mu Q/a^2 b$$

Where:

τ_w = Wall shear stress (dyne/cm^2)

μ = Coefficient of viscosity (Poise)

Q = Volumetric flow rate (ml/s)

a = Channel height (cm)

b = Channel width (cm)

Constant volumetric flow (0.0095 ml/s) was used for these experiments using a programmable syringe pump. The suspension containing the cells (1 x 10^6/ml) and its temperature determine the viscosity coefficient. At a constant temperature of 37^{o}C, PBS had a viscosity of 0.011 Poise. The channel height and width of the flow chamber were constant at 0.04 cm and 0.38 cm respectively. A wall shear stress of 1.031 dyne/cm^2 was generated.

A Nikon Eclipse TE3000 microscope fitted with x4, x10, x20 and x40 phase contrast objectives was used for these experiments. PMNs were perfused over the monolayer for 8 minutes and six 10 second frames were captured from random areas using a Q-imaging RetigaEXi digital camera (Q-imaging, Surry, Canada) and recorded in Image-Pro Plus capture software (Media Cybernetics, Rockville, USA) (Figure 2.13). The sequences were subsequently analysed using Image-ProPlus software package (Media Cybernetics, Rockville, USA) where PMNs were manually tagged and observed for migration. The total number of PMNs in the field during the 10 second sequences was recorded as “captured” and these were subsequently divided into rolling and adherent (mobile through the field and stationary respectively) as described previously[286].

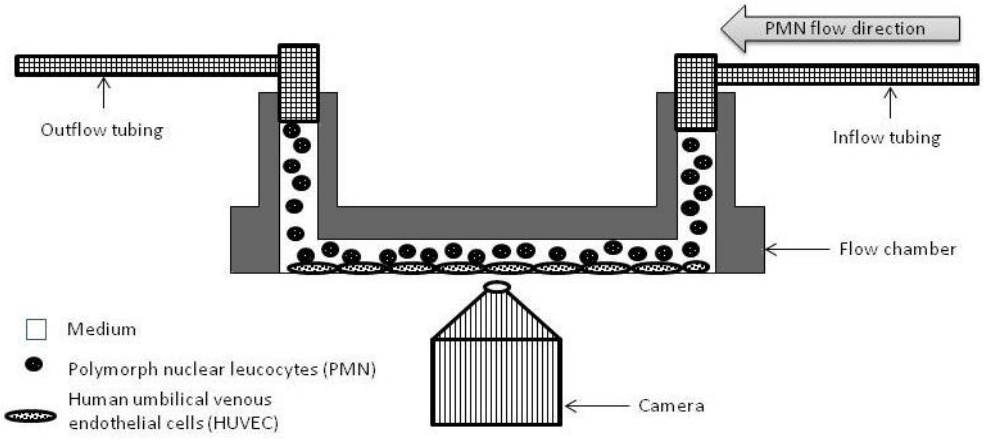


Figure 2.13.Schematic illustrating the flow chamber experiment. Medium containing polymorph-nuclear leucocytes (PMN) was perfused using a syringe pump into a flow chamber pre-seeded with a confluent layer of human umbilical cord venous endothelium (HUVEC) cells. A digital camera was positioned below the chamber to record the interaction between the two cell populations.

2.5.5 Human umbilical vein endothelial cells monolayer permeability assay

HUVEC were seeded into 8 well strip tissue culture inserts with 0.2µm porous membrane (NUNC, Denmark) at a final concentration of 5 x 10^4cells suspended in 60 µl of complete medium per well, as per the manufacturer's instructions. The insert was placed in a 96 well plate, where each well contained 150 µl of complete medium (Figure 2.14). During placement the insert was tilted to avoid air bubbles being trapped underneath. The whole set was placed over night in an incubator containing 5% CO_2 at 37^{o}C to ensure confluence.

Incomplete HUVEC medium containing MP at a concentration of 1 – 2 x 106/ml with or without 100ng/ml of lipopolysaccharide was used to replace the medium inside the insert wells. The incomplete medium was used to fill the 96 well plates. The set was placed in an incubator containing 5% CO_2 at a 37^{o}C for 4 hours.

The medium inside the insert wells was aspirated and replaced with the same volume of incomplete medium containing fluorescein isothiocyanate (FITC) labelled Dextran MW 40000 (Sigma-Aldrich, Poole, UK). Fresh incomplete medium was added to the bottom wells. This set was re-incubated.

The insert was then removed and 50µl of the medium in the bottom plate was removed and placed into a fresh 96 well plate. Medium without FITC – dextran was added to the plate to act as a negative control.

The NOVOstar plate reader (BMH LabTech, Ortenberg, Germany) was used to detect the fluorescence (Excitation filter: 485 and Emission filter: 520) for each treatment in the 96 well plate and the results were analysed using the NOVOstar software package.

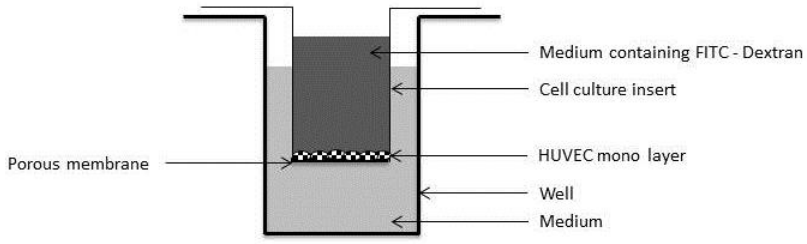


Figure 2.14. Schematic illustrating the human umbilical vein endothelial cell (HUVEC) monolayer permeability assay.

2.5.6 Human umbilical vein endothelial cells reactive oxygen species production assay

HUVEC were seeded in a 96 well plate at a concentration of 1 x 10^5/well. The plates were pre-coated with 0.5% gelatine and incubated at 37^{o}C for 20 minutes prior to seeding. The cells were placed over-night at in an incubator at 37^{o}C containing 5% CO_2 to reach confluence.

The cells were then incubated for 30 minutes with 20 µM of 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) (Invitrogen, Paisley, UK) suspended in incomplete HUVEC medium. The medium containing H_2DCFDA was aspirated and replaced by incomplete medium containing MP (1 -2 x 10^6/ml) in the absence or presence of 100ng/ml of lipopolysaccharide. The cells were re-incubated at the same conditions as above for 4 hours. The cells were washed twice with phosphate buffered saline (PBS). PBS containing 20 µM H_2DCFDA was used as a negative control.

NOVOstar plate reader (BMH LabTech, Ortenberg, Germany) was used to detect the fluorescence (Excitation filter: 485 and Emission filter: 520) for each treatment in the 96 well plate and the results were analysed using NOVOstar software package.

The negative control fluorescence values were subtracted from the treatment fluorescence.

2.5.7 Human umbilical vein endothelial cells nitrite production assay

HUVEC were seeded in a 96 well plate at a concentration of 2 x 10^5/well. The plates were pre-coated with 0.5% gelatine and incubated at 37^{o}C for 20 minutes prior to seeding. The cells were placed over night in an incubator containing 5% CO_2 at a 37^{o}C to reach confluence.

The medium was replaced with incomplete HUVEC medium containing MP (1 -2 x 10^6/ml) in the absence or presence of 100ng/ml of lipopolysaccharide and the cells were incubated in the same conditions as above for 4 hours.

A 150 µl aliquots of the supernatant was aspirated and plated in a flat bottom 96 well plate (Costar) for each treatment as well as 150 µl of phosphate buffered saline solution acting as negative control. The volume was made up to 280 µl with deionised water and 20 µl of Griess Reagent (Molecular Probes, Paisely, UK) was added in line with the manufacturer's guidance. The plate was re-incubated for a further 30 minutes and subsequently NOVOstar plate reader (BMH LabTech, Ortenberg, Germany) was used to detect the absorbance (wavelength A492) and the results were analysed using the NOVOstar software package.

2.5.8 Human umbilical vein endothelial cells cytokine production assay

HUVEC were seeded in a 96 well plate at a concentration of 5 x 10^4/well. The plates were pre-coated with 0.5% gelatine and incubated at 37^{o}C for 20 minutes prior to

seeding. The cells placed over night in an incubator containing 5% CO_2 at a 37^{o}C to reach confluence.

The medium was replaced with incomplete HUVEC medium containing MP (1 -2 x 10^6/ml) in the absence or presence of 100ng/ml of lipopolysaccharide. The cells were re-incubated at the same conditions as above for 4 hours. The supernatant was then collected and stored at -80^{o}C until assayed.

Commercially available enzyme-linked immunosorbent assay (ELISA) development kit was used for cytokine detection (Peprotech, London, UK). One hundred microliters of cytokine specific (IL8 and TNF α) capture antibody (0.5 µg/ml) was used to coat each well in a 96 well plate. The plate was sealed and incubated overnight at room temperature. Subsequently, the wells were aspirated and washed with 300 µL of wash buffer (0.05% Tween-20 in PBS) 4 times. Following the last wash, the plate was inverted and blotted to remove any residual buffer. Then, 300 µL of block buffer (1% bovine serum albumin in PBS) was added to each well and incubated at room temperature for 1 hour. After incubation with block buffer, the plate was washed again 4 times with wash buffer. In each well, 100 µL of sample was added and incubated for 2 hours at room temperature; the plate was then washed again 4 times. Detection antibody diluted in diluent (0.05% Tween-20, 0.1%BSA in PBS) to a concentration of 0.5 µg/ml and 100 µL was added to each well and incubated for another 2 hours at room temperature. The plate was washed 4 times and 100 µL of Avidin-HRP conjugate (0.5 µg/ml in diluent) was added to each well

and incubated for 30 minutes. Afterwards 100 µL of 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) liquid substrate was added to each well and incubated for 30 minutes at room temperature.

The plate was then read using a NOVOstar plate reader (BMH LabTech, Ortenberg, Germany) to detect the absorbance (wavelength A405nm) and the results were analysed using the NOVOstar software package.

2.5.9 Detection of microparticle uptake by human umbilical vein endothelial cells using confocal microscopy

HUVEC were seeded on 13 mm glass coverslips in a 24 well plate. The cover slips were coated with 0.5% gelatine containing 10 µg/ml of fibronectin (Millipore, Watford, UK) and incubated at 37^{o}C for 2 hours. The HUVEC cells were then seeded on the coverslips at a concentration of 1 x 10^{5}per slip. The coverslips in the plate were placed over night in an incubator containing 5% CO_2 at 37^{o}C for 24 to 48 hours to achieve confluence.

MP concentrate were stained with labelledboron-dipyrromethene (BODIPY) (2.5 µM) for 10 minutes and subsequently the suspension was centrifuged at 19000 x *g* for 30 minutes at 4^{o}C to pellet the MP. The supernatant containing the excess BODIPY was discarded and the MP pellet was re-suspended in the same original volume of PBS.

The medium was then replaced with 0.5 ml of incomplete HUVEC medium containing fluorescent MP at a concentration of 1 to 2 x 10^6/ml and incubated for 4 hours in an incubator containing 5% CO_2 at 37^{o}C. Subsequently, the coverslips were washed with phosphate buffered saline (PBS) twice and the cells were fixed with 1% paraformaldehyde (PFA) (200 µl/coverslip) for 10 minutes. Then, PFA was aspirated and the coverslips were washed thrice with PBS. Subsequently, PBS containing 1% bovine serum albumin (BSA) was added on top of each coverslip acting as a blocking buffer for thirty minutes. The blocking buffer was aspirated and the cells were washed twice with PBS.

To stain the HUVEC cell wall, wheat germ agglutinin (WGA) conjugated to Alexa Flour 633 (Life technologies, Paisley, UK) was used at a concentration of 5 µg/ml in PBS of which 200 µl was used to stain the cells in each coverslip. The plate containing the coverslip was re-incubated for a further 30 minutes. Following cell staining, the coverslips were washed twice with PBS.

The coverslips were carefully removed from the plate wells, inverted and placed on microscope slides where a 10 µl mountant (Prolong gold anti-fade mountant, Life technologies, Paisley, UK) containing 4',6-diamidino-2-phenylindole (DAPI - for staining HUVEC nuclei) was used to adhere the coverslips to the slide. The slides were incubated for a further 30 minutes in the same conditions as above. The slips were then stored at 4^{o}C at least over-night prior to microscopy.

A Zeiss Laser scanning microscope LSM 710 was used for microscopy of the HUVEC using Zeiss Zen software. The following excitation/emission conditions were used in conjunction with x63 or x100 magnification oil immersion objectives: DAPI 364/475-525 nm, BODIPY 488/505-530 and AlexFlour 633 at 638/658 in separate channels.

The images acquired were analysed using supplied Zen and ImageJ software packages. Distinct BODIPY fluorescent (green) points identified by eye in the micrographs were considered as distinct MP. The software packages were used to measure sizes of structures such as nuclei or MP.

2.5.10 Detection of microparticle uptake by human umbilical vein endothelial cells using imaging flow-cytometry

HUVEC were seeded in a 6 well plate at a concentration of 5 x 10^5/well. The plates were pre-coated with 0.5% gelatine and incubated at 37^{o}C for 20 minutes prior to seeding. The cells were in an incubator containing 5% CO_2 at 37^{o}C over night to reach confluence.

MP concentrate was incubated with fluorescein isothiocyanate (FITC) labeled boron-dipyrromethene (BODIPY) for 10 minutes. The suspension was then centrifuged at 19000 x *g* for 30 minutes, the supernatant containing the excess BODIPY was aspirated and discarded and the MP pellet was re-suspended in sterile double filtered phosphate buffered saline.

The medium was replaced with incomplete HUVEC medium containing BODIPY stained MP at a concentration of 1 – 2 x 10^6/ml and the cells were incubated in the same conditions as above for 4 hours.

Imagestream X mark II (Aminis, Seattle, USA) was used to detect the presence of microparticles inside HUVEC cells.

2.5.11 Inhibition of microparticle uptake by human umbilical vein endothelial cells

MP concentrates, prepared as above were incubated with Fluorescein isothiocyanate (FITC) labeled boron-dipyrromethene (BODIPY) for 10 minutes. The suspension was then centrifuged at 19000 x g for 30 minutes, the supernatant containing the excess BODIPY was aspirated and discarded and re-suspended in sterile double filtered phosphate buffered saline. The MP were then incubated with HUVEC cells without or without cytochalasin B (10 μM) to inhibit MP uptake.

Imagestream X mark II (Aminis, Seattle, USA) was used to detect the presence of microparticles inside HUVEC cells.

2.6. Polymorphonuclear cells

2.6.1 Isolation of human polymorphonuclear cells (PMN) from whole blood

A 21-gaage needle was used to obtain venous blood (50 ml) from consenting healthy volunteers and transferred to 50ml falcon tubes. Each tube contained 3.2% sodium citrate solution acting as an anticoagulant at 1/10th of the donated blood volume. Platelet rich plasma was separated through centrifugation at 137 x *g* for 20 minutes and discarded. PBS (10ml) and 6% dextran (8ml) were gently layered respectively on top of blood cells. The solution was mixed gently to ensure homogeneity and left to stand for 30 minutes resulting in red cells sedimenting at the bottom of the tube. The top layer containing the leucocytes was then removed, layered over 10ml of Histopaque 1077 solution in a 50 ml falcon tube and centrifuged at 483 x *g* for 30 minutes where layers are formed as demonstrated in Figure 2.15.

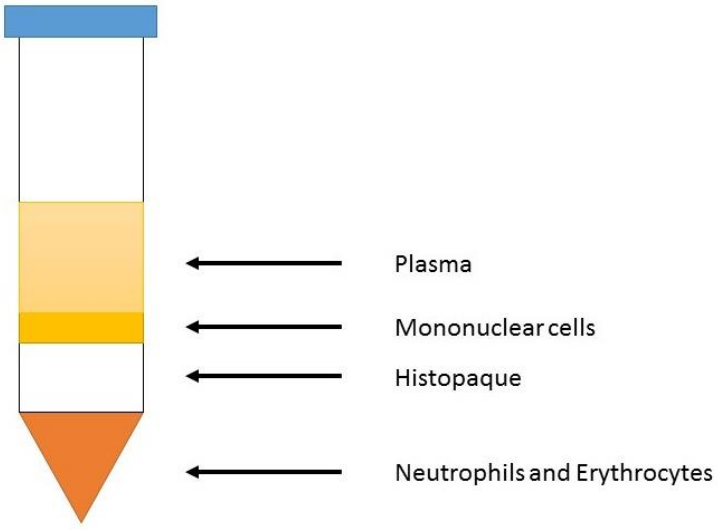


Figure 2.15. Isolation of polymorphonuclear cells from whole blood using dextran sedimentation.

The peripheral blood mononuclear cells (PBMC) were collected and the other layers were discarded leaving the bottom layer containing PMN and erythrocytes. Ice-cold water (9ml) was mixed with the bottom layer to lyse the erythrocytes and this mixture was then mixed with 1 ml of 10x Hanks balanced solution to restore the isotonic state. PBS was added up to a volume of 50ml and centrifuged at 215 x *g* for 10 minutes to pellet the PMNs. The cells were then re-suspended in 3 to 6 ml of PBS depending on the pellet size. A haemocytometer was used for counting, where 5 µl of the suspension was added to 495 µl of Turks stain and 10 µl was used for counting (Figure 2.16).

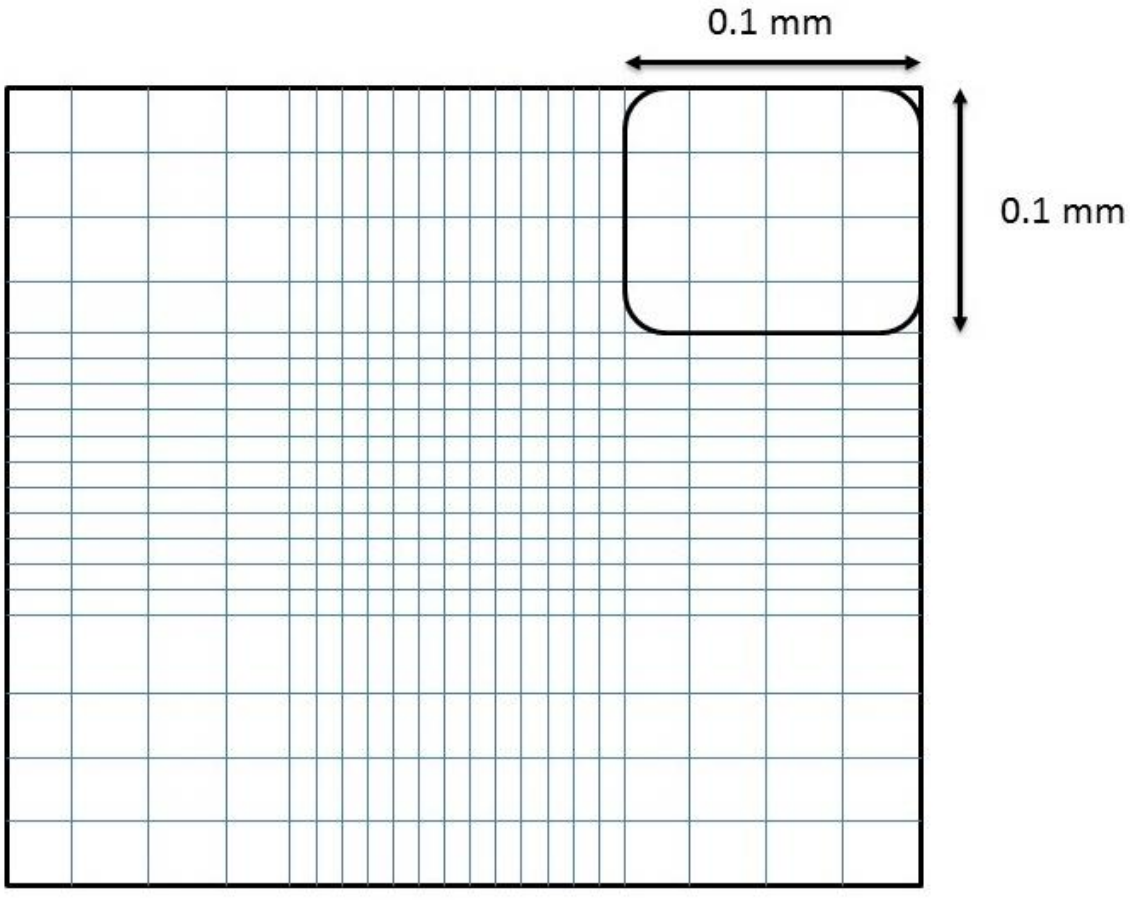


Figure 2.16. Neubauer Haemocytometer counting grid. PMN were counted in each corner as illustrated.

The total number of PMNs was calculated using the following formula:

$$\frac{\boldsymbol{Total\ number\ of\ cells\ in\ all\ 4\ corners}}{\boldsymbol{4}} \times \boldsymbol{10^4} \times \boldsymbol{dilution\ factor} = \boldsymbol{cells\ per\ ml}$$

Phosphate buffered solution was used to dilute the suspension to reach the desired concentration.

2.6.2 Polymorphonuclear cells stimulation using microparticles

PMNs isolated as above were suspended in 1ml of PBS at a concentration of 1 x 10^6/ml. MP were added to the suspension at a concentration of 1 to 2 x 10^6/ml with or without lipopolysaccharide (LPS) at a final concentration of 100ng/ml. The suspension was incubated in a 37^{o}C water bath for 1 hour.

2.6.3 Polymorphonuclear cells adhesion molecule assay using flow-cytometry

Following stimulation of PMNs, the cells were pelleted by centrifugation at 300 x *g* for 10 minutes. The pellet was then re-suspended in 400 µl of PBS and subsequently plated. The cells were then washed, re-suspended in solutions containing CD11b and CD62L fluorescent monoclonal antibodies (Allophycocyanin (APC) and Phycoerthryin-Cy5 (PE-Cy5) respectively) and incubated in the dark, on ice for 30 minutes.

Following staining, the cells were washed twice and re-suspended in 200 µl of 1% paraformaldehyde fixative (PFA) solution ready for analysis using flow-cytometry. Isotype controls (IgG1) were used for accurate calibration of the flow-cytometer. Adhesion molecules were expressed as median florescence intensity (MFI) units in the respective channel.

2.6.4 Polymorphonuclear cells reactive oxygen species (ROS) production assay

The 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA) (Invitrogen, Paisley, UK) is a stable and non-fluorescent compound that easily permeates into PMNs. Once inside the cell, H2DCFDA is cleaved by intra-cellular enzymes into the non-fluorescent dichlorodihydrofluorescein which is then rapidly oxidised in the presence of intra-cellular ROS (e.g. hydrogen peroxide) to the fluorescent 2',7'-dichlorodihydrofluorescein. One million neutrophils suspended in 1ml of phosphate buffered saline were incubated with microparticles ($1 – 2 \times 10^6$/ml) in the presence or absence of lipopolysaccharide (100ng/ml) (H_2DCFDA) (Invitrogen, Paisley, UK) was added at a final concentration of 5µM. The suspension was then incubated in a 37^{o}C water bath for one hour.

A 96 well plate was used for plating of the treatments. Each treatment was plated in quadruplicates (200×10^3 PMNs in each well). H_2DCFDA; PBS with no cells was used as a negative control.

A NOVOstar plate reader (BMH LabTech, Ortenberg, Germany) was used to detect the fluorescence (Excitation filter: 485 and Emission filter: 520) for each treatment in the 96 well plate and the results were analysed using the NOVOstar software package.

The negative control fluorescence values were subtracted from the samples fluorescence.

2.6.5 Polymorphonuclear cells phagocytosis assay

Heat killed Escherichia coli strain K12 (EC1-5G, Sigma-Aldrich, Poole, UK) were weighed and dissolved in phosphate buffered saline at a concentration of 8 mg/ml. The suspension was then incubated with fluorescent boron-dipyrromethene (BODIPY) 576/589 (Invitrogen, Paisley, UK) at a final concentration of 1μM for 5 minutes in the dark. Following incubation, the suspension was centrifuged at 300 x *g* for 10 minutes to pellet the fluorescent *E. Coli* and the supernatant containing the excess BODIPY was discarded. The *E. Coli* was then re-suspended in sterile filtered phosphate buffered saline at the same original concentration.

A 96 well plate was used for plating the PMN. Each treatment was plated in quadruplicate (2 x 10^5 PMNs /well) in a 200μl volume. One million neutrophils suspended in 1ml of phosphate buffered saline were incubated with microparticles (1 – 2 x 10^6/ml) in the presence or absence of lipopolysaccharide (100ng/ml). The suspension was then incubated in a 37^{o}C water bath for one hour. The supernatant

was subsequently discarded and the fluorescent *E. Coli* were then added to each well at a final concentration of 1 mg/ml. PMN with no *E. Coli* were also plated to act as negative control. The plate was then placed for 30 minutes in an incubator containing 5% CO_2 at 37^{o}C. Following incubation the supernatant was removed to discard the excess fluorescent *E. Coli* and the cells were subsequently washed twice with PBS.

A NOVOstar plate reader (BMH LabTech, Ortenberg, Germany) was used to detect the fluorescence (Excitation filter: 560 and Emission filter: 590) for each treatment in the 96 well plate and the results were analysed using the NOVOstar software package. The negative control fluorescence values were subtracted from the samples fluorescence.

2.6.6 Polymorphonuclear cells chemotaxis assay

As described before[287], a NeuroprobeChemoTx®plate96-well plate (Receptor Technologies Ltd, Adderbury, U.K.) with polycarbonate membrane filters and 3µm pores in the membrane was used for this assay (Figure 2.17). PMNs suspended in PBS were treated as indicated (4 x 10^6/ml). Formyl-Methionyl-Leucyl-Phenylalanine (fMLP) was used as chemo-attractant at a final concentration of 1 µM. fMLP or medium was added to the bottom wells (27 µl), the filters were placed on top of the wells and 25 µl PMNs cell suspension was added on the upper surface of the filter membrane in quadruplets (Figure 2.18). The plates were incubated for 60 minutes in a 37^{o}C incubator containing 5% CO_2.

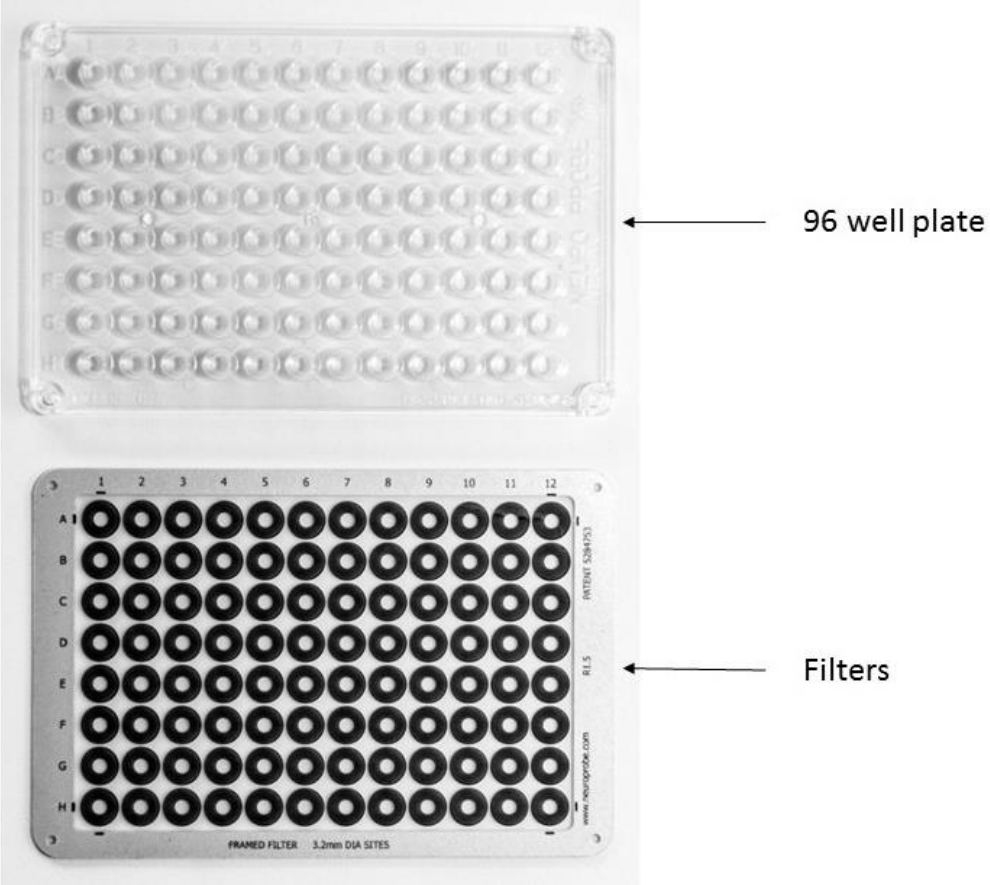


Figure 2.17. Polymorph nuclear cells migration assay plate. NeuroprobeChemoTx®plate 96-well plate (Receptor Technologies Ltd, Adderbury, U.K.) and polycarbonate membrane filters with 3µm pores were used for polymorph nuclear leucocytes chemotaxis assay. Formyl-Methionyl-Leucyl-Phenylalanine (fMLP) was used as a chemo-attractant at final concentration 1 µM. fMLP or medium was added to the bottom wells (27 µl), the filters were placed on top of the wells and 25µl PMNs cells suspension was added on the upper surface of the filter membrane in quadruplicates.

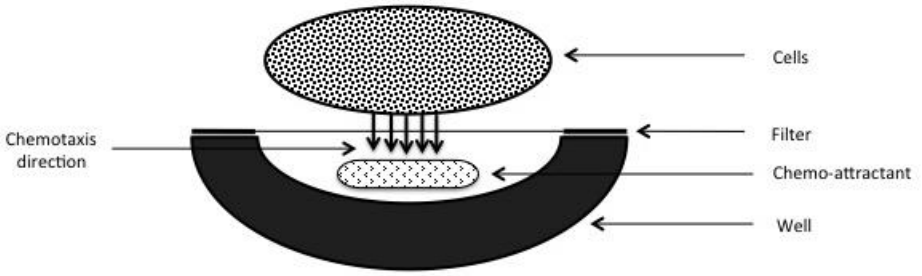


Figure 2.18. Schematic for polymorph nuclear leucocytes (PMN) chemotaxis assay using Neuroprobe ChemoTx®plate. Formyl-Methionyl-Leucyl-Phenylalanine (fMLP) was added to the wells (27 µl) at a final concentration of 1 µM acting as a chemo-attractant and 3 µm pore polycarbonate membrane filters were placed on top of the wells. Subsequently 25 µl of the PMN suspension (4 x 10^6/ml final concentration) was placed on top of the filters. The PMNs migrated across the filter pores towards the fMLP.

Following incubation, excess cells were removed by absorption using cotton buds and the surface of the filter was washed with 25 µl PBS per well and subsequently removed.

Centrifugation (312 x *g* for 1 minute) was used to pellet the cells. Subsequently the filter was removed and the cells were re-suspended. PrestoBlue® fluorescent dye (Life Technologies, U.K.) solution (30 µl of pre-diluted dye 1 in 5 in PBS) was mixed with 20 µl cells aliquots in quadruplicates in a 96-well plate. PrestoBlue® is a cell viability reagent which is modified by the reducing environment of the cell, turning a highly fluorescent red.

A standard curve was constructed using 0 to 4 x 10^6 PMNs and processed using the same method as above.

A NOVOstar plate reader (BMH LabTech, Ortenberg, Germany) was used to detect the fluorescence (Excitation filter: 530 and Emission filter: 590) and the results were analysed using the NOVOstar software package. The standard curve was used to interpolate the unknown values.

2.7 Statistical analysis

Data was expressed as mean ± standard deviation or mean ± standard error of the mean for normally distributed data. For mean comparisons two tailed unpaired Student's t tests were used. One or Two way ANOVA was used for comparison of

normally distributed data and the Bonferroni test was used for column comparisons. When data not normally distrusted, the data was represented as median. Mann-Whitney U, Kurskal-Wallis and Dunn's tests were used for comparisons of this data as appropriate. Comparison of proportions was performed using two-tailed Fisher's exact method.

In all cases a p value of < 0.05 was considered statistically significant to reject the null hypothesis.

GraphPad prism 4 for Macintosh (La Jolla, Ca, USA) was the software package used for statistical analysis.

Chapter 3: Microparticles characterisation in severe sepsis patients' plasma

3.1: Protocol optimisation

3.1.1 Preparation of plasma for microparticle characterisation

This project started by performing preliminary experiments to identify the optimum methodology for characterising microparticles (MP) in plasma. Literature review revealed that several centrifugation strategies were used to produce PFP from whole blood for the purpose of MP characterisation[288]. The most commonly used centrifugation protocol in studies similar to mine consisted of two steps, the first was centrifugation at 1500 x *g* for 15 minutes to produce platelet rich plasma (PRP) and then 13000 x *g* for 2 minutes to produce PFP [289, 290]. When this protocol was tested PFP stained with anti-CD66b (PE conjugated) there was a clear separation between stained sample and isotype controls (Figure 3.1). Therefore this differential centrifugation protocol was adopted for all subsequent experiments.

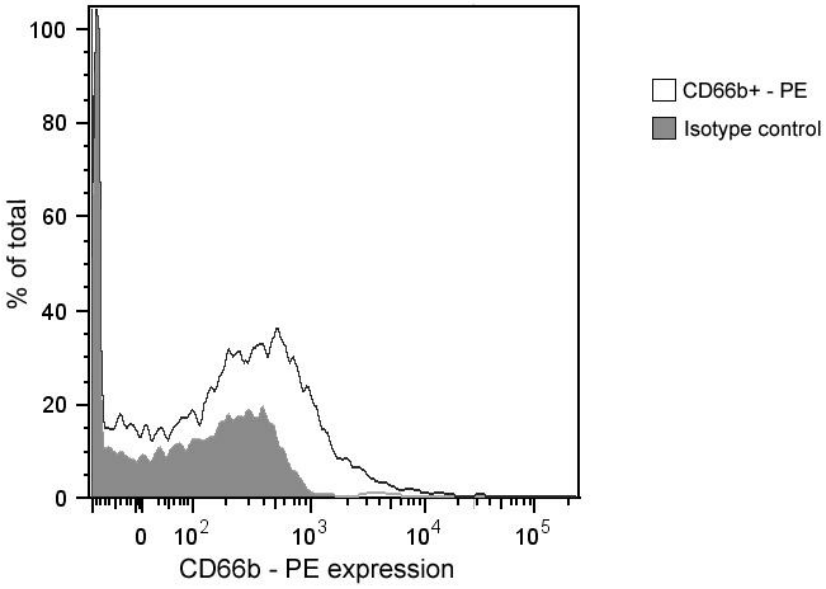


Figure 3.1. Representative flow-cytometry histogram of microparticles analysed in PFP of healthy volunteers produced through an optimized centrifugation protocol (1500 x *g* for 15 minutes followed by 13000 x *g* for 2 minutes). Microparticles were stained within PFP (5 µl) using Anti-CD66b PE (93.75 ng/ml) for 30 minutes at room temperature and analysed by flow-cytometry. The overlaid histograms of CD66b+ microparticles and isotype control show a clear separation using this centrifugation protocol.

3.1.2 Antibody Titration

Using blood from healthy volunteers, the optimum concentrations of each monoclonal antibody with relevant isotype controls were identified by titrations. Table 3.1 lists these antibodies whilst Figure 3.2 reports the data obtained from these analyses.

Monoclonal Antibody	Optimum concentration
Annexin V	200 ng/ml
Anti-CD66b	93.75 ng/ml
Anti-CD41	250 ng/ml
Anti-CD14	500 ng/ml
Anti-CD235	750 ng/ml
Anti-CD3	375 ng/ml
Anti-CD31	93.75 ng/ml
Anti-CD51	500 ng/ml
Anti-CD146	375 ng/ml
Anti-alpha-2-macrgolobulin	500 ng/ml
Anti-LAIR-1	90 ng/ml
Anti-Annexin A7	187.5 ng/ml

Table 3.1. Optimum concentrations of the monoclonal antibodies employed for microparticle detection. LAIR1: Leucocyte associated immunoglobulin like receptor-1.

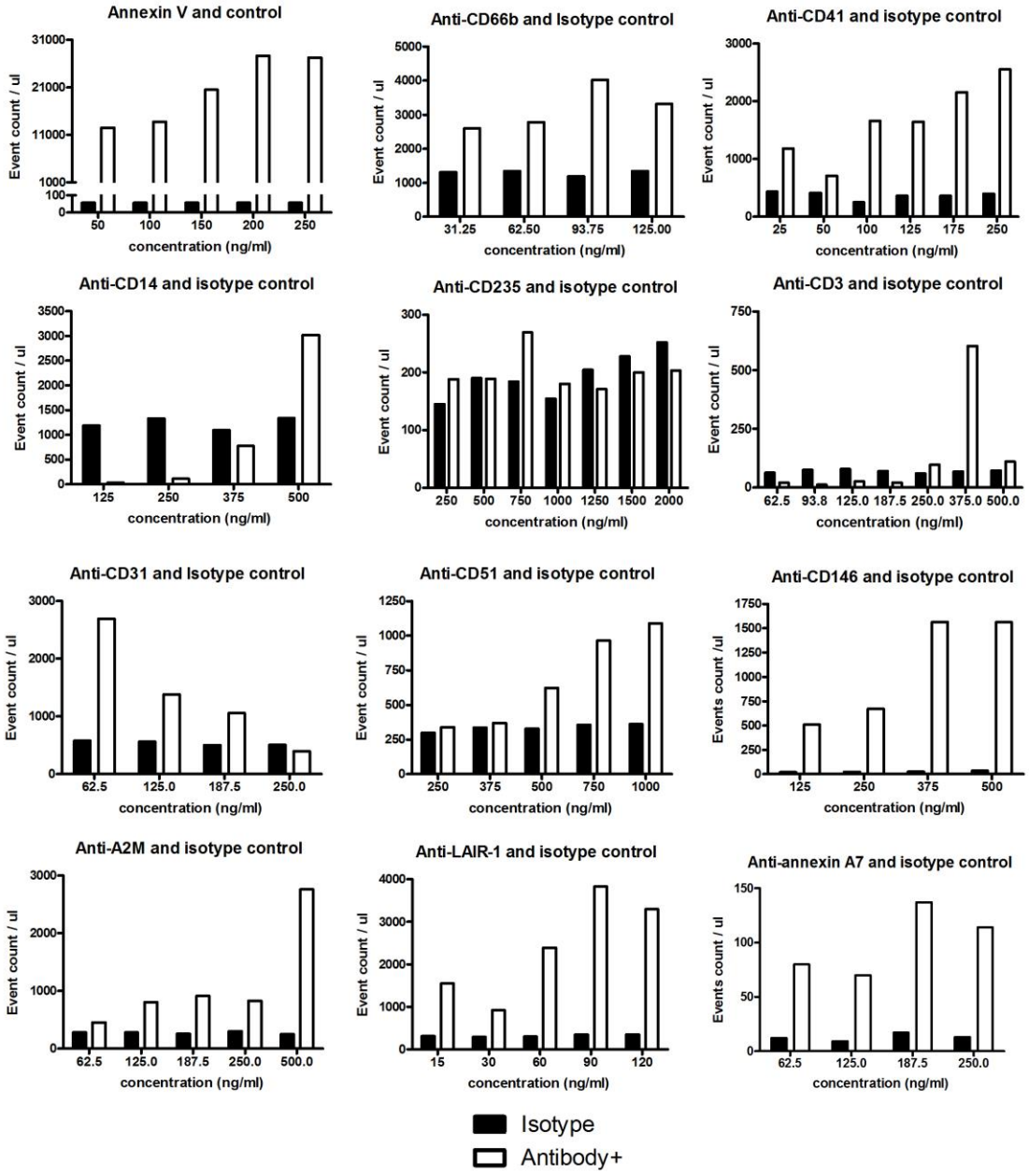


Figure 3.2. Antibody titrations for the various monoclonal antibodies used for microparticle detection. Platelet free plasma from healthy volunteers was stained using increasing concentrations of the respective antibody and its isotype control. Data is expressed as microparticle count per microliter of plasma. A2M: Alpha-2-macroglobulin, LAIR1: Leucocyte associated immunoglobulin like receptor 1.

3.1.3 Optimisation of annexin V binding to microparticles

Calcium is required for annexin V binding to phosphatidylserine. The advice of the

manufacturer is to use buffer containing calcium (2.5 mM), but this advice relates to binding cells rather than MP. Therefore a literature review was performed, which revealed that annexin V binding to MP is enhanced by increasing calcium concentrations, noting also how the medium composition may interfere with binding[291, 292]. In preliminary experiments three different buffers containing 2.5mM Calcium were tested, two of which were prepared locally and one of which was commercially available. I could not detect any significant difference between the three buffers in the annexin V positive events counted (Table 3.2).

Medium	Annexin V+ events
PBS + $CaCl_2$ 2.5mM	461 / µl
Hanks + $CaCl_2$ 2.5mM	472 / µl
Commercial annexin V binding buffer	458 / µl

Table 3.2. Various media and commercial Annexin V binding buffers showing no significant difference in the number of Annexin V positive events. Platelet free plasma from healthy volunteers was used for this experiment. Annexin V was used for staining at 200 ng/ml final concentration and events were detected using flow-cytometry. Data are expressed as micropartricle count per microliter of platelet free plasma. PBS: Phosphate buffered saline solution.

In the next series of preliminary experiments the commercially available annexin V buffer (10x) containing 25 mM calcium was used to test if increasing the calcium concentration in the medium would increase annexin V binding. The following concentration of calcium: 2.5, 5, 15, 20 and 25mM were tested. The results showed that higher calcium concentration in the buffer enhanced the total count of annexin V positive events (Table 3.3).

Calcium concentration	Annexin V+ events
2.5 mM	458 / µl
5 mM	399 / µl
10 mM	1584 / µl
15 mM	15762 / µl
20 mM	16213 / µl
25 mM	16817 / µl

Table 3.3. Titration of calcium concentration and the resulting annexin V positive events demonstrating an increase in count with increasing calcium concentration. Platelet free plasma from healthy volunteers was used for this experiment. Annexin V was used for staining at 200ng/ml final concentration and events were detected using flow-cytometry. Data is expressed as microparticle count per microliter of platelet free plasma.

3.1.4 Effect of anticoagulant on annexin V binding

The anticoagulant used when the blood is collected could interfere with the positive effect of calcium on annexin V. Heparin has been previously found to bind and inhibit the uptake of MP[293] and thus its use as an anticoagulant in MP studies is discouraged by the international society for extra-cellular vesicles[294]. In preliminary experiments the effect of the three most commonly used anticoagulants (sodium citrate, heparin and ethylenediaminetetraacetic acid (EDTA)) were tested on the number of annexin V events detected in healthy volunteers' plasma (Figure 3.3). Interestingly, only heparin seemed to suppress annexin V binding to MP even though, unlike sodium citrate and EDTA heparin is not known to bind to calcium.

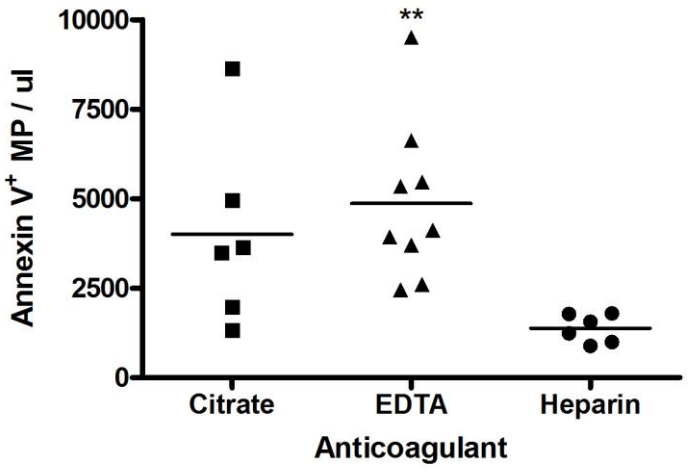


Figure 3.3. The effect of commonly used anticoagulants on annexin V binding to microparticles. Blood from healthy volunteers (n=21) was anticoagulated using 3.2% sodium citrate, 15% ethylenediaminetetra acetic (EDTA) or heparin (17 units/ml). Platelet free plasma was was incubated with annexin V for 30 minutes at room temperature. Annexin V positive microparticles were counted using flow-cytometry. Data is expressed as microparticle count per microliter of platelet free plasma. Data compared using Kurskal-Wallis test and Dunn's comparisons. **: $p < 0.01$

3.2 Characterisation of microparticles in plasma from patient with severe sepsis

3.2.1 Patient characteristics

One hundred patients recruited to the Genomic Advances in Sepsis (GAinS) study with severe sepsis due to community-acquired pneumonia (CAP) or faecal peritonitis (FP) were included in this study. By selection, half of each cohort survived beyond 28 days.

The baseline demographic characteristics, comorbidities, severity scores and cell counts on admission to intensive care unit were comparable between both patients' cohorts (Table 3.4). Healthy volunteers (HV) were significantly younger than both patient groups and their PMN count was significantly lower, while the HV lymphocytes were significantly higher in HV than in both patient groups.

When comparing CAP non-survivors to survivors of the CAP cohort, the non-survivors were older and had higher severity scores on admission (Table 3.5). Similarly, the FP non-survivors were also significantly older and had higher severity scores compared to FP survivors (Table 3.6).

	Community acquired pneumonia (n=60)	Faecal Peritonitis (n=40)	Healthy volunteers
Age in years mean ± SD	64.4 ± 16.3	68.6 ± 17.2	40.2 ± 14.7 ***
Gender F/M ratio	25/35	20/20	5/5
Comorbidities			
Cardiovascular			
1. Angina, n (%)	5 (8.3%)	4 (10%)	
2. Arrhythmia, n (%)	5 (8.3%)	4 (10%)	
3. Valvular, n (%)	1 (1.7%)	2 (5%)	
4. MI, n (%)	6 (10%)	0	
5. CHF, n (%)	4 (6.7%)	1 (2.5%)	
6. PVD, n (%)	0	2 (5%)	
Respiratory			
7. Asthma, n (%)	5 (8.3%)	2 (5%)	
8. COPD, n (%)	16 (26.7%)	6 (15%)	
Neurological			
9. Stroke, n (%)	2 (3.3%)	3 (7.5%)	
10. Dementia, n (%)	0	0	
Rheumatological			
11. RA, n (%)	3 (5%)	0	
12. SLE, n (%)	0	0	
Gastrointestinal, n (%)	4 (6.7%)	5 (12.5%)	
Diabetes Mellitus, n (%)	11 (18.3%)	9 (22.5%)	
Renal, n (%)	4 (6.7%)	3 (7.5%)	
Malignancy, n (%)	6 (10%)	9 (22.5%)	
Causative organism			
1. S. pneumoniae, n	14		
2. S. aureus, n	2		
3. H. influenza, n	3		
4. E. coli, n	1		
5. Legionella, n	2		
6. Viral, n	2		
7. Unknown, n	36		
Severity score on admission mean ± SD			
1. APACHE II	18 ± 5.8	17 ± 6.8	
2. SOFA	8 ± 4	9 ± 4.3	
Cell count on admission mean ± SD			
1. PMN x 10^9/L	12.1 ± 6.6	9.6 ± 7.4	3.2 ± 1.3 ***
2. Monocytes x 10^9/L	0.63 ± 0.52	0.54 ± 0.55	0.85 ± 0.68
3. Lymphocytes x 10^9/L	0.76 ± 0.54	0.75 ± 0.47	1.36 ± 0.68 **
4. Platelets x 10^9/L	205 ± 119.7	211 ± 128.6	238 ± 60.4

Table 3.4. Baseline patient parameters on admission to intensive care. Continuous variable were expressed as mean ± standard deviation (SD) and two-tail unpaired Student t test or one-way ANOVA was used to compare the means. Quantities are expressed as ratio or percentage and compared using Fischer's exact method. Differences are considered statistically significant when $p < 0.05$. APACHE II: acute physiological and chronic health evaluation II, COPD: Chronic obstructive pulmonary disease, CHF: congestive heart failure, E. coli: Escherichia coli, H. influenza: Haemophilus influenza, MI: myocardial infarction, NS: not significant, PMN: polymorph-nuclear leucocytes, PVD: peripheral vascular disease, RA: rheumatoid arthritis, S. aureus: staphylococcus aureus, S. pneumonia: streptococcal pneumoniae, SLE: systemic lupus erythematosus. $P < 0.05$ was considered statistically significant. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

	Non-survivors of community acquired pneumonia (n=30)	Survivors of community acquired pneumonia (n=30)
Age in years mean ± SD	70.26 ± 14.07 *	58.3 ± 16.74
Gender F/M ratio	9/21	16/14
Comorbidities		
Cardiovascular		
5. Angina, n (%)	3 (10%)	2 (6.7%)
6. Arrhythmia, n (%)	5 (16.7%)	0
7. Valvular, n (%)	1 (3.3%)	0
8. MI, n (%)	3 (10%)	3 (10%)
9. CHF, n (%)	3 (10%)	1 (3.3%)
10. PVD, n (%)	0	0
Respiratory		
11. Asthma, n (%)	5 (16.7%)	11 (36.7%)
12. COPD, n (%)	0	5 (16.7%)
Neurological		
13. Stroke, n (%)	2 (6.7%)	0
14. Dementia, n (%)	0	0
Rheumatological		
15. RA, n (%)	1 (3.3%)	2 (6.7%)
16. SLE, n (%)	0	0
Gastrointestinal, n (%)	1 (3.3%)	3 (10%)
Diabetes Mellitus, n (%)	6 (20%)	5 (16.7%)
Renal, n (%)	2 (6.7%)	2 (6.7%)
Malignancy, n (%)	2 (6.7%)	4 (13.3%)
Causative organism		
17. S. pneumoniae, n	10 (33.3%)	4 (13.3%)
18. S. aureus, n	2 (6.7%)	0
19. H. influenza, n	0	3 (10%)
20. E. coli, n	1 (3.3%)	0
21. Legionella, n	0	2 (6.7%)
22. Viral, n	2 (6.7%)	0
23. Unknown, n	15 (50%)	21 (70%)
Severity score on admission mean ± SD		
24. APACHE II	21 ± 6.37	16.43 ± 4.2 **
25. SOFA	9.19 ± 4.24	6.93 ± 3.47 *
Cell count on admission mean ± SD		
26. PMN x 10^9/L	10.23 ± 6.9	13.61 ± 6.0
27. Monocytes x 10^9/L	0.54 ± 0.42	0.71 ± 0.59
28. Lymphocytes x 10^9/L	0.68 ± 0.54	0.96 ± 0.57
29. Platelets x 10^9/L	198.5 ± 137.1	212.5 ± 100.6

Table 3.5. Baseline community acquired pneumonia patient (non-survivors and survivors) demographics on admission to intensive care. Continuous variables are expressed as mean ± standard deviation (SD) and two-tail unpaired Student t test is used to compare the means. Quantities are expressed as ratio or percentage and compared using Fischer's exact method. Differences are considered statistically significant when $p < 0.05$. APACHE II: acute physiological and chronic health evaluation II, COPD: Chronic obstructive pulmonary disease, CHF: congestive heart failure, E. coli: Escherichia coli, H. influenza: haemophilus influenza, MI: myocardial infarction, NS: not significant, PMN: peripheral mononuclear leucocytes, PVD: peripheral vascular disease, RA: rheumatoid arthritis, S. aureus: staphylococcal aureus, S. pneumonia: streptococcus pneumoniae, SLE: systemic lupus erythematosus. $P < 0.05$ was considered statistically significant. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

	Non-survivors of faecal peritonitis	Survivors of faecal peritonitis
Age in years mean ± SD	74.4 ± 15.77 *	62.7 ± 16.92
Gender F/M ratio	9/11	11/9
Comorbidities		
Cardiovascular		
30. Angina, n (%)	3 (7.5%)	1 (2.5%)
31. Arrhythmia, n (%)	2 (5%)	2 (5%)
32. Valvular, n (%)	2 (5%)	0
33. MI, n (%)	0	0
34. CHF, n (%)	1 (2.5%)	0
35. PVD, n (%)	0	2 (5)
Respiratory		
36. Asthma, n (%)	1 (2.5%)	1 (2.5%)
37. COPD, n (%)	2 (5%)	4 (10%)
Neurological		
38. Stroke, n (%)	2 (5%)	1 (2.5%)
39. Dementia, n (%)	0	0
Rheumatological		
40. RA, n (%)	0	0
41. SLE, n (%)	0	0
Gastrointestinal, n (%)	4 (10%)	1 (2.5)
Diabetes Mellitus, n (%)	4 (10%)	4 (10%)
Renal, n (%)	0	3 (7.5%)
Malignancy, n (%)	6 (15%)	3 (7.5%)
Severity score on admission mean ± SD		
42. APACHE II	21.53 ± 7.13	13.8 ± 3.72 ***
43. SOFA	9.4 ± 3.25	4.9 ± 2.88 ***
Cell count on admission mean ± SD		
44. PMN x 10^9/L	8.64 ± 8.17	10.59 ± 6.51
45. Monocytes x 10^9/L	0.48 ± 0.63	0.61 ± 0.47
46. Lymphocytes x 10^9/L	0.69 ± 0.42	0.81 ± 0.52
47. Platelets x 10^9/L	172.2 ± 99	250.2 ± 144.6

Table 3.6. Baseline faecal peritonitis patient (non-survivors and survivors) demographics on admission to intensive care. Continuous variables are expressed as mean ± standard deviation (SD) and two-tail unpaired Student t test is used to compare the means. Quantities are expressed as ratio or percentage and compared using Fischer's exact method. Differences are considered statistically significant when $p < 0.05$. APACHE II: acute physiological and chronic health evaluation II, COPD: Chronic obstructive pulmonary disease, CHF: congestive heart failure, MI: myocardial infarction, NS: not significant, PMN: peripheral mononuclear leucocytes, PVD: peripheral vascular disease, RA: rheumatoid arthritis, SLE: systemic lupus erythematosus. $P < 0.05$ was considered statistically significant. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

3.2.2 Effect of severe sepsis on plasma microparticles at day 1 post admission to ICU

MP were measured in platelet free plasma of all three cohorts (CAP, FP and HV). The plasma was collected on day 1 of admission to intensive care and the MP were characterised according to their cell of origin. Following quantification of the MP

according to their cells of origin using reliable markers, secondary staining was also performed to attempt further stratification. Thus, MP derived from blood cells were further characterised according to their expression of the protein alpha-2-macroglobulin (A2M) as we have previously in found in a small cohort of CAP severe sepsis patients that plasma levels of granulocyte derived MP expressing A2M were higher in survivors[191]. Here a larger cohort of CAP patients was examined and compared to another cohort of patients with severe sepsis secondary to FP as well as to HV. Also, the profiles of MP expressing A2M derived from other blood cells (monocytes, lymphocyte, platelets and erythrocytes) was investigated as well as examining the total population of A2M MP from all sources.

Even though there was no significant difference in granulocyte counts between the three cohorts, granulocyte MP (620±101/µl) were significantly higher in CAP compared to FP (34±7/µl) and HV (11±3/µl) with $p < 0.001$. Monocyte and lymphocyte count was observed to be lower in CAP and FP compared to HV, despite that MP number derived from these two cell lines was significantly higher in CAP (112±67/µl, 130±42/µl) compared to the other two cohorts ($p < 0.001$), while FP (9±8/µl, 46±7/µl) numbers were not significantly different in comparison to HV (3±1/µl, 83±14/µl) with p of < 0.001. Platelet and erythrocyte MP numbers did not vary significantly between the cohorts, but there was trend towards higher numbers in CAP (2370±1473/µl, 249±369/µl) and FP (1020±955/µl, 160±40/µl) in comparison to HV (2027±486/µl, 155±39/µl). MP derived from endothelial cells was significantly elevated in CAP patients (142±121/µl) compared to HV (52±5/µl), while in FP there

was a trend towards higher count (103±22/µl) but it did not reach significance. There was some overlap between CAP and FP in the endothelial MP numbers (Figure 3.4). This would suggest that MP numbers does not directly correlate to the number of their cell of origin but perhaps to these cells state of activation. It was also observed that, even though monocytes and lymphocytes were lower in number, they produced more MP relative to their number (Table 3.4) compared to granulocytes suggesting that monocytes and lymphocytes were more efficient in producing MP.

MP were then further characterised according not only to their cell of origin and but also according to their A2M expression. CAP had a significantly higher number of $A2M^+$ MP (371±137/µl) from all sources compared to FP (90±22/µl) and HV (107±17/µl) with p value < 0.01 and < 0.001 respectively. There was some overlap between CAP and FP in the total $A2M^+$ MP numbers despite the significant difference. The profile observed in granulocyte, monocyte and lymphocyte MP was replicated here where the $A2M^+$ MP derived from these cells was significantly higher in CAP (11±10/µl, 9±21/µl, 35±48/µl) compared to the other FP (3±1/µl, 1±1/µl, 1±6/µl) and HV (3±1/µl, 0±0/µl, 0±1/µl) despite some overlap. Interestingly, $A2M^+$ MP derived from platelets and erythrocyte were also significantly higher in CAP (62±22/µl, 54 ±46/µl) compared to FP (9±3/µl, 5±13/µl) and HV (8±2/µl, 4±2/µl) despite the lack of significant difference between MP subsets (positive and negative for A2M) derived from these cells (Figure 3.5). Also, $A2M^+$ MP number from all sources was higher than cell derived $A2M^+$ MP characterised here suggesting that $A2M^+$ MP were derived from sources other the cell sources described here.

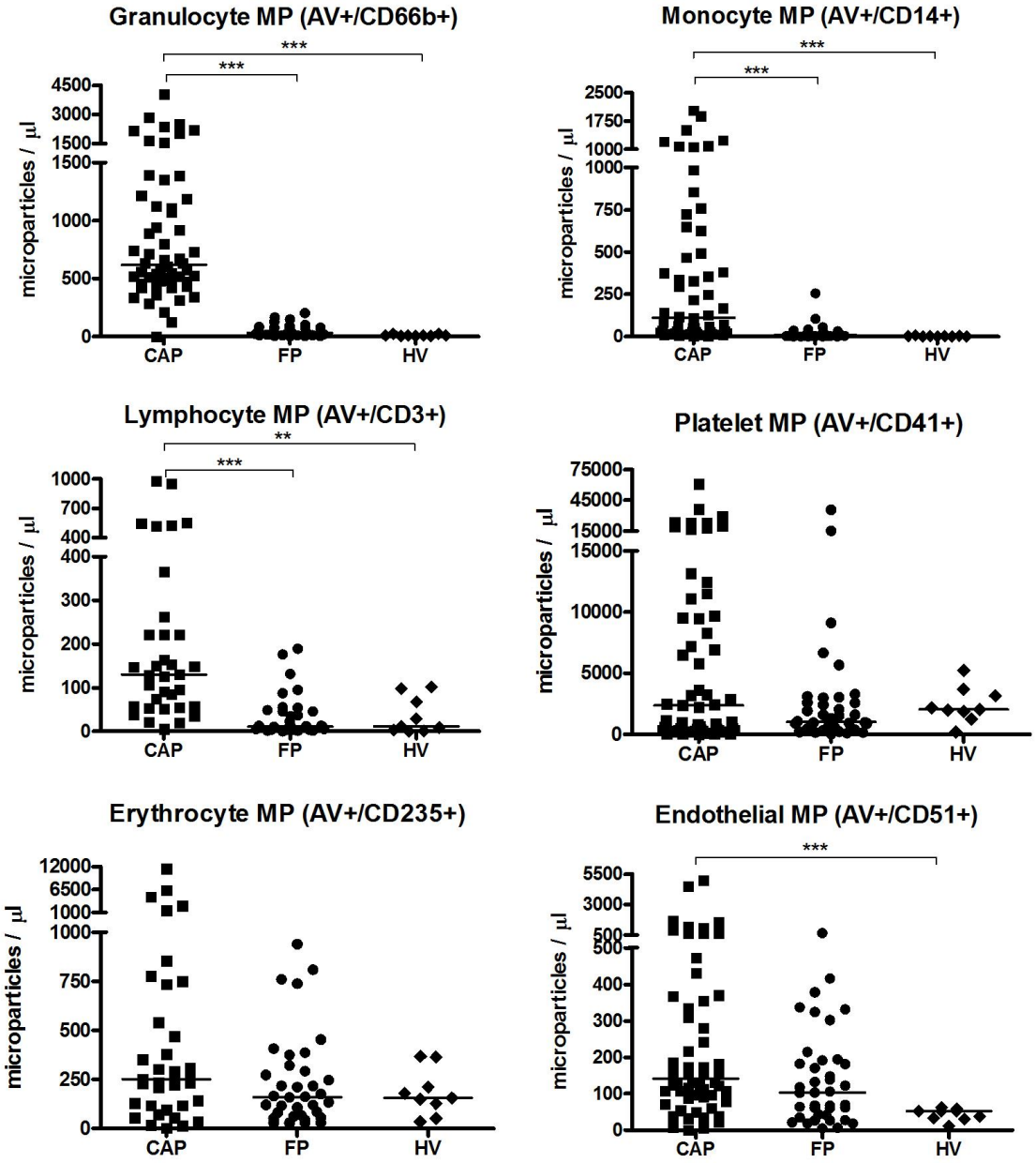


Figure 3.4. Microparticle subsets in patient with severe sepsis secondary to community acquired pneumonia or faecal peritonitis and in healthy volunteers at day 1 post admission to ICU. Platelet free plasma from all three cohorts was obtained by differential centrifugation of whole blood. The plasma (5 µL per test) was incubated with cell specific fluorochrome conjugated monoclonal antibodies as indicated and annexin V for half an hour at room temperature and the reaction was stopped by adding binding buffer up to a volume of 200 µL. The samples were subsequently acquired using flow-cytometry. Kruskal-Wallis and Dunn's tests were used for comparisons. AV: annexin V, CAP: community acquired pneumonia, FP: faecal peritonitis, HV: healthy volunteers, MP: microparticles. *: $p < 0.05$, **: $p< 0.01$. ***: $p < 0.001$.

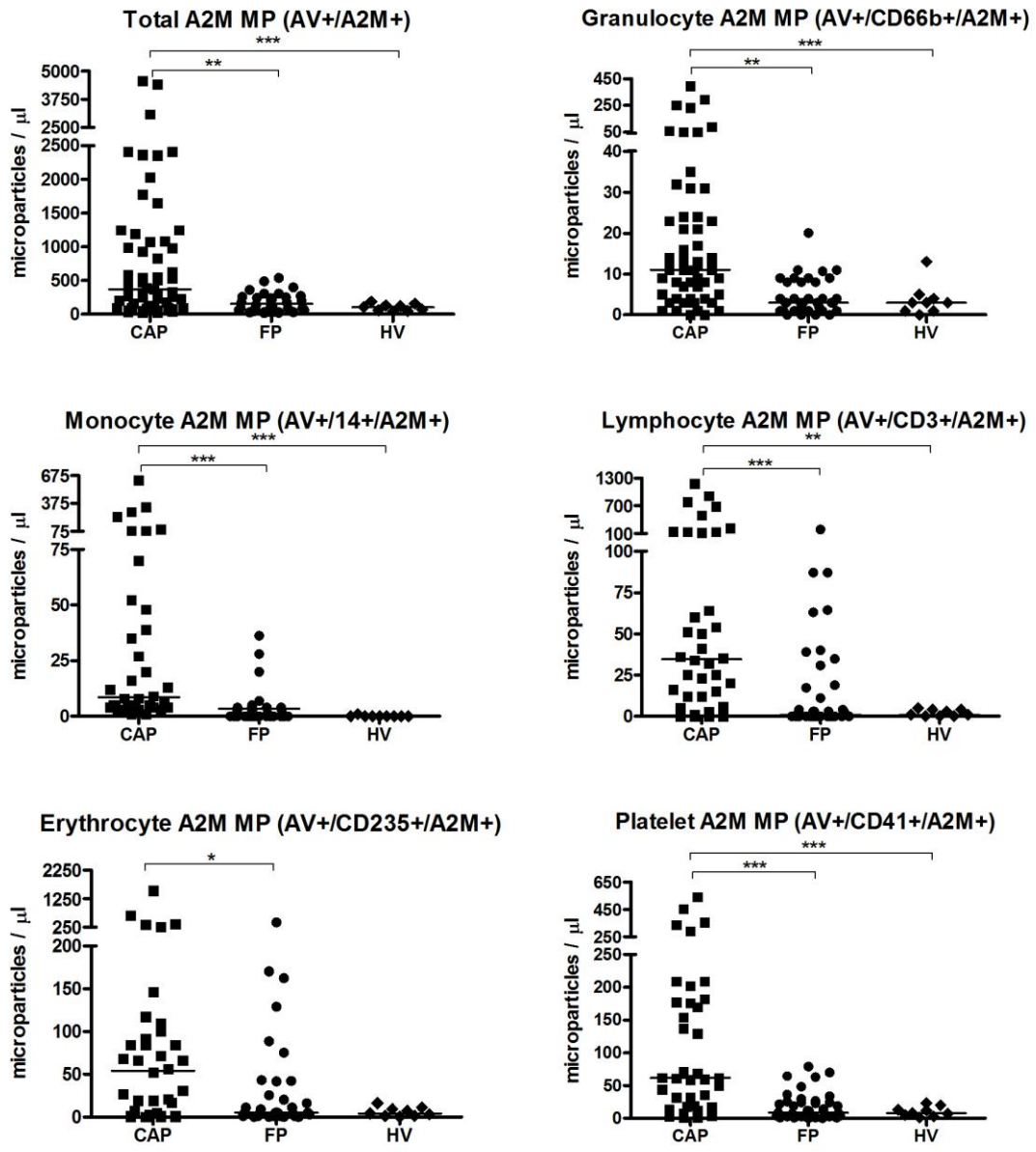


Figure 3.5. Number of alpha-2-macroglobulin positive microparticles in patients severe sepsis secondary to community acquired pneumonia or faecal peritonitis and in healthy volunteers at day 1 post admission to ICU. Platelet free plasma from all three cohorts was obtained by differential centrifugation of whole blood. The plasma (5 µL per test) was incubated with anti alpha-2-macroglobulin monoclonal antibody with or without cell specific fluorochrome conjugated monoclonal antibodies as indicated and annexin V for half an hour at room temperature. The reaction was stopped by adding binding buffer up to a volume of 200 µL. The samples were then acquired using flow-cytometry. The results are expressed as microparticles count / µL of platelet free plasma. Kruskal-Wallis and Dunn's tests were used for comparison. A2M: alpha-2-macroglobulin, AV: annexin V, CAP: community acquired pneumonia, FP: faecal peritonitis, HV: healthy volunteers, MP: microparticles. *: $p < 0.05$, **: $p < 0.01$. ***: $p < 0.001$.

3.2.3 Association between plasma microparticle subsets at day 1 post admission to ICU and mortality in severe sepsis secondary to community acquired pneumonia

Next the CAP cohort was divided into non-survivors and survivors; patients were considered survivors when they were alive at 28 days post admission to ICU. Then cell derived MP subsets of these two cohorts were compared. Granulocyte MP was higher in survivors (694±120/μl) compared to non-survivors (528±122/μl) despite no difference in their cell of origin (Table 3.5), but this trend did not reach significance. On the other hand, endothelial MP (AV+/CD51+) was found to be significantly higher in non-survivors (173±203/μl) compared to survivors (113±144/μl) with a $p < 0.05$. The comparison revealed no significant difference in the other MP subsets (Figure 3.6).

The number of $A2M^+$ MP from all sources was significantly higher in survivors (541±213/μl) compared to non-survivors (254±148/μl) with a $p < 0.05$. Granulocyte and monocyte derived $A2M^+$ MP were also significantly higher in survivors (14±17/μl, 37±35/μl) compared to non-survivors (6±9/μl, 5±4/μl) with p values < 0.01 and < 0.01 respectively. There was no difference in the number of MPs positive for A2M derived from lymphocytes, platelets or erythrocytes between survivors (16±57/μl, 62±20/μl, 27±72/μl) and non-survivors (51±84/μl, 61±36/μl, 66±45/μl) (Figure 3.7).

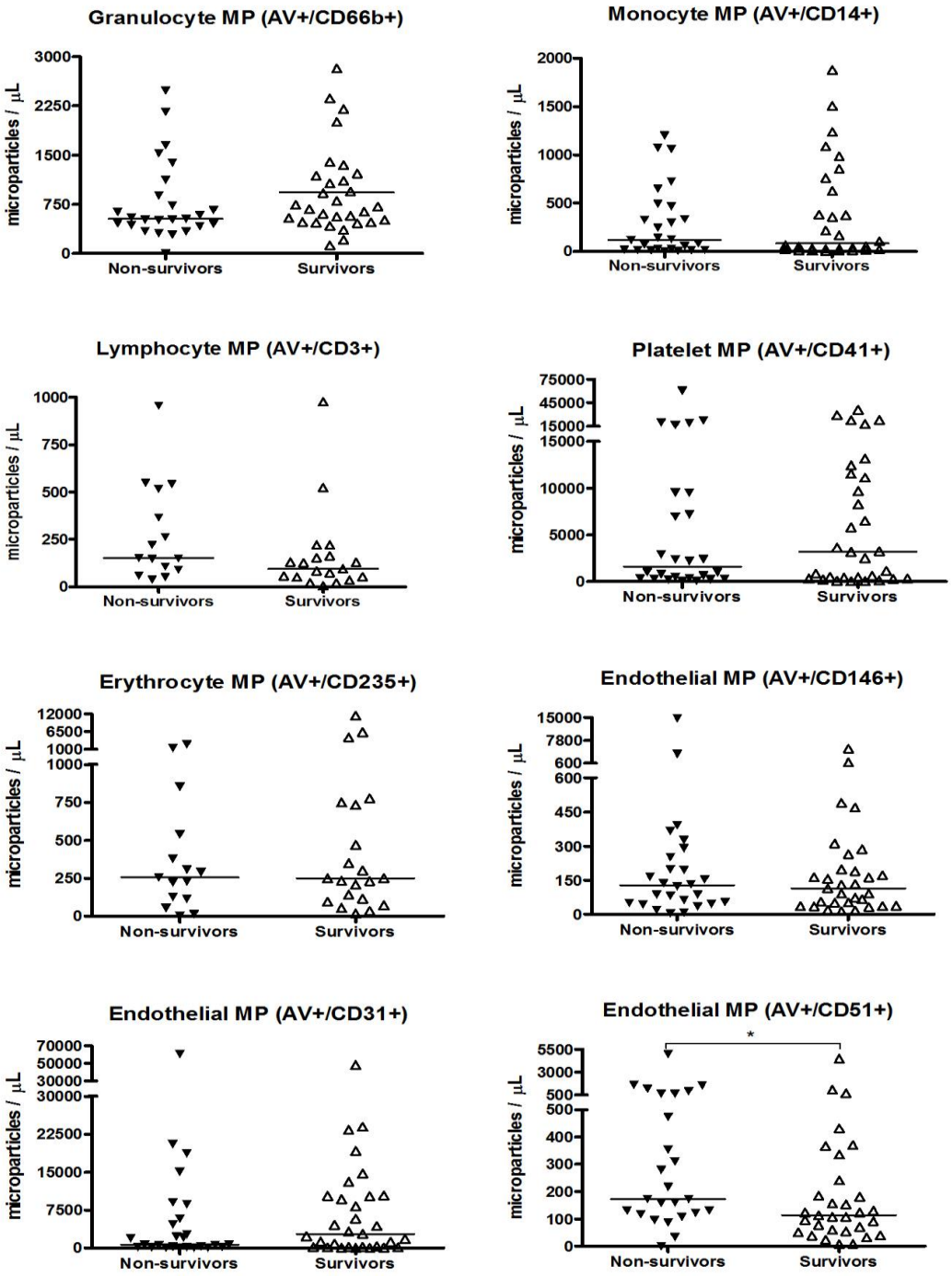


Figure 3.6. Microparticle subsets in patient in with severe sepsis secondary to community acquired pneumonia 28 day non-survivors compared to survivors at day 1 post admission to ICU. Platelet free plasma from the two cohorts was obtained by differential centrifugation of whole blood. The plasma (5 µL) was incubated with cell specific fluorochrome conjugated monoclonal antibodies as indicated and annexin V for half an hour at room temperature. The reaction was stopped by adding binding buffer up to a volume of 200 µL. The samples were subsequently acquired using flow-cytometry. The results are expressed as microparticle count/µL of platelet free plasma. Mann Whitney test was used for comparisons. AV: annexin V, MP: microparticles. *: $p < 0.05$.

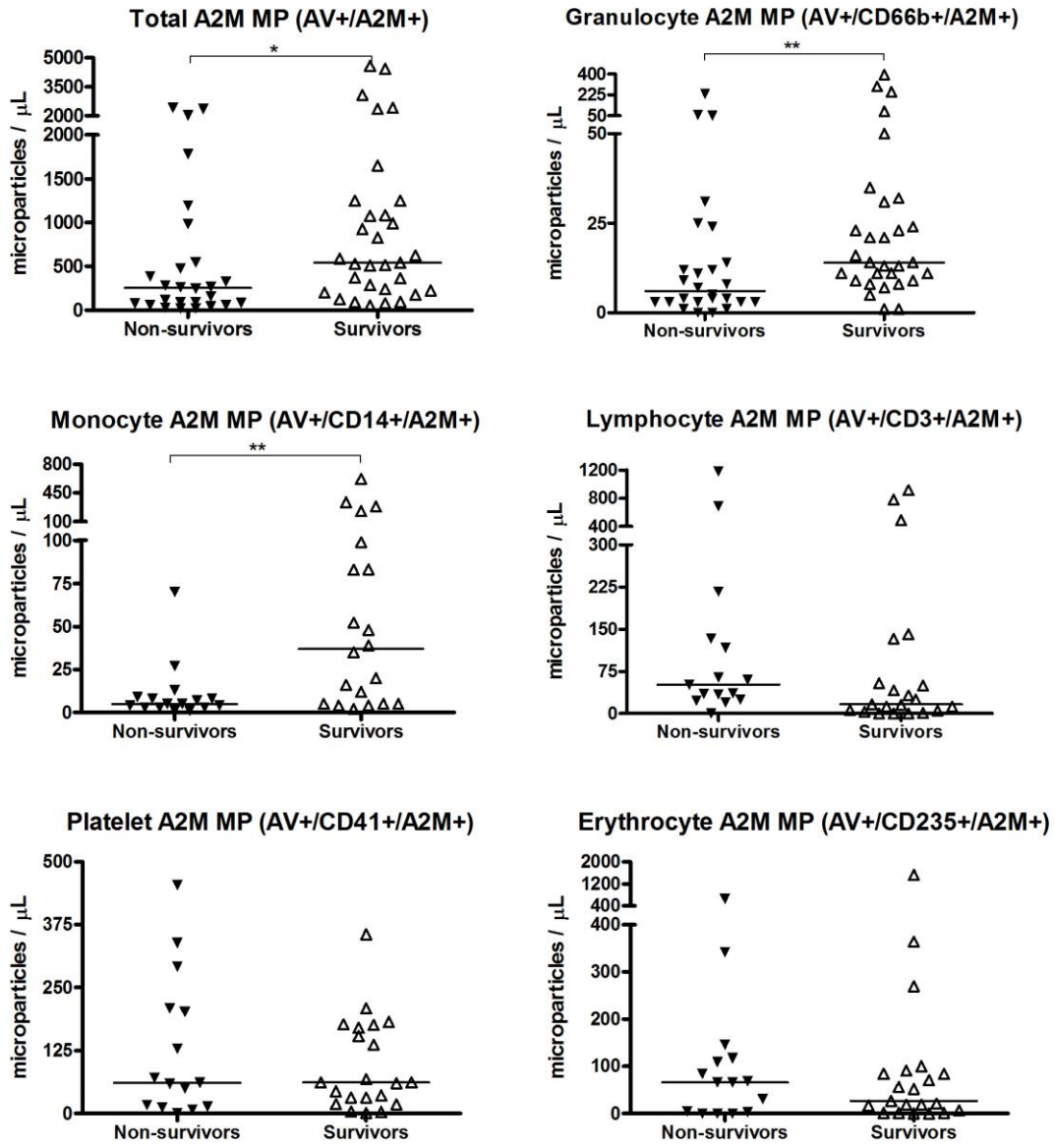


Figure 3.7. Number of alpha-2-macroglobulin positive microparticles in patients with severe sepsis secondary to community acquired pneumonia 28 day non-survivors compared to survivors at day 1 post admission to ICU. Platelet free plasma from the two cohorts was obtained by differential centrifugation of whole blood. The plasma (5 µL per test) was incubated with cell specific fluorochrome conjugated monoclonal antibodies as indicated and annexin V for half an hour at room temperature. The reaction was stopped by adding binding buffer up to a volume of 200 µL. The samples were subsequently acquired using flow-cytometry. The results are expressed as microparticles count/µL of platelet free plasma. Mann Whitney test was used for comparisons. A2M: alpha-2-macroglobulin, AV: annexin V, MP: microparticles. *: $p < 0.05$, **: $p < 0.01$.

3.2.4 Association between plasma microparticle subsets at day 1 post admission to ICU and outcome in severe sepsis secondary to faecal peritonitis

Patients who did not survive severe sepsis secondary to FP had significantly higher levels of granulocyte MP (81±28/μl) in comparison to those who survived the disease (28±9/μl) with a $p < 0.01$ (Figure 3.8). Interestingly, erythrocyte derived MP was also significantly higher in non-survivors (231±58/μl) compared to survivors (107±55/μl) with a $p < 0.05$. Granulocyte count did not vary between both cohorts (Table 3.6). Erythrocyte $A2M^+$ MP was significantly higher in non-survivors (10±11/μl) compared to survivors (2±11/μl) with a $p < 0.05$. Platelet derived $A2M^+$ MP showed a trend towards a higher count in non-survivors, but this did not reach statistical significance (Figure 3.9). Here also, there was no difference between cells of origin between FP severe sepsis non-survivors and survivors (Table 3.6).

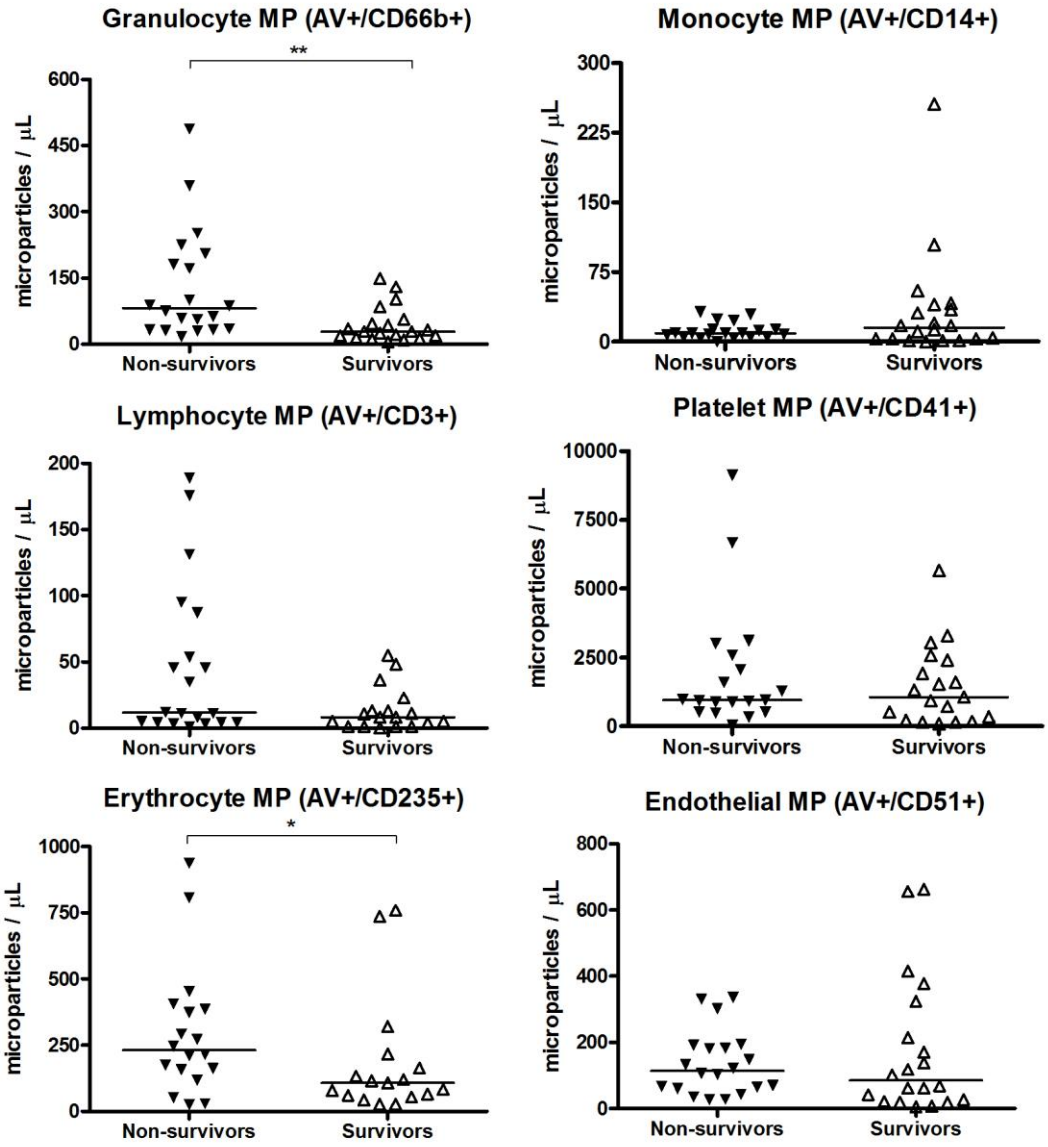


Figure 3.8. Microparticle subsets in patient in with severe sepsis secondary to faecal peritonitis 28 day non-survivors compared to survivors at day 1 post admission to ICU. Platelet free plasma from the two cohorts was obtained by differential centrifugation of whole blood. The plasma (5 µL) was incubated with cell specific fluorochrome conjugated monoclonal antibodies as indicated and annexin V for half an hour at room temperature. The reaction was stopped by adding binding buffer up to a volume of 200 µL. The samples were subsequently acquired using flow-cytometry. The results are expressed as microparticle count/µL of platelet free plasma. Mann Whitney test was used for comparisons. AV: annexin V, MP: microparticles. *: $p < 0.05$, **: $p < 0.01$.

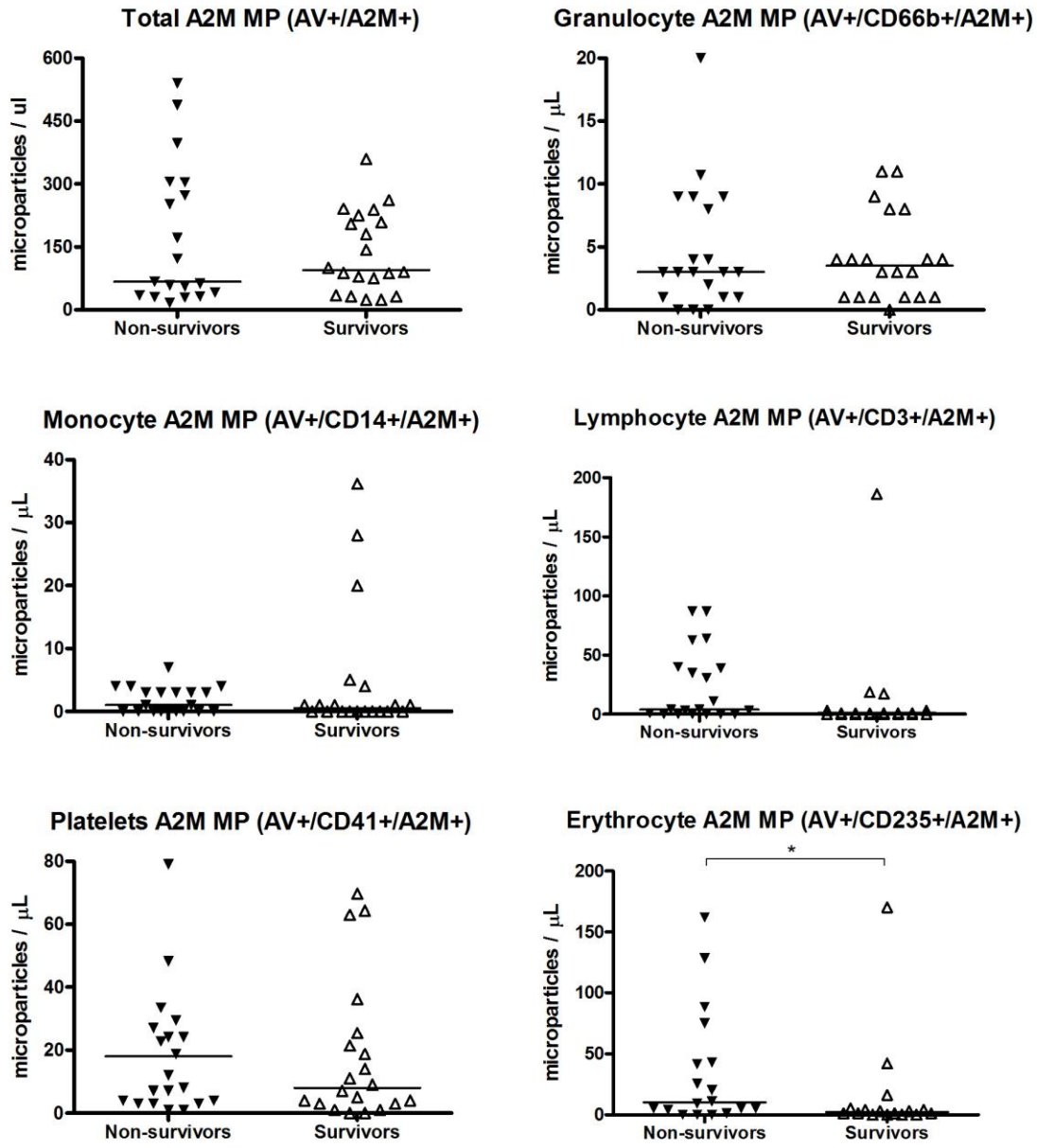


Figure 3.9. Number of alpha-2-macroglobulin positive microparticles in patients with severe sepsis secondary to faecal peritonitis 28 day non-survivors compared to survivors at day 1 post admission to ICU. Platelet free plasma from the two cohorts was obtained by differential centrifugation of whole blood. The plasma (5 µL per test) was incubated with cell specific fluorochrome conjugated monoclonal antibodies as indicated and annexin V for half an hour at room temperature. The reaction was stopped by adding binding buffer up to a volume of 200 µL. The samples were subsequently acquired using flow-cytometry. The results are expressed as microparticles count/µL of platelet free plasma. Mann Whitney test was used for comparisons. A2M: alpha-2-macroglobulin, AV: annexin V, MP: microparticles. *: $p < 0.05$.

3.2.5 Sequential changes in plasma microparticle subsets in patients with severe sepsis secondary to community acquired pneumonia; comparison between 28 day non-survivors and survivors

Subsequently, sequential changes in cell derived MP concentrations in non-survivors and survivors of severe sepsis secondary to community acquired pneumonia were examined. The results initially did not show clear trends, but when the numbers were log transformed clearer trends started to emerge. At day 1, survivors had a significantly higher granulocyte MP count a difference which persisted at day 3 and day 5. Although, the non-survivors had a higher lymphocyte MP count at day 1, this difference did not persist over the subsequent two time points. One subset of endothelial MP (AV+/CD51+) was higher in non-survivors at day 1 and day 5, although at day 3 this subset of MP was almost identical between the two cohorts (Figure 3.10).

When MP were further characterized according to alpha-2-macroglobulin expression a clearer picture emerged. Survivors had a higher count of total alpha-2-macroglobulin MP, as well as granulocyte and monocyte MP expressing A2M across all the time points, while the other MP subsets did not vary between the two cohorts at any time point (Figure 3.11).

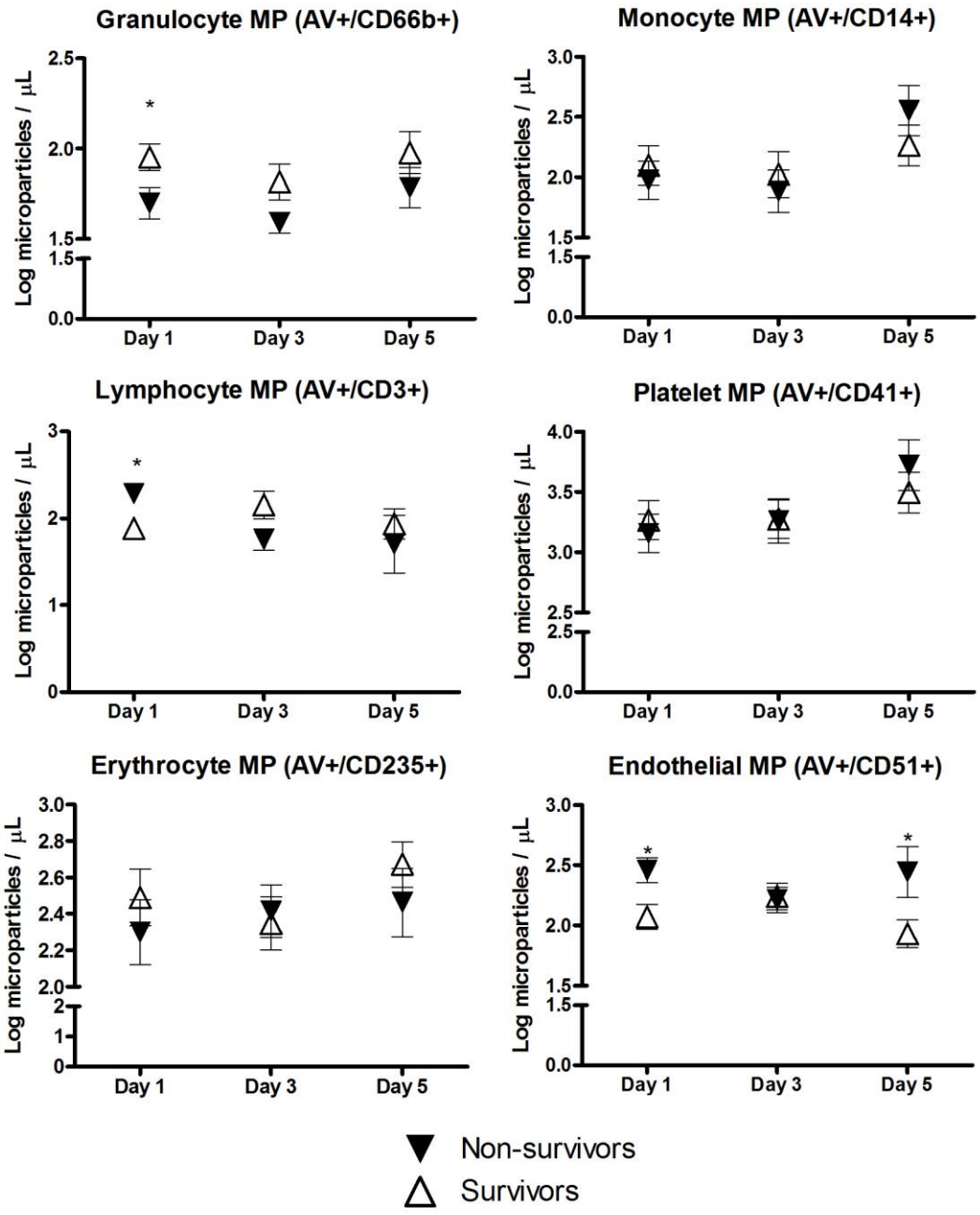


Figure 3.10. Sequential changes in microparticle subsets in patients with severe sepsis secondary to community acquired pneumonia; comparison between 28 day non-survivors and survivors. Platelet free plasma from the two cohorts was obtained by differential centrifugation of whole blood collected at day 1, day 3 and day 5 post admission to ICU**.** The plasma (5 µL per test) was incubated with cell specific fluorochrome conjugated monoclonal antibodies as indicated and annexin V for half an hour at room temperature and the reaction was stopped by adding binding buffer up to a volume of 200 µL (n=60). Non-survivors: Day 1 (n=30), Day 3 (n=25), Day 5 (n=13), Survivors Day 1, 3, 5 (n=30). The samples were subsequently acquired using flow-cytometry. The results were log transformed and compared using unpaired t test. AV: annexin V, MP: microparticles, *: $p < 0.05$.

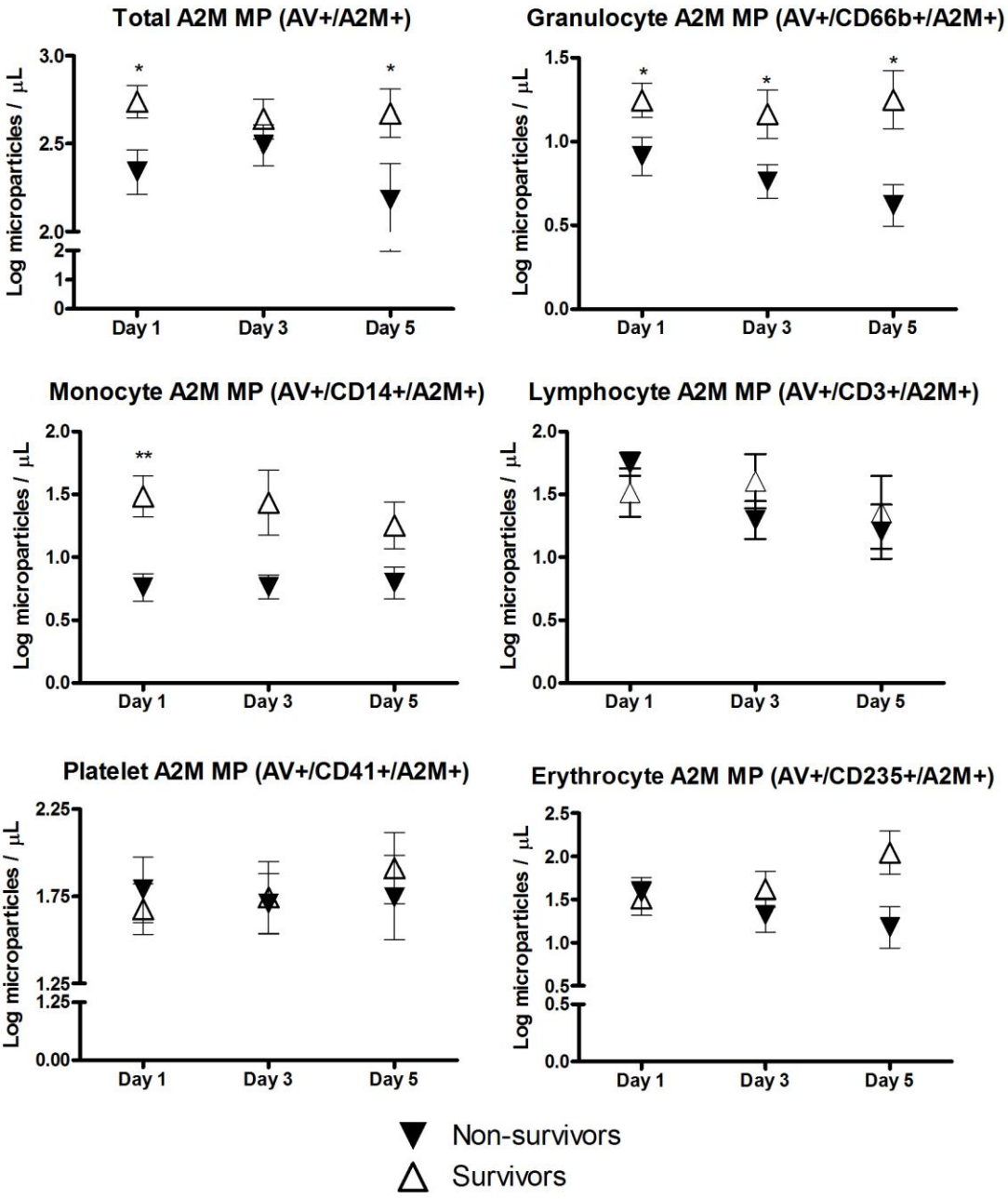


Figure 3.11. Sequential changes in microparticle subsets expressing alpha-2-macroglobulin in patients with severe sepsis secondary to community acquired pneumonia; comparison between 28 day non-survivors and survivors. Platelet free plasma from the two cohorts was obtained by differential centrifugation of whole blood collected at day 1, day 3 and day 5 post admission to ICU. The plasma (5 µL per test) was incubated with cell specific fluorochrome conjugated monoclonal antibodies as indicated, anti alpha-2-macroglobulin antibody and annexin V for half an hour at room temperature and the reaction was stopped by adding binding buffer up to a volume of 200 µL (n=60). Non-survivors: Day 1 (n=30), Day 3 (n=25), Day 5 (n=13), Survivors Day 1, 3, 5 (n=30). The samples were subsequently acquired using flow-cytometry. The results were log transformed and compared using unpaired t test. A2M: alpha-2-macroglobulin. AV: annexin V, MP: microparticles, *: $p < 0.05$, **: $p < 0.01$.

In summary, these result demonstrate that cell derived MP and $A2M^+$ MP profiles differed significantly between CAP and FP patients at day 1 post admission to ICU. CAP patients had significantly higher granulocyte, monocyte and lymphocyte derived MP compared to FP and HV at day 1, while FP patients MP profile was not significantly different comparted to HV. CAP patients also had a higher $A2M^+$ MP compared to FP and HV at day 1 post admission.

When each cohort was divided into survivors and non-survivors comparisons at day 1 revealed that endothelial MP were significantly higher in non-survivors of CAP while total $A2M^+$ MP, granulocyte derived A2M+ MP and monocyte derived $A2M^+$ MP were higher in survivors of CAP. In FP, granulocyte and erythrocyte derived MP were significantly higher in non-survivors of FP as well as erythrocyte $A2M^+$ MP were also significantly higher in non-survivors.

Endothelial MP remained significantly raised in non-survivors of CAP at day 5 post admission to ICU. Total $A2M^+$ MP, granulocyte $A2M^+$ MP and monocyte $A2M^+$ MP were consistently raised in CAP survivors across day 1, 3 and 5 post admission to ICU.

Chapter 4: The effect of granulocyte macrophage colony stimulating factor (GM-CSF) and interferon-gamma (IFN-γ) on whole blood microparticles phenotype

GM-CSF and IFN-γ have been tested as adjuvant therapies in severe sepsis and their immune-stimulating effects are similar to the effects we previously found for MP expressing alpha-2-macroglobulin ($A2M^+$ MP)[191, 254]. Therefore, in this project the effects of whole blood stimulation with GM-CSF and IFN-γ were examined to establish whether they could modulate $A2M^+$ MP production. The functional activities of the MP produced in this way were tested using human umbilical vein endothelial cells and neutrophils.

4.1 Concentration response and time course for microparticle production from whole blood stimulated with granulocyte-macrophage colony stimulating factor or interferon-γ.

To test the effects of GM-CSF and IFN-γ on production of $A2M^+$MP, whole blood obtained from healthy volunteers was incubated with increasing concentrations of these immune stimulants, above and below the concentrations used previously to produce MP[192] (10, 50 or 100 ng/ml) and subsequently measured $A2M^+$MP at various time points (30, 60, or 180 min). Vehicle was used as control. GM-CSF produced the highest number of $A2M^+$MP when whole blood was incubated with 100 ng/ml for 30 minutes, while incubation with IFN-γ 50 ng/ml for 180 minutes produced the highest number of MP. The experiments were repeated at the selected time points and concentration ranges to ensure reproducibility (Figure 4.1).

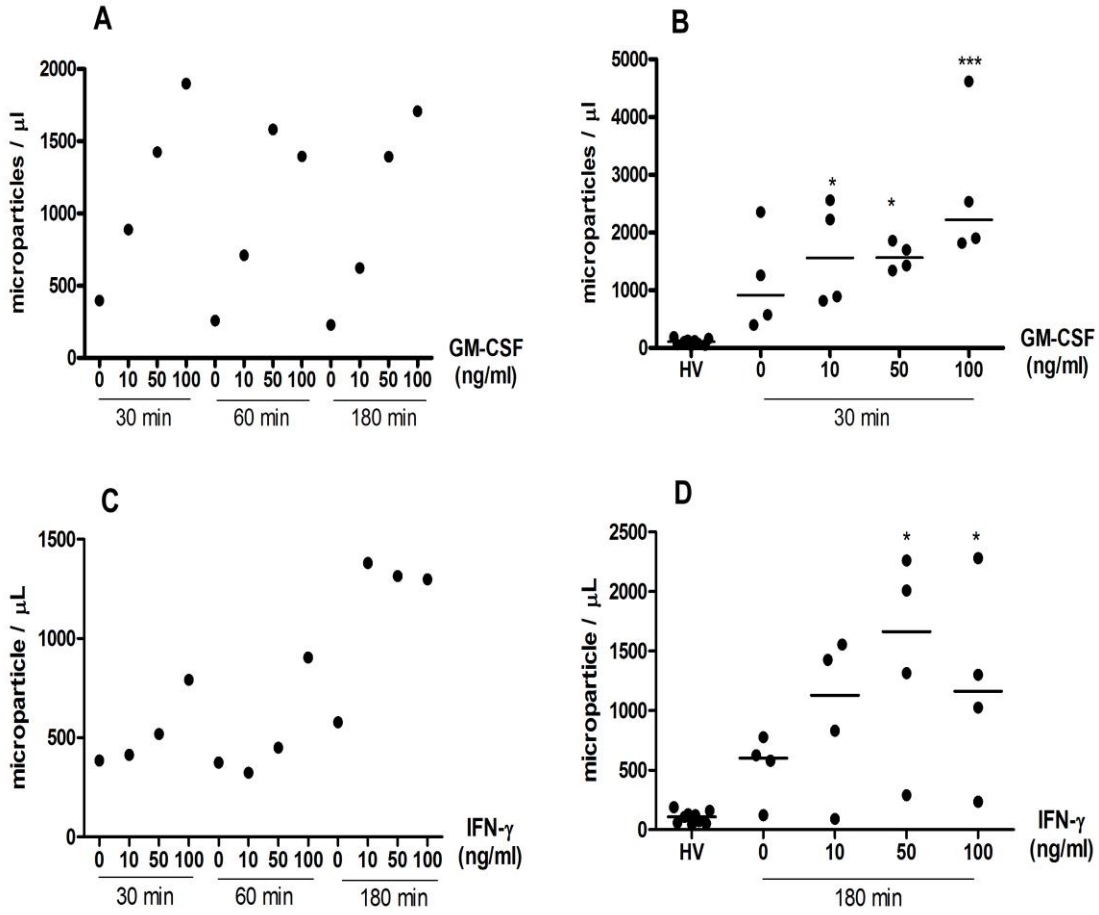


Figure 4.1. Effect of whole blood stimulation with either granulocyte macrophage colony-stimulating factor (GM-CSF) or interferon-γ (IFN-γ) on the number of microparticles expressing alpha-2-macroglobulin ($A2M^+MP$). Whole blood obtained from healthy volunteers was incubated at 37°C with or without GM-CSF or IFN-γ using increasing concentrations (10, 50 or 100 ng/ml). The number of $A2M^+MP$ was determined at various time points (30, 60 or 180 min). Differential centrifugation was employed to produce platelet free plasma from whole blood, which was subsequently stained with Annexin V and anti-alpha-2-macroglobulin antibody and processed using flow-cytometry. Experiments were repeated at the identified time points to ensure reproducibility. A: Effect of increasing concentrations of GM-CSF at various time points on $A2M^+MP$ production from whole blood (n=1). B: Effect of increasing concentrations of GM-CSF at 30 minutes (n=4). C: Effect of increasing concentrations of IFN-γ at various time points on $A2M^+MP$ production from whole blood (n=1). D: Effect of increasing concentrations of IFN-γ at 180 minutes (n=4). Data are expressed as microparticles count per microliter for platelet free plasma. Values are compared with these previously found in plasma of healthy volunteers. Kruskal-Wallis and Dunn's tests were used for comparisons. Ctrl: control, HV: healthy volunteers *: $p<0.05$, ***: $p<0.001$.

4.2 Identification of cell specific microparticles generated from whole blood stimulated with granulocyte macrophage colony-stimulating factor or interferon-γ.

As GM-CSF and IFN-γ have been and continue to be tested as adjuvant therapies in severe sepsis[133, 168], in this project it was aimed to establish whether these cytokines modulate MP phenotype upon addition to human whole blood. In the previous set of experiments, it was demonstrated that GM-CSF and IFN-γ enhance the production of MP expressing A2M from whole blood as well as defining the optimum concentration and incubation time. Subsequently, the effect of these two agents at the identified concentration and incubation period on blood cell MP profiles as well as the cellular origin of the $A2M^+$ MP cellular origin was examined.

Whole blood aliquots from healthy volunteers was incubated with GMCSF (100 ng/ml for 30 min) or IFN-γ (50 ng/ml for 180 minutes) and MP were characterised according to their cell of origin and A2M expression. In these experiments stimulation with GM-CSF and IFN-γ led to a significant increase in the number of granulocyte and monocyte MP and a trend towards increase in the number of lymphocyte and erythrocyte MP which did not reach statistical significance (Figure 4.2). When MP were characterised according to their cell of origin and A2M expression, both GM-CSF and IFN-γ produced more granulocyte, monocyte, erythrocyte and platelet MP expressing A2M, while IFN-γ produced more lymphocyte derived $A2M^+$MP compared to control (Figure 4.3).

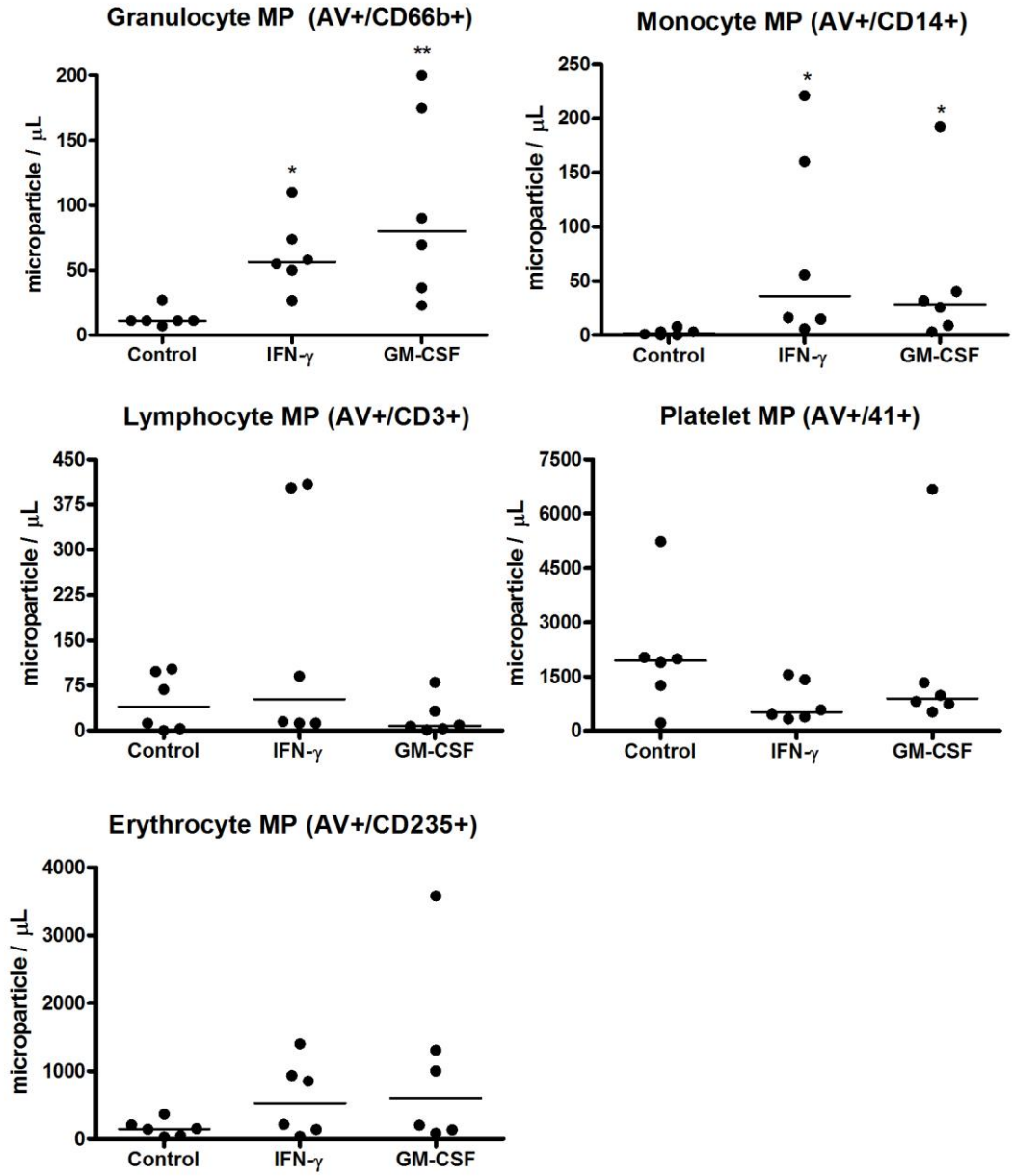


Figure 4.2. Identification of cell-specific microparticles in whole blood following stimulation with granulocyte macrophage colony-stimulating factor (GM-CSF) or interferon-γ (IFN-γ). Whole blood aliquots obtained from healthy volunteers were incubated at 37°C with or without GM-CSF or IFN-γ (100 ng/ml for 30 min, 50 ng/ml for 180 min respectively). Differential centrifugation was employed to produce platelet free plasma from whole blood, which was subsequently stained with annexin V and cell specific monoclonal antibodies for granulocytes, monocytes, lymphocytes, erythrocytes and platelet (CD66b, CD14, CD3, CD235, CD41 respectively) and processed using flow-cytometry (n=6). Data are expressed as microparticle count per microliter of platelet free plasma. Kruskal-Wallis and Dunn's tests were used for comparisons. GM-CSF: granulocyte macrophage colony-stimulating factor, IFN-γ: interferon-γ, MP: microparticles. *: $p<0.05$, **: $p<0.01$.

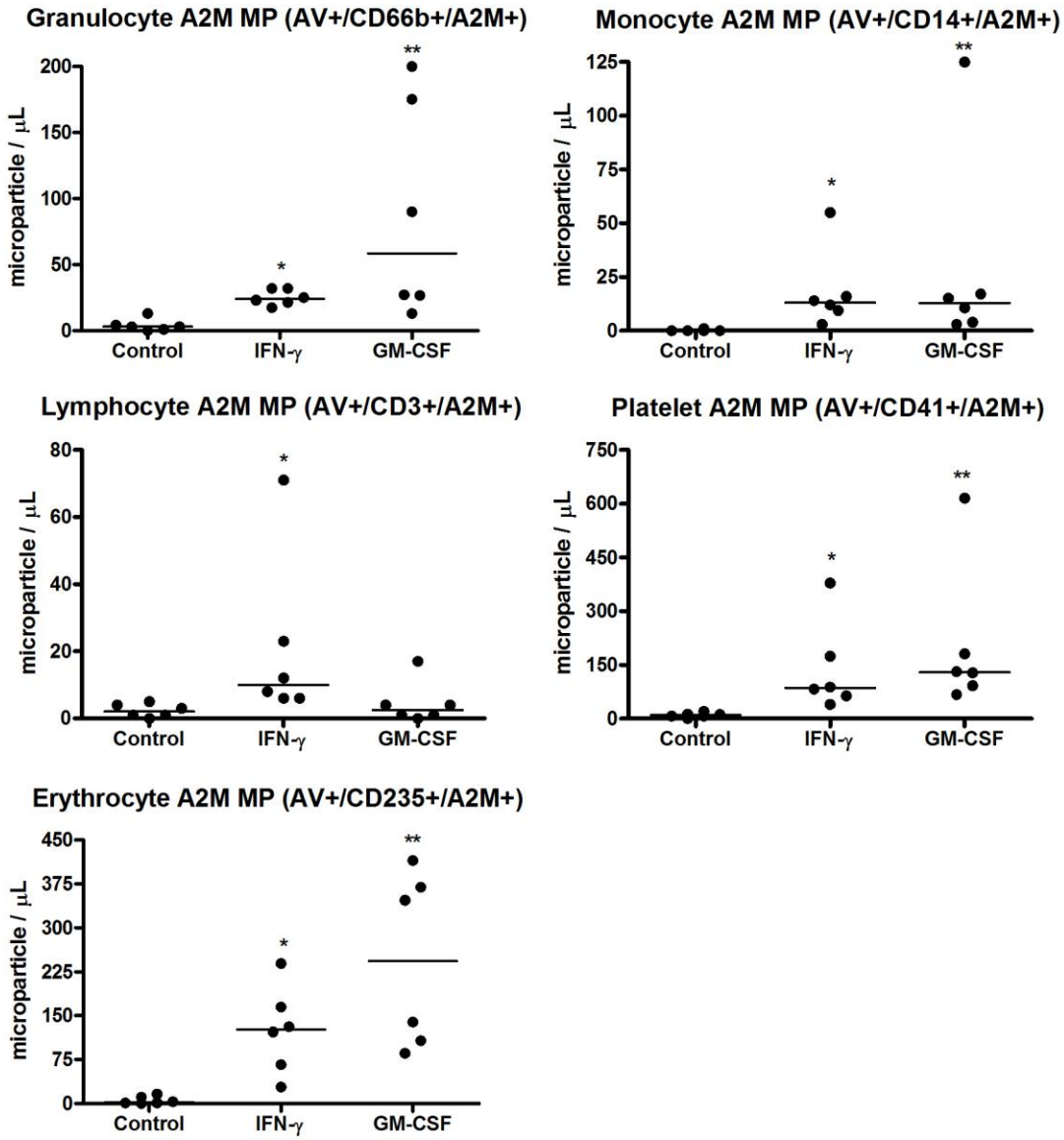


Figure 4.3. Identification of cell-specific microparticles positive for alpha-2-macroglobulin (A2M) in whole blood following stimulation with granulocyte macrophage colony-stimulating factor (GM-CSF) or interferon-γ (IFN-γ). Whole blood obtained from healthy volunteers was incubated at 37°C with or without GM-CSF or IFN-γ (100 ng/ml for 30 min, 50 ng/ml for 180 min respectively). Differential centrifugation was employed to produce platelet free plasma from whole blood, which was subsequently stained with annexin V, cell specific monoclonal antibodies for granulocytes, monocytes, lymphocytes, erythrocytes and platelet (CD66b, CD14, CD3, CD235, CD41 respectively) and anti-A2M antibody and processed using flow-cytometry (n=6 experiments with distinct donors run in triplicates). Data are expressed as microparticle count per microliter of platelet free plasma. Kruskal-Wallis and Dunn's tests were used for comparison. A2M: alpha-2-macroglobulin, GM-CSF: granulocyte macrophage colony-stimulating factor, IFN-γ: interferon-γ, MP: microparticles. *: $p<0.05$, **: $p<0.01$.

4.3 Microparticle uptake by human umbilical vein endothelial cells (HUVEC)

The endothelium plays an integral role in homeostasis and is an essential element in the host's defence against invading pathogens. In severe sepsis, the endothelium becomes severely dysfunctional. Because the endothelium may be the microenvironment in which GM-CSF and IFN-γ exert some of their actions, Iit was examined whether HUVEC would take up MP derived from whole blood stimulated with these agents. Whole blood MP were collected, concentrated and stained using boron-dipyrromethene (BODIPY). The MP (1 – 2 x 10^6/ml) were subsequently incubated with HUVEC (5 x 10^5) for 4 hours. In some cases, HUVECs were pre-treated with cytochalasin B (10 μM) to inhibit their endocytic pathways before adding the fluorescent MP. In addition unstained MP were also incubated with HUVEC to act as a control for baseline fluorescence. Cells were examined using imaging flow-cytometry to determine the percentage of HUVEC which had engulfed MP and also the level of fluorescent staining.

More than 85% of HUVEC incubated with labelled MP were FITC positive, whereas only 15% of HUVEC pre-treated with cytochalasin B were positive. HUVEC incubated with control MP which exhibited almost no fluorescence (0.05%).

The same pattern was observed when the fluorescence intensity was measured indicating that HUVEC uptake of MP was maximal without cytochalasin B (Figure 4.4).

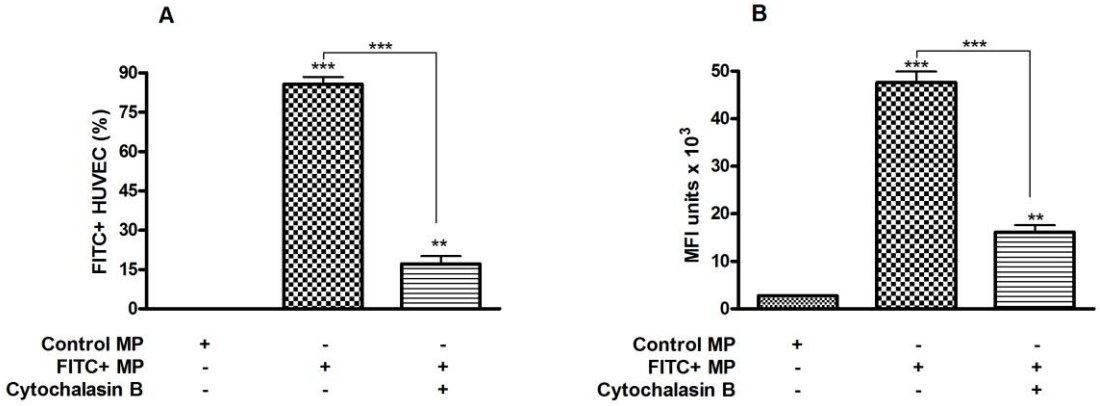


Figure 4.4. Human umbilical vein endothelial cells (HUVEC) uptake of microparticles. Whole blood from healthy volunteers was incubated with GM-CSF (100 ng/ml) for 30 minutes at 37^{o}C and platelet free plasma was obtained by differential centrifugation. Microparticles were pelleted and washed with phosphate buffered saline (PBS) twice and re-suspended in 1/10th of the original plasma volume in PBS. The microparticle (MP) concentrate was stained with fluorescein isothiocyanate (FITC) labelled boron-dipyrromethene (BODIPY) for 15 minutes and subsequently the MP were pelleted and re-suspended in PBS to wash off excess dye. The FITC^{+} MP were incubated with HUVEC (5 x 10^{5}) at a concentration of 1 – 2 x 10^{6} /ml in 6 well plates. The cells were incubated for 4 hours at 37^{o}C with 5% CO_2. The cells were then trypsinised, fixed, suspended in PBS and processed using imaging flow-cytometry. Another set of HUVEC were pre-treated for 10 minutes with 10 µM cytochalasin B to inhibit phagocytosis and subsequently treated as above. Another group of HUVEC cells were incubated with unstained microparticles to act as control (n=3). A. HUVEC uptake of FITC fluorescent MP (% of positive cells). B. Fluorescence intensity of the FITC^{+} HUVEC treated with FITC stained MP. The results are expressed as mean ± SEM of n=4 experiments performed in triplicates of median fluorescence intensity units as quantified by flow-cytometry. One way ANOVA and Bonferroni tests were used for comparison. FITC: fluorescein isothiocyanate, MFI: median fluorescence intensity, MP: microparticles. **: p<0.01, ***: p<0.001.

In another set of experiments designed to confirm the HUVEC uptake of MP, HUVEC were incubated with fluorescent MP for 2 hours and then the cells were then fixed and stained with Alexa-Flor 633 labelled wheat germ agglutinin to identify the cell membrane. Then counterstained with 4',6-diamidino-2-phenylindole (DAPI) to identify the nuclei. The slides were then visualised using confocal microscopy, showing that MP were present within the HUVEC (Figure 4.5, 4.6, 4.7).

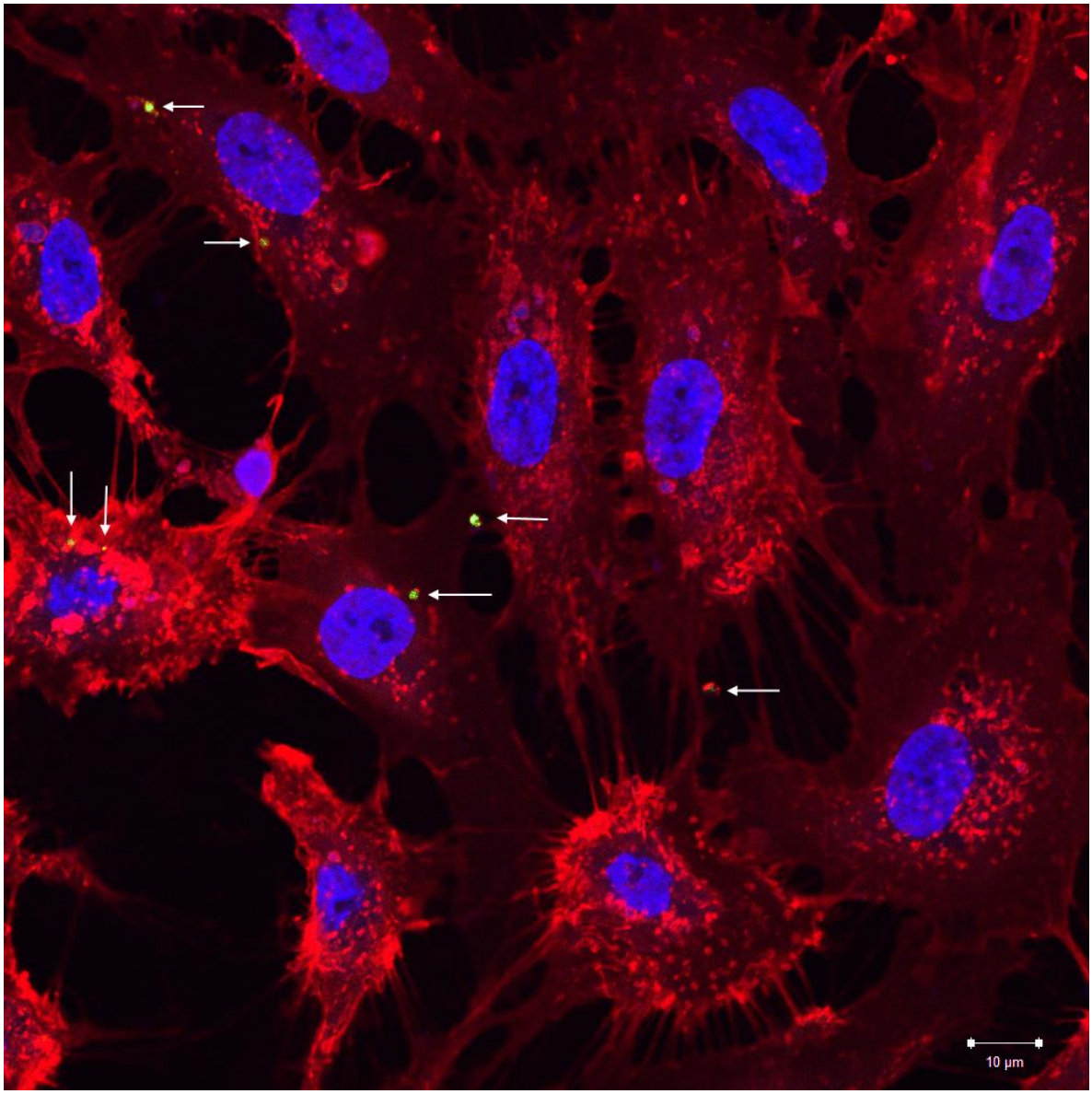


Figure 4.5. Confocal image of human umbilical vein endothelial cells (HUVEC) to assess the intracellular uptake of microparticles. Whole blood from healthy volunteers was incubated with GM-CSF (100 ng/ml) for 30 minutes and platelet free plasma was produced by differential centrifugation. Microparticles were pelleted and washed with phosphate buffered saline (PBS) twice and re-suspended in 1/10th of the original plasma volume in PBS. The MP concentrate was stained with Fluorescein isothiocyanate (FITC) labelled boron-dipyrromethene (BODIPY) for 15 minutes and subsequently the MP were pelleted and re-suspended in PBS. The fluorescent MP were incubated with HUVEC seeded on 13mm slide coverslips for 2 hours in protein free medium at a concentration of 1 - 2 x 10^{6}/ml. Alexa-flor 633 labelled wheat germ agglutinin and 4',6-diamidino-2-phenylindole (DAPI) were used for staining the cell wall and nuclei of the fixed HUVEC red and blue respectively. The slide were then visualised using confocal microscopy. Experiments were repeated three time to ensure reproducibility. Multiple HUVEC containing microparticles. The microparticles are annotated with arrows.

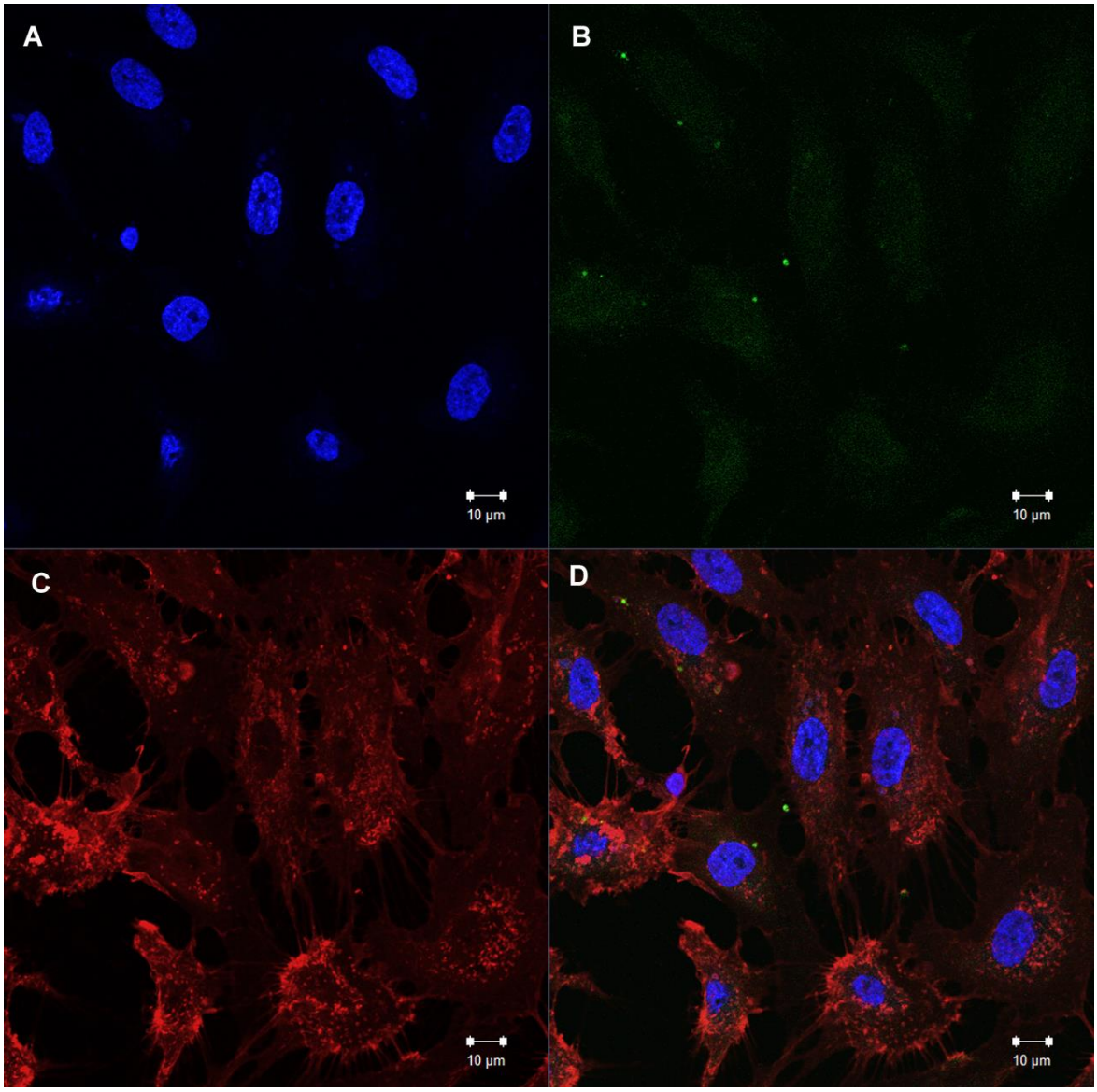


Figure 4.6. Confocal split images of human umbilical vein endothelial cells (HUVEC) to assess the intracellular uptake of microparticles. Whole blood from healthy volunteers was incubated with GM-CSF (100 ng/ml) for 30 minutes and platelet free plasma was produced by differential centrifugation. Microparticles were pelleted and washed with phosphate buffered saline (PBS) twice and re-suspended in 1/10th of the original plasma volume in PBS. The MP concentrate was stained with Fluorescein isothiocyanate (FITC) labelled boron-dipyrromethene (BODIPY) for 15 minutes and subsequently the MP were pelleted and re-suspended in PBS. The fluorescent MP were incubated with HUVEC seeded on 13mm slide coverslips for 2 hours in protein free medium at a concentration of 1 - 2 x 10^6/ml. Alexa-flor 633 labelled wheat germ agglutinin and 4',6-diamidino-2-phenylindole (DAPI) were used for staining the cell wall and nuclei of the fixed HUVEC red and blue respectively. The slide were then visualised using confocal microscopy. Experiments were repeated three time to ensure reproducibility. HUVEC containing microparticles in split channels and overlaid. Blue: DAPI stained nuclei, Green: BODIPY stained microparticles, Red: AlexaFlor 633 stained cell wall.

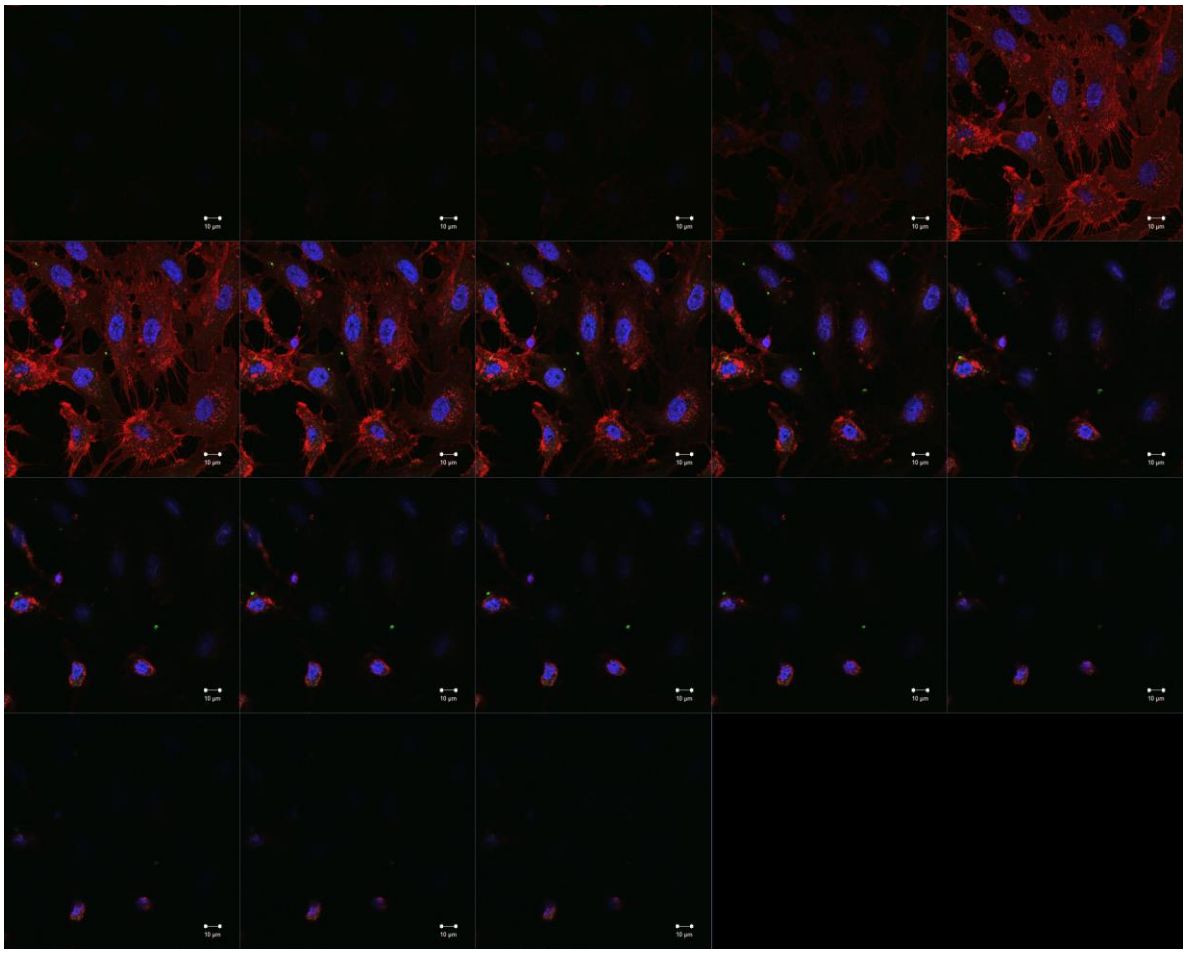


Figure 4.7. Confocal Z-stack analysis of human umbilical vein endothelial cells (HUVEC) to assess the uptake of microparticles. Whole blood from healthy volunteers was incubated with GM-CSF (100 ng/ml) for 30 minutes and platelet free plasma was produced by differential centrifugation. Microparticles were pelleted and washed with phosphate buffered saline (PBS) twice and re-suspended in 1/10th of the original plasma volume in PBS. The MP concentrate was stained with Fluorescein isothiocyanate (FITC) labelled boron-dipyrromethene (BODIPY) for 15 minutes and subsequently the MP were pelleted and re-suspended in PBS. The fluorescent MP were incubated with HUVEC seeded on 13mm slide coverslips for 2 hours in protein free medium at a concentration of 1 - 2 x 10^{6}/ml. Alexa-flor 633 labelled wheat germ agglutinin and 4',6-diamidino-2-phenylindole (DAPI) were used for staining the cell wall and nuclei of the fixed HUVEC red and blue respectively. The slide were then visualised using confocal microscopy. Experiments were repeated three time to ensure reproducibility. Serial confocal images taken through the HUVEC from top to bottom showing the microparticles to be inside the cells.

4.4 Effect of whole blood microparticles on human umbilical vein endothelial cells adhesion molecule and tissue factor expression

Then the effect of whole blood MP on HUVEC expression of adhesion molecules and tissue factor was.

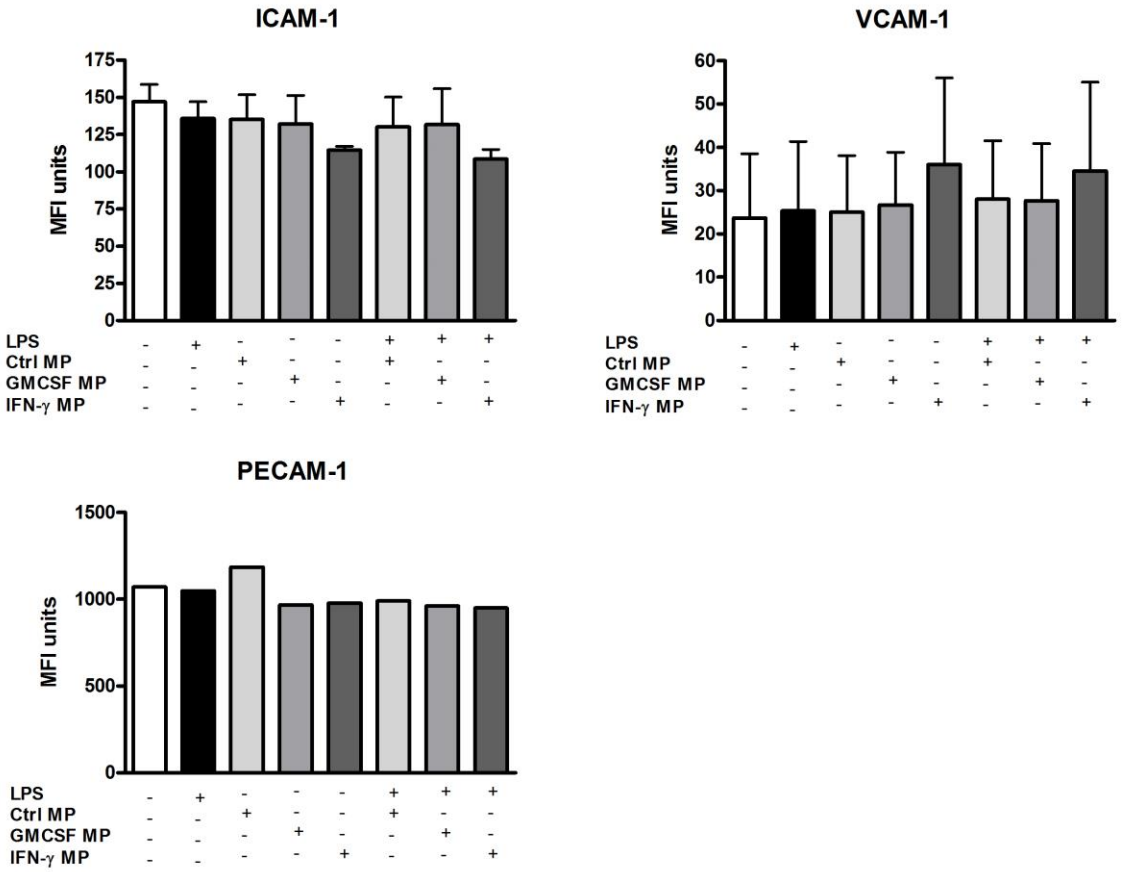


Figure 4.8. Effect of whole blood microparticles on human umbilical vein endothelial cells (HUVEC) adhesion molecule. Whole blood obtained from healthy volunteers was incubated with or without either granulocyte macrophage colony-stimulating factor (GM-CSF 100ng/ml) or interferon γ (IFN-γ 50ng/ml) for 30 and 180 min respectively. Platelet free plasma was obtained by differential centrifugation and microparticles were pelleted, washed twice and suspended in 1/10th of original plasma volume of PBS. HUVEC were seeded in 12 well plates at a concentration of 2 x 10^5 / well and the microparticles were added to them at a concentration of 1 – 2 x 10^6 / ml in protein free medium. The plates were incubated for 4 hours in 37^{o}C incubator containing 5% CO_2. Subsequently, HUVEC were trypsinised, pelleted, plated in 96 well plates and stained with fluorescent monoclonal antibodies against intracellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), platelet-endothelial cell adhesion molecule 1 (PECAM-1), E-selectin, low density lipoprotein receptor-related protein 1 (LRP-1) and tissue factor (TF). Data are expressed as mean and standard error of the mean. One way ANOVA and Bonferroni tests were used for comparison. Experiments were repeated at least 4 times with distinct HUVEC and MP donors. Data expressed as mean and SEM. Ctrl: Control, GM-CSF: granulocyte macrophage colony-stimulating factor, IFN-γ: interferon-γ, LPS: lipopolysaccharide, MP: microparticles.

HUVEC were incubated for 4 hours with vehicle, Control MP, GM-CSF MP or IFN-γ MP with or without lipopolysaccharide (LPS – 100 ng/ml). The cells were

subsequently stained for adhesion molecules (ICAM-1, VCAM-1, PECAM-1, E-selectin, lipoprotein receptor-related protein – 1 (LRP-1, receptor for alpha-2-macroglobulin) and tissue factor (TF) using fluorochrome conjugated monoclonal antibodies and examined using flow-cytometry. The MP did not increase ICAM-1, VCAM-1 and PECAM-1 compared to baseline, while E-selectin, LRP-1 and TF were not expressed (Figure 4.8).

4.5 Effect of whole blood microparticles on human umbilical vein endothelial cell production of reactive oxygen species (ROS) and nitric oxide (NO)

The effect of whole blood microparticles on HUVEC ROS and NO production was also tested. HUVEC were plated in 96 well plates at a concentration of 1×10^5 / well and MP with or without lipopolysaccharide (LPS – 100 ng/ml) were added at a concentration of $1 – 2 \times 10^6$ / ml of protein free medium. The plates were incubated for 4 hours in a 37^{o}C incubator containing 5% CO_2. GM-CSF MP elicited significantly more ROS irrespective of the presence or absence of LPS. MP obtained from whole blood stimulated with IFN-γ showed a trend towards production of more ROS but this did not reach statistical significance.

GM-CSF and IFN-γ microparticles alone did not stimulate the production NO. When the cells were incubated with GM-CSF or IFN-γ MP together with LPS NO production was increased significantly (Figure 4.9).

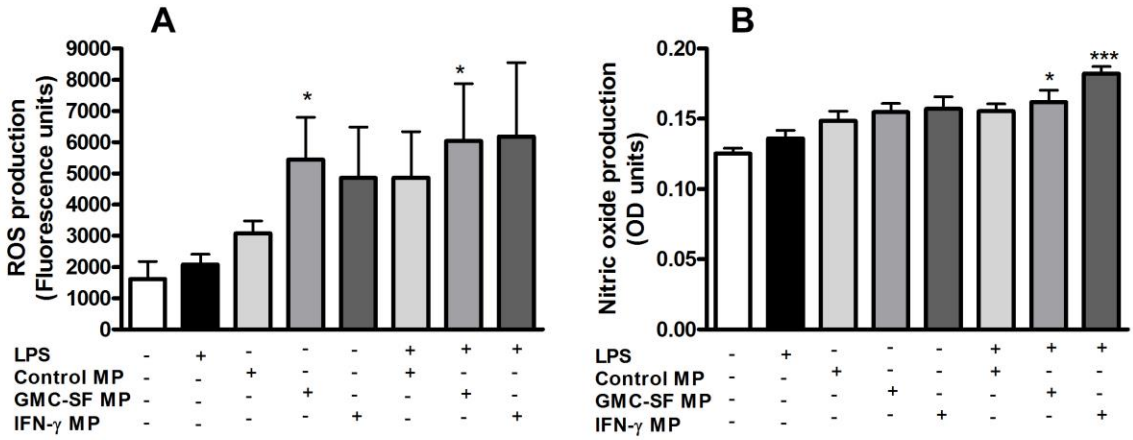


Figure 4.9. Effect of whole blood microparticles on human umbilical vein endothelial cell (HUVEC) production of reactive oxygen species (ROS) and nitiric oxide (NO). Whole blood obtained from healthy volunteers was incubated with or without either granulocyte macrophage colony-stimulating factor (GM-CSF) or interferon γ (IFN-γ) for 30 and 180 min respectively. Platelet free plasma was obtained by differential centrifugation and microparticles were pelleted, washed twice and suspended in 1/10th of original plasma volume of PBS. HUVEC were seeded in 96 well plates at a concentration of 1 x 10^5 cells per well and the microparticles were added at a concentration of 1 – 2 x 10^6 /ml in protein free medium with and without lipopolysaccharide (LPS – 100 ng/ml). The plates were incubated for 4 hours in 37^{o}C incubator containing 5% CO_2. For the reactive oxygen species experiments, HUVEC cells were pre-incubated for 30 minutes with 20 μM of 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA), washed and incubated with microparticles. For nitric oxide experiments, HUVEC supernatant was incubated with Griess reagent for a further 30 minutes. Plate reader was used to read fluorescence and absorbance for ROS and NO respectively. The results are expressed as arbitrary units. Experiments were repeated at least 4 times. Data expressed as mean and SEM. A: Effect of whole blood microparticles on HUVEC ROS production. B. Effect of whole blood microparticles on HUVEC NO production. One way ANOVA and Bonferroni tests were used for comparison. Ctrl: Control, GM-CSF: granulocyte macrophage colony-stimulating factor, IFN-γ: interferon-γ, LPS: lipopolysaccharide, MP: microparticles. *: p<0.05, ***: p<0.001

4.6 Effect of whole blood microparticles on human umbilical vein endothelial cell cytokine production

Next, the effect of whole blood MP on HUVEC TNF-α and IL-8 production was examined. HUVEC were plated in 96 well plates at a concentration of 5 x 10^4 cells per well and incubated with MP with or without LPS (100 ng/ml) at a concentration of 1 – 2 x 10^6 / ml of protein free medium. The plates were incubated for 4 hours in a 37^{o}C incubator containing 5% CO_2. The supernatant was collected and the concentration of TNF-α and IFN-γ was measured using enzyme-linked

immunosorbent assay (ELISA). GMCSF and IFN-γ MP increased production of TNF-α and IL-8 with and without LPS compared to LPS alone. Interestingly, Control MP with LPS also produced significantly higher concentrations of IL-8 (Figure 4.10).

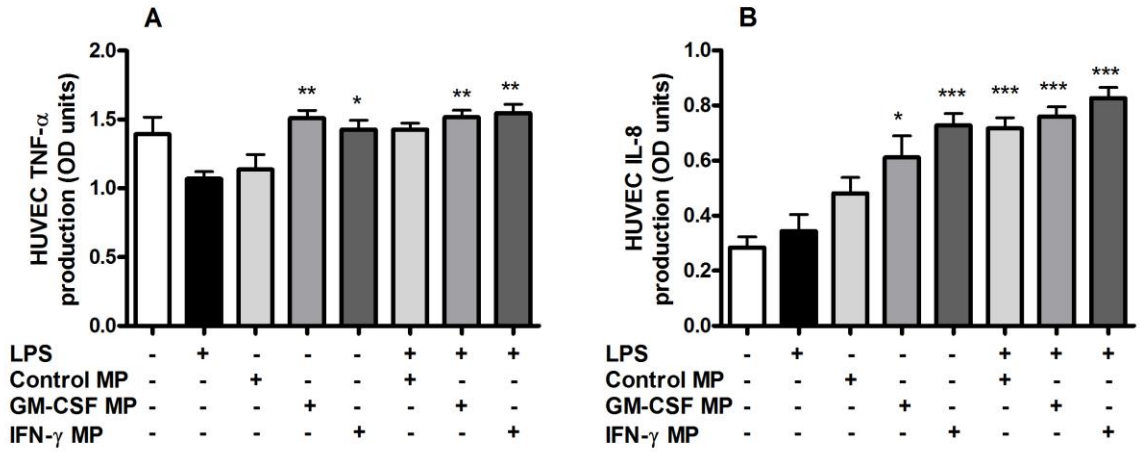


Figure 4.10. Effect of whole blood microparticles on the human umbilical vein endothelial cells (HUVEC) cytokine production. Whole blood obtained from healthy volunteers was incubated with or without either granulocyte macrophage colony-stimulating factor (GM-CSF) or interferon γ (IFN-γ) for 30 and 180 min respectively. Platelet free plasma was obtained by differential centrifugation and microparticles were pelleted, washed twice and suspended in 1/10th of original plasma volume of PBS. HUVEC were seeded in 96 well plates at a concentration of 1 x 10^5 /well and the microparticles were added to them at a concentration of 1 – 2 x 10^6 /ml in protein free medium with and without lipopolysaccharide (LPS – 100 ng/ml). The plates were incubated for 4 hours in 37^{o}C incubator containing 5% CO_2. TNF-αand IL-8 were measured in the supernatant in duplicates using enzyme-linked immunosorbent assay (ELISA) (n=4). A: Effect of whole blood microparticles on HUVEC TNF-αproduction. B: Effect of whole blood microparticles on HUVEC IL-8 production. Experiments were repeated at least 4 times and data expressed as mean and SEM. One way ANOVA and Bonferroni tests were used for comparison to LPS. GM-CSF: granulocyte macrophage colony-stimulating factor, IFN-γ: interferon-γ, LPS: lipopolysaccharide, MP: microparticles. *: p<0.05, **: p<0.01, ***: p<0.001.

4.7 Effect of whole blood microparticles on human umbilical vein endothelial cell monolayer permeability

An important function of the endothelium is to control the passage of solute from the blood to the tissues, ensuring the appropriate translocation of nutrients without precipitating tissue oedema. Inflammation increases endothelial permeability. For this experiment HUVEC were seeded on tissue culture insert, grew them until confluent and subsequently stimulated with whole blood microparticles in the

presence or absence of LPS. Addition of fluorescein isothiocyanate (FITC) labelled dextran to the top well was used to assess endothelial monolayer permeability, by quantifying the recovery of the tracer from the bottom well. In the presence of LPS, GM-CSF and IFN-γ microparticles significantly reduced the HUVEC monolayer permeability. Control microparticles did not have any significant effect (Figure 4.11).

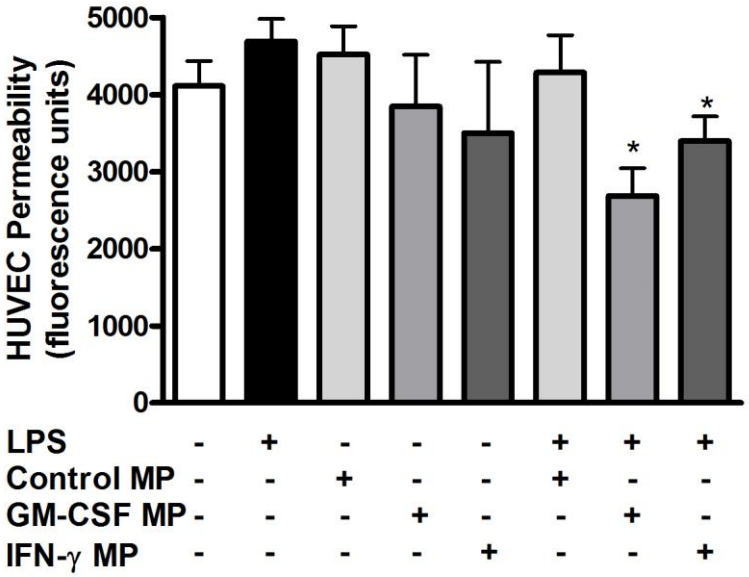


Figure 4.11. Effect of whole blood microparticles on human umbilical vein endothelial cells (HUVEC) monolayer permeability. Whole blood obtained from healthy volunteers was incubated with or without either granulocyte macrophage colony-stimulating factor (GM-CSF) or interferon γ (IFN-γ) for 30 and 180 min respectively. Platelet free plasma was obtained by differential centrifugation and microparticles were pelleted, washed twice and suspended in 1/10th of original plasma volume of PBS. HUVEC were seeded in tissue culture inserts at a concentration of 5 x 10^4cells per well and the microparticles were added at a concentration of 1 – 2 x 10^6 /ml in protein free medium with and without lipopolysaccharide (LPS – 100 ng/ml). The plates were incubated for 4 hours in 37^{o}C incubator containing 5% CO_2. Fluorescent dextran (4 x 10^4 kDa) was added to the tissue culture inserts on top of the HUVEC monolayer and incubated for further 30 minutes in new plates. Amount of fluorescence dextran under the tissue culture insert as measured using plate reader. Experiments were repeated at least 4 times and data expressed as mean and SEM. One way ANOVA and Bonferroni tests were used for comparison to LPS. GM-CSF: granulocyte macrophage colony-stimulating factor, IFN-γ: interferon-γ, LPS: lipopolysaccharide, MP: microparticles. *: $p<0.05$.

4.8 Effect of whole blood microparticles on neutrophil function

As it was previously demonstrated that A2M expressing MP stimulate neutrophil activity[191, 254], the effect of whole blood MP stimulated with GM-CSF and IFN-γ on neutrophil function by measuring CD11b adhesion molecule expression, reactive

oxygen species (ROS) production, phagocytosis and chemotaxis were examined. Neutrophils from healthy volunteers were incubated with or without MP in the presence or absence of LPS. In the presence of LPS, GM-CSF and IFN-γ whole blood MP enhanced the expression of CD11b as well as neutrophil phagocytosic function, but they had no significant effect on their own, Control MP did not affect these neutrophil functions (Figure 4.12, 4.13). GM-CSF and IFN-γ MP did not affect neutrophil ROS production or chemotaxis (Figure 4.14, 4.15).

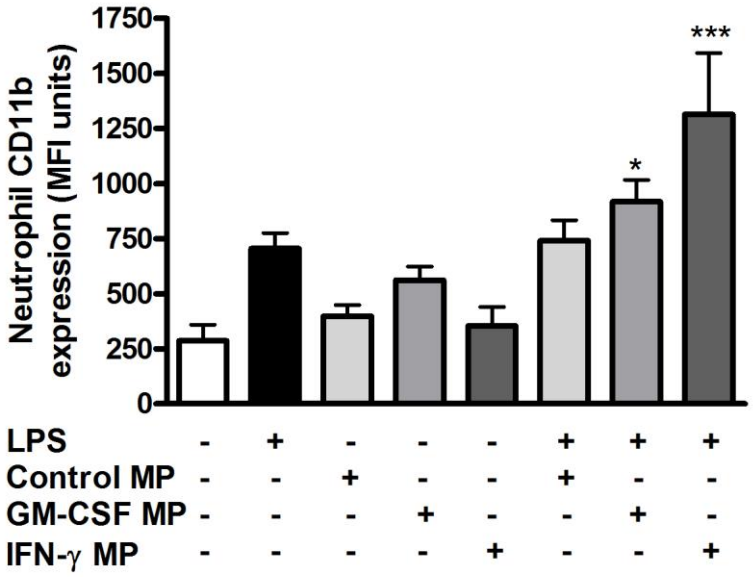


Figure 4.12. Effect of whole blood microparticles on neutrophil CD11b adhesion molecule expression. Whole blood from healthy volunteers (HV) was incubated at 37°C with or without GM-CSF (100ng/ml for 30 min) or IFN-γ (50ng/ml for 180 min). Platelet free plasma (PFP) was produced from whole blood by differential centrifugation. Subsequently, the MP were pelleted, washed twice and suspended in 1/10th of the original PFP volume. Neutrophils were isolated from HV whole blood and incubated for 1 hour (1 x 10^6/ml) with whole blood microparticles (1 – 2 x 10^6/ml). The stimulated neutrophils were stained with fluorescent monoclonal antibody against CD11b for 30 minutes and processed using flow-cytometry. Experiments were repeated at least 4 times with distinct neutrophil and MP donors. Data are expressed as mean ± SEM of median fluorescence intensity (MFI) unit as quantified by flow-cytometry. Experiments were repeated at least 4 times. One way ANOVA and Bonferroni tests were used for comparison to base. GM-CSF: granulocyte macrophage colony-stimulating factor, IFN-γ: interferon γ, LPS: lipopolysaccharide, MFI: median fluorescence intensity, MP: microparticles. *: p<0.05, ***: p<0.001.

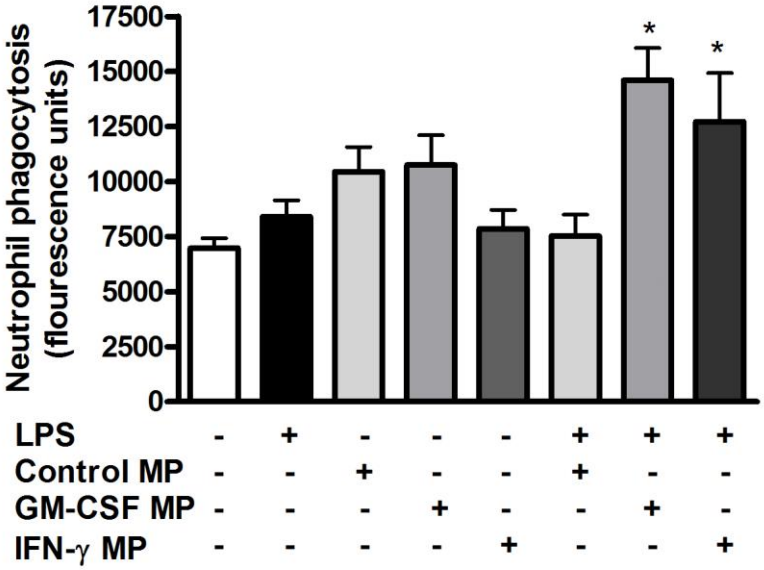


Figure 4.13. Effect of whole blood microparticles on neutrophil phagocytosis. Whole blood from healthy volunteers (HV) was incubated at 37°C with or without GM-CSF (100ng/ml for 30 min) or IFN-γ (50ng/ml for 180 min). Platelet free plasma (PFP) was produced from whole blood by differential centrifugation. Subsequently, the MP were pelleted, washed twice and suspended in 1/10th of the original PFP volume. Neutrophils were isolated from HV whole blood and incubated for 1 hour (1 x 10^6/ml) with whole blood microparticles (1 – 2 x 10^6/ml). Stimulated neutrophils were washed and incubated with fluorescent E. Coli (1 mg/ml) for half an hour in quadruplicated and the fluorescence of neutrophils as subsequently measured using a plate reader. Experiments were repeated at least 4 times with distinct neutrophil and MP donors. Data are expressed as mean ± SEM of fluorescence units as quantified by plate reader. One way ANOVA and Bonferroni tests were used for comparison to base. GM-CSF: granulocyte macrophage colony-stimulating factor, IFN-γ: interferon γ, LPS: lipopolysaccharide, MP: microparticles. *: p<0.05.

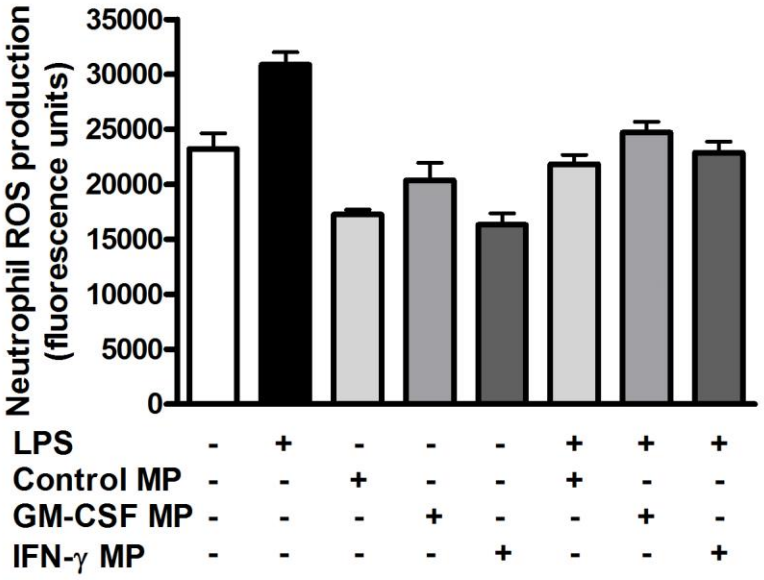


Figure 4.14. Effect of whole blood microparticles on neutrophil reactive oxygen species (ROS) production. Whole blood from healthy volunteers (HV) was incubated at 37°C with or without GM-CSF (100ng/ml for 30 min) or IFN-γ (50ng/ml for 180 min). Platelet free plasma (PFP) was produced from whole blood by differential centrifugation. Subsequently, the MP were pelleted, washed twice and suspended in 1/10th of the original PFP volume. Neutrophils were isolated from HV whole blood and incubated for 1 hour (1 x 10^6/ml) with whole blood microparticles (1 – 2 x 10^6/ml). Stimulated neutrophils were washed and incubated with 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA) for half an hour in quadruplicates and the fluorescence of neutrophils was subsequently measured using a plate reader. Experiments were repeated at least 4 times with distinct neutrophil and MP donors. Data are expressed as mean ± SEM of fluorescence units as quantified by plate reader. One way ANOVA and Bonferroni tests were used for comparison to control. GM-CSF: granulocyte macrophage colony-stimulating factor, IFN-γ: interferon γ, LPS: lipopolysaccharide, ROS: reactive oxygen species, MP: microparticles.

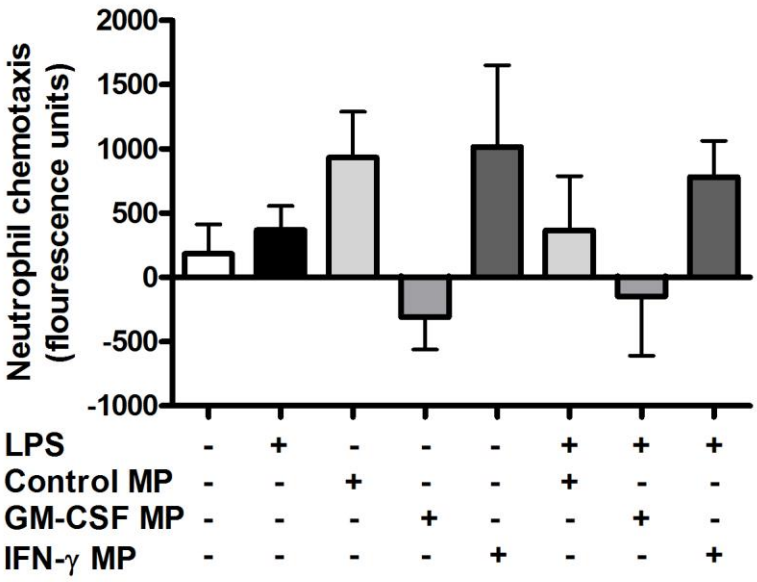


Figure 4.15. Effect of whole blood microparticles on neutrophil chemotaxis. Whole blood from healthy volunteers (HV) was incubated at 37°C with or without GM-CSF (100ng/ml for 30 min) or IFN-γ (50ng/ml for 180 min). Platelet free plasma (PFP) was produced from whole blood by differential centrifugation. Subsequently, the MP were pelleted, washed twice and suspended in 1/10th of the original PFP volume. Neutrophils were isolated for HV whole blood and incubated for 1 hour (1 x 10^6/ml) with whole blood microparticles (1 – 2 x 10^6/ml). Stimulated neutrophils were washed. Formyl-Methionyl-Leucyl-Phenylalanine (FMLP) was used as chemo-attractant at 1μM final concentration. fMLP or medium was added to the bottom wells (27μl), filters were placed on top of the wells and 25μl neutrophils were added on the upper surface of the filter membrane in quadruplets The plates were incubated for 60 minutes in a 37°C incubator containing 5% CO_2. Centrifugation (312 x *g* for 1 minute) was used to pellet the cells, subsequently the filter was removed and the cells were re-suspended. PrestoBlue® fluorescent dye solution (30μl of pre-diluted dye 1 in 5 in PBS) was mixed with 20μl cells aliquots in quadruplets in a 96-well plate. The neutrophils were then quantified using plate reader.Experiments were repeated at least 4 times with distinct neutrophil and MP donors. Data are expressed as mean ± SEM of fluorescence units as quantified by plate reader. One way ANOVA and Bonferroni tests were used from comparisons. GM-CSF: granulocyte macrophage colony-stimulating factor, IFN-γ: interferon γ, LPS: lipopolysaccharide, ROS: reactive oxygen species, MP: microparticles.

4.9 Effect of whole blood microparticles on neutrophil-endothelial interaction under flow conditions

Finally, these studies were concluded by investigating the effect of whole blood MP on neutrophil-endothelial interaction under flow conditions. HUVEC seeded on μ-slides were stimulated with LPS with or without MP for 4 hours. Subsequently, neutrophils were perfused over the HUVEC monolayers. Granulocyte macrophage colony-stimulating factor (GM-CSF) and interferon-γ (IFN-γ) whole blood MP enhanced the capture of neutrophils as well as their adhesion and rolling over the

HUVEC monolayer; this effect was significantly greater than was seen with LPS alone. Also the GM-CSF and IFN-γ MP significantly increased the number of neutrophils transmigrating through the HUVEC monolayer when compared to LPS alone. Control MP did not have an effect (Figure 4.16).

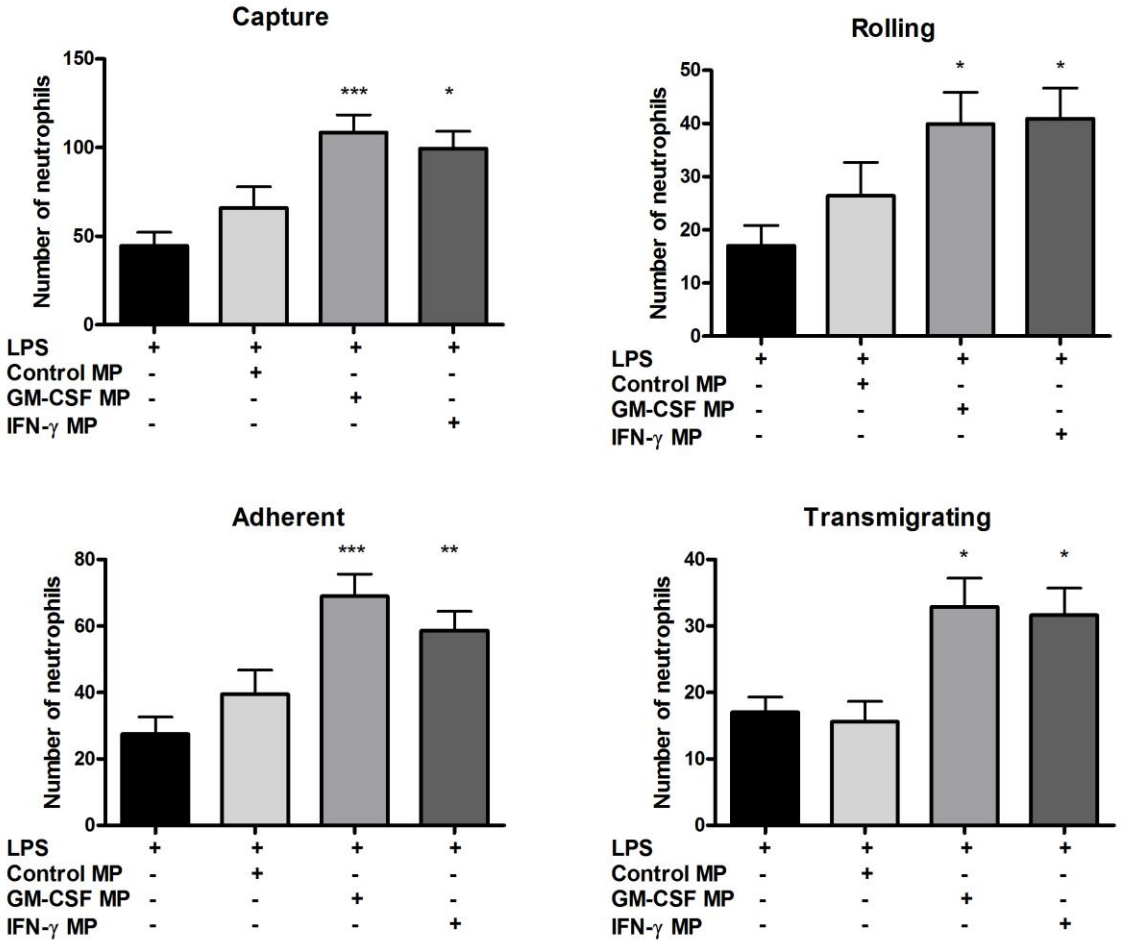


Figure 4.16. Effect of whole blood microparticles on neutrophil-endothelial interaction under flow conditions. Whole blood from healthy volunteers (HV) was incubated at 37°C with or without GM-CSF (100ng/ml for 30 min) or IFN-γ (50ng/ml for 180 min). Platelet free plasma (PFP) was produced from whole blood by differential centrifugation. Subsequently, the MP were pelleted, washed twice and suspended in 1/10th of the original PFP volume. To assess neutrophil-endothelial interaction, human umbilical cord endothelial cells (HUVEC) were plated onto µ-Slides and incubated at 37°C until confluent and stimulated with lipopolysaccharide (LPS) with or without MP for 4 hours. Human neutrophils from HV were isolated and perfused over the HUVEC monolayer at 1 dyne/cm^2 for 8 min. Six random fields were recorded for 10 s. Neutrophils were quantified as captured, adherent, rolling or transmigrating. Experiments were repeated at least 4 times with distinct HUVEC, neutrophil and MP donors. Data are expressed as mean ± SEM. One way ANOVA and Bonferroni tests were used for comparisons. GM-CSF: granulocyte macrophage colony-stimulating factor, IFN-γ: interferon γ, LPS: lipopolysaccharide, MP: microparticles. *: p<0.05, **: p<0.01, ***: p<0.001.

In summary, GMCSF or IFN-γ stimulation of whole blood resulted in enhanced production of MP particularly from granulocytes and monocytes. They also enhanced the production of $A2M^+$ MP as well as the production of $A2M^+$ MP from all blood cell lines.

These MP were found to be endocytosed by HUVEC. From a functional point of view they enhanced ROS and NO production as well as TNF-α and IL-8 production. They also reduced HUVEC monolayer permeability. These MP had no effect of HUVEC adhesion molecule expression nor on tissue factor expression. They also had an effect on neutrophils where they enhanced CD11b expression and neutrophil phagocytosis. They may have reduced neutrophil ROS production as well neutrophil chemotaxis. These MP were found to enhance neutrophil-endothelial interaction under flow conditions.

Taken together these results suggest that MP produced by GMCSF and IFN-γ may play a role in the potential beneficial effect of these cytokines when utilised as adjuvant therapies in sepsis.

Chapter 5: Discussion

In this study, MP profile varied according to the cause of severe sepsis where CAP patients had higher concentrations of MP compared to FP. There was no clear association between blood cell derived MP and outcome. Endothelial derived MP was associated with worse outcome in CAP patients at day 1 post admission to ICU. All blood cell derived MP expressed A2M. There was no clear associated between parent cell concentration and MP production. Monocytes and lymphocytes were more efficient in producing $A2M^+$ MP compared to neutrophils. Higher plasma concentration of $A2M^+$ MP in the first 24 hours post admission to intensive care was associated with 28 day survival in patients with severe sepsis secondary to CAP. This rise was sustained over the time points investigated (day 1, 3 and 5). GM-CSF and IFN-γ enhanced the production of $A2M^+$ MP from whole blood and these MP were endocytosed by HUVEC. GM-CSF and IFN-γ MP enhanced some pathogen clearance function of HUVEC and neutrophils, as well as neutrophil-endothelial interaction under flow conditions.

Definition and detection of microparticles. MP are small in size, ranging from 0.1 to 1 μm in diameter and are therefore difficult to detect. The method most often employed to characterise MP is flow-cytometry; MP are stained with fluorescent antibodies that are within the detection range of the available flow-cytometer. This process is fraught with difficulties; including sources of interference and the danger of false positives[295-297]. Due to the variability in the capabilities of flow-cytometers, the chosen methodologies have to be tailored to ensure maximum confidence in the

results. In this project, considerable time was dedicated to formulating a protocol for MP detection that would be reliable using the equipment available (BD LSRFortessa).

The first step was to define what would constitute a MP for the purpose of this project. A microparticle was defined as a particle detected by flow-cytometer ranging in size between 0.1 and 1µm in diameter, positive for annexin V (AV) and at least one other marker. Adopting AV as a definitive general marker for MP risked discounting AV negative events but on balance it was concluded that defining MP using a single marker (e.g. CD66b+ for neutrophil derived MP) risked misclassifying cell debris as MP. Another advantage of using AV as a marker is that the results could be easily compared to other published studies[236, 241, 242]. Other factors considered in the preliminary experiments were the choice of anticoagulant, centrifugation protocol, size calibration and resolution, reducing noise and antibody titration. As result a robust protocol was established that has now been adopted by the host group and exploited for parallel projects.

Microparticle profiles in patients with sepsis. Severe sepsis and its catastrophic consequences was initially thought to be due to an excessive, disseminated pro-inflammatory response, but more recently it has be recognised as being related to a dysregulated host response often characterised by co-existent immune-paresis[47]. Given this state of immune dissonance understanding the role of MP as cell-to-cell communicators becomes even more pertinent. A pragmatic approach was applied

to this project and the first step would be the detailed characterisation of MP profiles in septic patients. Previous attempts to profile MP in severe sepsis, have yielded conflicting results, both in comparison to healthy volunteers and in relation to outcome[235, 236, 238, 239]. In most of previous studies, researchers have included patients with multiple and heterogeneous causes of sepsis (e.g. urinary tract infections, soft tissue infections, trauma and more). The results described here shed some light on the pathophysiological heterogeneity of sepsis populations as we have demonstrated that MP derived from immune cells and endothelium were significantly higher in CAP sepsis in comparison to FP patients and HV. This observation was particularly interesting given that CAP and FP cohorts were similar in terms of demographics, co-morbidities, severity scores and cells counts. Surprisingly, apart from granulocyte, the MP profile of FP patients was not significantly different from HV, even though FP patients were older, had a major illness requiring intensive care and higher cell counts. One cannot exclude that other circulating MP subtypes or from different cells of origin might be different between FP and HV. In any case, in association with outcome, CAP non-survivors had higher levels of endothelial MP while non-survivors of FP had higher granulocytes and erythrocytes derived MP.

This data indicate that previous studies that have focused on establishing variations in MP derived from different cells have probably missed an important point, an issue that was addressed here which is the heterogeneity of MP. It was gratifying to observe that A2M staining afforded much more clear cut differences, when present, and this was only in part associated with the cell of origin. Coupled to the study that

paved the way to this studentship[254], the new data presented herein give substance to the need of stratifying MP for at least 2 or more markers if one has to attempt meaningful association with outcome.

Heterogeneity of sepsis. The discrepancies seen here may be due to the different natural course of each disease; CAP patients tend to harbour the disease for a few days prior to ICU presentation in comparison to FP patients who generally have a distinct and fairly recent onset of illness prior to presentation. There are also important aetiological and pathological differences between the two conditions. CAP is generally caused by specific pathogens and is commonly preceded by viral infection which enhances bacterial adherence to alveolar type II cells and promotes the release of cytokines, facilitating the development of pneumonia. The cell walls of dividing bacteria bind to epithelial, endothelial and local immune cells resulting in production of IL-1 and the separation of endothelial cells, with leakage and accumulation of serous fluid in the lungs in the early stages of consolidation (called "engorgement")[298]. "Red hepatisation" is the next stage where there is leakage of erythrocytes into the alveoli, with expression of tissue factor and platelet activating factor by endothelial cells resulting in a localised pro-coagulant state and recruitment of platelets and leucocytes to the area. Leucocytes are further recruited via selectin-CD18 integrin, platelet activating factor and alternative complement pathways as well as via complement in a stage called "grey hepatisation"[299]. As leucocytes kill the invading bacteria there is release of microbial cell wall products and other components resulting in further local inflammation and a cytotoxic effect on

pulmonary cells. When neutrophils begin to phagocytose and kill the pathogen the process of consolidation resolution ensues during which monocytes are recruited to the area to clear debris, fibrin and apoptotic neutrophils and the lung eventually returns to normal function[300]. The outcome of this infection will to a certain extent depend on the host's ability to withstand and resolve the inflammation which could be exaggerated and dysregulated resulting in severe sepsis picture[301, 302]. FP, on the other hand is caused by spillage of large intestinal content into the peritoneal cavity through anastomosis breakdown, penetrating wounds or perforated diverticulitis. Faecal material be localised around the affected viscous causing an abscess or may cause generalised peritonitis. The resident peritoneal macrophages encounter bacteria or bacterial components resulting in the production of pro-inflammatory cytokines such TNF-α, IL-1, IL-6, IL-8 and IFN-γ about 2 hours following the initial insult[303]. These cytokines are found in the peritoneal exudates and reach the systemic circulation *via* lymphatic drainage. These cytokines in the peritoneum induce the production of nitric oxide and arachidonic acid metabolites resulting in activation and neutrophil recruitment[304, 305]. It has been shown that the concentration of these cytokines is much higher than in plasma, suggesting that there is a considerable degree of "compartmentalisation"[303, 306, 307].

Moreover, the majority of the causative organisms in CAP are Gram positive[308] while FP is usually due to peritoneal inflammation and contamination with a mixture of Gram negative and anaerobic organisms[309]. The causative organism influences the disease course through multiple mechanisms. For example secreted toxins may

exacerbate the inflammatory response. Panton-Valentine leukocidin secreted by staphylococci contributes to the development of haemorrhagic pneumonia in some patients. Other toxins include toxic shock syndrome toxin 1 and exfoliative toxin secreted by *Staphylococcus aureus*[310]. Another mechanism by which the causative organism can influence the course of sepsis is *via* release of cell wall components. Toll like receptors (TLR) expressed on the surface of cells of the innate immune system recognise bacterial cell wall components. For example, LPS and lipotecichoic acid are present in the cell walls of Gram negative and Gram positive bacteria and they activate TLR-4 and TLR-2 respectively. There is some overlap in the TLR receptors by the intact micro-organism but activation by Gram negative and Gram positive bacteria remains dominantly TLR-4 and TLR-2 dependent respectively. The activation of these receptors results in the up-regulation of nuclear factor-κB (NF-κB), activator protein-1 and interferon response factor-1 resulting in the transcription of pro-inflammatory genes. This overlap in the downstream pathways is not complete and the response can vary between the two categories of bacterial infections. For example, heat killed Escherichia coli produces more IL-10 and less interferon-gamma (IFN-γ) than staphylococci, as well as stimulating the production of more IL-6, IL-8, IL-1β and tumour necrosis factor (TNF)-α than heat killed streptococci in a whole blood model. In vivo, Gram negative infections produce more TNF-α and IL10 and less IFN-γ than are produced by Gram positive organisms[310].

An external factor by which the course of the illness may be affected is the choice of antibiotics as the recommended empirical agents used for CAP differ from those used for FP. Also the specific antibiotic therapy that is used subsequently once the organism has been identified will differ between the two forms of severe sepsis. Macrolides such as clarithromycin are frequently used in the treatment of pneumonia and rarely used for FP. Recently, these compounds have been shown to possess anti-inflammatory actions beyond their anti-microbial properties[311]. Macrolides reduce the production of TNF-α and IL-1, inhibit the NF-κB pathway, suppress TLR expression and induce neutrophil apoptosis[311]. Moxifloxacin a quinolone antibiotic can reduce IL-8, IL-1β and TNF-α induction by LPS in human monocytes in vitro and has been shown to inhibit NF-κB and the mitogen activated protein kinases (JNK and ERK) pathways[312].

Another important consideration is that in Gram negative sepsis, circulating levels of LPS vary according to the source of infection. Patients with CAP were found to have higher plasma levels of LPS in comparison to patients with intra-abdominal sepsis[313]. Monocyte HLA-DR expression also varied according to the source of infection as well as apoptosis of natural killer cells and lymphocytes[314].

It was interesting and perhaps surprising to observe the variation in erythrocyte derived MP between non-survivors and survivors of FP sepsis which was not demonstrated in CAP. This would suggest some participation of erythrocytes MP in the immune process in FP. Erythrocyte MP have been previously investigated in the

context of stored erythrocytes for transfusion. Stored erythrocytes have been demonstrated to produce large number of MP in storage with a distinct proteomic profile[315]. These MP were shown in another study to have a pro-inflammatory effect when injected in mice[316]. The stored erythrocyte MP also enhanced human neutrophils CD11b expression, ROS production and phagocytosis in vitro[316]. They were found to activate monocytes in vitro inducing pro-inflammatory cytokines production and this effect was attributed more to exosomes rather than the large microparticles[317]. Another study demonstrated a pro-coagulant effect of stored erythrocyte MP mediated through initiation of thrombin generation[318]. Also these MP were shown to express phosphatidylserine and this expression increased as the age of the stored blood increased exerting a pro-thrombotic effect[319]. These studies consolidate the notion that erythrocyte MP have a role to play in inflammation.

Taken together these findings suggest that CAP and FP are quite different diseases, albeit with a similar clinical phenotype presenting as severe sepsis and this may explain the differences in MP profiles described in this thesis and the inconsistent findings in previous studies.

Role of microparticles expressing alpha-2-macroglobulin (A2M) in sepsis. A2M is a tetrameric glycoprotein consisting of four identical subunits. The main function of this acute phase reactant is the entrapment and inhibition of proteinases by preventing the attachment of large molecular weight substrates to the active site. A2M entraps the proteinases produced by neutrophils and other cells during inflammation as well

as regulating clotting and fibrinolysis via inhibition of proteolytic activity[320]. A2M also protects the host by entrapping proteases produced by invading pathogens. Chymase is a protease secreted by mast cells that is involved in the activation of many important mediators such as angiotensin, IL-1β, endothelin and collagenase, as well as the induction of basement membrane degradation and inhibition of thrombin receptor. A2M has also been shown to inhibit and regulate chymase activity. Transferrin is an iron transporting protein that requires A2M for its uptake by cells. A2M also acts as a scavenger of inflammatory mediators such as defensins in order to regulate the inflammatory process and also binds cytokines such as TNF-α, IL-1β and IL-6 in order to protect them from degradation and deliver them to cells through A2M receptor pathways. A2M plays a similar role in binding, protecting and delivering hormones such as hepcidin (regulates transmembrane iron transport) and leptin (regulates appetite and energy expenditure) to cells. Some of the effects of A2M are mediated *via* the receptor low density lipoprotein receptor (LRP). Activation of this receptor on macrophages reduces apoptosis and induces production of eicosanoids. Activation of LRP inhibits the production of reactive oxygen species by granulocytes.

It is intriguing that A2M has been considered as a potential biomarker for various diseases such as liver fibrosis, cardiac hypertrophy, myocardial infarction in diabetic patients, diabetes mellitus, pancreatitis and inflammatory bowel disease[270], however these studies did not investigate whether this acute phage protein was encapsulated in vesicles or free floating in the plasma.

Our group has previously demonstrated that MP produced from neutrophils adherent to an endothelial monolayer contained and expressed A2M among other proteins[191]. The proteome of these adherent MP differed from that derived from neutrophils in suspension. The A2M expressing adherent MP up-regulated pro-inflammatory gene expression when incubated with endothelial cells and similar A2M expressing MP were found in the plasma of patients with sepsis[191]. Injection of A2M expressing MP into septic mice was associated with a significant reduction in mortality as well as a reduction in pro-inflammatory and an increase in pro-resolution lipid mediators in their peritoneal exudate. These A2M enriched MP also reduced local and systemic bacterial load and protected against hypothermia. The neutrophil count and level of pro-inflammatory cytokines in the peritoneal cavity were also reduced without a reduction in the monocyte/macrophage count. The A2M MP were also found to enhance neutrophil phagocytosis, the production of reactive oxygen species and neutrophil endothelial interaction[254]. The A2M MP exerted their effects through LRP-1[254], a widely expressed receptor protein that has a role in cellular endocytosis and signal transduction. In the lungs this receptor has a pro-inflammatory role where it triggers macrophage phagocytosis against foreign bodies and cytokines release. This receptor is also expressed on endothelial cells, where it promotes endothelial interaction with leucocytes and maintains vascular integrity[321]. Data from our group showed that neutrophils also express LRP-1. Another property of A2M was its transfer *via* MP to endothelial cells where it promotes endothelial neutrophil interaction through binding the LRP-1 on the surface of neutrophils[254]. Taken

together these experiments demonstrated an activating role for A2M expressing MP on the innate immune response which could have beneficial effects against the immune-paresis typical of severe sepsis. These observations were extended in the work reported in this thesis.

First, it was observed that $A2M^+$ MP are derived from all blood cells, not just neutrophils, an observation that is supported by previous proteomic analyses of MP derived from monocytes, lymphocytes, platelets and erythrocytes showing that these MP subsets do indeed contain A2M[190, 322-324]. The results of this study also suggest that monocytes and lymphocytes are more efficient in producing $A2M^+$ MP, at the single cell level than neutrophils in patients with severe sepsis.

Second, it was demonstrated that patients with CAP had higher total $A2M^+$ MP and A2M MP derived from granulocytes, monocytes, lymphocytes, platelets and erythrocytes in comparison to patients with FP and healthy volunteers. This result reinforces the increasing recognition that CAP and FP are different diseases, albeit with a similar clinical picture of severe sepsis. There were no significant differences in all these $A2M^+$ MP subsets between FP and HV.

Third, when MP were further characterised according to their A2M expression in relation to outcome, significant differences emerged. CAP survivors had higher total $A2M^+$ MP from and increased levels of granulocyte and monocyte MP expressing A2M compared to non-survivors, whereas in FP patients there was a significant

increase in erythrocyte $A2M^+$ MP in non-survivors compared to non-survivors. The higher levels of $A2M^+$ MP in CAP survivors was sustained across all three time points examined in comparison to non-survivors, while non-survivors had either persistently low or decreasing $A2M^+$ MP levels. This was particularly evident in the case of total $A2M^+$ MP as well as granulocyte and monocyte derived MP expressing A2M.

The higher level of MP expressing this acute phase reactant (A2M) may point towards the need for a more potent, targeted inflammatory response to overcome an invasive infection with more generalised effects. This suggestion is supported by the immuno-modulatory effects attributed to A2M expressing MP previously reported by our group [191] and in the latter part of this project.

Immune-paresis in sepsis. A favourable outcome in severe sepsis is associated with the ability to maintain an effective immune response in the face of continuing and added infections as well as the ability to resolve inflammation once the offending pathogen has been eliminated. The antigen presenting molecule human leucocyte antigen (HLA)-DR, which is expressed by monocytes and is a marker of the ability to mount a response against invading pathogens has been shown to be linked to the development and outcome of severe sepsis. Reduced expression of this molecule on monocytes is associated with an inability to mount a TNF-α response when challenged with LPS (i.e. the monocytes are “deactivated”) [325, 326]. Following major surgery, HLA-DR expression falls, but those patients in whom who HLA-DR

expression is rapidly restored survive without superadded infections, whereas those in whom HLA-DR expression is restored more slowly developed infection and in those whom HLA-DR expression never recovered developed severe sepsis and died[327]. This observation was confirmed in a study in trauma patients in which it was found that patients with lower HLA-DR expression were more likely to develop severe sepsis[327]. In severe sepsis secondary to CAP, reduced HLA-DR expression has been associated with worse outcome[328]. Higher levels of the anti-inflammatory cytokine IL-10 have also been associated with worse outcomes and IL-10 mRNA expression in leucocytes might be a useful marker for the onset of immune-paresis. The sustained production of IL-10 beyond the initial phase of sepsis and decreasing levels of the pro-inflammatory tumour necrosis factor (TNF)-α were found to be associated with worse outcome; thus in the same cohort a high IL-10 to TNF-α ratio was associated with non-survival[329]. This observation was confirmed in severe sepsis patients with CAP in whom higher levels of IL-10 were again associated with worse outcome[328, 330]. These observations support the concept that following the initial hyper-immune phase in severe sepsis a persistent hypo-immune response is associated with worse outcome.

Further confirmation of the importance of immune-paresis in determining sepsis outcomes comes from clinical trials that attempted to neutralise pro-inflammatory cytokines and in effect induce hypo-immunity, a clinical approach that in many cases resulted in a worse outcomes[331]. On the other hand attempts to reverse the hypo-immune state seen in sepsis patients have resulted in some promising results. As

outlined in the introduction chapter of this thesis, granulocyte-macrophage colony stimulating factor (GM-CSF) and interferon-γ (IFN-γ) have been shown to reverse the monocyte deactivation seen in sepsis and restore their ability to produce TNF-α, with some beneficial effects in clinical trials particularly in patients with low monocyte HLA-DR expression[331]. This results demonestrate that patients who are able to produce and maintain a higher level of the pro-inflammatory $A2M^+$ MP were more likely to survive is in keeping with the concept that an effective therapeutic strategy in sepsis may entail the boosting of the immune response.

GM-CSF and IFN-γ clinical trials in sepsis have shown some beneficial effects particularly in those with biomarkers of hypo-immunity (i.e. low monocyte HLA-DR expression) where they were able to reverse the monocyte deactivation state[133, 168]. In the experiments reported here, GM-CSF or IFN-γ were used to stimulate whole blood obtained from healthy volunteers, observing that these treatments induced the production of $A2M^+$ MP. These experiments demonstrated that these MP elicited a pro-inflammatory effect on neutrophils and endothelial cells as well as enhancing the neutrophil-endothelial interactions. The effects are consistent with previous observations that $A2M^+$ MP enhance pathogen clearance[191, 254]. These results indicate that some of the potential beneficial effects of GM-CSF and IFN-γ in sepsis could be mediated by MP.

Functional effects of GM-CSF or IFN-γ induced microparticles. MP interact with target cells via various mechanisms including binding to membrane receptors and

internalisation. It has been demonstrated previously that endothelial cells uptake of MP depends on the phosphatidylserine expressed on their surface[332, 333] and is independent of adhesion molecule expression on the endothelial cell surface[334]. The mechanism for this internalisation has been proposed to be via phagocytosis and macropinocytosis[334]. In this study, it was demonstrated that the vast majority of endothelial cells have taken up MP after 4 hours and this uptake can be largely prevented by cytochalasin B which inhibits the actin filament polymerisation required for endocytosis[335]. These results were corroborated further by in-vitro analyses by measuring the effect of cytokine-induced MP on the release of specific immune-modulatory mediators.

Reactive oxygen species (ROS) are produced by various cells as a means of eliminating pathogens, on the other hand excessive release can lead to tissue damage. MP have been shown previously to increase ROS for example by T-cells stimulated with actinomycin D for 24 hours[336] or by circulating MP obtained from hypoxic mice[216]. In other reports MP reduced endothelial ROS production, including MP obtained from T-cells stimulated by phytohemagglutinin for 72 hours followed by phorbol-12-myristate-13 and actinomycin D for a further 24 hours[337] and circulating MP from all sources obtained from patients with metabolic syndrome[338]. The same discrepancies are seen in sepsis where MP from septic rats induced ROS production in the aortas and hearts of healthy rats in one study[250] while in another MP from septic patients did not alter endothelial ROS production in vitro[237]. This study has demonstrated that MP produced from whole blood stimulated by GM-CSF or IFN-γ

enhance ROS production in endothelial cells and that this was further enhanced in the presence of LPS. The same MP reduced neutrophil ROS production. These findings suggest that the functional effect of MP depends on the stimulus for their production, the context and the target cell. In the context of a severe sepsis induced hypo-immune state, a robust production of ROS by endothelial cells could help fend off invasive pathogens while reduced neutrophil ROS production could reduce the risk of acute respiratory distress syndrome (ARDS). Indeed a reduction in hospital acquired infections and lung injury were two of the effects seen in clinical trials examining GM-CSF or IFN-γ as adjuvant therapies in severe sepsis[133-135, 171].

Nitiric oxide (NO) is a vasodilating agent produced by the endothelium and other cells *via* endothelial nitric oxide synthase (eNOS) or inducible nitric oxide synthase (iNOS) respectively. Severe sepsis and experimental endotoxaemia initially both reduce production of NO *via* suppression of eNOS and later in the course of the disease there is increased production of NO *via* activation of (iNOS). The initial reduction in endothelial NO production contributes to the vascular injury seen in sepsis while the later increase in NO results in excessive vasodilation and hypotension[9]. MP play a role in regulating endothelial NO production. T-cell MP, depending on the stimulus for their production can either increase or decrease endothelial NO production[336, 337]. Metabolic syndrome MP reduce NO levels [338], while the MP from Crohn's disease patients cause over production of NO *via* enhanced iNOS activity[339]. MP derived from septic rats reduced eNOS activity and enhanced the effect of iNOS in the aortae and hearts of healthy rats[250]. Here, MP

produced in response to GM-CSF and IFN-γ enhanced the production of NO from endothelial cells, presumably due to up-regulation of eNOS. This effect might help to restore some endothelial function in sepsis patients.

TNF-α is one of the principal cytokines implicated in the pathogenesis of severe sepsis. A surge in TNF-α levels is observed during the early systemic inflammatory response syndrome (SIRS) phase; this is followed by lower levels during the compensatory anti-inflammatory response syndrome (CARS) hypo-immune phase[52]. In clinical trials, GM-CSF and IFN-γ restored TNF-α levels and the response to endotoxin in patients with severe sepsis, as well as reducing the incidence of hospital acquired infection in some patients[131, 133, 169, 340]. IL-8 is another cytokine with an important role in inflammation and pathogen elimination *via* chemotactic attraction of neutrophils to the site of the insult. In severe sepsis, IL-8 production from whole blood in response to endotoxins was subdued[341], a response that could be restored by GM-CSF[131]. MP were also found to have an effect on the production of these two cytokines; for example platelet MP produced by shear stress increased TNF-α and IL-8 production by endothelial cells[342], as so did atheromatous plaque MP[343]. On the other hand circulating MP from patients with metabolic syndrome did not influence the production of these cytokines by endothelial cells[338]. In the present study the whole blood MP produced in response to GM-CSF or IFN-γ enhanced TNF-α and IL-8 production by endothelial cells and this effect was more marked in the presence of LPS. On the other hand these MP did not enhance neutrophil chemotaxis. These observations again highlight the role played by MP in the

potential beneficial effects seen in clinical trials of GM-CSF or IFN-γ and may help to shed some light on the mechanism of reduced neutrophil sequestration in the lungs.

Increased endothelial permeability in severe sepsis results in tissue oedema which contributes to multi-organ dysfunction and eventually failure[72]. This effect is particularly pertinent in the lungs where it can lead to acute lung injury (ALI) or acute respiratory distress syndrome (ARDS). In experimental models of sepsis LPS was found to increase endothelial permeability both systemically and in the lungs resulting in ALI/ARDS in a murine model[344-346]. The effect of LPS was mediated *via* the enhanced expression of caveolin-1[344] (facilities albumin endothelial transcytosis[347]) and angiotensin II[345] (enhances the expression of VGEF which remodel the extra-cellular matrix resulting in increased permeability[348]). MP can influence endothelial permeability. Endothelial derived MP increased permeability[346], whereas circulating MP from septic patients treated with activated protein C reduced permeability [349] and surprisingly LPS induced monocyte derived MP did not alter permeability[350]. GM-CSF has been shown to improve gas exchange in sepsis related respiratory dysfunction[135] and as shown in a separate clinical investigation, its administration can reduce the number of days on mechanical ventilation[133]. Also GM-CSF has been demonstrated to reduce vascular permeability in burns which has been attributed to RhoA inhibition and maintaining VE-cadherin which enhances the adherens junctions between endothelial cells[351]. In this study it was demonstrated that MP produced from whole blood stimulated by GM-CSF or IFN-γ reduced

endothelial monolayer permeability despite increasing TNF-α levels. This result mirrors, and may contribute to the effects of GM-CSF found previously.

Neutrophil-endothelial interaction is vital for pathogen clearance as it facilitates neutrophil migration to the site of infection. This interaction is impaired in sepsis, where there is reduction in rolling, adherence and transmigration of neutrophils across the endothelium which results in failure of neutrophil migration to the site of infection and therefore reduced in pathogen clearance[57]. As a consequence, inflammation persists, resolution is delayed and organ damage is perpetuated. Our group has shown previously that $A2M^+$ MP enhance neutrophil-endothelial interaction, as well as enhancing bacterial clearance and resolution of inflammation in a murine model of sepsis[191, 254]. These potential beneficial effects were found not to be dependent on endothelial adhesion molecule up-regulation but on A2M transfer via MP to endothelial cells and interactions with the LRP-1 receptor expressed on the surface of neutrophils[254]. GM-CSF or IFN-γ whole blood MP seem to have a similar effect, enhancing neutrophil-endothelial interaction under flow without increasing the expression of endothelial adhesion molecules.

Limitation of the studies reported in this thesis. The study was performed retrospectively and MP were characterised in stored plasma even though it was previously demonstrated that storage and freeze/thaw cycles had no effect of the concentration of MP in plasma[352]. The study only examined two causes of severe sepsis. The results were compared to healthy volunteers only and less severe forms

of sepsis were not included and neither were causes of systemic inflammatory response syndrome other than infection. The experiments were conducted by a single operator and the operator was not blinded to outcome. Whole blood model used for GM-CSF and IFN-γ MP production. In this study, the incubation periods for whole blood was relatively short (30 or 180 minutes) but whole blood once outside the body starts to become hypoxic, hypoglycaemic and acidic which might influence MP production. Unstimulated whole blood incubated in vitro was used to produce control MP to mitigate the risks of the whole blood model.

Mechanisms behind the effect of whole blood MP effects were not investigated. Blocking of A2M expressed on MP would have shed some light on the mechanisms however our group has previously demonstrated that blocking A2M expressed on MP using monoclonal antibodies abrogated many of the MP effects[191, 254].

Future work. The differences observed between CAP and FP MP profiles would lead to investigating the functional effects of CAP and FP MP on neutrophils, monocytes and endothelial cells in order to investigate whether these MP contribute to the immune process of these diseases. Also investigating whether there is a difference in functional effect of MP between survivors and non-survivors of CAP and the role of A2M in this function. This could be taken a step further by sorting the MP according to cell of origin in order to pin point which cell line is responsible for the effects seen. This could be followed by investigating the proteomic profiles of these MP populations as well as their microRNA content. If a specific microRNA is identified,

this could blocked to investigate whether the effect of these MP is microRNA dependent.

The results of the functional effects of GM-CSF and IFN-γ would lead to investigating the proteomic profile of the MP as well as their microRNA content and whether the function of these MP is dependent on microRNA.

Chapter 6: Conclusions

Microparticle profiles in relation to source of infection. Microparticle (MP) profiles differed significantly according to the source of infection. Patients with severe sepsis due to community acquired pneumonia patients had higher plasma concentrations of MP in comparison to those with faecal peritonitis and healthy volunteers; this finding was consistent across all the measured MP subsets. Surprisingly there was no significant difference in MP concentrations between faecal peritonitis patients and healthy volunteers. This finding adds to the increasing body of evidence indicating that sepsis is a heterogeneous condition, emphasising the importance of studying more homogenous cohorts of patients and developing source targeted treatments.

Microparticle profiles relation to outcome. Community acquired pneumonia non-survivors had levels higher of endothelial MP while faecal peritonitis non-survivors had higher granulocyte and erythrocyte derived MP counts at day 1 post admission to intensive care. When MP were further characterised according to alpha-2-macroglobulin (A2M) expression a new picture emerged. The total plasma concentration of $A2M^+$ MP, as well as granulocyte and monocyte $A2M^+$ MP plasma concentrations were higher in survivors of community acquired pneumonia sepsis compared to non-survivors at day 1; this difference was sustained across the examined time points (day 3 and 5). This data highlights the potential for MP profiles to be used as a biomarker in severe sepsis when they are characterised not only according to their cell of origin but also according to protein expression. MP expressing the protein A2M have the potential as novel biomarkers in patients with severe sepsis due to community acquired pneumonia. It is envisage that a more

analytical follow up studies where MP from septic patients are sorted according to their cell of origin marker and then used for unbiased analyses (e.g. proteomics, lipidomics, microRNA) could allow the identification of more markers of disease to be used alone or in association with the A2M+ MP characterised here.

Microparticles produced by stimulating whole blood with granulocyte-macrophage colony stimulating factor (GM-CSF) or interferon-gamma (IFN-γ) functional effects. The MP produced by stimulating whole blood with GM-CSF or IFN-γ had a pro pathogen clearance effect on neutrophils and endothelial cells, as well as enhancing neutrophil-endothelial interaction under flow conditions. These results shed light on the role of MP in the potential beneficial effect of GM-CSF and IFN-γ seen in clinical trials when these cytokines were used as adjuvant immune-stimulatory therapies in severe sepsis.

Chapter 7: References

References:

1. Medzhitov R. Origin and physiological roles of inflammation. *Nature*. 2008;454:428-435
2. Kolaczkowska E, Kubes P. Neutrophil recruitment and function in health and inflammation. *Nature reviews. Immunology*. 2013;13:159-175
3. Sundd P, Gutierrez E, Koltsova EK, Kuwano Y, Fukuda S, Pospieszalska MK, Groisman A, Ley K. 'Slings' enable neutrophil rolling at high shear. *Nature*. 2012;488:399-403
4. Phillipson M, Heit B, Colarusso P, Liu L, Ballantyne CM, Kubes P. Intraluminal crawling of neutrophils to emigration sites: A molecularly distinct process from adhesion in the recruitment cascade. *The Journal of experimental medicine*. 2006;203:2569-2575
5. Burns AR, Bowden RA, MacDonell SD, Walker DC, Odebunmi TO, Donnachie EM, Simon SI, Entman ML, Smith CW. Analysis of tight junctions during neutrophil transendothelial migration. *Journal of cell science*. 2000;113 (Pt 1):45-57
6. Phillipson M, Kaur J, Colarusso P, Ballantyne CM, Kubes P. Endothelial domes encapsulate adherent neutrophils and minimize increases in vascular permeability in paracellular and transcellular emigration. *PloS one*. 2008;3:e1649
7. Wang S, Voisin MB, Larbi KY, Dangerfield J, Scheiermann C, Tran M, Maxwell PH, Sorokin L, Nourshargh S. Venular basement membranes contain specific matrix protein low expression regions that act as exit points for emigrating neutrophils. *The Journal of experimental medicine*. 2006;203:1519-1532
8. Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, Weinrauch Y, Zychlinsky A. Neutrophil extracellular traps kill bacteria. *Science*. 2004;303:1532-1535
9. Ait-Oufella H, Maury E, Lehoux S, Guidet B, Offenstadt G. The endothelium: Physiological functions and role in microcirculatory failure during severe sepsis. *Intensive care medicine*. 2010;36:1286-1298
10. Pober JS, Sessa WC. Evolving functions of endothelial cells in inflammation. *Nature reviews. Immunology*. 2007;7:803-815
11. Sessa WC. Enos at a glance. *Journal of cell science*. 2004;117:2427-2429
12. Bazzoni G, Dejana E. Endothelial cell-to-cell junctions: Molecular organization and role in vascular homeostasis. *Physiological reviews*. 2004;84:869-901
13. McEver RP, Beckstead JH, Moore KL, Marshall-Carlson L, Bainton DF. Gmp-140, a platelet alpha-granule membrane protein, is also synthesized by vascular endothelial cells and is localized in weibel-palade bodies. *The Journal of clinical investigation*. 1989;84:92-99
14. Wolff B, Burns AR, Middleton J, Rot A. Endothelial cell "memory" of inflammatory stimulation: Human venular endothelial cells store interleukin 8 in weibel-palade bodies. *The Journal of experimental medicine*. 1998;188:1757-1762
15. Matsushita K, Morrell CN, Cambien B, Yang SX, Yamakuchi M, Bao C, Hara MR, Quick RA, Cao W, O'Rourke B, Lowenstein JM, Pevsner J, Wagner DD, Lowenstein

CJ. Nitric oxide regulates exocytosis by s-nitrosylation of n-ethylmaleimide-sensitive factor. *Cell*. 2003;115:139-150
16. Stevens T, Garcia JG, Shasby DM, Bhattacharya J, Malik AB. Mechanisms regulating endothelial cell barrier function. *American journal of physiology. Lung cellular and molecular physiology*. 2000;279:L419-422
17. Lorant DE, Patel KD, McIntyre TM, McEver RP, Prescott SM, Zimmerman GA. Coexpression of gmp-140 and paf by endothelium stimulated by histamine or thrombin: A juxtacrine system for adhesion and activation of neutrophils. *The Journal of cell biology*. 1991;115:223-234
18. Martin MU, Wesche H. Summary and comparison of the signaling mechanisms of the toll/interleukin-1 receptor family. *Biochimica et biophysica acta*. 2002;1592:265-280
19. Petrache I, Birukova A, Ramirez SI, Garcia JG, Verin AD. The role of the microtubules in tumor necrosis factor-alpha-induced endothelial cell permeability. *American journal of respiratory cell and molecular biology*. 2003;28:574-581
20. Li JH, Pober JS. The cathepsin b death pathway contributes to tnf plus ifn-gamma-mediated human endothelial injury. *J Immunol*. 2005;175:1858-1866
21. Bombeli T, Karsan A, Tait JF, Harlan JM. Apoptotic vascular endothelial cells become procoagulant. *Blood*. 1997;89:2429-2442
22. Nawroth PP, Stern DM. Modulation of endothelial cell hemostatic properties by tumor necrosis factor. *The Journal of experimental medicine*. 1986;163:740-745
23. Ortega-Gomez A, Perretti M, Soehnlein O. Resolution of inflammation: An integrated view. *EMBO molecular medicine*. 2013;5:661-674
24. Dean RA, Cox JH, Bellac CL, Doucet A, Starr AE, Overall CM. Macrophage-specific metalloelastase (mmp-12) truncates and inactivates elr+ cxc chemokines and generates ccl2, -7, -8, and -13 antagonists: Potential role of the macrophage in terminating polymorphonuclear leukocyte influx. *Blood*. 2008;112:3455-3464
25. Mantovani A, Bonecchi R, Locati M. Tuning inflammation and immunity by chemokine sequestration: Decoys and more. *Nature reviews. Immunology*. 2006;6:907-918
26. D'Amico G, Frascaroli G, Bianchi G, Transidico P, Doni A, Vecchi A, Sozzani S, Allavena P, Mantovani A. Uncoupling of inflammatory chemokine receptors by il-10: Generation of functional decoys. *Nature immunology*. 2000;1:387-391
27. van den Berg JM, Weyer S, Weening JJ, Roos D, Kuijpers TW. Divergent effects of tumor necrosis factor alpha on apoptosis of human neutrophils. *Journal of leukocyte biology*. 2001;69:467-473
28. Perretti M, Chiang N, La M, Fierro IM, Marullo S, Getting SJ, Solito E, Serhan CN. Endogenous lipid- and peptide-derived anti-inflammatory pathways generated with glucocorticoid and aspirin treatment activate the lipoxin a4 receptor. *Nature medicine*. 2002;8:1296-1302
29. Bournazou I, Pound JD, Duffin R, Bournazos S, Melville LA, Brown SB, Rossi AG, Gregory CD. Apoptotic human cells inhibit migration of granulocytes via release of lactoferrin. *The Journal of clinical investigation*. 2009;119:20-32
30. Fadok VA, Bratton DL, Konowal A, Freed PW, Westcott JY, Henson PM. Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory

cytokine production through autocrine/paracrine mechanisms involving tgf-beta, pge2, and paf. *The Journal of clinical investigation*. 1998;101:890-898
31. Bystrom J, Evans I, Newson J, Stables M, Toor I, van Rooijen N, Crawford M, Colville-Nash P, Farrow S, Gilroy DW. Resolution-phase macrophages possess a unique inflammatory phenotype that is controlled by camp. *Blood*. 2008;112:4117-4127
32. Stables MJ, Shah S, Camon EB, Lovering RC, Newson J, Bystrom J, Farrow S, Gilroy DW. Transcriptomic analyses of murine resolution-phase macrophages. *Blood*. 2011;118:e192-208
33. Rajakariar R, Lawrence T, Bystrom J, Hilliard M, Colville-Nash P, Bellingan G, Fitzgerald D, Yaqoob MM, Gilroy DW. Novel biphasic role for lymphocytes revealed during resolving inflammation. *Blood*. 2008;111:4184-4192
34. Schif-Zuck S, Gross N, Assi S, Rostoker R, Serhan CN, Ariel A. Saturated-efferocytosis generates pro-resolving cd11b low macrophages: Modulation by resolvins and glucocorticoids. *European journal of immunology*. 2011;41:366-379
35. Saclier M, Yacoub-Youssef H, Mackey AL, Arnold L, Ardjoune H, Magnan M, Sailhan F, Chelly J, Pavlath GK, Mounier R, Kjaer M, Chazaud B. Differentially activated macrophages orchestrate myogenic precursor cell fate during human skeletal muscle regeneration. *Stem cells*. 2013;31:384-396
36. Leask A, Abraham DJ. Tgf-beta signaling and the fibrotic response. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*. 2004;18:816-827
37. Barrientos S, Stojadinovic O, Golinko MS, Brem H, Tomic-Canic M. Growth factors and cytokines in wound healing. *Wound repair and regeneration : official publication of the Wound Healing Society [and] the European Tissue Repair Society*. 2008;16:585-601
38. Kim J, Hematti P. Mesenchymal stem cell-educated macrophages: A novel type of alternatively activated macrophages. *Experimental hematology*. 2009;37:1445-1453
39. Serhan CN. Pro-resolving lipid mediators are leads for resolution physiology. *Nature*. 2014;510:92-101
40. Filippi CM, von Herrath MG. Il-10 and the resolution of infections. *The Journal of pathology*. 2008;214:224-230
41. Ouyang W, Rutz S, Crellin NK, Valdez PA, Hymowitz SG. Regulation and functions of the il-10 family of cytokines in inflammation and disease. *Annual review of immunology*. 2011;29:71-109
42. Skrupky LP, Kerby PW, Hotchkiss RS. Advances in the management of sepsis and the understanding of key immunologic defects. *Anesthesiology*. 2011;115:1349-1362
43. Harrison DA, Welch CA, Eddleston JM. The epidemiology of severe sepsis in england, wales and northern ireland, 1996 to 2004: Secondary analysis of a high quality clinical database, the icnarc case mix programme database. *Crit Care*. 2006;10:R42
44. Dellinger RP, Levy MM, Rhodes A, Annane D, Gerlach H, Opal SM, Sevransky JE, Sprung CL, Douglas IS, Jaeschke R, Osborn TM, Nunnally ME, Townsend SR,

Reinhart K, Kleinpell RM, Angus DC, Deutschman CS, Machado FR, Rubenfeld GD, Webb S, Beale RJ, Vincent JL, Moreno R, Surviving Sepsis Campaign Guidelines Committee including The Pediatric S. Surviving sepsis campaign: International guidelines for management of severe sepsis and septic shock, 2012. *Intensive care medicine*. 2013;39:165-228
45. Marti-Carvajal AJ, Sola I, Gluud C, Lathyris D, Cardona AF. Human recombinant protein c for severe sepsis and septic shock in adult and paediatric patients. *Cochrane Database Syst Rev*. 2012;12:CD004388
46. Minnich DJ, Moldawer LL. Anti-cytokine and anti-inflammatory therapies for the treatment of severe sepsis: Progress and pitfalls. *The Proceedings of the Nutrition Society*. 2004;63:437-441
47. Oberholzer A, Oberholzer C, Moldawer LL. Sepsis syndromes: Understanding the role of innate and acquired immunity. *Shock*. 2001;16:83-96
48. Opal SM, Huber CE. Bench-to-bedside review: Toll-like receptors and their role in septic shock. *Crit Care*. 2002;6:125-136
49. Censoplano N, Epting CL, Coates BM. The role of the innate immune system in sepsis. *Clinical Pediatric Emergency Medicine*. 2014
50. Boomer JS, Green JM, Hotchkiss RS. The changing immune system in sepsis: Is individualized immuno-modulatory therapy the answer? 2014
51. Aziz M, Jacob A, Yang WL, Matsuda A, Wang P. Current trends in inflammatory and immunomodulatory mediators in sepsis. *Journal of leukocyte biology*. 2013;93:329-342
52. Osuchowski MF, Welch K, Siddiqui J, Remick DG. Circulating cytokine/inhibitor profiles reshape the understanding of the sirs/cars continuum in sepsis and predict mortality. *J Immunol*. 2006;177:1967-1974
53. Ward NS, Casserly B, Ayala A. The compensatory anti-inflammatory response syndrome (cars) in critically ill patients. *Clinics in chest medicine*. 2008;29:617-625, viii
54. Reddy RC, Standiford TJ. Effects of sepsis on neutrophil chemotaxis. *Current opinion in hematology*. 2010;17:18-24
55. Brealey D, Brand M, Hargreaves I, Heales S, Land J, Smolenski R, Davies NA, Cooper CE, Singer M. Association between mitochondrial dysfunction and severity and outcome of septic shock. *Lancet*. 2002;360:219-223
56. Skoutelis AT, Kaleridis V, Athanassiou GM, Kokkinis KI, Missirlis YF, Bassaris HP. Neutrophil deformability in patients with sepsis, septic shock, and adult respiratory distress syndrome. *Critical care medicine*. 2000;28:2355-2359
57. Benjamim CF, Silva JS, Fortes ZB, Oliveira MA, Ferreira SH, Cunha FQ. Inhibition of leukocyte rolling by nitric oxide during sepsis leads to reduced migration of active microbicidal neutrophils. *Infection and immunity*. 2002;70:3602-3610
58. Benjamim CF, Ferreira SH, Cunha FQ. Role of nitric oxide in the failure of neutrophil migration in sepsis. *The Journal of infectious diseases*. 2000;182:214-223
59. Freitas A, Alves-Filho JC, Secco DD, Neto AF, Ferreira SH, Barja-Fidalgo C, Cunha FQ. Heme oxygenase/carbon monoxide-biliverdin pathway down regulates neutrophil rolling, adhesion and migration in acute inflammation. *British journal of pharmacology*. 2006;149:345-354

60. Cummings CJ, Martin TR, Frevert CW, Quan JM, Wong VA, Mongovin SM, Hagen TR, Steinberg KP, Goodman RB. Expression and function of the chemokine receptors cxcr1 and cxcr2 in sepsis. *J Immunol*. 1999;162:2341-2346
61. Souto FO, Alves-Filho JC, Turato WM, Auxiliadora-Martins M, Basile-Filho A, Cunha FQ. Essential role of ccr2 in neutrophil tissue infiltration and multiple organ dysfunction in sepsis. *American journal of respiratory and critical care medicine*. 2011;183:234-242
62. Weighardt H, Holzmann B. Role of toll-like receptor responses for sepsis pathogenesis. *Immunobiology*. 2007;212:715-722
63. Xiong Y, Medvedev AE. Induction of endotoxin tolerance in vivo inhibits activation of irak4 and increases negative regulators irak-m, ship-1, and a20. *Journal of leukocyte biology*. 2011;90:1141-1148
64. Mashimo H, Ohguro N, Nomura S, Hashida N, Nakai K, Tano Y. Neutrophil chemotaxis and local expression of interleukin-10 in the tolerance of endotoxin-induced uveitis. *Investigative ophthalmology & visual science*. 2008;49:5450-5457
65. Clark SR, Ma AC, Tavener SA, McDonald B, Goodarzi Z, Kelly MM, Patel KD, Chakrabarti S, McAvoy E, Sinclair GD, Keys EM, Allen-Vercoe E, Devinney R, Doig CJ, Green FH, Kubes P. Platelet tlr4 activates neutrophil extracellular traps to ensnare bacteria in septic blood. *Nature medicine*. 2007;13:463-469
66. Hakkim A, Furnrohr BG, Amann K, Laube B, Abed UA, Brinkmann V, Herrmann M, Voll RE, Zychlinsky A. Impairment of neutrophil extracellular trap degradation is associated with lupus nephritis. *Proceedings of the National Academy of Sciences of the United States of America*. 2010;107:9813-9818
67. Kovach MA, Standiford TJ. The function of neutrophils in sepsis. *Current opinion in infectious diseases*. 2012;25:321-327
68. Reidy MA, Schwartz SM. Endothelial injury and regeneration. Iv. Endotoxin: A nondenuding injury to aortic endothelium. *Laboratory investigation; a journal of technical methods and pathology*. 1983;48:25-34
69. Leclerc J, Pu Q, Corseaux D, Haddad E, Decoene C, Bordet R, Six I, Jude B, Vallet B. A single endotoxin injection in the rabbit causes prolonged blood vessel dysfunction and a procoagulant state. *Critical care medicine*. 2000;28:3672-3678
70. Polunovsky VA, Wendt CH, Ingbar DH, Peterson MS, Bitterman PB. Induction of endothelial cell apoptosis by tnf alpha: Modulation by inhibitors of protein synthesis. *Experimental cell research*. 1994;214:584-594
71. Hebert MJ, Gullans SR, Mackenzie HS, Brady HR. Apoptosis of endothelial cells is associated with paracrine induction of adhesion molecules: Evidence for an interleukin-1beta-dependent paracrine loop. *The American journal of pathology*. 1998;152:523-532
72. Lee WL, Slutsky AS. Sepsis and endothelial permeability. *The New England journal of medicine*. 2010;363:689-691
73. Nooteboom A, van der Linden CJ, Hendriks T. Modulation of adhesion molecule expression on endothelial cells after induction by lipopolysaccharide-stimulated whole blood. *Scandinavian journal of immunology*. 2004;59:440-448

74. Sessler CN, Windsor AC, Schwartz M, Watson L, Fisher BJ, Sugerman HJ, Fowler AA, 3rd. Circulating icam-1 is increased in septic shock. *American journal of respiratory and critical care medicine*. 1995;151:1420-1427
75. Kayal S, Jais JP, Aguini N, Chaudiere J, Labrousse J. Elevated circulating e-selectin, intercellular adhesion molecule 1, and von willebrand factor in patients with severe infection. *American journal of respiratory and critical care medicine*. 1998;157:776-784
76. Asaduzzaman M, Lavasani S, Rahman M, Zhang S, Braun OO, Jeppsson B, Thorlacius H. Platelets support pulmonary recruitment of neutrophils in abdominal sepsis. *Critical care medicine*. 2009;37:1389-1396
77. MacGregor IR, Perrie AM, Donnelly SC, Haslett C. Modulation of human endothelial thrombomodulin by neutrophils and their release products. *American journal of respiratory and critical care medicine*. 1997;155:47-52
78. Moore KL, Esmon CT, Esmon NL. Tumor necrosis factor leads to the internalization and degradation of thrombomodulin from the surface of bovine aortic endothelial cells in culture. *Blood*. 1989;73:159-165
79. Fischer LG, Freise H, Hilpert JH, Wendholt D, Lauer S, Van Aken H, Sielenkamper AW. Modulation of hypoxic pulmonary vasoconstriction is time and nitric oxide dependent in a peritonitis model of sepsis. *Intensive care medicine*. 2004;30:1821-1828
80. Wu L, Mayeux PR. Effects of the inducible nitric-oxide synthase inhibitor l-n(6)-(1-iminoethyl)-lysine on microcirculation and reactive nitrogen species generation in the kidney following lipopolysaccharide administration in mice. *The Journal of pharmacology and experimental therapeutics*. 2007;320:1061-1067
81. Li JM, Shah AM. Endothelial cell superoxide generation: Regulation and relevance for cardiovascular pathophysiology. *American journal of physiology. Regulatory, integrative and comparative physiology*. 2004;287:R1014-1030
82. Marechal X, Favory R, Joulin O, Montaigne D, Hassoun S, Decoster B, Zerimech F, Neviere R. Endothelial glycocalyx damage during endotoxemia coincides with microcirculatory dysfunction and vascular oxidative stress. *Shock*. 2008;29:572-576
83. Peterson TE, Poppa V, Ueba H, Wu A, Yan C, Berk BC. Opposing effects of reactive oxygen species and cholesterol on endothelial nitric oxide synthase and endothelial cell caveolae. *Circulation research*. 1999;85:29-37
84. Cerwinka WH, Cooper D, Krieglstein CF, Feelisch M, Granger DN. Nitric oxide modulates endotoxin-induced platelet-endothelial cell adhesion in intestinal venules. *American journal of physiology. Heart and circulatory physiology*. 2002;282:H1111-1117
85. Gertzberg N, Neumann P, Rizzo V, Johnson A. Nad(p)h oxidase mediates the endothelial barrier dysfunction induced by tnf-alpha. *American journal of physiology. Lung cellular and molecular physiology*. 2004;286:L37-48
86. Kirchhofer D, Tschopp TB, Hadvary P, Baumgartner HR. Endothelial cells stimulated with tumor necrosis factor-alpha express varying amounts of tissue factor resulting in inhomogenous fibrin deposition in a native blood flow system.

Effects of thrombin inhibitors. *The Journal of clinical investigation*. 1994;93:2073-2083
87. Szotowski B, Antoniak S, Poller W, Schultheiss HP, Rauch U. Procoagulant soluble tissue factor is released from endothelial cells in response to inflammatory cytokines. *Circulation research*. 2005;96:1233-1239
88. Egorina EM, Sovershaev MA, Bjorkoy G, Gruber FX, Olsen JO, Parhami-Seren B, Mann KG, Osterud B. Intracellular and surface distribution of monocyte tissue factor: Application to intersubject variability. *Arteriosclerosis, thrombosis, and vascular biology*. 2005;25:1493-1498
89. Camerer E, Huang W, Coughlin SR. Tissue factor- and factor x-dependent activation of protease-activated receptor 2 by factor viia. *Proceedings of the National Academy of Sciences of the United States of America*. 2000;97:5255-5260
90. Paulus P, Jennewein C, Zacharowski K. Biomarkers of endothelial dysfunction: Can they help us deciphering systemic inflammation and sepsis? *Biomarkers : biochemical indicators of exposure, response, and susceptibility to chemicals*. 2011;16 Suppl 1:S11-21
91. O'Brien M. The reciprocal relationship between inflammation and coagulation. *Topics in companion animal medicine*. 2012;27:46-52
92. Leclerc F, Hazelzet J, Jude B, Hofhuis W, Hue V, Martinot A, Van der Voort E. Protein c and s deficiency in severe infectious purpura of children: A collaborative study of 40 cases. *Intensive care medicine*. 1992;18:202-205
93. Semeraro F, Ammollo CT, Semeraro N, Colucci M. Tissue factor-expressing monocytes inhibit fibrinolysis through a tafi-mediated mechanism, and make clots resistant to heparins. *Haematologica*. 2009;94:819-826
94. Maugeri N, Brambilla M, Camera M, Carbone A, Tremoli E, Donati MB, de Gaetano G, Cerletti C. Human polymorphonuclear leukocytes produce and express functional tissue factor upon stimulation. *Journal of thrombosis and haemostasis : JTH*. 2006;4:1323-1330
95. Moosbauer C, Morgenstern E, Cuvelier SL, Manukyan D, Bidzhekov K, Albrecht S, Lohse P, Patel KD, Engelmann B. Eosinophils are a major intravascular location for tissue factor storage and exposure. *Blood*. 2007;109:995-1002
96. Panes O, Matus V, Saez CG, Quiroga T, Pereira J, Mezzano D. Human platelets synthesize and express functional tissue factor. *Blood*. 2007;109:5242-5250
97. Egorina EM, Sovershaev MA, Olsen JO, Osterud B. Granulocytes do not express but acquire monocyte-derived tissue factor in whole blood: Evidence for a direct transfer. *Blood*. 2008;111:1208-1216
98. Pawlinski R, Mackman N. Cellular sources of tissue factor in endotoxemia and sepsis. *Thrombosis research*. 2010;125 Suppl 1:S70-73
99. Asakura H, Suga Y, Yoshida T, Ontachi Y, Mizutani T, Kato M, Ito T, Morishita E, Yamazaki M, Miyamoto K, Nakao S. Pathophysiology of disseminated intravascular coagulation (dic) progresses at a different rate in tissue factor-induced and lipopolysaccharide-induced dic models in rats. *Blood coagulation & fibrinolysis : an international journal in haemostasis and thrombosis*. 2003;14:221-228

100. Erlich J, Fearns C, Mathison J, Ulevitch RJ, Mackman N. Lipopolysaccharide induction of tissue factor expression in rabbits. *Infection and immunity*. 1999;67:2540-2546
101. Semeraro N, Ammollo CT, Semeraro F, Colucci M. Sepsis, thrombosis and organ dysfunction. *Thrombosis research*. 2012;129:290-295
102. Zeerleder S, Schroeder V, Hack CE, Kohler HP, Wuillemin WA. Tafi and pai-1 levels in human sepsis. *Thrombosis research*. 2006;118:205-212
103. Mavrommatis AC, Theodoridis T, Economou M, Kotanidou A, El Ali M, Christopoulou-Kokkinou V, Zakynthinos SG. Activation of the fibrinolytic system and utilization of the coagulation inhibitors in sepsis: Comparison with severe sepsis and septic shock. *Intensive care medicine*. 2001;27:1853-1859
104. Campbell RA, Overmyer KA, Selzman CH, Sheridan BC, Wolberg AS. Contributions of extravascular and intravascular cells to fibrin network formation, structure, and stability. *Blood*. 2009;114:4886-4896
105. Mutch NJ, Engel R, Uitte de Willige S, Philippou H, Ariens RA. Polyphosphate modifies the fibrin network and down-regulates fibrinolysis by attenuating binding of tpa and plasminogen to fibrin. *Blood*. 2010;115:3980-3988
106. Hutchins NA, Unsinger J, Hotchkiss RS, Ayala A. The new normal: Immunomodulatory agents against sepsis immune suppression. *Trends in molecular medicine*. 2014;20:224-233
107. Francisco-Cruz A, Aguilar-Santelises M, Ramos-Espinosa O, Mata-Espinosa D, Marquina-Castillo B, Barrios-Payan J, Hernandez-Pando R. Granulocyte–macrophage colony-stimulating factor: Not just another haematopoietic growth factor. *Medical Oncology*. 2014;31:1-14
108. de Groot RP, Coffer PJ, Koenderman L. Regulation of proliferation, differentiation and survival by the il-3/il-5/gm-csf receptor family. *Cellular signalling*. 1998;10:619-628
109. Watanabe S, Itoh T, Arai K. Roles of jak kinases in human gm-csf receptor signal transduction. *The Journal of allergy and clinical immunology*. 1996;98:S183-191
110. Sakai I, Kraft AS. The kinase domain of jak2 mediates induction of bcl-2 and delays cell death in hematopoietic cells. *The Journal of biological chemistry*. 1997;272:12350-12358
111. Fleetwood AJ, Lawrence T, Hamilton JA, Cook AD. Granulocyte-macrophage colony-stimulating factor (csf) and macrophage csf-dependent macrophage phenotypes display differences in cytokine profiles and transcription factor activities: Implications for csf blockade in inflammation. *J Immunol*. 2007;178:5245-5252
112. O'Mahony DS, Pham U, Iyer R, Hawn TR, Liles WC. Differential constitutive and cytokine-modulated expression of human toll-like receptors in primary neutrophils, monocytes, and macrophages. *International journal of medical sciences*. 2008;5:1-8
113. Tanimoto A, Murata Y, Wang KY, Tsutsui M, Kohno K, Sasaguri Y. Monocyte chemoattractant protein-1 expression is enhanced by granulocyte-macrophage colony-stimulating factor via jak2-stat5 signaling and inhibited by atorvastatin in human monocytic u937 cells. *The Journal of biological chemistry*. 2008;283:4643-4651

114. Takahashi GW, Andrews DF, 3rd, Lilly MB, Singer JW, Alderson MR. Effect of granulocyte-macrophage colony-stimulating factor and interleukin-3 on interleukin-8 production by human neutrophils and monocytes. *Blood*. 1993;81:357-364
115. Yong KL, Linch DC. Differential effects of granulocyte- and granulocyte-macrophage colony-stimulating factors (g- and gm-csf) on neutrophil adhesion in vitro and in vivo. *European journal of haematology*. 1992;49:251-259
116. Bober LA, Grace MJ, Pugliese-Sivo C, Rojas-Triana A, Waters T, Sullivan LM, Narula SK. The effect of gm-csf and g-csf on human neutrophil function. *Immunopharmacology*. 1995;29:111-119
117. Mudzinski SP, Christian TP, Guo TL, Cirenza E, Hazlett KR, Gosselin EJ. Expression of hla-dr (major histocompatibility complex class ii) on neutrophils from patients treated with granulocyte-macrophage colony-stimulating factor for mobilization of stem cells. *Blood*. 1995;86:2452-2453
118. Hornell TM, Beresford GW, Bushey A, Boss JM, Mellins ED. Regulation of the class ii mhc pathway in primary human monocytes by granulocyte-macrophage colony-stimulating factor. *J Immunol*. 2003;171:2374-2383
119. Krausgruber T, Blazek K, Smallie T, Alzabin S, Lockstone H, Sahgal N, Hussell T, Feldmann M, Udalova IA. Irf5 promotes inflammatory macrophage polarization and th1-th17 responses. *Nature immunology*. 2011;12:231-238
120. Cantrell MA, Anderson D, Cerretti DP, Price V, McKereghan K, Tushinski RJ, Mochizuki DY, Larsen A, Grabstein K, Gillis S, et al. Cloning, sequence, and expression of a human granulocyte/macrophage colony-stimulating factor. *Proceedings of the National Academy of Sciences of the United States of America*. 1985;82:6250-6254
121. Burgess AW, Begley CG, Johnson GR, Lopez AF, Williamson DJ, Mermod JJ, Simpson RJ, Schmitz A, DeLamarter JF. Purification and properties of bacterially synthesized human granulocyte-macrophage colony stimulating factor. *Blood*. 1987;69:43-51
122. Hussein AM, Ross M, Vredenburgh J, Meisenberg B, Hars V, Gilbert C, Petros WP, Coniglio D, Kurtzberg J, Rubin P, et al. Effects of granulocyte-macrophage colony stimulating factor produced in chinese hamster ovary cells (regramostim), escherichia coli (molgramostim) and yeast (sargramostim) on priming peripheral blood progenitor cells for use with autologous bone marrow after high-dose chemotherapy. *European journal of haematology*. 1995;55:348-356
123. Ganser A, Heil G. Use of hematopoietic growth factors in the treatment of acute myelogenous leukemia. *Current opinion in hematology*. 1997;4:191-195
124. Lane TA, Law P, Maruyama M, Young D, Burgess J, Mullen M, Mealiffe M, Terstappen LW, Hardwick A, Moubayed M, et al. Harvesting and enrichment of hematopoietic progenitor cells mobilized into the peripheral blood of normal donors by granulocyte-macrophage colony-stimulating factor (gm-csf) or g-csf: Potential role in allogeneic marrow transplantation. *Blood*. 1995;85:275-282
125. Brosbøl-Ravnborg A, Hvas CL, Agnholt J, Dahlerup JF, Vind I, Till A, Rosenstiel P, Höllsberg P. Toll-like receptor-induced granulocyte-macrophage colony-stimulating factor secretion is impaired in crohn's disease by nucleotide

oligomerization domain 2-dependent and-independent pathways. *Clinical & Experimental Immunology*. 2009;155:487-495
126. Korzenik JR, Dieckgraefe BK, Valentine JF, Hausman DF, Gilbert MJ, Sargramostim in Crohn's Disease Study G. Sargramostim for active crohn's disease. *The New England journal of medicine*. 2005;352:2193-2201
127. Valentine JF, Fedorak RN, Feagan B, Fredlund P, Schmitt R, Ni P, Humphries TJ. Steroid-sparing properties of sargramostim in patients with corticosteroid-dependent crohn's disease: A randomised, double-blind, placebo-controlled, phase 2 study. *Gut*. 2009;58:1354-1362
128. Kowanko IC, Ferrante A, Harvey DP, Carman KL. Granulocyte-macrophage colony-stimulating factor augments neutrophil killing of torulopsis glabrata and stimulates neutrophil respiratory burst and degranulation. *Clinical and experimental immunology*. 1991;83:225-230
129. Newman SL, Gootee L. Colony-stimulating factors activate human macrophages to inhibit intracellular growth of histoplasma capsulatum yeasts. *Infection and immunity*. 1992;60:4593-4597
130. Roilides E, Mertins S, Eddy J, Walsh TJ, Pizzo PA, Rubin M. Impairment of neutrophil chemotactic and bactericidal function in children infected with human immunodeficiency virus type 1 and partial reversal after in vitro exposure to granulocyte-macrophage colony-stimulating factor. *The Journal of pediatrics*. 1990;117:531-540
131. Flohe S, Borgermann J, Dominguez FE, Majetschak M, Lim L, Kreuzfelder E, Obertacke U, Nast-Kolb D, Schade FU. Influence of granulocyte-macrophage colony-stimulating factor (gm-csf) on whole blood endotoxin responsiveness following trauma, cardiopulmonary bypass, and severe sepsis. *Shock*. 1999;12:17-24
132. Flohe S, Lendemans S, Selbach C, Waydhas C, Ackermann M, Schade FU, Kreuzfelder E. Effect of granulocyte-macrophage colony-stimulating factor on the immune response of circulating monocytes after severe trauma. *Critical care medicine*. 2003;31:2462-2469
133. Meisel C, Schefold JC, Pschowski R, Baumann T, Hetzger K, Gregor J, Weber-Carstens S, Hasper D, Keh D, Zuckermann H, Reinke P, Volk HD. Granulocyte-macrophage colony-stimulating factor to reverse sepsis-associated immunosuppression: A double-blind, randomized, placebo-controlled multicenter trial. *American journal of respiratory and critical care medicine*. 2009;180:640-648
134. Hall MW, Knatz NL, Vetterly C, Tomarello S, Wewers MD, Volk HD, Carcillo JA. Immunoparalysis and nosocomial infection in children with multiple organ dysfunction syndrome. *Intensive care medicine*. 2011;37:525-532
135. Presneill JJ, Harris T, Stewart AG, Cade JF, Wilson JW. A randomized phase ii trial of granulocyte-macrophage colony-stimulating factor therapy in severe sepsis with respiratory dysfunction. *American journal of respiratory and critical care medicine*. 2002;166:138-143
136. Schroder K, Hertzog PJ, Ravasi T, Hume DA. Interferon-gamma: An overview of signals, mechanisms and functions. *Journal of leukocyte biology*. 2004;75:163-189

137. Munder M, Mallo M, Eichmann K, Modolell M. Murine macrophages secrete interferon gamma upon combined stimulation with interleukin (il)-12 and il-18: A novel pathway of autocrine macrophage activation. *The Journal of experimental medicine*. 1998;187:2103-2108
138. Salazar-Mather TP, Hamilton TA, Biron CA. A chemokine-to-cytokine-to-chemokine cascade critical in antiviral defense. *The Journal of clinical investigation*. 2000;105:985-993
139. Schindler H, Lutz MB, Rollinghoff M, Bogdan C. The production of ifn-gamma by il-12/il-18-activated macrophages requires stat4 signaling and is inhibited by il-4. *J Immunol*. 2001;166:3075-3082
140. D'Andrea A, Aste-Amezaga M, Valiante NM, Ma X, Kubin M, Trinchieri G. Interleukin 10 (il-10) inhibits human lymphocyte interferon gamma-production by suppressing natural killer cell stimulatory factor/il-12 synthesis in accessory cells. *The Journal of experimental medicine*. 1993;178:1041-1048
141. Umland SP, Nahrebne DK, Razac S, Beavis A, Pennline KJ, Egan RW, Billah MM. The inhibitory effects of topically active glucocorticoids on il-4, il-5, and interferon-gamma production by cultured primary cd4+ t cells. *The Journal of allergy and clinical immunology*. 1997;100:511-519
142. Delsing CE, Gresnigt MS, Leentjens J, Preijers F, Frager FA, Kox M, Monneret G, Venet F, Bleeker-Rovers CP, van de Veerdonk FL, Pickkers P, Pachot A, Kullberg BJ, Netea MG. Interferon-gamma as adjunctive immunotherapy for invasive fungal infections: A case series. *BMC infectious diseases*. 2014;14:166
143. Steimle V, Siegrist CA, Mottet A, Lisowska-Grospierre B, Mach B. Regulation of mhc class ii expression by interferon-gamma mediated by the transactivator gene ciita. *Science*. 1994;265:106-109
144. Boehm U, Klamp T, Groot M, Howard JC. Cellular responses to interferon-gamma. *Annual review of immunology*. 1997;15:749-795
145. Yoshida A, Koide Y, Uchijima M, Yoshida TO. Ifn-gamma induces il-12 mrna expression by a murine macrophage cell line, j774. *Biochemical and biophysical research communications*. 1994;198:857-861
146. Hsieh CS, Macatonia SE, Tripp CS, Wolf SF, O'Garra A, Murphy KM. Development of th1 cd4+ t cells through il-12 produced by listeria-induced macrophages. *Science*. 1993;260:547-549
147. Marodi L, Schreiber S, Anderson DC, MacDermott RP, Korchak HM, Johnston RB, Jr. Enhancement of macrophage candidacidal activity by interferon-gamma. Increased phagocytosis, killing, and calcium signal mediated by a decreased number of mannose receptors. *The Journal of clinical investigation*. 1993;91:2596-2601
148. Marchi LF, Sesti-Costa R, Ignacchiti MD, Chedraoui-Silva S, Mantovani B. In vitro activation of mouse neutrophils by recombinant human interferon-gamma: Increased phagocytosis and release of reactive oxygen species and pro-inflammatory cytokines. *International immunopharmacology*. 2014;18:228-235
149. Chung HK, Lee IK, Kang H, Suh JM, Kim H, Park KC, Kim DW, Kim YK, Ro HK, Shong M. Statin inhibits interferon-gamma-induced expression of intercellular adhesion molecule-1 (icam-1) in vascular endothelial and smooth muscle cells. *Experimental & molecular medicine*. 2002;34:451-461

150. Struyf S, Van Collie E, Paemen L, Put W, Lenaerts JP, Proost P, Opdenakker G, Van Damme J. Synergistic induction of mcp-1 and -2 by il-1beta and interferons in fibroblasts and epithelial cells. *Journal of leukocyte biology*. 1998;63:364-372
151. Kasama T, Strieter RM, Lukacs NW, Lincoln PM, Burdick MD, Kunkel SL. Interferon gamma modulates the expression of neutrophil-derived chemokines. *Journal of investigative medicine : the official publication of the American Federation for Clinical Research*. 1995;43:58-67
152. Proost P, Verpoest S, Van de Borne K, Schutyser E, Struyf S, Put W, Ronsse I, Grillet B, Opdenakker G, Van Damme J. Synergistic induction of cxcl9 and cxcl11 by toll-like receptor ligands and interferon-gamma in fibroblasts correlates with elevated levels of cxcr3 ligands in septic arthritis synovial fluids. *Journal of leukocyte biology*. 2004;75:777-784
153. Miller CH, Maher SG, Young HA. Clinical use of interferon-γ. *Annals of the New York Academy of Sciences*. 2009;1182:69-79
154. Giannopoulos A, Constantinides C, Fokaeas E, Stravodimos C, Giannopoulou M, Kyroudi A, Gounaris A. The immunomodulating effect of interferon-gamma intravesical instillations in preventing bladder cancer recurrence. *Clinical cancer research : an official journal of the American Association for Cancer Research*. 2003;9:5550-5558
155. Marth C, Windbichler GH, Hausmaninger H, Petru E, Estermann K, Pelzer A, Mueller-Holzner E. Interferon-gamma in combination with carboplatin and paclitaxel as a safe and effective first-line treatment option for advanced ovarian cancer: Results of a phase i/ii study. *International journal of gynecological cancer : official journal of the International Gynecological Cancer Society*. 2006;16:1522-1528
156. Alberts DS, Marth C, Alvarez RD, Johnson G, Bidzinski M, Kardatzke DR, Bradford WZ, Loutit J, Kirn DH, Clouser MC, Markman M, Consortium GCT. Randomized phase 3 trial of interferon gamma-1b plus standard carboplatin/paclitaxel versus carboplatin/paclitaxel alone for first-line treatment of advanced ovarian and primary peritoneal carcinomas: Results from a prospectively designed analysis of progression-free survival. *Gynecologic oncology*. 2008;109:174-181
157. Condos R, Raju B, Canova A, Zhao BY, Weiden M, Rom WN, Pine R. Recombinant gamma interferon stimulates signal transduction and gene expression in alveolar macrophages in vitro and in tuberculosis patients. *Infection and immunity*. 2003;71:2058-2064
158. Condos R, Rom WN, Schluger NW. Treatment of multidrug-resistant pulmonary tuberculosis with interferon-gamma via aerosol. *Lancet*. 1997;349:1513-1515
159. Lau JY, Lai CL, Wu PC, Chung HT, Lok AS, Lin HJ. A randomised controlled trial of recombinant interferon-gamma in chinese patients with chronic hepatitis b virus infection. *Journal of medical virology*. 1991;34:184-187
160. Katayama K, Kasahara A, Sasaki Y, Kashiwagi T, Naito M, Masuzawa M, Katoh M, Yoshihara H, Kamada T, Mukuda T, Hijioka T, Hori M, Hayashi N. Immunological response to interferon-gamma priming prior to interferon-alpha treatment in refractory chronic hepatitis c in relation to viral clearance. *Journal of viral hepatitis*. 2001;8:180-185

161. Weng HL, Cai WM, Liu RH. Animal experiment and clinical study of effect of gamma-interferon on hepatic fibrosis. *World journal of gastroenterology : WJG*. 2001;7:42-48
162. Muir AJ, Sylvestre PB, Rockey DC. Interferon gamma-1b for the treatment of fibrosis in chronic hepatitis c infection. *Journal of viral hepatitis*. 2006;13:322-328
163. Pappas PG, Bustamante B, Ticona E, Hamill RJ, Johnson PC, Reboli A, Aberg J, Hasbun R, Hsu HH. Recombinant interferon- gamma 1b as adjunctive therapy for aids-related acute cryptococcal meningitis. *The Journal of infectious diseases*. 2004;189:2185-2191
164. Riddell LA, Pinching AJ, Hill S, Ng TT, Arbe E, Lapham GP, Ash S, Hillman R, Tchamouroff S, Denning DW, Parkin JM. A phase iii study of recombinant human interferon gamma to prevent opportunistic infections in advanced hiv disease. *AIDS research and human retroviruses*. 2001;17:789-797
165. Rigato O, Salomao R. Impaired production of interferon-gamma and tumor necrosis factor-alpha but not of interleukin 10 in whole blood of patients with sepsis. *Shock*. 2003;19:113-116
166. Ono S, Ueno C, Aosasa S, Tsujimoto H, Seki S, Mochizuki H. Severe sepsis induces deficient interferon-gamma and interleukin-12 production, but interleukin-12 therapy improves survival in peritonitis. *American journal of surgery*. 2001;182:491-497
167. Boomer JS, Shuherk-Shaffer J, Hotchkiss RS, Green JM. A prospective analysis of lymphocyte phenotype and function over the course of acute sepsis. *Crit Care*. 2012;16:R112
168. Docke WD, Randow F, Syrbe U, Krausch D, Asadullah K, Reinke P, Volk HD, Kox W. Monocyte deactivation in septic patients: Restoration by ifn-gamma treatment. *Nature medicine*. 1997;3:678-681
169. Kox WJ, Bone RC, Krausch D, Docke WD, Kox SN, Wauer H, Egerer K, Querner S, Asadullah K, von Baehr R, Volk HD. Interferon gamma-1b in the treatment of compensatory anti-inflammatory response syndrome. A new approach: Proof of principle. *Archives of internal medicine*. 1997;157:389-393
170. Wasserman D, Ioannovich JD, Hinzmann RD, Deichsel G, Steinmann GG. Interferon-gamma in the prevention of severe burn-related infections: A european phase iii multicenter trial. The severe burns study group. *Critical care medicine*. 1998;26:434-439
171. Dries DJ, Jurkovich GJ, Maier RV, Clemmer TP, Struve SN, Weigelt JA, Stanford GG, Herr DL, Champion HR, Lewis FR, et al. Effect of interferon gamma on infection-related death in patients with severe injuries. A randomized, double-blind, placebo-controlled trial. *Archives of surgery*. 1994;129:1031-1041; discussion 1042
172. Boomer JS, Green JM, Hotchkiss RS. The changing immune system in sepsis: Is individualized immuno-modulatory therapy the answer? *Virulence*. 2014;5:45-56
173. Wolf P. The nature and significance of platelet products in human plasma. *British journal of haematology*. 1967;13:269-288

174. van der Pol E, Hoekstra AG, Sturk A, Otto C, van Leeuwen TG, Nieuwland R. Optical and non-optical methods for detection and characterization of microparticles and exosomes. *Journal of thrombosis and haemostasis : JTH*. 2010;8:2596-2607
175. Nomura S, Ozaki Y, Ikeda Y. Function and role of microparticles in various clinical settings. *Thrombosis research*. 2008;123:8-23
176. Nieuwland R, Sturk A. Why do cells release vesicles? *Thrombosis research*. 2010;125 Suppl 1:S49-51
177. Montoro-Garcia S, Shantsila E, Marin F, Blann A, Lip GY. Circulating microparticles: New insights into the biochemical basis of microparticle release and activity. *Basic research in cardiology*. 2011;106:911-923
178. van Meer G, Voelker DR, Feigenson GW. Membrane lipids: Where they are and how they behave. *Nature reviews. Molecular cell biology*. 2008;9:112-124
179. Roelants FM, Baltz AG, Trott AE, Fereres S, Thorner J. A protein kinase network regulates the function of aminophospholipid flippases. *Proceedings of the National Academy of Sciences of the United States of America*. 2010;107:34-39
180. Cerella C, Diederich M, Ghibelli L. The dual role of calcium as messenger and stressor in cell damage, death, and survival. *International journal of cell biology*. 2010;2010:546163
181. Manno S, Takakuwa Y, Mohandas N. Identification of a functional role for lipid asymmetry in biological membranes: Phosphatidylserine-skeletal protein interactions modulate membrane stability. *Proceedings of the National Academy of Sciences of the United States of America*. 2002;99:1943-1948
182. Yano Y, Shiba E, Kambayashi J, Sakon M, Kawasaki T, Fujitani K, Kang J, Mori T. The effects of calpeptin (a calpain specific inhibitor) on agonist induced microparticle formation from the platelet plasma membrane. *Thrombosis research*. 1993;71:385-396
183. Crespin M, Vidal C, Picard F, Lacombe C, Fontenay M. Activation of pak1/2 during the shedding of platelet microvesicles. *Blood coagulation & fibrinolysis : an international journal in haemostasis and thrombosis*. 2009;20:63-70
184. Abramovici H, Mojtabaie P, Parks RJ, Zhong XP, Koretzky GA, Topham MK, Gee SH. Diacylglycerol kinase zeta regulates actin cytoskeleton reorganization through dissociation of rac1 from rhogdi. *Molecular biology of the cell*. 2009;20:2049-2059
185. Curtis AM, Wilkinson PF, Gui M, Gales TL, Hu E, Edelberg JM. P38 mitogen-activated protein kinase targets the production of proinflammatory endothelial microparticles. *Journal of thrombosis and haemostasis : JTH*. 2009;7:701-709
186. Sebbagh M, Renvoize C, Hamelin J, Riche N, Bertoglio J, Breard J. Caspase-3-mediated cleavage of rock i induces mlc phosphorylation and apoptotic membrane blebbing. *Nature cell biology*. 2001;3:346-352
187. Sapet C, Simoncini S, Loriod B, Puthier D, Sampol J, Nguyen C, Dignat-George F, Anfosso F. Thrombin-induced endothelial microparticle generation: Identification of a novel pathway involving rock-ii activation by caspase-2. *Blood*. 2006;108:1868-1876
188. Tramontano AF, O'Leary J, Black AD, Muniyappa R, Cutaia MV, El-Sherif N. Statin decreases endothelial microparticle release from human coronary artery

endothelial cells: Implication for the rho-kinase pathway. *Biochemical and biophysical research communications*. 2004;320:34-38
189. Morel O, Jesel L, Freyssinet JM, Toti F. Cellular mechanisms underlying the formation of circulating microparticles. *Arteriosclerosis, thrombosis, and vascular biology*. 2011;31:15-26
190. Dean WL, Lee MJ, Cummins TD, Schultz DJ, Powell DW. Proteomic and functional characterisation of platelet microparticle size classes. *Thrombosis and haemostasis*. 2009;102:711-718
191. Dalli J, Montero-Melendez T, Norling LV, Yin X, Hinds C, Haskard D, Mayr M, Perretti M. Heterogeneity in neutrophil microparticles reveals distinct proteome and functional properties. *Molecular & cellular proteomics : MCP*. 2013;12:2205-2219
192. Ismail N, Wang Y, Dakhlallah D, Moldovan L, Agarwal K, Batte K, Shah P, Wisler J, Eubank TD, Tridandapani S, Paulaitis ME, Piper MG, Marsh CB. Macrophage microvesicles induce macrophage differentiation and mir-223 transfer. *Blood*. 2013;121:984-995
193. Sims PJ, Faioni EM, Wiedmer T, Shattil SJ. Complement proteins c5b-9 cause release of membrane vesicles from the platelet surface that are enriched in the membrane receptor for coagulation factor va and express prothrombinase activity. *The Journal of biological chemistry*. 1988;263:18205-18212
194. Fourcade O, Simon MF, Viode C, Rugani N, Leballe F, Ragab A, Fournie B, Sarda L, Chap H. Secretory phospholipase a2 generates the novel lipid mediator lysophosphatidic acid in membrane microvesicles shed from activated cells. *Cell*. 1995;80:919-927
195. Pluskota E, Woody NM, Szpak D, Ballantyne CM, Soloviev DA, Simon DI, Plow EF. Expression, activation, and function of integrin alphambeta2 (mac-1) on neutrophil-derived microparticles. *Blood*. 2008;112:2327-2335
196. Jimenez JJ, Jy W, Mauro LM, Soderland C, Horstman LL, Ahn YS. Endothelial cells release phenotypically and quantitatively distinct microparticles in activation and apoptosis. *Thrombosis research*. 2003;109:175-180
197. Shedden K, Xie XT, Chandaroy P, Chang YT, Rosania GR. Expulsion of small molecules in vesicles shed by cancer cells: Association with gene expression and chemosensitivity profiles. *Cancer research*. 2003;63:4331-4337
198. Safaei R, Larson BJ, Cheng TC, Gibson MA, Otani S, Naerdemann W, Howell SB. Abnormal lysosomal trafficking and enhanced exosomal export of cisplatin in drug-resistant human ovarian carcinoma cells. *Molecular cancer therapeutics*. 2005;4:1595-1604
199. Moskovich O, Fishelson Z. Live cell imaging of outward and inward vesiculation induced by the complement c5b-9 complex. *The Journal of biological chemistry*. 2007;282:29977-29986
200. Hugel B, Martinez MC, Kunzelmann C, Freyssinet JM. Membrane microparticles: Two sides of the coin. *Physiology*. 2005;20:22-27
201. Jy W, Jimenez JJ, Mauro LM, Ahn YS, Newton KR, Mendez AJ, Arnold PI, Schultz DR. Agonist-induced capping of adhesion proteins and microparticle shedding in cultures of human renal microvascular endothelial cells. *Endothelium : journal of endothelial cell research*. 2002;9:179-189

202. Del Conde I, Shrimpton CN, Thiagarajan P, Lopez JA. Tissue-factor-bearing microvesicles arise from lipid rafts and fuse with activated platelets to initiate coagulation. *Blood*. 2005;106:1604-1611
203. Perez-Casal M, Downey C, Fukudome K, Marx G, Toh CH. Activated protein c induces the release of microparticle-associated endothelial protein c receptor. *Blood*. 2005;105:1515-1522
204. Herrera MB, Fonsato V, Gatti S, Deregibus MC, Sordi A, Cantarella D, Calogero R, Bussolati B, Tetta C, Camussi G. Human liver stem cell-derived microvesicles accelerate hepatic regeneration in hepatectomized rats. *Journal of cellular and molecular medicine*. 2010;14:1605-1618
205. Bruno S, Grange C, Deregibus MC, Calogero RA, Saviozzi S, Collino F, Morando L, Busca A, Falda M, Bussolati B, Tetta C, Camussi G. Mesenchymal stem cell-derived microvesicles protect against acute tubular injury. *Journal of the American Society of Nephrology : JASN*. 2009;20:1053-1067
206. Gatti S, Bruno S, Deregibus MC, Sordi A, Cantaluppi V, Tetta C, Camussi G. Microvesicles derived from human adult mesenchymal stem cells protect against ischaemia-reperfusion-induced acute and chronic kidney injury. *Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association*. 2011;26:1474-1483
207. Pisetsky DS, Ullal AJ, Gauley J, Ning TC. Microparticles as mediators and biomarkers of rheumatic disease. *Rheumatology (Oxford)*. 2012;51:1737-1746
208. Boilard E, Nigrovic PA, Larabee K, Watts GF, Coblyn JS, Weinblatt ME, Massarotti EM, Remold-O'Donnell E, Farndale RW, Ware J, Lee DM. Platelets amplify inflammation in arthritis via collagen-dependent microparticle production. *Science*. 2010;327:580-583
209. Biro E, Nieuwland R, Tak PP, Pronk LM, Schaap MC, Sturk A, Hack CE. Activated complement components and complement activator molecules on the surface of cell-derived microparticles in patients with rheumatoid arthritis and healthy individuals. *Annals of the rheumatic diseases*. 2007;66:1085-1092
210. Gyorgy B, Szabo TG, Turiak L, Wright M, Herczeg P, Ledeczi Z, Kittel A, Polgar A, Toth K, Derfalvi B, Zelenak G, Borocz I, Carr B, Nagy G, Vekey K, Gay S, Falus A, Buzas EI. Improved flow cytometric assessment reveals distinct microvesicle (cell-derived microparticle) signatures in joint diseases. *PloS one*. 2012;7:e49726
211. Guiducci S, Distler JH, Jungel A, Huscher D, Huber LC, Michel BA, Gay RE, Pisetsky DS, Gay S, Matucci-Cerinic M, Distler O. The relationship between plasma microparticles and disease manifestations in patients with systemic sclerosis. *Arthritis and rheumatism*. 2008;58:2845-2853
212. Nielsen CT, Ostergaard O, Johnsen C, Jacobsen S, Heegaard NH. Distinct features of circulating microparticles and their relationship to clinical manifestations in systemic lupus erythematosus. *Arthritis and rheumatism*. 2011;63:3067-3077
213. Helal O, Defoort C, Robert S, Marin C, Lesavre N, Lopez-Miranda J, Riserus U, Basu S, Lovegrove J, McMonagle J, Roche HM, Dignat-George F, Lairon D. Increased levels of microparticles originating from endothelial cells, platelets and erythrocytes in subjects with metabolic syndrome: Relationship with oxidative

stress. *Nutrition, metabolism, and cardiovascular diseases : NMCD*. 2011;21:665-671
214. Chen J, Chen S, Chen Y, Zhang C, Wang J, Zhang W, Liu G, Zhao B, Chen Y. Circulating endothelial progenitor cells and cellular membrane microparticles in db/db diabetic mouse: Possible implications in cerebral ischemic damage. *American journal of physiology. Endocrinology and metabolism*. 2011;301:E62-71
215. Martinez MC, Tual-Chalot S, Leonetti D, Andriantsitohaina R. Microparticles: Targets and tools in cardiovascular disease. *Trends in pharmacological sciences*. 2011;32:659-665
216. Tual-Chalot S, Guibert C, Muller B, Savineau JP, Andriantsitohaina R, Martinez MC. Circulating microparticles from pulmonary hypertensive rats induce endothelial dysfunction. *American journal of respiratory and critical care medicine*. 2010;182:261-268
217. Chahed S, Leroyer AS, Benzerroug M, Gaucher D, Georgescu A, Picaud S, Silvestre JS, Gaudric A, Tedgui A, Massin P, Boulanger CM. Increased vitreous shedding of microparticles in proliferative diabetic retinopathy stimulates endothelial proliferation. *Diabetes*. 2010;59:694-701
218. Nomura S, Inami N, Shouzu A, Omoto S, Kimura Y, Takahashi N, Tanaka A, Urase F, Maeda Y, Ohtani H, Iwasaka T. The effects of pitavastatin, eicosapentaenoic acid and combined therapy on platelet-derived microparticles and adiponectin in hyperlipidemic, diabetic patients. *Platelets*. 2009;20:16-22
219. Mobarrez F, He S, Broijersen A, Wiklund B, Antovic A, Antovic J, Egberg N, Jorneskog G, Wallen H. Atorvastatin reduces thrombin generation and expression of tissue factor, p-selectin and gpiiia on platelet-derived microparticles in patients with peripheral arterial occlusive disease. *Thrombosis and haemostasis*. 2011;106:344-352
220. Esposito K, Ciotola M, Giugliano D. Pioglitazone reduces endothelial microparticles in the metabolic syndrome. *Arteriosclerosis, thrombosis, and vascular biology*. 2006;26:1926
221. Murakami T, Horigome H, Tanaka K, Nakata Y, Ohkawara K, Katayama Y, Matsui A. Impact of weight reduction on production of platelet-derived microparticles and fibrinolytic parameters in obesity. *Thrombosis research*. 2007;119:45-53
222. Yun CH, Jung KH, Chu K, Kim SH, Ji KH, Park HK, Kim HC, Lee ST, Lee SK, Roh JK. Increased circulating endothelial microparticles and carotid atherosclerosis in obstructive sleep apnea. *J Clin Neurol*. 2010;6:89-98
223. Abid Hussein MN, Boing AN, Sturk A, Hau CM, Nieuwland R. Inhibition of microparticle release triggers endothelial cell apoptosis and detachment. *Thrombosis and haemostasis*. 2007;98:1096-1107
224. Andreola G, Rivoltini L, Castelli C, Huber V, Perego P, Deho P, Squarcina P, Accornero P, Lozupone F, Lugini L, Stringaro A, Molinari A, Arancia G, Gentile M, Parmiani G, Fais S. Induction of lymphocyte apoptosis by tumor cell secretion of fasl-bearing microvesicles. *The Journal of experimental medicine*. 2002;195:1303-1316
225. Huber V, Fais S, Iero M, Lugini L, Canese P, Squarcina P, Zaccheddu A, Colone M, Arancia G, Gentile M, Seregni E, Valenti R, Ballabio G, Belli F, Leo E, Parmiani G,

Rivoltini L. Human colorectal cancer cells induce t-cell death through release of proapoptotic microvesicles: Role in immune escape. *Gastroenterology*. 2005;128:1796-1804
226. Keryer-Bibens C, Pioche-Durieu C, Villemant C, Souquere S, Nishi N, Hirashima M, Middeldorp J, Busson P. Exosomes released by ebv-infected nasopharyngeal carcinoma cells convey the viral latent membrane protein 1 and the immunomodulatory protein galectin 9. *BMC cancer*. 2006;6:283
227. Janowska-Wieczorek A, Wysoczynski M, Kijowski J, Marquez-Curtis L, Machalinski B, Ratajczak J, Ratajczak MZ. Microvesicles derived from activated platelets induce metastasis and angiogenesis in lung cancer. *International journal of cancer. Journal international du cancer*. 2005;113:752-760
228. van Doormaal FF, Kleinjan A, Di Nisio M, Buller HR, Nieuwland R. Cell-derived microvesicles and cancer. *The Netherlands journal of medicine*. 2009;67:266-273
229. Zahra S, Anderson JA, Stirling D, Ludlam CA. Microparticles, malignancy and thrombosis. *British journal of haematology*. 2011;152:688-700
230. Baj-Krzyworzeka M, Szatanek R, Weglarczyk K, Baran J, Urbanowicz B, Branski P, Ratajczak MZ, Zembala M. Tumour-derived microvesicles carry several surface determinants and mrna of tumour cells and transfer some of these determinants to monocytes. *Cancer immunology, immunotherapy : CII*. 2006;55:808-818
231. Graves LE, Ariztia EV, Navari JR, Matzel HJ, Stack MS, Fishman DA. Proinvasive properties of ovarian cancer ascites-derived membrane vesicles. *Cancer research*. 2004;64:7045-7049
232. Al-Nedawi K, Meehan B, Micallef J, Lhotak V, May L, Guha A, Rak J. Intercellular transfer of the oncogenic receptor egfrviii by microvesicles derived from tumour cells. *Nature cell biology*. 2008;10:619-624
233. Helley D, Banu E, Bouziane A, Banu A, Scotte F, Fischer AM, Oudard S. Platelet microparticles: A potential predictive factor of survival in hormone-refractory prostate cancer patients treated with docetaxel-based chemotherapy. *European urology*. 2009;56:479-484
234. Nieuwland R, Berckmans RJ, McGregor S, Boing AN, Romijn FP, Westendorp RG, Hack CE, Sturk A. Cellular origin and procoagulant properties of microparticles in meningococcal sepsis. *Blood*. 2000;95:930-935
235. Ogura H, Kawasaki T, Tanaka H, Koh T, Tanaka R, Ozeki Y, Hosotsubo H, Kuwagata Y, Shimazu T, Sugimoto H. Activated platelets enhance microparticle formation and platelet-leukocyte interaction in severe trauma and sepsis. *The Journal of trauma*. 2001;50:801-809
236. Mostefai HA, Meziani F, Mastronardi ML, Agouni A, Heymes C, Sargentini C, Asfar P, Martinez MC, Andriantsitohaina R. Circulating microparticles from patients with septic shock exert protective role in vascular function. *American journal of respiratory and critical care medicine*. 2008;178:1148-1155
237. Janiszewski M, Do Carmo AO, Pedro MA, Silva E, Knobel E, Laurindo FR. Platelet-derived exosomes of septic individuals possess proapoptotic nad(p)h oxidase activity: A novel vascular redox pathway. *Critical care medicine*. 2004;32:818-825

238. Soriano AO, Jy W, Chirinos JA, Valdivia MA, Velasquez HS, Jimenez JJ, Horstman LL, Kett DH, Schein RM, Ahn YS. Levels of endothelial and platelet microparticles and their interactions with leukocytes negatively correlate with organ dysfunction and predict mortality in severe sepsis. *Critical care medicine*. 2005;33:2540-2546
239. Joop K, Berckmans RJ, Nieuwland R, Berkhout J, Romijn FP, Hack CE, Sturk A. Microparticles from patients with multiple organ dysfunction syndrome and sepsis support coagulation through multiple mechanisms. *Thrombosis and haemostasis*. 2001;85:810-820
240. Fujimi S, Ogura H, Tanaka H, Koh T, Hosotsubo H, Nakamori Y, Kuwagata Y, Shimazu T, Sugimoto H. Activated polymorphonuclear leukocytes enhance production of leukocyte microparticles with increased adhesion molecules in patients with sepsis. *The Journal of trauma*. 2002;52:443-448
241. Woth G, Tokes-Fuzesi M, Magyarlaki T, Kovacs GL, Vermes I, Muhl D. Activated platelet-derived microparticle numbers are elevated in patients with severe fungal (candida albicans) sepsis. *Annals of clinical biochemistry*. 2012;49:554-560
242. Tokes-Fuzesi M, Woth G, Ernyey B, Vermes I, Muhl D, Bogar L, Kovacs GL. Microparticles and acute renal dysfunction in septic patients. *Journal of critical care*. 2012
243. van Ierssel SH, Van Craenenbroeck EM, Hoymans VY, Vrints CJ, Conraads VM, Jorens PG. Endothelium dependent vasomotion and in vitro markers of endothelial repair in patients with severe sepsis: An observational study. *PloS one*. 2013;8:e69499
244. Delabranche X, Boisrame-Helms J, Asfar P, Berger A, Mootien Y, Lavigne T, Grunebaum L, Lanza F, Gachet C, Freyssinet JM, Toti F, Meziani F. Microparticles are new biomarkers of septic shock-induced disseminated intravascular coagulopathy. *Intensive care medicine*. 2013;39:1695-1703
245. Prakash PS, Caldwell CC, Lentsch AB, Pritts TA, Robinson BR. Human microparticles generated during sepsis in patients with critical illness are neutrophil-derived and modulate the immune response. *The journal of trauma and acute care surgery*. 2012;73:401-406; discussion 406-407
246. Wang JG, Manly D, Kirchhofer D, Pawlinski R, Mackman N. Levels of microparticle tissue factor activity correlate with coagulation activation in endotoxemic mice. *Journal of thrombosis and haemostasis : JTH*. 2009;7:1092-1098
247. Aras O, Shet A, Bach RR, Hysjulien JL, Slungaard A, Hebbel RP, Escolar G, Jilma B, Key NS. Induction of microparticle- and cell-associated intravascular tissue factor in human endotoxemia. *Blood*. 2004;103:4545-4553
248. Ovstebo R, Aass HC, Haug KB, Troseid AM, Gopinathan U, Kierulf P, Berg JP, Brandtzaeg P, Henriksson CE. Lps from neisseria meningitidis is crucial for inducing monocyte- and microparticle-associated tissue factor activity but not for tissue factor expression. *Innate immunity*. 2012;18:580-591
249. Hellum M, Ovstebo R, Brusletto BS, Berg JP, Brandtzaeg P, Henriksson CE. Microparticle-associated tissue factor activity correlates with plasma levels of

bacterial lipopolysaccharides in meningococcal septic shock. *Thrombosis research*. 2014;133:507-514
250. Mortaza S, Martinez MC, Baron-Menguy C, Burban M, de la Bourdonnaye M, Fizanne L, Pierrot M, Cales P, Henrion D, Andriantsitohaina R, Mercat A, Asfar P, Meziani F. Detrimental hemodynamic and inflammatory effects of microparticles originating from septic rats. *Critical care medicine*. 2009;37:2045-2050
251. Mastronardi ML, Mostefai HA, Meziani F, Martinez MC, Asfar P, Andriantsitohaina R. Circulating microparticles from septic shock patients exert differential tissue expression of enzymes related to inflammation and oxidative stress. *Critical care medicine*. 2011;39:1739-1748
252. Boisrame-Helms J, Delabranche X, Degirmenci SE, Zobairi F, Berger A, Meyer G, Burban M, Mostefai HA, Levy B, Toti F, Meziani F. Pharmacological modulation of procoagulant microparticles improves haemodynamic dysfunction during septic shock in rats. *Thrombosis and haemostasis*. 2014;111:154-164
253. Dalli J, Montero Melendez T, Norling LV, Yin X, Hinds C, Haskard D, Mayr M, Perretti M. Heterogeneity in neutrophil microparticles reveals distinct proteome and functional properties. *Molecular & cellular proteomics : MCP*. 2013
254. Dalli J, Norling LV, Montero-Melendez T, Federici Canova D, Lashin H, Pavlov AM, Sukhorukov GB, Hinds CJ, Perretti M. Microparticle alpha-2-macroglobulin enhances pro-resolving responses and promotes survival in sepsis. *EMBO molecular medicine*. 2014;6:27-42
255. van der Pol E, Boing AN, Harrison P, Sturk A, Nieuwland R. Classification, functions, and clinical relevance of extracellular vesicles. *Pharmacological reviews*. 2012;64:676-705
256. Mathivanan S, Ji H, Simpson RJ. Exosomes: Extracellular organelles important in intercellular communication. *Journal of proteomics*. 2010;73:1907-1920
257. Wubbolts R, Leckie RS, Veenhuizen PT, Schwarzmann G, Mobius W, Hoernschemeyer J, Slot JW, Geuze HJ, Stoorvogel W. Proteomic and biochemical analyses of human b cell-derived exosomes. Potential implications for their function and multivesicular body formation. *The Journal of biological chemistry*. 2003;278:10963-10972
258. Bilyy RO, Shkandina T, Tomin A, Munoz LE, Franz S, Antonyuk V, Kit YY, Zirngibl M, Furnrohr BG, Janko C, Lauber K, Schiller M, Schett G, Stoika RS, Herrmann M. Macrophages discriminate glycosylation patterns of apoptotic cell-derived microparticles. *The Journal of biological chemistry*. 2012;287:496-503
259. Munoz LE, Lauber K, Schiller M, Manfredi AA, Herrmann M. The role of defective clearance of apoptotic cells in systemic autoimmunity. *Nature reviews. Rheumatology*. 2010;6:280-289
260. Turiak L, Misjak P, Szabo TG, Aradi B, Paloczi K, Ozohanics O, Drahos L, Kittel A, Falus A, Buzas EI, Vekey K. Proteomic characterization of thymocyte-derived microvesicles and apoptotic bodies in balb/c mice. *Journal of proteomics*. 2011;74:2025-2033
261. Zernecke A, Bidzhekov K, Noels H, Shagdarsuren E, Gan L, Denecke B, Hristov M, Koppel T, Jahantigh MN, Lutgens E, Wang S, Olson EN, Schober A, Weber C.

Delivery of microrna-126 by apoptotic bodies induces cxcl12-dependent vascular protection. *Science signaling*. 2009;2:ra81
262. Zhang Q, Li Y, Tsien RW. The dynamic control of kiss-and-run and vesicular reuse probed with single nanoparticles. *Science*. 2009;323:1448-1453
263. Clark NA, Lunacek JH, Benedek GB. A study of brownian motion using light scattering. *Am. J. Phys*. 1970;38:575-585
264. Filella M, Zhang J, Newman ME, Buffle J. Analytical applications of photon correlation spectroscopy for size distribution measurements of natural colloidal suspensions: Capabilities and limitations. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*. 1997;120:27-46
265. Dragovic RA, Gardiner C, Brooks AS, Tannetta DS, Ferguson DJ, Hole P, Carr B, Redman CW, Harris AL, Dobson PJ, Harrison P, Sargent IL. Sizing and phenotyping of cellular vesicles using nanoparticle tracking analysis. *Nanomedicine : nanotechnology, biology, and medicine*. 2011;7:780-788
266. Hein B, Willig KI, Hell SW. Stimulated emission depletion (sted) nanoscopy of a fluorescent protein-labeled organelle inside a living cell. *Proceedings of the National Academy of Sciences of the United States of America*. 2008;105:14271-14276
267. Pisitkun T, Shen RF, Knepper MA. Identification and proteomic profiling of exosomes in human urine. *Proceedings of the National Academy of Sciences of the United States of America*. 2004;101:13368-13373
268. Siedlecki CA, Wang IW, Higashi JM, Kottke-Marchant K, Marchant RE. Platelet-derived microparticles on synthetic surfaces observed by atomic force microscopy and fluorescence microscopy. *Biomaterials*. 1999;20:1521-1529
269. Rahman M. Introduction to flow cytometry. 2006
270. Rehman AA, Ahsan H, Khan FH. Alpha-2-macroglobulin: A physiological guardian. *Journal of cellular physiology*. 2012
271. Schroder AK, Uciechowski P, Fleischer D, Rink L. Crosslinking of cd66b on peripheral blood neutrophils mediates the release of interleukin-8 from intracellular storage. *Human immunology*. 2006;67:676-682
272. Kielian TL, Blecha F. Cd14 and other recognition molecules for lipopolysaccharide: A review. *Immunopharmacology*. 1995;29:187-205
273. Privratsky JR, Newman DK, Newman PJ. Pecam-1: Conflicts of interest in inflammation. *Life sciences*. 2010;87:69-82
274. Shih IM. The role of cd146 (mel-cam) in biology and pathology. *The Journal of pathology*. 1999;189:4-11
275. Horstman LL, Jy W, Jimenez JJ, Ahn YS. Endothelial microparticles as markers of endothelial dysfunction. *Frontiers in bioscience : a journal and virtual library*. 2004;9:1118-1135
276. Sinning JM, Losch J, Walenta K, Bohm M, Nickenig G, Werner N. Circulating cd31+/annexin v+ microparticles correlate with cardiovascular outcomes. *European heart journal*. 2011;32:2034-2041
277. Angus DC, Marrie TJ, Obrosky DS, Clermont G, Dremsizov TT, Coley C, Fine MJ, Singer DE, Kapoor WN. Severe community-acquired pneumonia: Use of intensive care services and evaluation of american and british thoracic society

diagnostic criteria. *American journal of respiratory and critical care medicine*. 2002;166:717-723
278. Bone RC, Sibbald WJ, Sprung CL. The accp-sccm consensus conference on sepsis and organ failure. *Chest*. 1992;101:1481-1483
279. Bernard GR, Artigas A, Brigham KL, Carlet J, Falke K, Hudson L, Lamy M, Legall JR, Morris A, Spragg R. The american-european consensus conference on ards. Definitions, mechanisms, relevant outcomes, and clinical trial coordination. *American journal of respiratory and critical care medicine*. 1994;149:818-824
280. Vincent JL, Moreno R, Takala J, Willatts S, De Mendonca A, Bruining H, Reinhart CK, Suter PM, Thijs LG. The sofa (sepsis-related organ failure assessment) score to describe organ dysfunction/failure. On behalf of the working group on sepsis-related problems of the european society of intensive care medicine. *Intensive care medicine*. 1996;22:707-710
281. Macey MG, Enniks N, Bevan S. Flow cytometric analysis of microparticle phenotype and their role in thrombin generation. *Cytometry. Part B, Clinical cytometry*. 2011;80:57-63
282. Strasser EF, Happ S, Weiss DR, Pfeiffer A, Zimmermann R, Eckstein R. Microparticle detection in platelet products by three different methods. *Transfusion*. 2013;53:156-166
283. Chen YL, Chen CH, Wallace CG, Wang HT, Yang CC, Yip HK. Levels of circulating microparticles in patients with chronic cardiorenal disease. *Journal of atherosclerosis and thrombosis*. 2015;22:247-256
284. Jaffe EA, Nachman RL, Becker CG, Minick CR. Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. *The Journal of clinical investigation*. 1973;52:2745-2756
285. Lawrence MB, McIntire LV, Eskin SG. Effect of flow on polymorphonuclear leukocyte/endothelial cell adhesion. *Blood*. 1987;70:1284-1290
286. Patel KD. Mechanisms of selective leukocyte recruitment from whole blood on cytokine-activated endothelial cells under flow conditions. *J Immunol*. 1999;162:6209-6216
287. Frevert CW, Wong VA, Goodman RB, Goodwin R, Martin TR. Rapid fluorescence-based measurement of neutrophil migration in vitro. *Journal of immunological methods*. 1998;213:41-52
288. Jy W, Horstman LL, Jimenez JJ, Ahn YS, Biro E, Nieuwland R, Sturk A, Dignat-George F, Sabatier F, Camoin-Jau L, Sampol J, Hugel B, Zobairi F, Freyssinet JM, Nomura S, Shet AS, Key NS, Hebbel RP. Measuring circulating cell-derived microparticles. *Journal of thrombosis and haemostasis : JTH*. 2004;2:1842-1851
289. Dignat-George F, Camoin-Jau L, Sabatier F, Arnoux D, Anfosso F, Bardin N, Veit V, Combes V, Gentile S, Moal V, Sanmarco M, Sampol J. Endothelial microparticles: A potential contribution to the thrombotic complications of the antiphospholipid syndrome. *Thrombosis and haemostasis*. 2004;91:667-673
290. Walenta K, Schwarz V, Schirmer SH, Kindermann I, Friedrich EB, Solomayer EF, Sliwa K, Labidi S, Hilfiker-Kleiner D, Bohm M. Circulating microparticles as indicators of peripartum cardiomyopathy. *European heart journal*. 2012;33:1469-1479

291. Montoro-Garcia S, Shantsila E, Orenes-Pinero E, Lozano ML, Lip GY. An innovative flow cytometric approach for small-size platelet microparticles: Influence of calcium. *Thrombosis and haemostasis*. 2012;108:373-383
292. Connor DE, Exner T, Ma DD, Joseph JE. The majority of circulating platelet-derived microparticles fail to bind annexin v, lack phospholipid-dependent procoagulant activity and demonstrate greater expression of glycoprotein ib. *Thrombosis and haemostasis*. 2010;103:1044-1052
293. Maguire CA, Balaj L, Sivaraman S, Crommentuijn MH, Ericsson M, Mincheva-Nilsson L, Baranov V, Gianni D, Tannous BA, Sena-Esteves M, Breakefield XO, Skog J. Microvesicle-associated aav vector as a novel gene delivery system. *Molecular therapy : the journal of the American Society of Gene Therapy*. 2012;20:960-971
294. Witwer KW, Buzas EI, Bemis LT, Bora A, Lasser C, Lotvall J, Nolte-'t Hoen EN, Piper MG, Sivaraman S, Skog J, Thery C, Wauben MH, Hochberg F. Standardization of sample collection, isolation and analysis methods in extracellular vesicle research. *Journal of extracellular vesicles*. 2013;2
295. Kim HK, Song KS, Lee ES, Lee YJ, Park YS, Lee KR, Lee SN. Optimized flow cytometric assay for the measurement of platelet microparticles in plasma: Pre-analytic and analytic considerations. *Blood coagulation & fibrinolysis : an international journal in haemostasis and thrombosis*. 2002;13:393-397
296. Shah MD, Bergeron AL, Dong J-F, López JA. Flow cytometric measurement of microparticles: Pitfalls and protocol modifications. *Platelets*. 2008;19:365-372
297. Lacroix R, Judicone C, Poncelet P, Robert S, Arnaud L, Sampol J, DIGNAT-GEORGE F. Impact of pre-analytical parameters on the measurement of circulating microparticles: Towards standardization of protocol. *Journal of Thrombosis and Haemostasis*. 2012;10:437-446
298. Harford CG, Hara M, Technical Assistance of Alice H. Pulmonary edema in influenzal pneumonia of the mouse and the relation of fluid in the lung to the inception of pneumococcal pneumonia. *The Journal of experimental medicine*. 1950;91:245-260
299. Loosli CG, Baker RF. Acute experimental pneumococcal (type i) pneumonia in the mouse: The migration of leucocytes from the pulmonary capillaries into the alveolar spaces as revealed by the electron microscope. *Transactions of the American Clinical and Climatological Association*. 1962;74:15-28
300. Johnston RB, Jr. Pathogenesis of pneumococcal pneumonia. *Reviews of infectious diseases*. 1991;13 Suppl 6:S509-517
301. Tuomanen EI, Austrian R, Masure HR. Pathogenesis of pneumococcal infection. *The New England journal of medicine*. 1995;332:1280-1284
302. Singh YD. Pathophysiology of community acquired pneumonia. *The Journal of the Association of Physicians of India*. 2012;60 Suppl:7-9
303. Holzheimer RG, Schein M, Wittmann DH. Inflammatory response in peritoneal exudate and plasma of patients undergoing planned relaparotomy for severe secondary peritonitis. *Archives of surgery*. 1995;130:1314-1319; discussion 1319-1320
304. Hogaboam CM, Steinhauser ML, Schock H, Lukacs N, Strieter RM, Standiford T, Kunkel SL. Therapeutic effects of nitric oxide inhibition during experimental

fecal peritonitis: Role of interleukin-10 and monocyte chemoattractant protein 1. *Infection and immunity*. 1998;66:650-655
305. Matsukawa A, Hogaboam CM, Lukacs NW, Lincoln PM, Strieter RM, Kunkel SL. Endogenous monocyte chemoattractant protein-1 (mcp-1) protects mice in a model of acute septic peritonitis: Cross-talk between mcp-1 and leukotriene b4. *J Immunol*. 1999;163:6148-6154
306. Johnson CC, Baldessarre J, Levison ME. Peritonitis: Update on pathophysiology, clinical manifestations, and management. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America*. 1997;24:1035-1045; quiz 1046-1037
307. Schein M, Wittmann DH, Holzheimer R, Condon RE. Hypothesis: Compartmentalization of cytokines in intraabdominal infection. *Surgery*. 1996;119:694-700
308. Marik PE. The clinical features of severe community-acquired pneumonia presenting as septic shock. Norasept ii study investigators. *Journal of critical care*. 2000;15:85-90
309. Krepel CJ, Gohr CM, Edmiston CE, Condon RE. Surgical sepsis: Constancy of antibiotic susceptibility of causative organisms. *Surgery*. 1995;117:505-509
310. Gao H, Evans TW, Finney SJ. Bench-to-bedside review: Sepsis, severe sepsis and septic shock - does the nature of the infecting organism matter? *Crit Care*. 2008;12:213
311. Salih W, Short PM, Schembri S. Macrolides as anti-inflammatory agents in cap. *European Respiratory Monograph 63: Community-Acquired Pneumonia*. 2014;63
312. Weiss T, Shalit I, Blau H, Werber S, Halperin D, Levitov A, Fabian I. Anti-inflammatory effects of moxifloxacin on activated human monocytic cells: Inhibition of nf-kappab and mitogen-activated protein kinase activation and of synthesis of proinflammatory cytokines. *Antimicrobial agents and chemotherapy*. 2004;48:1974-1982
313. Kritselis I, Tzanetakou V, Adamis G, Anthopoulos G, Antoniadou E, Bristianou M, Kotanidou A, Lignos M, Polyzos K, Retsas T, Sassopoulou P, Papaioannou AI, Sinapidis D, Sereti K, Vittoros V, Ghanas P, Gogos C, Giamarellos-Bourboulis EJ. The level of endotoxemia in sepsis varies in relation to the underlying infection: Impact on final outcome. *Immunology letters*. 2013;152:167-172
314. Gogos C, Kotsaki A, Pelekanou A, Giannikopoulos G, Vaki I, Maravitsa P, Adamis S, Alexiou Z, Andrianopoulos G, Antonopoulou A, Athanassia S, Baziaka F, Charalambous A, Christodoulou S, Dimopoulou I, Floros I, Giannitsioti E, Gkanas P, Ioakeimidou A, Kanellakopoulou K, Karabela N, Karagianni V, Katsarolis I, Kontopithari G, Kopterides P, Koutelidakis I, Koutoukas P, Kranidioti H, Lignos M, Louis K, Lymberopoulou K, Mainas E, Marioli A, Massouras C, Mavrou I, Mpalla M, Michalia M, Mylona H, Mytas V, Papanikolaou I, Papanikolaou K, Patrani M, Perdios I, Plachouras D, Pistiki A, Protopapas K, Rigaki K, Sakka V, Sartzi M, Skouras V, Souli M, Spyridaki A, Strouvalis I, Tsaganos T, Zografos G, Mandragos K, Klouva-Molyvdas P, Maggina N, Giamarellou H, Armaganidis A, Giamarellos-Bourboulis EJ. Early alterations of the innate and adaptive immune statuses in sepsis according to the type of underlying infection. *Crit Care*. 2010;14:R96

315. Rubin O, Crettaz D, Canellini G, Tissot JD, Lion N. Microparticles in stored red blood cells: An approach using flow cytometry and proteomic tools. *Vox sanguinis*. 2008;95:288-297
316. Belizaire RM, Prakash PS, Richter JR, Robinson BR, Edwards MJ, Caldwell CC, Lentsch AB, Pritts TA. Microparticles from stored red blood cells activate neutrophils and cause lung injury after hemorrhage and resuscitation. *Journal of the American College of Surgeons*. 2012;214:648-655; discussion 656-647
317. Danesh A, Inglis HC, Jackman RP, Wu S, Deng X, Muench MO, Heitman JW, Norris PJ. Exosomes from red blood cell units bind to monocytes and induce proinflammatory cytokines, boosting t-cell responses in vitro. *Blood*. 2014;123:687-696
318. Gao Y, Lv L, Liu S, Ma G, Su Y. Elevated levels of thrombin-generating microparticles in stored red blood cells. *Vox sanguinis*. 2013;105:11-17
319. Sweeney J, Kouttab N, Kurtis J. Stored red blood cell supernatant facilitates thrombin generation. *Transfusion*. 2009;49:1569-1579
320. Steiner JP, Migliorini M, Strickland DK. Characterization of the reaction of plasmin with alpha 2-macroglobulin: Effect of antifibrinolytic agents. *Biochemistry*. 1987;26:8487-8495
321. May P, Herz J, Bock HH. Molecular mechanisms of lipoprotein receptor signalling. *Cellular and molecular life sciences : CMLS*. 2005;62:2325-2338
322. Kadiu I, Narayanasamy P, Dash PK, Zhang W, Gendelman HE. Biochemical and biologic characterization of exosomes and microvesicles as facilitators of hiv-1 infection in macrophages. *J Immunol*. 2012;189:744-754
323. Meckes DG, Jr., Gunawardena HP, Dekroon RM, Heaton PR, Edwards RH, Ozgur S, Griffith JD, Damania B, Raab-Traub N. Modulation of b-cell exosome proteins by gamma herpesvirus infection. *Proceedings of the National Academy of Sciences of the United States of America*. 2013;110:E2925-2933
324. Bosman GJ, Lasonder E, Groenen-Dopp YA, Willekens FL, Werre JM. The proteome of erythrocyte-derived microparticles from plasma: New clues for erythrocyte aging and vesiculation. *Journal of proteomics*. 2012;76 Spec No.:203-210
325. Volk H-D, Reinke P, Falck P, Staffa G, Briedigkeit H, V BAEHR R. Diagnostic value of an immune monitoring program for the clinical management of immunosuppressed patients with septic complications. *Clinical transplantation*. 1989;3:246-252
326. Volk H, Thieme M, Ruppe U, Heym S, Döcke W, Manger D, Zuckermann S, Golosubow A, Nieter B, Klug H. Alterations in function and phenotype of monocytes from patients with septic disease: Predictive value and new therapeutic strategies. *Host defense dysfunction in trauma, shock and sepsis*. Springer; 1993:365-371.
327. Das U. Hla-dr expression, cytokines and bioactive lipids in sepsis. *Archives of medical science : AMS*. 2014;10:325-335
328. Lekkou A, Karakantza M, Mouzaki A, Kalfarentzos F, Gogos CA. Cytokine production and monocyte hla-dr expression as predictors of outcome for patients with community-acquired severe infections. *Clinical and diagnostic laboratory immunology*. 2004;11:161-167

329. Gogos CA, Drosou E, Bassaris HP, Skoutelis A. Pro- versus anti-inflammatory cytokine profile in patients with severe sepsis: A marker for prognosis and future therapeutic options. *The Journal of infectious diseases*. 2000;181:176-180
330. Kellum JA, Kong L, Fink MP, Weissfeld LA, Yealy DM, Pinsky MR, Fine J, Krichevsky A, Delude RL, Angus DC, Gen IMSI. Understanding the inflammatory cytokine response in pneumonia and sepsis: Results of the genetic and inflammatory markers of sepsis (genims) study. *Archives of internal medicine*. 2007;167:1655-1663
331. Kox WJ, Volk T, Kox SN, Volk HD. Immunomodulatory therapies in sepsis. *Intensive care medicine*. 2000;26 Suppl 1:S124-128
332. Rautou PE, Leroyer AS, Ramkhelawon B, Devue C, Duflaut D, Vion AC, Nalbone G, Castier Y, Leseche G, Lehoux S, Tedgui A, Boulanger CM. Microparticles from human atherosclerotic plaques promote endothelial icam-1-dependent monocyte adhesion and transendothelial migration. *Circulation research*. 2011;108:335-343
333. Terrisse AD, Puech N, Allart S, Gourdy P, Xuereb JM, Payrastre B, Sie P. Internalization of microparticles by endothelial cells promotes platelet/endothelial cell interaction under flow. *Journal of thrombosis and haemostasis : JTH*. 2010;8:2810-2819
334. Faille D, El-Assaad F, Mitchell AJ, Alessi MC, Chimini G, Fusai T, Grau GE, Combes V. Endocytosis and intracellular processing of platelet microparticles by brain endothelial cells. *Journal of cellular and molecular medicine*. 2012;16:1731-1738
335. MacLean-Fletcher S, Pollard TD. Mechanism of action of cytochalasin b on actin. *Cell*. 1980;20:329-341
336. Mostefai HA, Agouni A, Carusio N, Mastronardi ML, Heymes C, Henrion D, Andriantsitohaina R, Martinez MC. Phosphatidylinositol 3-kinase and xanthine oxidase regulate nitric oxide and reactive oxygen species productions by apoptotic lymphocyte microparticles in endothelial cells. *J Immunol*. 2008;180:5028-5035
337. Agouni A, Mostefai HA, Porro C, Carusio N, Favre J, Richard V, Henrion D, Martinez MC, Andriantsitohaina R. Sonic hedgehog carried by microparticles corrects endothelial injury through nitric oxide release. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*. 2007;21:2735-2741
338. Agouni A, Lagrue-Lak-Hal AH, Ducluzeau PH, Mostefai HA, Draunet-Busson C, Leftheriotis G, Heymes C, Martinez MC, Andriantsitohaina R. Endothelial dysfunction caused by circulating microparticles from patients with metabolic syndrome. *The American journal of pathology*. 2008;173:1210-1219
339. Leonetti D, Reimund JM, Tesse A, Viennot S, Martinez MC, Bretagne AL, Andriantsitohaina R. Circulating microparticles from crohn's disease patients cause endothelial and vascular dysfunctions. *PloS one*. 2013;8:e73088
340. Nierhaus A, Montag B, Timmler N, Frings DP, Gutensohn K, Jung R, Schneider CG, Pothmann W, Brassel AK, Schulte Am Esch J. Reversal of immunoparalysis by recombinant human granulocyte-macrophage colony-stimulating factor in patients with severe sepsis. *Intensive care medicine*. 2003;29:646-651

341. Marie C, Fitting C, Muret J, Payen D, Cavaillon JM. Interleukin 8 production in whole blood assays: Is interleukin 10 responsible for the downregulation observed in sepsis? *Cytokine*. 2000;12:55-61
342. Nomura S, Tandon NN, Nakamura T, Cone J, Fukuhara S, Kambayashi J. High-shear-stress-induced activation of platelets and microparticles enhances expression of cell adhesion molecules in thp-1 and endothelial cells. *Atherosclerosis*. 2001;158:277-287
343. Canault M, Leroyer AS, Peiretti F, Leseche G, Tedgui A, Bonardo B, Alessi MC, Boulanger CM, Nalbone G. Microparticles of human atherosclerotic plaques enhance the shedding of the tumor necrosis factor-alpha converting enzyme/adam17 substrates, tumor necrosis factor and tumor necrosis factor receptor-1. *The American journal of pathology*. 2007;171:1713-1723
344. Tiruppathi C, Shimizu J, Miyawaki-Shimizu K, Vogel SM, Bair AM, Minshall RD, Predescu D, Malik AB. Role of nf-kappab-dependent caveolin-1 expression in the mechanism of increased endothelial permeability induced by lipopolysaccharide. *The Journal of biological chemistry*. 2008;283:4210-4218
345. Zhang H, Sun GY. Lps induces permeability injury in lung microvascular endothelium via at(1) receptor. *Archives of biochemistry and biophysics*. 2005;441:75-83
346. Densmore JC, Signorino PR, Ou J, Hatoum OA, Rowe JJ, Shi Y, Kaul S, Jones DW, Sabina RE, Pritchard KA, Jr., Guice KS, Oldham KT. Endothelium-derived microparticles induce endothelial dysfunction and acute lung injury. *Shock*. 2006;26:464-471
347. Mehta D, Malik AB. Signaling mechanisms regulating endothelial permeability. *Physiological reviews*. 2006;86:279-367
348. Pupilli C, Lasagni L, Romagnani P, Bellini F, Mannelli M, Misciglia N, Mavilia C, Vellei U, Villari D, Serio M. Angiotensin ii stimulates the synthesis and secretion of vascular permeability factor/vascular endothelial growth factor in human mesangial cells. *Journal of the American Society of Nephrology : JASN*. 1999;10:245-255
349. Perez-Casal M, Thompson V, Downey C, Welters I, Wyncoll D, Thachil J, Toh CH. The clinical and functional relevance of microparticles induced by activated protein c treatment in sepsis. *Crit Care*. 2011;15:R195
350. Wen B, Combes V, Bonhoure A, Weksler BB, Couraud PO, Grau GE. Endotoxin-induced monocytic microparticles have contrasting effects on endothelial inflammatory responses. *PloS one*. 2014;9:e91597
351. Zhao J, Chen L, Shu B, Tang J, Zhang L, Xie J, Liu X, Xu Y, Qi S. Granulocyte/macrophage colony-stimulating factor attenuates endothelial hyperpermeability after thermal injury. *American journal of translational research*. 2015;7:474-488
352. Jayachandran M, Miller VM, Heit JA, Owen WG. Methodology for isolation, identification and characterization of microvesicles in peripheral blood. *Journal of immunological methods*. 2012;375:207-214