Exaggerated Neutrophil Immunosenescence in Sepsis and its Potential Modification with Simvastatin

By

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Abstract

Sepsis remains a common reason for hospital admission and is associated with a high mortality especially in the elderly. Neutrophils are one of the primary immune cells involved in the elimination of pathogens; however also contribute to the pathogenesis of multi-organ failure in sepsis. Previous studies have demonstrated an age-related decline in neutrophil migration and phagocytosis suggesting this as a mechanism for the poorer outcomes observed from sepsis in the elderly. Observational studies suggest that HMG-CoA reductase inhibitors (statins) are associated with improved outcomes from sepsis and potentially modulate neutrophil function.

This thesis demonstrates that high dose (80mg) simvastatin improved the migration of neutrophils in the healthy elderly without affecting other key neutrophil functions, such as phagocytosis and reactive oxygen species production (ROS). Studies in patients with sepsis, demonstrated that circulating neutrophils displayed features of immune-paresis and failed to migrate, but were activated with increased phagocytosis and ROS. This was accompanied by reduced neutrophil extracellular trap (NET) formation and delayed late apoptosis. The use of in-vitro simvastatin failed to modulate migration, whilst ROS and NET production was reduced by simvastatin. Simvastatin remains a potential immune-modulating drug for treating early infection in the elderly.

This thesis is dedicated to my role model, mentor and brother Nilay S. Patel, who suddenly passed away on May 13th 2014 at the age of 38. He remains a true inspiration and is missed terribly.

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Finally, thanks to all the patients who part took in my research without them none of this would have been possible.

Declaration

This thesis is submitted to the University of Birmingham to support my application for the degree of Doctor of Philosophy. I certify it has been entirely composed by myself and contains no material, which, has been accepted for the award of any other degree, or diploma in my name, in any University or other tertiary institution apart from the neutrophil function assays in Chapter 3, which were conducted in-conjunction with Dr Hannah Greenwood. To the best of my knowledge and belief the thesis contains no material previously published or written by another person, except where the due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for another degree or diploma.

TABLE OF CONTENTS

List of Figures

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Chapter 6

List of Tables

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Chapter 6

List of Abbreviations

AFU	Arbitrary fluorescent units
ALI	Acute lung injury
ALT	Alanine Transferase
AMU	Acute medical admissions units
ANOVA	One-way analysis of variance
APACHE	Acute Physiology and Chronic Health Evaluation
APAF-1	Apoptotic proteases activating factor-1
APC	Activated Protein C
APC	Allophycocyanin
ARDS	Acute Respiratory Distress Syndrome
AUC	Area under the curve
BALF	Broncho-alveolar lavage fluid
BMI	Body Mass Index
BPI	Bacterial permeability- increasing protein
C3a	Complement factor 3a
C5a	Complement factor 5a
CARS	Compensatory Anti-Inflammatory Response
CG	Cathepsin G
CGD	Chronic granulomatous disease
CK	Creatinine kinase
CLP	Caecal ligation and puncture
CMV	Cytomegalovirus

CO_2	Carbon Dioxide
COPD	Chronic Obstructive Pulmonary Disease
CR-	Complement receptor
CRP	C-reactive Protein
CVD	Cerebrovascular disease
CVP	Central venous pressure
CXCL-8	Interleukin-8
DAG	Diacylglycerol
DAMPS	Danger-Associated-Molecular-Patterns
DIC	Disseminated intravascular coagulation
DISC	Death inducing signalling complex
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPI	Diphenylene iodonium
E.Coli	*Escherichia Coli*
eNOS	Endogenous nitric oxide synthetase
ER	Endoplasmic reticulum
F	Factor (coagulation)
FEV1	Forced expiratory volume in 1 second
FiO_2	Inspired concentration of oxygen
FITC	Fluorescein Isothiocyanate
fMLP	N-formylmethionyl-leucyl-phenylalanine
FPP	Farnesylpyrophosphate
FVC	Forced Vital Capacity

G-CSF	Granulocyte colony stimulating growth factor
GCS	Glasgow Coma Scale
GM-CSF	Granulocyte macrophage colony stimulating growth factor
GPCR	G-protein coupled receptors
GPP	Geranylgeranylpyrophosphate
GRK	G-protein receptor kinase
H_2O_2	Hydrogen peroxide
HBSS	Hanks Balanced Salt Solution
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme
HMGB-1	High-Mobility Group Protein B1
HOCL	Hypochlorous Acid
HPEAC	Human pulmonary artery endothelial cell
ICAM	Intracellular adhesion molecules
ICU	Intensive Care Units
IL-	Interleukin
iNOS	Inducible nitric oxide synthetase
IPP	Isopentenyl pyrophospate
IQR	Inter-quartile range
ITAM	Immunoreceptor tyrosine-based activating motif
JAMs	Junctional adhesion molecules
LFA-1	Lymphocyte function associated-antigen 1
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid

MAC-1	Macrophage antigen 1
MAP	Mean arterial pressure
MFI	Median fluorescent intensity
MMP	Matrix metalloproteinases
Mnase	Micrococcal nuclease
MOF	Multi-organ failure
MPO	Myleoperxoidase
N/C	Nuclear cytoplasmic ratio
NAC	N-Acetylcysteine
NADPH	Nicotinamide adenine dinucleotide phosphate-oxidase
NE	Neutrophil Elastase
NETs	Neutrophil extracellular traps
NHS	National Health Service
NK	Natural killer
NO	Nitric Oxide
O_2	Oxygen
Pa	Partial pressure
PAD4	Peptidylarginine deiminase 4
PAI-1	Platelet-activation inhibitor-1
PAMPS	Pathogen-Associated Molecular Patterns
PAR	Protease-activated receptors
PBS	Phosphate buffered saline
PE	Phycoerythrin

PECAM-1	Platelet/endothelial adhesion molecule -1
PI	Phagocytic Index
PI3K	Phosphatidylinositol-3-kinase
PIP2	Phosphatidylinositol-4, 5-bisphosphate
PIP3	Phosphatidylinositol 3,4,5-triphosphate
PKC	Protein Kinase C
PLC	Phospholipase C
PMA	Phorbol-12-myristate-13-acetate
PMN	Polymorphonuclear
PPAR	Peroxisome proliferator-activated receptor
PRRs	Pattern recognition receptors
PR3	Proteinase 3
PYH	Pack year history
RNA	Ribonucleic acid
RNS	Reactive nitrogen oxygen
ROS	Reactive oxygen species
S.Aureus	*Staphylococcus Aureus*
SAPS	Simplified Acute Physiology Score
$ScV0_2$%	Central venous saturations
SGL-1	Selectin glycoprotein ligand 1
SIRS	Systemic Inflammatory Response
SLE	Systemic Lupus Erythematosus
SOD	Superoxide Dismutase
SOFA	Sequential organ failure assessment

SSCG	Surviving Sepsis Campaign Guidelines
TACE	Tumour necrosis factor alpha converting enzyme
TF	Tissue Factor
TFPI	Tissue Factor Pathway Inhibitor
TGF-β	Transforming growth factor –beta
TLRs	Toll-like receptors
TNF-α	Tumour necrosis factor alpha
tPA	Tissue plasminogen activator
TRAIL	Tumour necrosis factor alpha related apoptosis inducing ligand
UK	United Kingdom
VAP	Ventilator-associated pneumonia
vWF	von Willebrand Factor
χ2 test	Chi-squared test

Chapter 1: Introduction

1.1 The Neutrophil

Neutrophils are the most abundant polymorphonuclear (PMNs) cells making up 50-70% of the circulating pool of leucocytes, with peripheral blood counts of 2.5-7.5 $x10^6$/ml in health. Neutrophils are released from the bone marrow as terminally differentiated cells and have a short circulating half-life of 6-8 hours. The bone marrow generates and releases large numbers of neutrophils, with up to 5 $x10^{10}$ cells being produced daily. Neutrophils are an essential part of the innate immune system being one of the primary cells involved in pathogen containment and elimination. Once an invading pathogen has been detected neutrophils are rapidly recruited to the site of infection where their principal role is to kill and contain the responsible organism (1-3).

1.1.1 Differentiation, maturation and apoptosis

Neutrophils are produced within the bone marrow and the process of maturation from myeloid stem cells takes approximately two weeks and is principally under the controls of granulocyte colony stimulating growth factor (G-CSF), which in turn is regulated by the release of interleukin (IL-) 17A and IL-23 from regulator T-cells and dendritic cells (1, 4-7).

1.1.1.1 Maturation

The neutrophils pass through six distinct morphological stages in their maturation starting as nuclear dense myeloid stem cells, which differentiate to form promyelocytes then to myelocytes and again to metamyeloblasts. These then differentiate to immature neutrophils, called bands, and then finally differentiate into

mature segmented neutrophils characterised by their multi-lobar nucleus (see figure 1.1) (1).

During maturation and differentiation the nucleus changes structure and shape to become segmented with reductions in the nuclear cytoplasmic ratio (N/C) observed. Myeloid cells have a high N/C ratio of 80-85%, band cells 33-40% N/C ratio and mature segmented cells a N/C of 33%.

1.1.1.2. Granule formation

The proliferation and maturation of neutrophils prepares the cells for their functions once circulating. This involves the production of three distinct granule populations, azurophillic/primary granules, neutrophil specific/secondary granules and tertiary/small granules. The azurophillic granules are produced during early maturation as promyelocytes. At this stage myeloperoxidase (MPO), the three serine proteases, neutrophil elastase (NE), cathepsin G (CG) and proteinase 3 (PR3) and other antimicrobial proteins (defensins, lysozyme and azurocidin) are produced and stored within the cell. Neutrophil specific/secondary granules, such as lysozyme, lactoferrin, collagenase and various membrane receptors, are produced during the myelocytic phase of development. The tertiary granules are produced last when the cells are metamyelocytes. The small granules contain gelatinases (matrix metalloproteinases - MMP) and Cathepsin B and D (1). This is summarized in table 1.1 and figure 1.1.

The azurophillic granules fuse with phagosomes and release their granules within them to degrade and kill invading organisms. MPO is considered to be the most

effective agent that neutrophils have to neutralise invading organisms, whilst the defensins, perforins and bacterial permeability- increasing protein (BPI) target the membrane permeability of the pathogen.

Secondary granules are released extracellularly to kill pathogens (cytochrome b558), prevent pathogen proliferation (lactoferrin) and to initiate the inflammatory process (N-formylmethionyl-leucyl-phenylalanine {fMLP} receptors and plasminogen activator) by the further recruitment of immune cells.

Mature neutrophils leaving the bone marrow are capable of effective migration, phagocytosis and are armed with an arsenal of antimicrobial products to carry out their lethal functions. Mature neutrophils are released into the circulation in a resting, latent state and under normal conditions have a short half-life of 6-8hours. Neutrophils subsequently marginate into peripheral tissue pools where they undergo apoptosis and phagocytosis by macrophages.

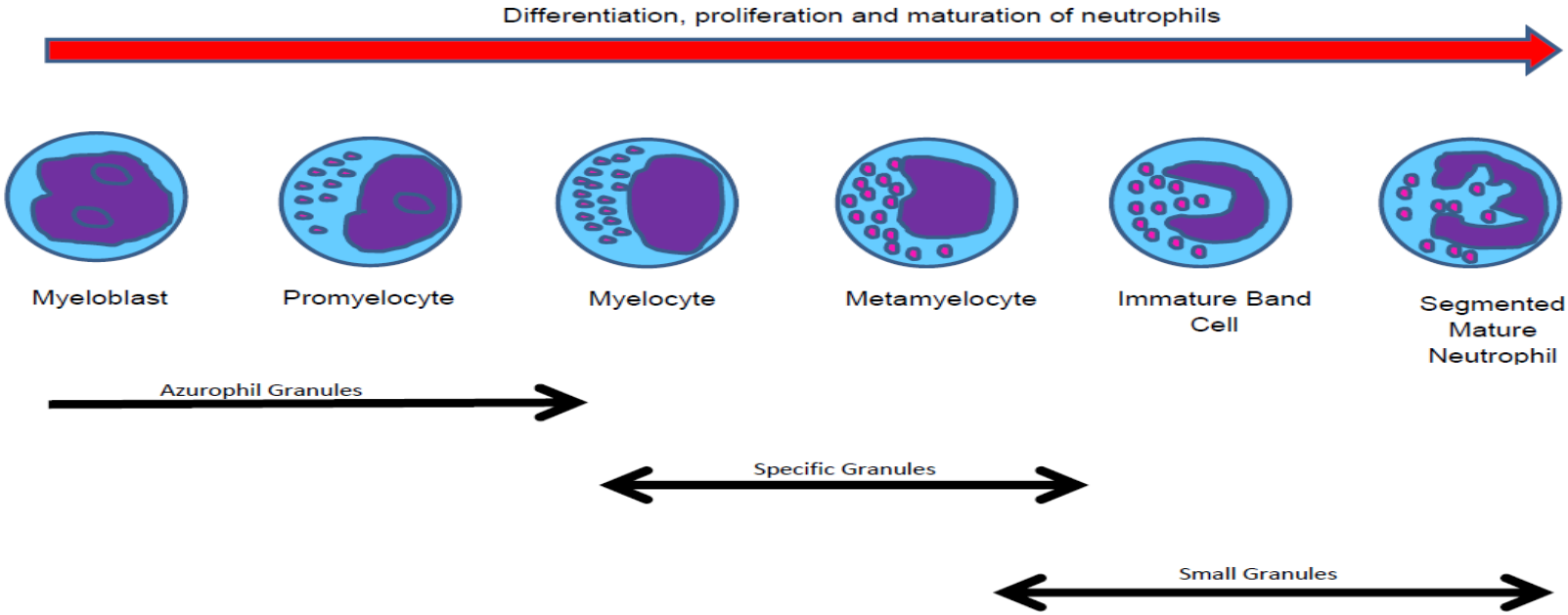


Figure 1.1. Differentiation and maturation of neutrophils from the bone marrow.

An illustration showing the progressive maturation phases from myeloblast through to a mature segmented neutrophil within the bone marrow in health. The various stages of granule formation are also shown. Neutrophils leaving the bone marrow are terminally differentiated mature cells equipped with all the tools required for them to carry out their cytotoxic functions.

Table 1.1. Types of neutrophil granules and their contents

	Granules		
Constituents	**Azurophillic**	**Specific**	**Small**
Antimicrobial	Myeloperoxidase Lysozyme Bacterial permeability-increasing protein (BPI) Defensins	Lysozyme Lactoferrin	
Neutral Proteinases	Elastase Cathepsin G Proteinase 3	Collagenase Complement activator	Gelatinase Plasminogen Activator
Acid hydrolases	Cathepsin D ß-D-Glucuronidase α-Mannosidase Phospholipase A2	Phospholipase A2	Cathepsin B & D ß-D-Glucuronidase α-Mannosidase
Cytoplasmic membrane receptors		CR3, CR4 fMLP receptors Laminin receptors	
Others	Chondroitin-4-sulphate	Cytochrome b558 Monocyte chemotactic factor Histaminase Vitamin B12 binding protein	Cytochrome b558

The granule, contents and actions of neutrophils.
fMLP: N-formylmethionyl-leucyl-phenylalanine
CR-: Complement receptor -

1.1.1.3 Neutrophil Apoptosis

Apoptosis is controlled and programmed cell death that eliminates cells from the circulation to maintain cellular homeostasis and prevent excessive morphogenesis, as well as modulating inflammation. Neutrophil apoptosis requires tight regulation to prevent the uncontrolled release of cytotoxic granules and unnecessary accumulation that can precipitate collateral tissue injury (8).

Neutrophil apoptosis is characterised by cell body shrinkage, notching of the cell membrane, vacuolated cytoplasm, mitochondrial depolarization and the fragmentation and condensation of deoxyribonucleic acid (DNA). Apoptotic neutrophils are biological inactive and show reduced migration, superoxide burst and phagocytosis. A balance of pro- and anti-apoptotic signals regulates the fate of neutrophils; these are summarised in table 1.2. (8, 9).

Three principal mechanisms of neutrophil apoptosis have been identified, the extrinsic death receptor pathway, the intrinsic (mitochondrial) pathway and the endoplasmic reticulum (ER) or stress induced pathway (8).

The extrinsic death receptor pathway is triggered by specific ligand and cell surface receptor binding resulting in the recruitment of various proteins that forms the death inducing signalling complex (DISC). Apoptosis is induced by FAS, Tumour Necrosis Factor alpha (TNF-α) and TNF-α –related apoptosis inducing ligand (TRAIL) receptors. These bind to their respectively ligands (FAS ligand, TNF-α and TRAIL) to

activate Caspase-8 and -3 which are responsible for the fragmentation and condensation of the DNA (8, 10-12).

The intrinsic death pathway is mediated via the production of reactive oxygen species (ROS), which causes mitochondrial depolarization. This results in the loss of cytochrome C inducing apoptotic proteases activating factor-1 (APAF-1) and subsequently activates Caspase 9 and -3 (8, 13-15).

ER stress can directly and indirectly lead to apoptosis and is initiated via physiological stress, such as hypoxia and acidaemia. Direct activation of apoptosis is via the activation of Caspase 12, which lies on the ER membrane and the subsequent activation of Caspase 9 and -3. Indirectly ER stress has been shown to trigger the release of cytochrome C from neutrophils thereby activating the intrinsic apoptotic pathway.

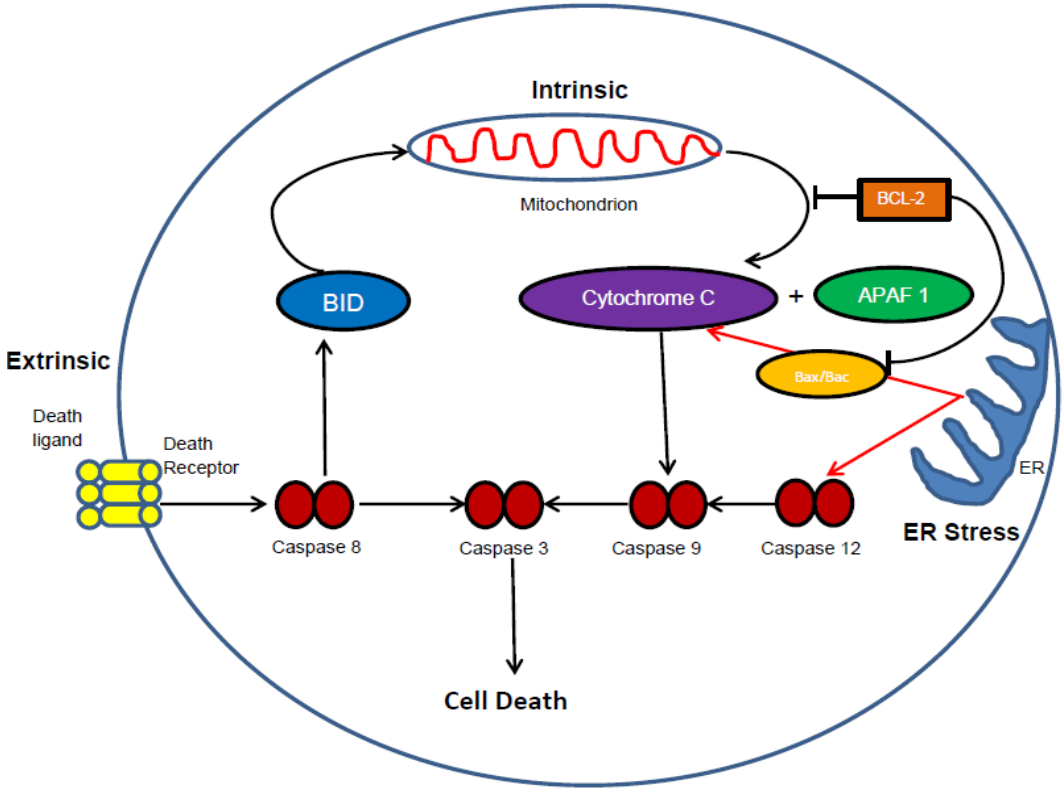


Figure 1.2. Pathways of Neutrophil Apoptosis

A schematic diagram showing the extrinsic, intrinsic and endoplasmic reticulum induced stress pathways that lead to neutrophil apoptosis. The extrinsic pathway is mediated by specific interactions between ligands and their receptors on the cell surface. This activates the Caspase pathways that are responsible for the condensation of cellular DNA. The intrinsic pathway is mediated via the release of reactive oxygen species (ROS) from mitochondria with the resultant loss of cytochrome C, which induces Caspase 9 and subsequent cell apoptosis. Hypoxia and acidosis induces endoplasmic reticulum stress with the resultant activation of Caspase 12 and subsequently Caspase 9 and 3 to induce apoptosis. ER stress also induces death via the intrinsic pathway through the release of cytochrome C.

Table 1.2. Intracellular and extracellular modulators of neutrophil apoptosis.

Modulators	Mechanisms
Intracellular modulator	
Reactive oxygen species	DNA damage. Activation of p38, NFKβ, JNK and MAPK
Caspases	Activation of Caspase cascade, PKC-δ and DNA cleavage
BCL-2 membrane proteins	Regulate mitochondrial membrane permeability and control cytochrome c release
Survival/Death pathways	
PKB/AKT	Activated Bax and NFKβ
P38 MAPK	Inhibits Caspase 8 and 3
PKC-δ	Unknown
Extracellular modulators	
Anti-apoptotic factors	
Type 1 and II interferons	Activate STAT 3
LPS	Phosphorylation of Akt and up regulation of Mcl-1 and A1
G-CSF and GM-CSF	Via up regulation of PI3 kinase/Akt
TNF-α (low doses)	Activation of PI3 kinase, CXCL-8 release
Pro-apoptotic	
Fas Ligand	Increased mitochondrial permeability and Caspase activation
TRAIL	Via TRAIL–R2 and R3 receptors
Phagocytosis	ROS production and caspase activation
TNF-α (high doses)	ROS production, activation of JNK and MAPK

The intracellular and extracellular modulators of neutrophil apoptosis with mechanisms of actions. Adapted from Luo, H.R. et al, 2008 (8).

1.1.2. Neutrophil Function in Health

Neutrophils form the primary cellular response to bacteria, viruses and fungi. Neutrophils are required to migrate (chemotax) to sites of infection by transmigrating the vascular endothelium. Once at the site of infection they must eliminate and contain the infectious source by engulfing them (phagocytosis) and generating ROS. Neutrophils also release their DNA into the extracellular space to ensnare and kill pathogens in a process termed neutrophil extracellular traps (NETs) formation (16). Finally, neutrophils undergo apoptosis and clearance from tissues (see figure 1.3).

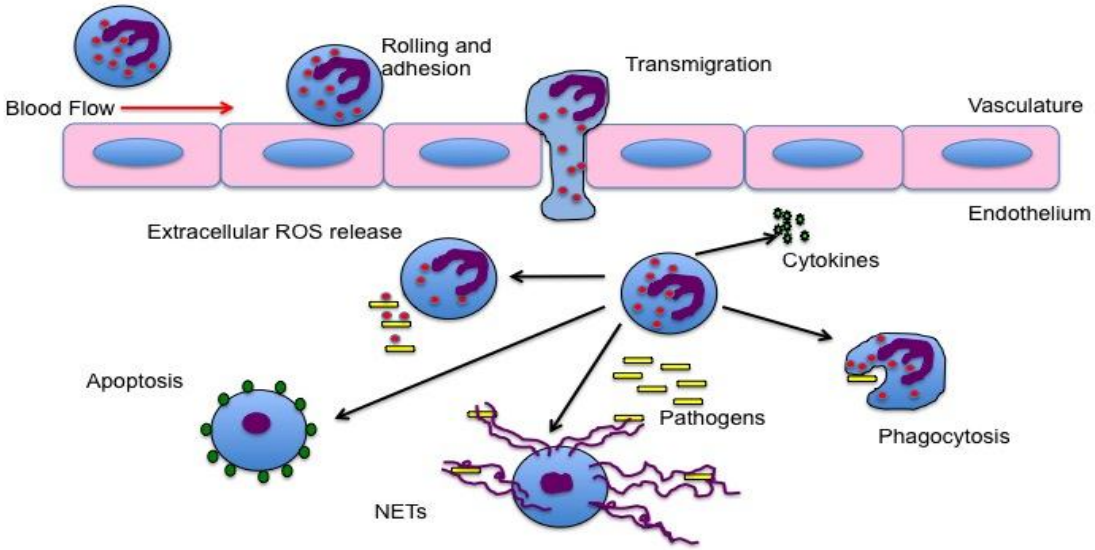


Figure 1.3. Neutrophil Function in Health.

Neutrophils tether, roll and adhere to the endothelium prior to transmigrating through the endothelium (chemotaxis). Once at the site of inflammation, they kill the invading pathogens by phagocytosis, extracellular reactive oxygen species (ROS) release and by producing neutrophil extracellular traps (NETS). They also produce cytokines, which attract other immune cells and modulates the inflammatory response. Once the neutrophils have carried out their function they die by apoptosis, a process that contributes to the resolution of the inflammation.

1.1.2.1. Neutrophil Chemotaxis

Chemotaxis is defined as the directional migration of leucocytes along a chemical gradient and is the term used to describe the process of neutrophil migration into tissues from the intravascular space. Neutrophils migrate towards a range of chemoattractants such as the bacterial peptide fMLP, complement factor 5a (C5a), leukotriene B (product of phospholipid metabolism) and cytokines such as IL-8, known as CXCL-8 (17, 18).

Five distinct stages of chemotaxis have been described, tethering, rolling, adhesion, crawling and transmigration (19). Leucocytes resident within tissues release a variety of cytokines, histamine and leukotrienes after contact with invading pathogens instigating neutrophil chemotaxis. The endothelial cells directly recognise pathogens via specific pattern recognition receptors (PRRs) such as the Toll-like receptor (TLR) family, to up regulate adhesion molecules. The adhesion molecule P-selectin is rapidly expressed by their release from Wiebal-Palade bodies in response to lipopolysaccharide (LPS), TNF-α and IL-1. E-selectin, another key adhesion molecule, is synthesised by endothelial cells and expressed with 90-120 minutes after endothelial cell activation. These two-adhesion molecules glycosylate to their specific ligand expressed by neutrophils, P-selectin glycoprotein ligand 1 (P-SGL1) and E-selectin glycoprotein-1 (E-SGL1) to capture (tether) flowing neutrophils to the endothelium (19, 20)

Tethering of the neutrophil to the endothelium via P-selectin and P-SGL1 is temporary, as the fast flowing neutrophil has to slow down. The neutrophil rolls along

the endothelial surface membrane by releasing the rear tether (P-selectin-P-SGL bond) and moving it to the leading edge on the cell, where it interacts with another P-selectin molecule. This repeated sling shooting of the cells is known as rolling and causes deceleration of the neutrophil (19-22).

As activated neutrophils roll in this sling shot manner they come into contact with an endothelium laden with chemokines, in particular CXCL-8, causing them to adhere to the endothelium. These chemokines are positively charged molecules bound to the endothelium by negatively charged heparan sulphates that anchor the neutrophil to prevent them being detached and create a chemotactic gradient. CXCL-8 signals via G-protein coupled receptors (GPCR) on the neutrophils (CXC-R1 and CXC-R2) causing conformational changes and increasing the expression of the adhesion molecules, lymphocyte function associated-antigen 1 (LFA1 also known as, CD11a/CD18) and macrophage antigen 1 (MAC-1: CD11b/CD18). These adhesion molecules bind to intracellular adhesion molecules (ICAM-1 and ICAM-2) resulting in the firm adhesion of neutrophils to the endothelial surface (19, 23-26).

Adherence of the neutrophils prepares the cell for transmigration. Transmigration requires the neutrophil to be adherent to the cell membrane at all times and is achieved through the binding of MAC-1 to ICAM-1. These bonds must be present at all times and crawling is in part achieved by the release and formation of new bonds. Crawling is initiated by the neutrophil forming pseudopods at random. Pseudopods that are correctly orientated in the direction of the chemokine gradient form the leading edge of the neutrophil. This pseudopod, with an actin-based leading edge

pulls the neutrophil forward, whilst a rear uropod (made from an actin/myosin structure) helps the rear to contract as the cell moves forwards (17, 19, 20, 27, 28).

The crawling of neutrophils along the endothelium mediated by a chemokine gradient allows pseudopods to sense the location of tight junctions between the endothelial cells to allow the paracellular (between the cells) migration of cells. The process of transmigration is not fully understood, however studies suggest that platelet/endothelial adhesion molecule -1 (PECAM-1) and junctional adhesion molecules (JAMs) play an essential role in “labelling” tight junctions and subsequently interacting with ICAM-1 and LFA-1 to allow neutrophils to pass through the endothelium and into the basement membrane (19, 20, 29, 30).

Once the neutrophils have transmigrated they continue to sense a chemokine gradient allowing the directional migration of cells through the extracellular membrane towards the sites of infection. These gradients are created by end-target chemoattractants, such as fMLP and C5a, which override the signals from the endothelial chemoattractants (IL-8 and LTA-4) (19, 29, 31).

The mechanism of migration through the extracellular membrane is not clear, as experimental studies investigating this in-vitro cannot replicate in-vivo conditions. Current opinion suggests that neutrophils seek out gaps between adjacent pericytes to aid migration but also release proteases (e.g. MMP-8 and MMP-9), NE and ROS to breakdown the laminin and collagen rich membrane to create a pathway through the membrane. The deformable structure of neutrophils created by actin mobilisation

at the leading edge and the actin/myosin “tail” helps to guide the cells through the membrane to the tissue either via gap junctions or the destructive lesions created by the neutrophils (32-34).

Traditionally, neutrophils have been thought to die at the site of infection, however emerging evidence from murine and human models suggest that some are capable of reverse transmigrating back into the blood stream from tissues. These cells are usually resistant to apoptosis and seem to maintain a phenotype similar to that seen in inflamed tissue (a pro-inflammatory phenotype) resulting in continued phagocytosis and ROS production within the systemic circulation. It is postulated that these cells may disseminate inflammation and infection and lead to distant organ damage (35-37).

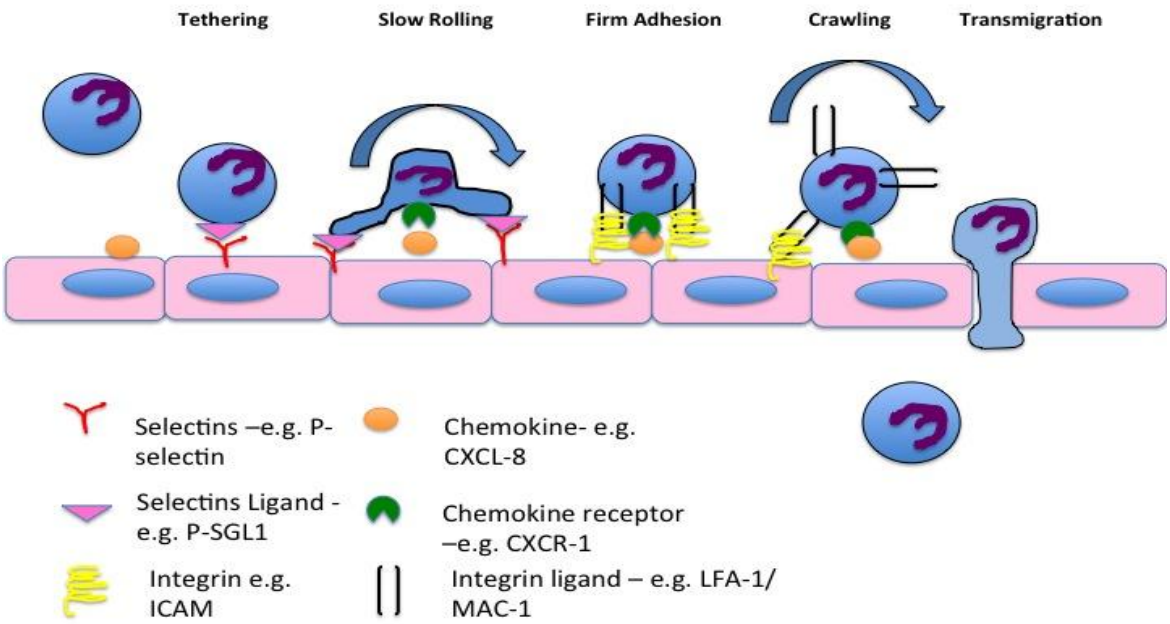


Figure 1.4. Neutrophil Chemotaxis

A schematic diagram of the process of neutrophil migration from the blood across the endothelium. Tethering and rolling is mostly selectin-dependent and binds to the specific ligands (e.g. P-selectin glycoprotein-1, P-SGL1). Adhesion, crawling and transmigration are dependent upon integrin interactions (e.g. Intracellular Adhesion Molecule -1, ICAM -1), which bind LFA-1 (lymphocyte function associated antigen-1) and MAC-1 (macrophage-1- antigen). The chemokines that line the endothelium activate rolling neutrophils and induce conformational changes of neutrophil integrins that instigate crawling and transmigration. Crawling neutrophils follow the chemokine gradient along the endothelium, which guides them to the preferential sites of transmigration.

1.1.2.2. Neutrophil phagocytosis

Phagocytosis is the process that describes the engulfing and elimination of pathogens and cell debris by neutrophils and other leucocytes. The mechanistic pathways involved in phagocytosis have been collated from experiments using macrophages, which unlike neutrophils, are amenable to manipulation allowing close analysis of molecular mechanisms (38). Therefore the mechanisms of neutrophil phagocytosis remain unclear with assumptions that the pathways involved in phagocytosis are similar in both cell types.

Neutrophils, like macrophages, are capable of internalising opsonised and non-opsonised pathogens. Opsonisation coats pathogen with antibodies (IgG predominantly) and complement (C3b and C4b) and attracts phagocytes to them. The Fab proportion of the antibody binds to the pathogen, whilst the Fc proportion is left exposed for binding to the phagocytes. On neutrophils the Fc receptors are CD32 (FcγRIIA), CD16 (FcγRIIIb) and the high affinity receptor CD64 (FcγRI). Complement is recognised by a variety of receptors on the cell surface including complement receptor (CR) -1 and CR-3 and the β-2 integrin, MAC-1 (Cd11b/CD18) (38-40).

The process of subsequent phagocytosis differs depending on the mechanism of detection. Bacteria recognised by Fc receptors leads to the rapid generation and entrapment by a pseudopod. Complement based detection leads to the sinking of the pathogen slowly into the cell (38).

Binding to Fc receptors on neutrophils leads to a series of complex cell signalling pathways that triggers the engulfment of bacteria. This is initiated by phosphorylation of the Fc receptors and immunoreceptor tyrosine-based activating motif (ITAM). This provides a binding site for the tyrosine kinase, Syk and is accompanied by the activation of phosphatidylinositol-3-kinase (PI3K). PI3K is responsible for the conversion of phosphatidylinositol-4, 5-bisphosphate (PIP_2) to phosphatidylinositol 3,4,5-triphosphate (PIP_3) at the phagosomal cup. Although PIP_2 acts as the substrate for PI3K and is utilised heavily in phagocytosis, the levels increase, as it is also acts as a substrate for phospholipase C (PLC) that generates diacylglycerol (DAG). This in turn activates isoforms of protein kinase C (PKC), which are recruited to the phagosome and are vital for the engulfing of pathogens (41-44).

Activation of these proteins and kinases causes accumulation and polymerisation of F-actin and membrane remodelling to allow vacuole formation. The family of Rac GTPases are involved in the assembly of actin via Fc receptor stimulation, whilst the Rho family is involved in complement mediated activation (38, 40).

Once a vacuole has been formed around the particle/pathogen it is transported into the cell by a process known as endocytosis and the receptor recycled to the cell surface membrane. The internalised vacuole, known as the early endosome, contains the ingested species and is mildly acidic (pH 6.5-6.0). Maturation occurs due to lysosome delivery making the endosome increasingly acidic and creating a hostile environment for the pathogen. This process is mediated by increases in cytosolic calcium levels and a variety of protein kinases (38).

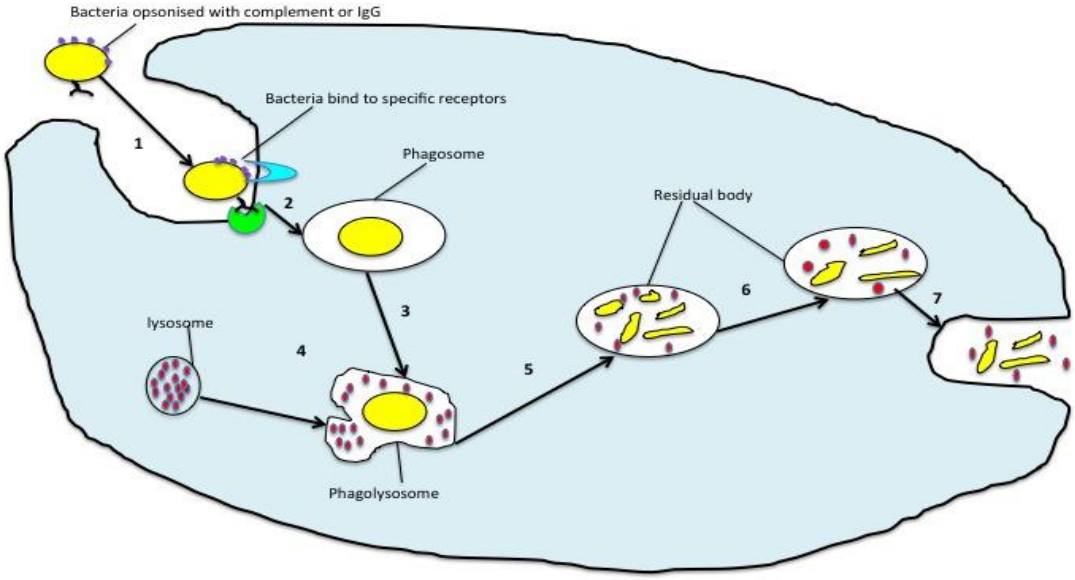


Figure 1.5. The phagocytosis of pathogens by neutrophils.

1.The bacteria are opsonised by IgG or complement and the neutrophil migrates towards it. 2. Bacteria bind to specific Fc receptors and are engulfed into the cell. 3. Formation of the phagosome. 4. The fusion of lysosome with the phagosome to form the phagolysosome. 5. The pathogens are digested by the release of enzymes from the lysosome. 6. Formation of the residual body containing indigestible products. 7. These products are then released into the extracellular space.

1.1.2.3. Reactive Oxygen Species (ROS) production and Neutrophil Degranulation

The enzyme, nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase), found on the inner cell surface membrane and in mitochondria, is responsible for the reduction-oxygenation (redox) reactions that occur to form water from oxygen to generate ROS. The initial reaction involves the reduction of oxygen by NADPH to form a superoxide oxygen anion. This is converted to hydrogen peroxide (H_2O_2) either spontaneously or by superoxide dismutase (SOD). H_2O_2 is then either converted by catalases to the free radical hydroxyl ion (OH-) or interacts with the primary granule, MPO, to produce the highly toxic substance hypochlorous acid (HOCL). The OH- ion is neutralised to water, whilst HOCL is converted to the toxic free radical singlet oxygen. In addition reactive nitrogen oxygen (RNS) are produced by inducible nitric oxide synthetase (iNOS) generating the free radical nitric oxide (NO) from L-arginine. NO combines with the oxygen superoxide anion to create peroxynitrite (ONOO-) another potent free radical (see figure 1.6) (1, 45-52).

Activation of latent circulating neutrophils by bacterial products (fMLP), cytokines (CXCL-8/TNF-α) and cell surface receptors (Fc, MAC-1) results in the mobilisation of stored vesicles. These granules bind to the phagosome or the cell membrane in a process known as exocytosis and release their antimicrobial load directly into the phagosome or the extracellular space. This release of enzymes and proteinases plays an essential role in intracellular and extracellular pathogen elimination. Neutrophil degranulation and exocytosis, like phagocytosis, is regulated via intracellular calcium and phospholipid (PIP_2 and PI3kinase) signalling (48, 51, 53-58).

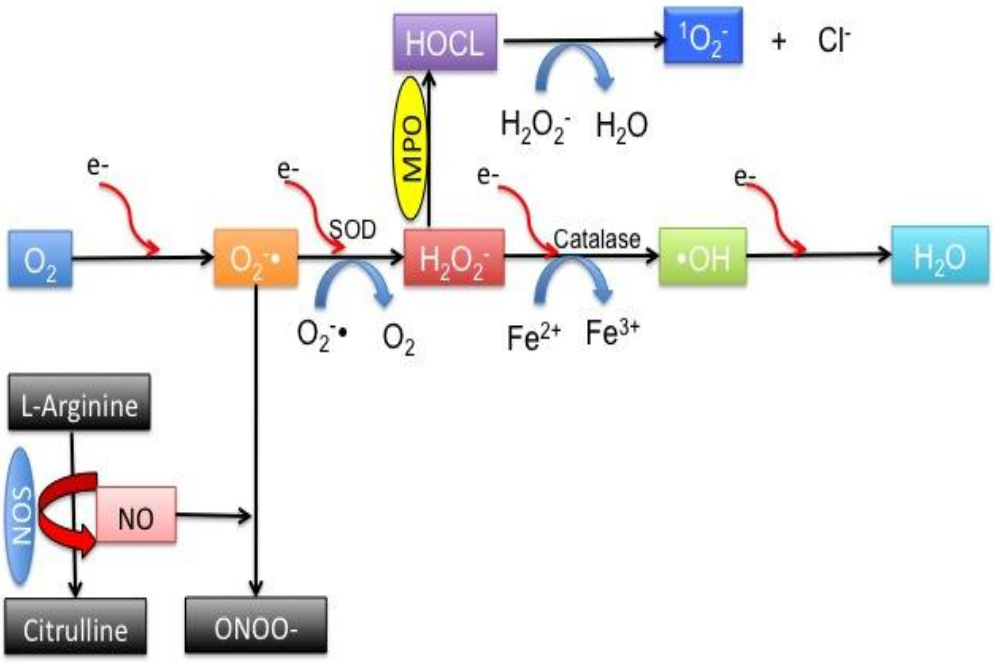


Figure 1.6 The Formation of Reactive Oxygen Species (ROS) and Reactive Nitrogen Species in neutrophils.

An illustration of the pathways leading to the generation of ROS. Oxygen is converted by nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) to form an oxygen superoxide ion. This is converted by superoxide dismutase (SOD) to hydrogen peroxide (H_2O_2) that is either converted to a hydroxyl ion (+OH) and water or interacts with myeloperoxidase (MPO) to produce hypochlorous acid (HOCL) and then singlet oxygen. The oxygen superoxide ion also interacts with nitric oxide (NO) to produce nitrogen free radicals.

1.1.2.4. Neutrophil Extracellular Trap (NETs) formation

The phagocytosis of invading organisms and the release of lytic enzymes by neutrophils has been regarded as the mechanism the innate immune system utilises to eliminate bacteria, fungi and viruses. Brinkmann and colleagues described the production of extracellular fibres from activated neutrophils, which created a mesh like structure and were capable of ensnaring and potentially killing bacteria. These are made up of a DNA backbone laden with neutrophil granule products, such as MPO, NE, BPI, CG and lactoferrin. This additional anti-microbial mechanism has been termed, neutrophil extracellular traps (NETs) with the process leading to their production described as NETosis (16, 59, 60).

Three independent pathways for NET formation have so far been described. The first pathway is an oxidant dependent pathway that was described in neutrophils activated with either live bacteria or phorbol-12- myristate-13-acetate (PMA) resulting in cells becoming highly phagocytic, generating ROS via NADPH-oxidase and leading to NET formation. The assembly of NETs follows a particular pattern with the initial loss of nuclear segregation (eu- and heterochromatin) and the fragmentation of the characteristic lobar nucleus. This is followed by the disintegration of the nuclear membrane into vesicles and the disappearance of the neutrophil granules. This process allows the nuclear chromatin to mix with the cytoplasm and granules. The final stage involves rupture of the cell membrane and the extrusion of the NETs. The authors demonstrated that the process of NET formation leads to the eventual death of the cell but is a distinct form of cell death separate from apoptosis and necrosis – termed NETosis (59, 60).

The second pathway describes an oxidant independent pathway of rapid NET production from activated neutrophils that does not lead to cellular death and allows the neutrophils to continue with their other actions. Neutrophils incubated with *Staphylococcus Aureus* (*S.Aureus*) were shown to condense their chromatin into discrete vesicles and pass through the cytoplasm to acquire granule products. These vesicles are exocytosed into the extracellular space where the chromatin is released in the form of a NET. Uniquely this process occurred rapidly (within 10minutes) in response to direct stimulation by *S.Aureus* and was not dependent on the formation of ROS (61).

The third pathway describes the release of mitochondrial DNA in an oxidant dependent manner from neutrophils that were primed with granulocyte macrophage colony-stimulating-factor (GM-CSF) and C5a. Neutrophils that release mitochondrial DNA in the form of NETs do not induce neutrophil death (NETosis) but instead demonstrate prolonged survival, allowing them to continue their anti-microbial actions. This pathway remains controversial with several authors suggesting that mitochondrial NETs are not released and that the identification of mitochondrial DNA is primarily due to cell lysis (62).

Two of the pathways that mediate NET formation are dependent on the production of ROS, with pharmacological blockade of NADPH-oxidase (with diphenylene iodonium-DPI) inhibiting NET formation. This is corroborated clinically in patients with inherited disorders of NADPH-oxidase (chronic granulomatous disease -CGD), whose neutrophils are unable to produce NETs upon stimulation. When given gene therapy

to replace NADPH-oxidase is provided to these patients NET generation is restored (59, 60, 63).

Another key biological process to the generation of NETs is the citrullination of histone H2A, H3 and H4 that occurs early in NET formation and forms the backbone of its structure. This process is catalysed by the enzyme peptidylarginine deiminase 4 (PAD4), which is abundant in neutrophils and converts histone arginine to citrulline. This is a vital step in the de-condensation of DNA. Inhibition of PAD4 pharmacologically and in mice deficient in PAD4 has demonstrated the failure of neutrophils to generate NETs, suggesting that this enzyme is crucial for their formation (64-68).

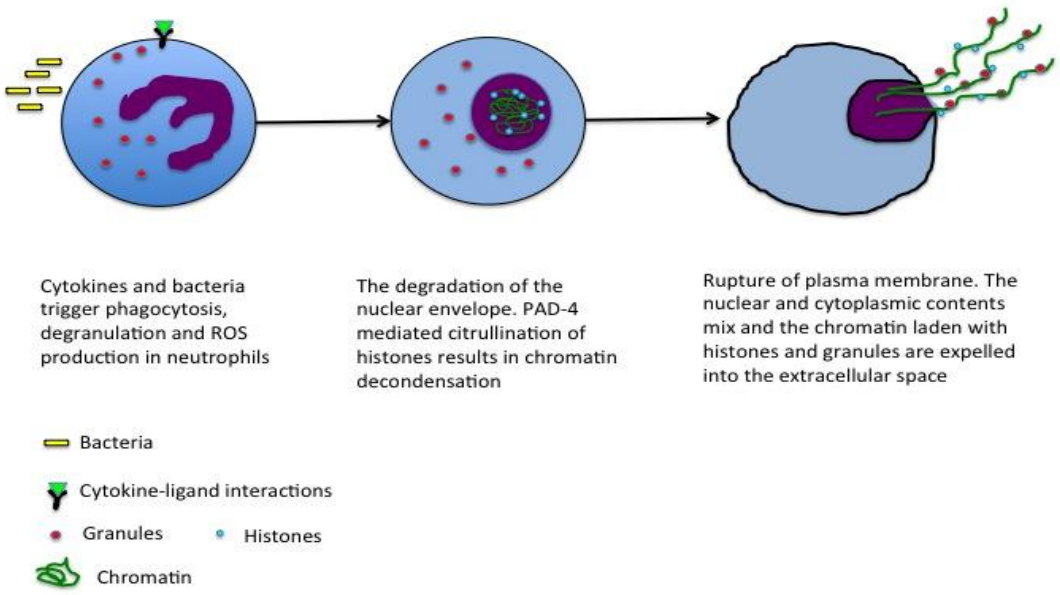


Figure 1.7. Schematic representation of the NETosis pathway.

A diagram showing the classical pathway of NETosis originally described by Brinkmann et al, whereby activation by bacteria or cytokines leads to activation of neutrophils and the subsequent degradation of the nuclear envelope and peptidylarginine deiminase 4 (PAD-4) mediated citrullination. Finally the plasma membrane ruptures and the DNA laden with histones and granule products are released extracellularly.

1.1.2.5. Summary

In health, neutrophils are efficient at sensing and migrating to sites of infection and once homed in on the target can exterminate the invading organism by phagocytosis, degranulation and ROS/RNS production. Neutrophils can kill extracellularly by releasing ROS/RNS, the exocytosis of granules and the formation of NETs. This makes well functioning neutrophils essential to the innate immunity.

However neutrophils are involved in the pathogenesis of inflammation, as each neutrophil function can potentially cause collateral tissue damage. For example the process of migration creates a temporary breach of the endothelium, the release of granules such as MPO and NE and ROS (H_2O_2 and HOCL) are highly toxic to tissues and the endothelium and NETs may cause tissue damage due to the granules that adhere to them (3, 69, 70).

Alterations in neutrophil functions and number are associated with poorer outcomes from many congenital (CGD) and acquired diseases (rheumatoid arthritis, systemic lupus erythematosus - SLE) with both poor and exaggerated function being implicated (3, 69).

1.2. Immunosenescence

Ageing is a modern health care challenge with the population living longer and consequently placing increasing demands on health and social care. The ageing process is associated with a progressive decline in immune function, referred to as immunosenescence. The changes that occur to both the adaptive and innate immune systems have been proposed to lead to the increased severity of infection, poor responses to vaccination, cancer, autoimmune diseases and other chronic disease of old age (e.g. atherosclerosis, Alzheimer's disease and type 2- diabetes) (71-76).

It has been suggested that immunosenescence is an adaptive process that occurs over a lifespan. One adaptation is "inflammaging", where chronic antigen exposure results in a persistent state of low level inflammation within the innate immunity. Additionally hormonal changes and alterations in neuro-endocrine function are thought to play a role in "inflammaging." Cytokine profiling in elderly people has demonstrated higher levels of pro-inflammatory cytokines, such as IL-6, IL-15, IL-18, C-reactive protein (CRP), TNF-α, and coagulation factors (fibrinogen and von Willebrand Factor (vWF)). The ability of the immune system to counteract inflammaging, by the production of anti-inflammatory cytokines (e.g. IL-10, Transforming growth factor –beta (TGF-β) and cortisol), is of great relevance to survival and longevity and is observed in cohorts of centenarians who have immune system profiles similar to those observed in younger adults (72, 73).

The changes in adaptive immunity have been well characterised, whilst innate immunity has only recently been investigated in terms of immunosenescence. A

summary of the changes observed is shown in the table 1.3 and figure 1.8. Overall, these changes result in a heightened baseline pro-inflammatory state, making the ageing individual prone to inflammatory diseases (e.g. atherosclerosis, rheumatoid arthritis) and potentially reduce their ability to fight new infections. Changes to adaptive immunity lead to reduced success of vaccinations, reactivation of latent viruses (e.g. cytomegalovirus (CMV)) and the increased prevalence of cancer in the elderly (73, 76-80).

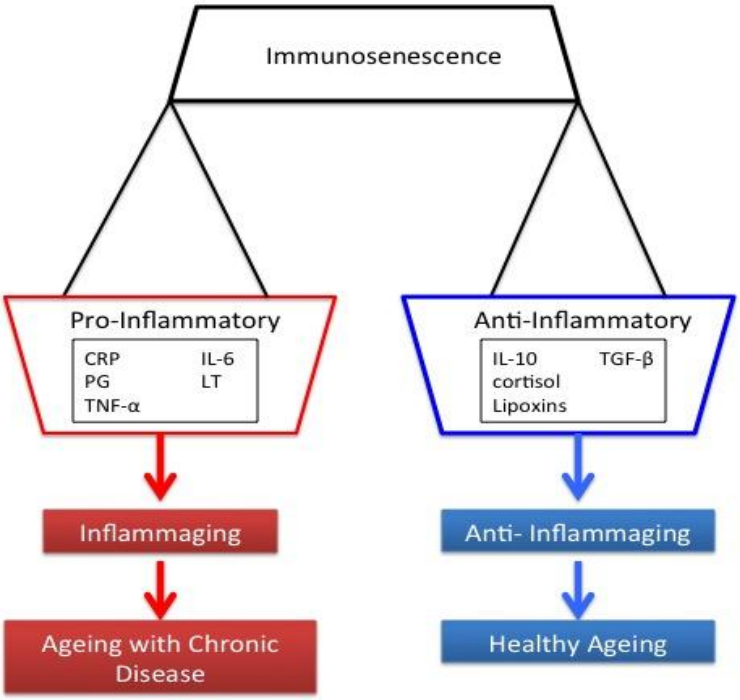


Figure 1.8. The balancing between pro- and anti-inflammatory agents and relationship to immunosenescence

A homeostatic balance exists between pro-inflammatory and anti-inflammatory factors in response to inflammation. Efficient pro-inflammatory responses lead to greater protection from infectious diseases in early life, but an increased susceptibility to inflammation-based diseases with advancing age. Conversely a predominant anti-inflammatory response, increases susceptible to infectious diseases in early life, but confers a survival advantage in old age. PG: prostaglandins; LT: leukotrienes; CRP: C-reactive protein; TNF-α: Tumour necrosis factor- alpha; TGF-β: Tumour growth factor- beta; IL-: Interleukin. Modified from Francheschi C. et al 2007 (73).

Table 1.3. The age–related changes that occur in the adaptive and innate immune systems.

	Cell Type	Age related increases	Age-related decreases
Innate Immunity	Neutrophils		Oxidative burst. Phagocytic capacity. Bactericidal activity
	Macrophages		Oxidative burst. Phagocytic capacity.
	NK cells	Total number of cells	Proliferative response to IL-2. Cytotoxicity.
	Dendritic cells		Capacity to stimulate antigen specific T cells. Lymph node homing
	Cytokines and Chemokines	Serum levels of IL-6, IL1β and TNF-α	
Adaptive Immunity	T-Lymphocytes	Number of memory and effector cells. Expanded clones of effector cells. Release of pro-inflammatory cytokines.	Number of native T-cells. Diversity of the T-cell repertoire. Expression of co-stimulatory molecules (CD28, CD27, CD40L). Proliferative capacity
	B-lymphocytes	Auto-reactive serum antibodies	Generation of B-cell precursors. Numbers of naïve B cells. Diversity of B-cell repertoire. Expression of co-stimulatory molecules (CD27, CD40) Antibody affinity Isotype Switch

The table summarizes factors that are increased and decreased due to the normal ageing process, termed immunosenescence.

TNF-α: Tumour necrosis factor- alpha; IL-: Interleukin; NK: Natural killer. Modified from Weiskopf, 2009 (76).

1.2.1. Impact of age on neutrophil function

Alterations in the ability of neutrophils to migrate to sites of infection and contain and eliminate bacteria are likely to contribute to sepsis, multi-organ failure (MOF) and death. Age contributes to the prognosis of sepsis, with increasing age associated with poorer outcomes. The impact of ageing upon innate immunity, in particular neutrophil functions is poorly defined (3, 78, 80-82).

The numbers of circulating neutrophils is not altered with age, nor is the ability of the bone marrow to generate an appropriate neutrophilla (increased neutrophil numbers) in response to G-CSF and GM-CSF. This implies that an adequate bone marrow response in the elderly is present and not a contributor to the poorer outcomes observed (78, 83). Functional studies on neutrophils from aged donors suggest that immunosenescence results in a reduced ability to migrate, phagocytose and generate a superoxide burst as described below.

1.2.1.1. Ageing and Neutrophil Chemotaxis

Studies on the impacts of age on neutrophil chemotaxis suggest that although neutrophil adherence is preserved with age, the ability of neutrophils to migrate to sites of infection is impaired. Sapey et al suggest that an age-related decline in neutrophil migration exists. This is based on data from 84 healthy controls between the ages of 25-94 showing a linear decline in directional migration with age (Spearman's r=-0.690 95% CI= -0.790- -0.554, p=0.001, see figure 1.9). Although neutrophils are capable of reaching sites of infection they take longer due altered directional migration (78, 82, 84-86).

This has two potential consequences for the host. Firstly bacteria are able to rapidly multiple at sites of infection increasing the severity of the insult and resulting in a slower resolution of inflammation. Secondly, inaccurate migration may lead to an increase in proteinase release by neutrophils (NE, MMP-8 and MMP-9) leading to an augmentation of collateral tissue damage (78, 79, 82, 86).

The mechanisms underpinning this are not fully understood, however Sapey E et al suggest that over-expression of PI3-kinase activity in elderly donor neutrophils is responsible for aberrant neutrophil chemotaxis. Inhibition of PI3-kinase with LY294002 corrected migration of neutrophils towards levels seen in healthy young donors (86).

1.2.1.2 Ageing and Pathogen Killing

Ageing reduces the ability of neutrophils to phagocytose bacteria with studies suggesting that the mechanistic ability to phagocytose is retained but with a reduced capability (less bacteria being ingested per cell). This has been demonstrated for both *S.Aureus* and *E.Coli*, both of which are responsible for significant infections in the elderly (81, 87, 88).

The impact of age on ROS production from neutrophils remains ambiguous, with some authors showing a reduction in ROS generation, whilst other have shown no difference between young and old controls (84, 87, 89).

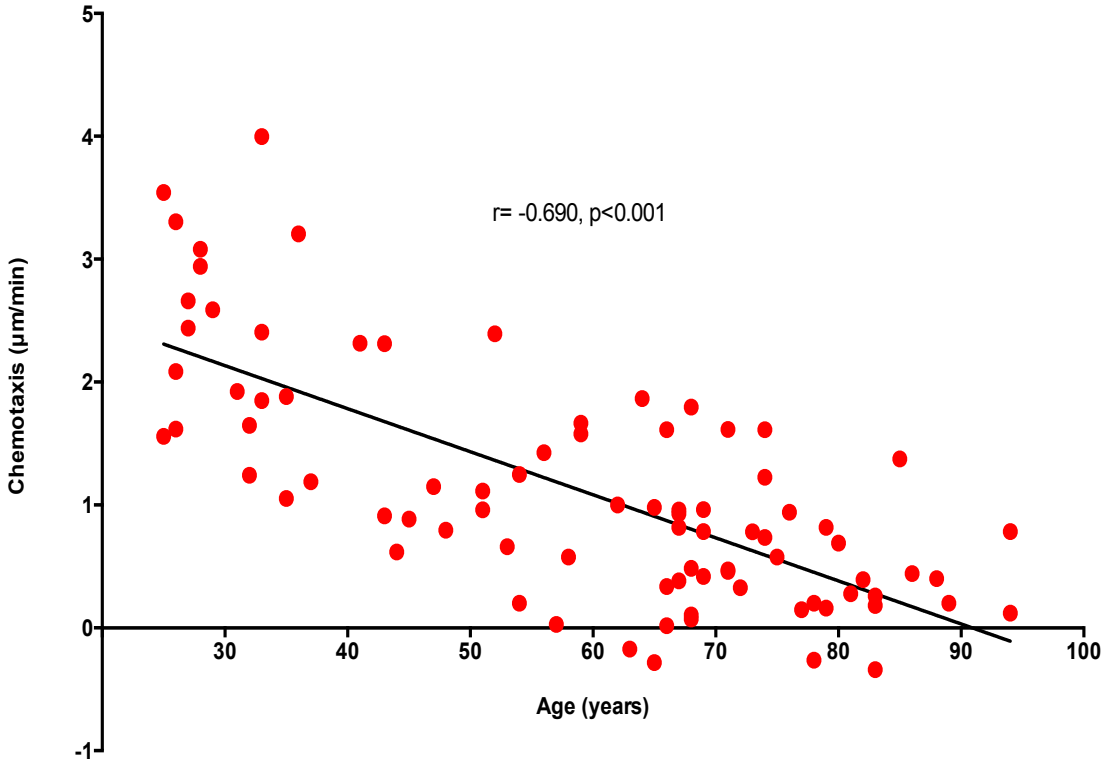


Figure 1.9. A correlation between age and neutrophil chemotaxis
A Spearman's correlation between age (in years) and neutrophil chemotaxis in 84 healthy controls between the ages of 25-94. Data courtesy of Dr E Sapey and reproduced with her permission. (86)

1.2.1.3. Ageing and Neutrophil Extracellular Traps

The impact of ageing on NETs has recently been investigated by Hazeldene et al, who demonstrated that primed (with TNF-α) neutrophils from elderly donors produced less NETs compared to young donors in response to fMLP and LPS. No differences were seen with PMA. They proposed the failure to produce NETs in the elderly as a potential mechanism for poorer containment and elimination of bacteria, which contributes to the poorer outcomes observed in the elderly (90).

1.2.1.4 Summary

The elderly are at increased risk of fatal sepsis and insights into innate immunity suggest that key neutrophil functions worsen with age, predisposing the elderly to increased severity of sepsis. The modulation of neutrophil responses by pharmacological agents towards a younger phenotype is a potential mechanism to improve outcomes in the elderly from infection and other neutrophil mediated chronic inflammatory diseases (80, 86).

1.3. Sepsis

Sepsis has been challenging medicine since the time of Hippocrates, who described sepsis as “by which flesh rots, swamps generate foul smells and wounds fester.” The development of the germ theory, by Pasteur suggested that sepsis resulted from the systemic dissemination of bacteria into the blood stream, termed septicaemia or “blood poisoning.” However, despite the advent of antibiotics and the adequate treatment of infection sepsis continues to develop and cause death. This lead to the concept of host mediated immune dysfunction in response to the invading pathogen driving the pathophysiology of sepsis (91, 92).

1.3.1. Definitions

Sepsis was formally defined by a consensus group of experts in 1992, who stated that sepsis was a systemic inflammatory response to infection. They introduced the terms sepsis, severe sepsis and septic shock to describe the progression of the initial inflammatory response to MOF and refractory hypotension that is observed (93). These definitions were adopted by the Surviving Sepsis Campaign Guidelines (SSCG) in 2003 and have become an international standard for the diagnosis of sepsis (see tables 1.4 and 1.5). These guidelines have been updated in 2008 and in 2013 and provide an evidence-based framework on the management of sepsis (94-96).

1.3.2. Epidemiology

The exact incidence of sepsis is difficult to quantify due to variability in the coding mechanism of admissions and outcomes from hospitalisation and the associated pathologies that patients' who develop sepsis present with. In the United States, sepsis is estimated to account for 2% of all hospital admissions and 10% of all cases admitted to Intensive Care Units (ICU), with a predicted incidence of 750,000 cases annually, whilst in the United Kingdom (UK) the annual incidence is approximately 102,000. Similar incidences have been reported from European and Australian/New Zealand studies, although this is thought to be an under-estimate, with data suggesting the incidence is rising by 8-13% per year. Extrapolated data provides a conservative estimate of 20-30 million cases of sepsis per year worldwide (91, 97-101). The rise in sepsis has been attributed to an ageing population, advances in the treatment of medical conditions predisposing patients to sepsis (e.g.: bone marrow

transplant and immunosuppressive agents) and an increased awareness of the condition (91, 102).

Thirty years ago the mortality from severe sepsis was as high as 80%, however increased access to ICU care, the introduction of the SSCG and the earlier initiation of antibiotics has helped to reduce sepsis-related mortality. In the UK, sepsis is thought to account for 7.7% of all deaths in 2010, representing an estimated 23700 people. In this UK based study, which used mortality data from the office of national statistics, mortality of sepsis peaked in 2006 and since an annual decrease in mortality has been observed despite an increasing incidence (91, 97, 102). A similar pattern in mortality was observed in an Australian & New Zealand study that analysed ICU mortality from sepsis over twelve years (2000-2012). They demonstrated that mortality from sepsis decreased by 16.7%, from 35% in 2000 to 18.4% in 2012, representing an absolute annual reduction of 1.3% (103).

Risk factors associated with the development of sepsis are extremes of age, with the very young (<1 year old) and old (>60 years old) having similarly high mortality from sepsis. Men also have poorer outcomes from sepsis compared with age-matched women and this is thought to be due to the protective effectives of oestrogens and the harmful effects of testosterone on inflammation. The presence of chronic diseases (chronic kidney disease and diabetes mellitus) and those patients that are immunosuppressed from disease (HIV and Cancer) or treatments (chemotherapy and steroids) predisposes them to sepsis and sepsis-related mortality (91, 97, 102, 104-106).

Table 1.4. The diagnostic criteria for the systemic inflammatory response syndrome (95).

2 or more of the following criteria
Fever of more than 38.3°C or less than 36°C
Heart rate of more than 90 beats per minute
Respiratory rate of more than 20 breaths per minute or a $PaCO_2$ level of less than 32 mm Hg
Abnormal white blood cell count (>12,000/μL or <4,000/μL or >10% bands)

Table 1.5. Surviving Sepsis Campaign Guideline Definitions of Sepsis(95).

Sepsis	SIRS + new/suspected infection
Severe Sepsis	Sepsis + sepsis-induced organ dysfunction
Organ Dysfunction	*Sepsis-induced hypotension* *Lactate > normal laboratory results* *Urine output <0.5 mL/kg hr. for >2 hrs.,* *ALI with PaO_2/FIO_2 <250 in the absence of pneumonia as infection source* *ALI with PaO_2/FIO_2 <200 in the presence of pneumonia as infection source* *Creatinine >176.8 mmol/L)* *Bilirubin >34.2 mmol/L)* *Platelet count >100,000/mm^3* *Coagulopathy (INR>1.5)*
Septic Shock	Severe sepsis + hypotension not reversed by fluid resuscitation

Sepsis places a considerable burden on health care resources with patients admitted to hospital having longer hospital and ICU lengths of stay and costing more than those not admitted with sepsis. In the US conservative estimates suggest that $16.7 billion is spent of treating sepsis, whilst in the UK, estimates based on the treatment of 100,000 patients with severe sepsis in ICU, suggests the National Health Service (NHS) spends £2.5billion/year managing sepsis (97, 99, 107).

1.3.3. Pathophysiology of Sepsis

Sepsis results from a host mediated immune response to infection. The pathogen load, its virulence and the subsequent host characteristics determine the extent of the response. Initial theories suggested that an exaggerated and dysregulated pro-inflammatory response generated a cytokine storm that led to clinical features of fever, tachycardia, hypotension and MOF. Recent advances in sepsis biology have challenged this, with the current models suggesting a combined pro-inflammatory response (SIRS) that is counteracted by a compensatory anti-inflammatory response syndrome (CARS). See figure 1.10 (91, 108-111).

1.3.3.1. Innate Immunity in Sepsis

The innate immune system is responsible for the recognition and immediate response to pathogen invasion. This is achieved through specific PRRs, such as the TLR family, C-type lectin receptors, retinoic acid inducible gene-1-like receptors and nucleotide-binding oligomerization domain-like receptors. These PRRs recognise specific pathogen-associated molecular patterns (PAMPs), such as LPS from gram-negative bacteria, which binds to TLR-4 and peptidoglycan from gram-positive bacteria, which binds TLR-2. PRRs also recognise host cellular damage from molecules known as danger-associated-molecular-patterns (DAMPs) or alarmins

such as high-mobility group protein B1 (HMGB-1), extracellular DNA, ribonucleic acid (RNA) and histones (91, 110, 112, 113).

The activation of the PRR, TLR-4 by the PAMP, LPS, triggers a serious of intracellular signalling mechanisms resulting in the up-regulation of genes coding for pro-inflammatory cytokine production, such as TNF-α, IL-1β and IL-6, chemotactic signals and ROS intermediates and iNOS. The net result of this is the recruitment of neutrophils and other innate immune cells to the sites of infection and the enhancement of local immune cell function. Activation of PRRs not only enhances pro- inflammatory gene transcription but also anti-inflammatory transcription (e.g. IL-10) ensuring a balanced immune response to contain and eliminate invading pathogen and minimising collateral tissue damage (91, 110, 114, 115).

The systemic spread into the vasculature of these very same mediators is responsible for the vasodilation and subsequent hypotension that precipitates the MOF characteristic of sepsis (115).

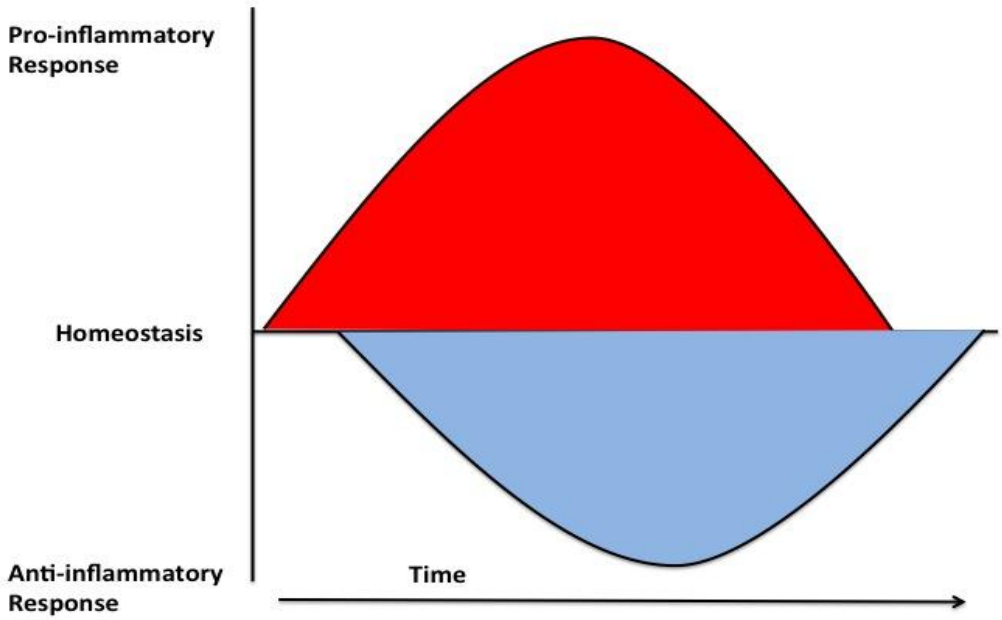


Figure 1.10. The resolution of sepsis.

Illustration of the current mechanism of sepsis resolution, showing an initial pro-inflammatory response, which is counteracted by a compensatory immune response.

1.3.3.2 Coagulation in sepsis

Disordered coagulation is a hallmark of severe sepsis characterised by platelet consumption and prolonged clotting times leading to disseminated intravascular coagulation (DIC) in 30-50% of patients with septic shock. The net result of sepsis upon the coagulation system is increased fibrin deposition due to increased thrombin generation, impairment of anticoagulation pathways and a reduction in the fibrinolytic system (91, 108, 116-118).

Sepsis results in a state of micro-vascular thrombosis driven by the exposure of tissue factor (TF) to circulating blood cells after activation by TNF-α and bacterial products. TF binds and activates Factor (F) VII, which activates FX and results in the conversion of prothrombin to thrombin. Thrombus formation decreases blood flow to septic tissues exacerbating hypo perfusion. The crucial role of this in sepsis has been demonstrated by the prevention of MOF and death by its inhibition in murine and primate models of LPS sepsis (91, 115, 118-121).

Concurrently mechanisms to prevent thrombus formation are impaired. These are controlled by antithrombin, TF pathway inhibitor (TFPI) and activated protein C (APC). Pro-inflammatory cytokines in sepsis inhibit the production of intermediaries and thus impair the function of antithrombin and TFPI. APC is an important anticoagulant mechanism in sepsis as it inactivates FVa and FVIIIa. Levels of APC are substantially reduced in sepsis due to decreased production by the liver, increased consumption and by reduced activation due to lower levels of thrombomodulin and endothelial protein C receptors (91, 108, 118).

The protease-activated receptors (PARs) form an important link between coagulation and inflammation. Four have been identified to date with PAR-1 being of greatest importance in sepsis. Thrombin is able to activate PAR1, whilst TF induces cell signalling via FXa and APC exerting cellular effects (117, 122).

The central role of the coagulation system, and specifically APC, in sepsis led to the development of recombinant APC (Xigris, Eli Lily) as a treatment of severe sepsis. Pre-clinical studies demonstrated improved outcomes in animal model of sepsis infused with APC with the phase III PROWESS and ADDRESS trials showing improved clinical outcomes in patients with severe sepsis. Interestingly it was the anti-inflammatory effects of recombinant APC mediated via PAR1 that were attributed to the improvements rather than direct actions upon the coagulation system. Recombinant APC was withdrawn from the market after a third Phase III trial (PROWESS-SHOCK) showed no benefit and an increase in fatal bleeding complications (91, 117, 118, 122-125).

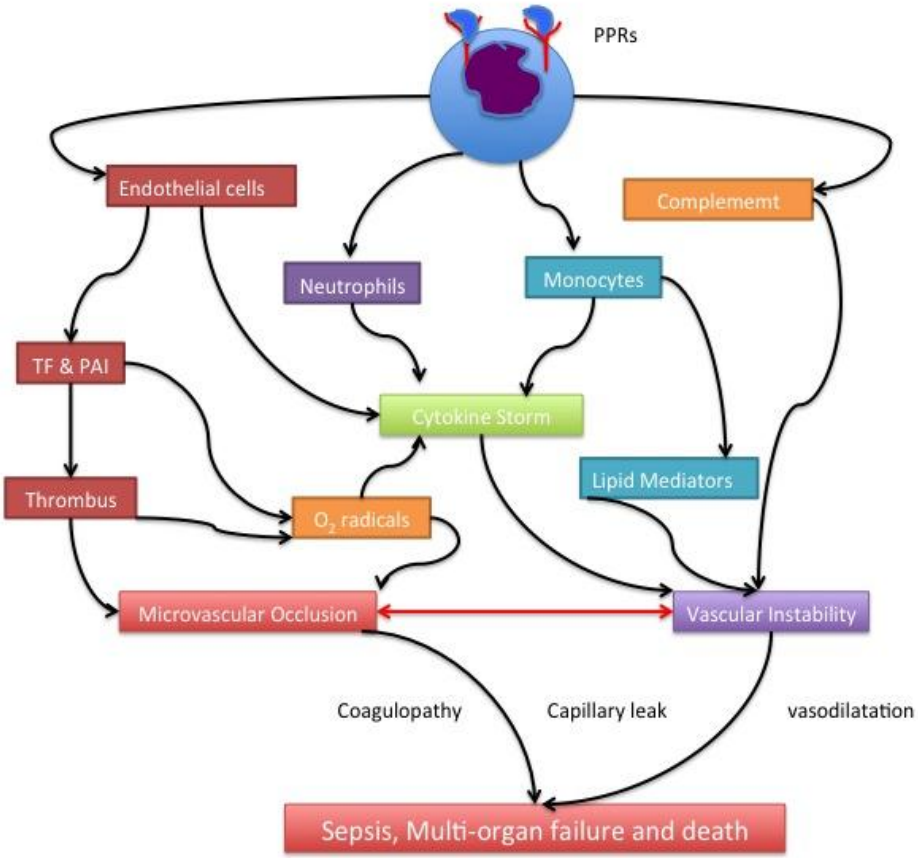


Figure 1.11: A simplified diagram of the innate immune response to infection and tissue injury involving the inflammatory cytokines and the coagulation cascade.

Pattern recognition receptors (PRRs), such as TLRs on endothelial cells, on neutrophils and monocytes bind bacterial products resulting in an inflammatory cascade involving the coagulation systems and up-regulated cytokine gene production. The resultant microvascular occlusion and vascular instability leads to the characteristic coagulopathy increased capillary leak and vasodilation seen in sepsis. Adapted from Cohen J 2002 (126).

1.3.3.3 Immunosuppression in Sepsis

There is increasing evidence that immunosuppression plays an important role in the pathogenesis of sepsis. It is uncommon for patients to die from sepsis in the pro-inflammatory phase commonly seen in young patients with meningococcal sepsis, as advances in medicine ensure such patients receive timely treatment. Deaths from sepsis tend to occur in-patients who have often survived the initial insult only to succumb to secondary opportunistic infections (91, 127, 128). This has been attributed to state of relative immunosuppression that follows the initial pro-inflammatory phase. Evidence from post-mortems and lung samples of patients with sepsis have shown that leucocytes develop a relative immunosuppressed phenotype and show reduced activity against bacteria. In addition death from sepsis is associated with apoptosis of both adaptive and innate immune cells (109, 127-129).

Common nosocomial infections that cause secondary sepsis are of relatively low virulence (*Strenotrophomonas spp, Actinobacter spp, Pseudomonas spp and Candida spp*) and there is a high incidence of reactivation of latent viruses (CMV and herpes simplex) adding to evidence that hospitalised patients with sepsis are relatively immunosuppressed (128, 130-132).

1.3.3.4 Multi-organ failure in Sepsis

Organ failure, in particular of the cardiovascular system, leads to death from sepsis. The exact mechanism of organ failure is still not fully known, with tissue hypo-perfusion, microvascular thrombi, endothelial, immune and organ cell death all involved. The increase in oxygen demand leads to oxidative stress, mitochondrial dysfunction and eventual death (91, 133, 134).

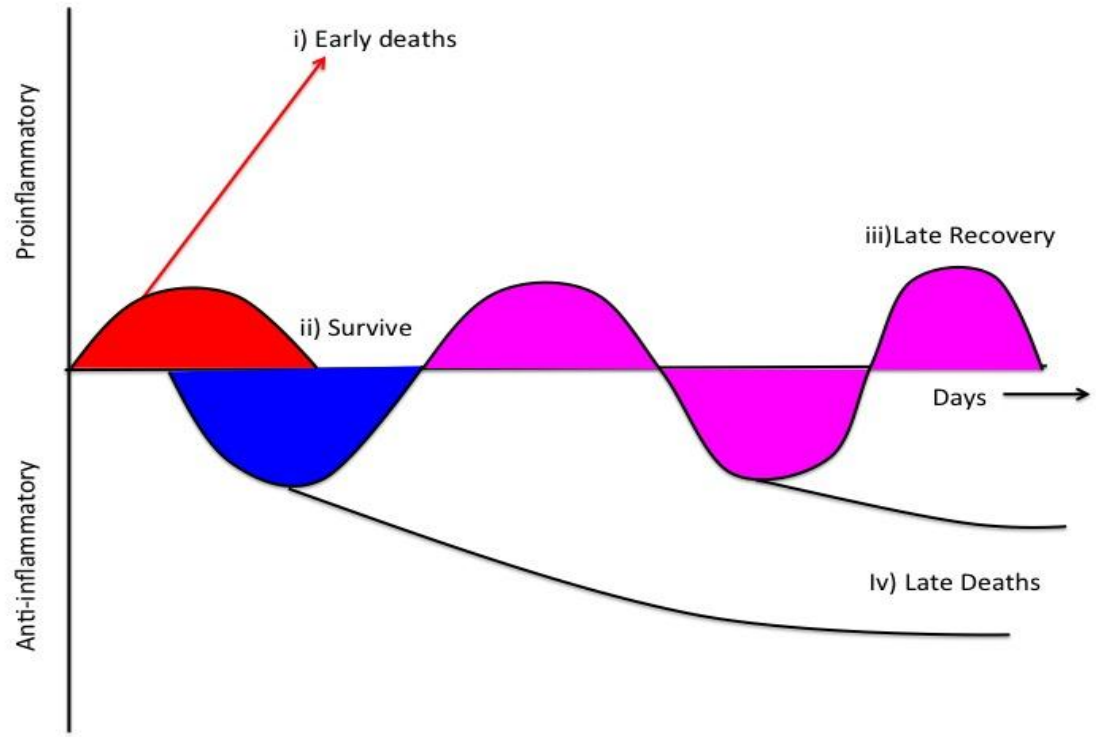


Figure 1.12 Potential inflammatory responses in sepsis

i) Early deaths from sepsis are driven by an overwhelming pro-inflammatory phase leading to refractory cardiovascular shock, metabolic derangements and multi-organ failure. ii) A large proportion of patients are elderly and present with complex comorbidities. These patients develop an initial blunted/absent pro-inflammatory response with an exaggerated anti-inflammatory phase. These patients may survive and recover or go onto develop a state of prolonged critical illness. iii) An additional suggested immunological response is characterised by cycling between pro-inflammatory and anti-inflammatory states. In this response patients have an initial proinflammatory response followed by a hypo-inflammatory state. With secondary infection, patients have a repeat pro-inflammatory response from which they may recover or develop a further an anti-inflammatory phase. iv) Late deaths from sepsis may arise due to significant immunosuppression, the reactivation of latent viruses and the development of nosocomial infection. Modified from Hotchkiss et al, Lancet Infectious Diseases 2013 (128).

1.3.4. Treatment

The SSCG break down sepsis management into bundles of care that have been shown to improve outcomes when applied. The initial sepsis bundle focuses on the resuscitation of the patients, source identification and control and the delivery of timely antibiotic therapy (see table 1.6) (94-96, 135-137). The importance of early detection of sepsis and the initiation of early antibiotic therapy is vital as delays in the initiation of appropriate therapy significantly increases mortality with one study suggesting that for every hour that antibiotics are delayed mortality is increased by 10% (96, 138-140).

A large part of sepsis management focuses on the restoration of circulating volume and the maximisation of oxygen delivery. This involves the administration of oxygen, intravenous fluids and where appropriate, the initiation of vasopressors and mechanical ventilation. The delivery of protocolised goal-directed therapy to achieve optimal oxygen delivery was proven by Rivers et al and has guided sepsis management since. However results from the PROCESS and ARISE trials has challenged this paradigm suggesting that targeting resuscitation to correct lactate levels is as effective as guiding resuscitation using central venous pressure (CVP) monitoring and central venous saturations ($ScV0_2$%) (96, 141-143).

The SSCG's second bundle incorporates management strategies on ICU and includes guidance on vasopressor use, mechanical ventilation, renal replacement therapy, sedation, nutritional support and the prevention of thrombo-embolic events and nosocomial infections (91, 96).

Trials on immunomodulatory drugs to treat sepsis have failed to show any benefits and at present, only a short course of hydrocortisone 200mg per day, has been suggested in patients who remain hypotensive despite adequate fluid resuscitation and appropriate vasopressor therapy to modulate immune responses in sepsis (91, 96, 123, 124, 144-147).

1.3.5. Summary

Sepsis is a global health problem affecting millions of people worldwide, causing significant morbidity and mortality and places a great economic burden on health services. The incidence of sepsis continues to rise in part due to an ageing population. Sepsis arises due to an imbalance in the pro-inflammatory and anti-inflammatory host response to infection and is a complex biological process. Treatment of sepsis is based on early recognition, initiation of antibiotics and optimisation of oxygen delivery. Although medical science has failed to develop any immunomodulatory therapies to tackle the immune dysfunction of sepsis, the application of the SSCG bundles and principles have seen sepsis-related mortality fall year upon year over the past decade (91, 96, 102, 103).

Table 1.6: Surviving Sepsis Campaign Guidelines for Initial Resuscitation

Surviving Sepsis Campaign Bundles
To be completed within 3 hours
1. Measure lactate level **2. Obtain blood culture prior to administration of antibiotics** **3. Administer broad spectrum antibiotics** 4. **Administer 30ml/kg crystalloid for hypotension or lactate >4mmol/L.**
To be completed within 6hours
5. Apply vasopressors (for hypotension that does not apply to initial fluid resuscitation • **In the event of persistent arterial hypotension despite volume resuscitation (septic shock) or initial lactate >4 mmol/L** • **Measure central venous pressure (CVP)*** • **Measure central venous oxygen saturation ($ScvO_2$)*** **6. Re-measure lactate if initial lactate was elevated***
***Targets for quantitative resuscitation included in the guidelines are CVP of ≥8 mmHg, $ScvO_2$ of 70%, and normalization of lactate.**

1.4. Neutrophil Function in Sepsis

Neutrophils are implicated in the pathogenesis of MOF in sepsis, with over activated neutrophils, at sites of infection, causing collateral tissue damage, whilst suppressed neutrophil function leads to ineffective pathogen elimination and dissemination of infection. Finally neutrophils may cause organ/tissue damage at sites remote to the infectious focus due to systemic activation of neutrophils leading to invasion and damage of highly vascular organs, such as the lungs, liver and kidney. The key changes in neutrophil function are summarised in table 1.7 (148-155).

1.4.1. Impact of sepsis on neutrophil chemotaxis

Neutrophil migration is essential for the elimination of pathogens, with impaired migration being linked with poorer outcomes from sepsis in murine models of sepsis and in humans. The mechanism is likely to be complex relationship between multiple pathways (2, 148, 154, 156-158).

The inflammatory response of severe sepsis results in increased neutrophil rigidity, alteration in the expressions of adhesion molecules, the internalisation of chemokine receptors and changes in intracellular signalling, all of which contribute to a state of neutrophil desensitisation and resultant decrease in migration (2, 148, 159). Furthermore, pro-inflammatory cytokines (TNF-α) and bacterial products (fMLP) cause increased neutrophil cell membrane rigidity via the deposition of F-actin just below the cell membrane resulting in reduced deformability, preventing effective rolling and leading to neutrophil sequestration into capillary beds, which exacerbates tissue hypoxia. This process is mediated via peroxisome proliferator-activated

receptor (PPAR) γ, a group of nuclear hormone receptors. Reddy et al demonstrated that in patients with sepsis PPARγ receptor levels are higher and are associated with defective neutrophil chemotaxis. They also demonstrated that inhibition of PPARγ in a murine model of sepsis prevents the deposition of F-Actin in response to fMLP and improves neutrophil migration (2, 148, 160, 161).

NO also plays a key role in altering the migratory dynamics of neutrophils in sepsis by reducing the expression of beta-2-integrins (LFA-1 and MAC-1) and selectins (L, P and E-Selectin) on neutrophils and endothelium adhesion molecules expression (ICAM-1 and VCAM-1). Studies in murine models of sepsis (caecal ligation and puncture {CLP}) have demonstrated that blocking iNOS either pharmacologically or genetically reverses the deleterious effects of sepsis on neutrophil migration, improves bacterial clearance and improves survival (2, 148, 154, 156, 162-164).

The TLR family are important in the initiation of innate immunity and act as PRRs, which detect PAMPs and thus cause increased transcription of pro-inflammatory cytokines, however they have also been implicated in aberrant chemotaxis. A CLP model of murine sepsis with mice deficient in TLR-4 and -2 demonstrated improved chemotaxis and survival when TLR function was inhibited (2, 154, 156, 165, 166).

A phenomenon observed in neutrophil studies in sepsis is a state of neutrophil paralysis or desensitisation leading to impairment of chemotaxis. It has been suggested that during sepsis neutrophils are persistently stimulated by high doses of pro-inflammatory cytokines leading to the activation of G-protein receptor kinase

(GRK). GRK-2 and GRK5 phosphorylate GPCRs, such as CXCR2 (CXCL-8 receptor), resulting in their desensitisation and internalisation. TLR-4 activation has also been implicated in neutrophil chemokine internalisation with blockade of TLR-4 using IL-33 preserving CXCR2 receptor expression and neutrophil chemotaxis in murine models of sepsis (148, 154, 155, 166-168).

1.4.2. Impact of sepsis on neutrophil phagocytosis

The effects of sepsis on neutrophil phagocytosis remain controversial, with evidence from the literature of reduced and increased phagocytosis. The ability of neutrophils to eliminate pathogens is crucial with several studies suggesting that neutrophils up-regulate phagocytic receptors (CD64/ FcγRI) enabling greater bacterial ingestion (3, 169-173). Studies have also suggested that the increased release of neutrophils in response to sepsis leads to an immature pool of neutrophils that are less capable than their mature counterparts. Selective isolation of these immature neutrophils (bands) has demonstrated reduced CD64/FcγRI expression and reduced phagocytosis (174-178). Efficient neutrophils phagocytosis relies on the opsonisation of bacteria by IgG, and the failure to do so has also been implicated in the reduced phagocytosis observed in patients with sepsis (176, 179).

1.4.3. Impact of sepsis on neutrophil degranulation and reactive oxygen species (ROS) production

In infection and sepsis ROS and RNS is up regulated to allow effective pathogen elimination. However, the products of ROS (H_2O_2, HOCL), RNS (peroxynitrite) and degranulation (MPO and NE) are highly toxic to DNA, lipid membranes and the endothelium. In homeostatic responses to infection naturally occurring anti-oxidants,

such as glutathione, vitamin E and another micronutrients, neutralise these toxic substances (45, 53, 180, 181).

In sepsis the generation of ROS is massively increased via stimulation of NADPH by pro-inflammatory cytokines and bacterial products. This combined with a state of tissue hypo-perfusion (hypoxia) and a consumption of anti-oxidants results in excessive ROS and RNS species, exacerbates cell-death and endothelial damage in sepsis. This has been termed oxidative stress and is implicated in the MOF observed in sepsis and other inflammatory conditions (46, 47, 133, 169, 182-184).

Interventions aimed at reducing oxidative stress by the supplementation of anti-oxidants directly or indirectly using N-Acetylcysteine (NAC) showed improved outcomes in murine models of sepsis and in phase II human trials. These have not translated into clinical practice as two large clinical trials with antioxidant therapy and a Cochrane Review on NAC suggest increased harm with their use (183, 185-191).

1.4.4. Impact of sepsis on neutrophil apoptosis

The apoptosis of neutrophils is a complex process that is regulated by pro and anti-apoptotic signals. The immune response of sepsis promotes both pro-apoptotic signalling via the ROS and phagocytosis and anti-apoptotic signalling via G-CSF, GM-CSF and LPS. Reduced neutrophil survival is associated with poorer outcome from sepsis as neutrophils are unable to adequately eliminate pathogens, whilst prolonged survival aggravates host tissue damage (8, 10, 192, 193).

Studies on neutrophils in patients from sepsis suggest that it is the anti-apoptotic signals that pre-dominate to prolong neutrophil survival, with high levels of G-CSF, GM-CSF, TNF-α and LPS inhibiting the process (192, 194-199).

Neutrophil apoptosis promotes the resolution of inflammation causing macrophages to change to an anti-inflammatory phenotype and promoting the release of IL-10. In a variety of animal models of sepsis inducing apoptosis results in enhanced resolution of inflammation, leading many to suggest that targeting apoptosis is a viable therapeutic intervention (192, 195, 197, 200-204).

1.4.5. Impact of sepsis on neutrophil extracellular trap (NET) formation

The role of NETs in health and disease remains unclear. There is increasing evidence that in chronic inflammatory conditions, such as vasculitis and rheumatoid arthritis, increased NET release is correlated with increased disease severity and may be involved directly in disease pathogenesis. Kessenbrook et al proposed that the release of double stranded DNA, MPO and NE by neutrophils triggers an autoimmune response and damages the endothelial that is characteristic of these diseases (205-207).

Current evidence suggests that NETs are a vital function of neutrophils, being found in a variety of infected sites, such as the lung, peritoneum and skin where they ensnare bacteria pathogens and deploy high concentrations of antimicrobial toxins. In murine models of sepsis mice unable to generate NETs demonstrated increased susceptibility to infection, reduced bacterial clearance, exacerbation of the original

pathology and decreased survival suggesting that NETs are an essential part of the innate immune response (16, 64, 208-210).

Human studies regarding NET production in sepsis are lacking. Neutrophil studies in infants show preserved ROS generation and phagocytosis, but impaired NET production suggesting this may be implicated in their poorer outcomes. Meng et al found that NETs were raised in patients admitted to hospital following major trauma at admission and decreased over time. However in patients who subsequently developed sepsis, NET values rose again (211-213).

NET formation is a process that requires tight homeostatic control, as the antimicrobial properties may be offset by unregulated NET production leading to collateral tissue damage from the proteases and histones that they contain. These molecules cause endothelial damage that is the hallmark of severe sepsis and subsequent MOF with evidence that NET production is involved in the pathogenesis of acute respiratory distress syndrome (ARDS), malaria falciparium and septic hepatic dysfunction (214-218).

1.4.6. Neutrophils, tissue damage and multi-organ failure.

Sepsis is characterised by immune dysfunction and tissue hypoperfusion that progresses to MOF. Although this process involves many facets of host innate and adaptive immunity, the role of the neutrophil is becoming increasingly evident (3, 91, 108).

Sepsis results in altered chemotaxis preventing neutrophils reaching sites of infection. However neutrophils in the circulation become primed due to high concentrations of circulating pro-inflammatory cytokines. The activated but "paralysed" circulating neutrophils, with their reduced deformability get trapped within the vasodilated vascular beds distant to the site of infection. This exacerbates tissue hypo-perfusion by reducing blood flow and causing microvascular occlusion through interactions with fibrin. Activated neutrophils release ROS, MPO and NE within these vascular beds causing endothelial cell damage and death leading to capillary leak and further tissue/organ hypo-perfusion. The interaction between activated platelets and NETs further damages tissues and increases thrombus formation (3, 219-222).

The net result is cells/organs downstream from this neutrophil-induced endothelial injury become increasingly hypoxic, aggravating oxidative stress, collateral tissue damage and eventually resulting in cell death and organ dysfunction (133, 134, 149, 220, 222, 223).

Within the pulmonary tissues the pattern of injury is different with a large numbers of neutrophils being extravasated into the lungs during systemic sepsis, directly injuring the lung tissue through degranulation, ROS generation, NET production and reduced apoptosis. These primed neutrophils have recently been shown to re-circulate within the systemic circulation and may be implicated in the distal organ damage seen in patients with severe pneumonia and ARDS (3, 198, 224-227).

This makes therapeutic intervention aimed at modifying neutrophil challenging, as benefits from enhanced neutrophil migration and microbicidal actions at sites of infection may be offset by systemic neutrophil activation within distal organs. In contrast therapies that reduce systemic neutrophil activation may reduce distal organ damage but at the expense of impairing the innate immune responses to infection (3). An ideal therapeutic agent would enhance migratory dynamics and neutrophil functions in the initial phases of infection to assist with pathogen elimination, but reduce neutrophil activation as the infection progresses to severe sepsis to avoid deleterious consequences.

Table 1.7. A summary of the changes in neutrophil function with sepsis

	Observed Changes	Mechanisms
Chemotaxis	Reduced/Inhibited	Internalisation and desensitisation of chemokine receptors. Increased cell membrane rigidity mediated by PPARγ Reduced adhesion receptor expression on neutrophils and on the endothelium. Overexpression of TLR-4 and -2.
Phagocytosis	Increased Decreased	Up regulation of phagocytic receptors e.g. CD64 Down regulation of CD64 receptor Overexpression of C5a with down regulation of CD88
Reactive Oxygen Species	Increased	Stimulation of NADPH by pro-inflammatory cytokines
Apoptosis	Delayed	Due to high levels of circulating G-CSF, GM-CSF, TNF- α and LPS
NETosis	Increased	Mechanisms unclear at present.

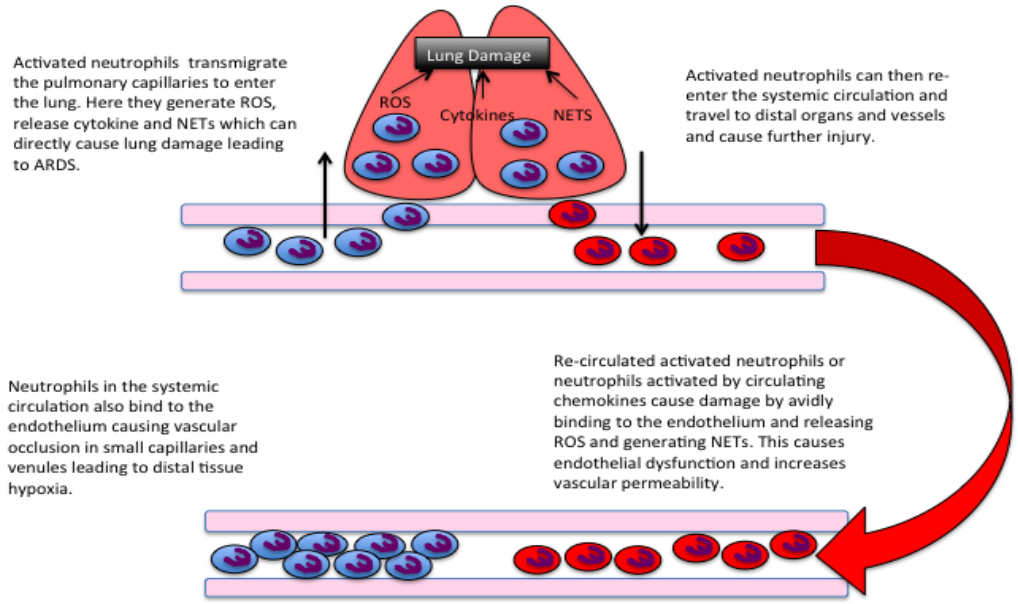


Figure 1.13: Proposed mechanisms of neutrophil-mediated organ damage in sepsis

The figure details potential mechanisms involved in the direct tissue damage caused by neutrophils within the lungs and the mechanisms thought to occur in the microvasculartur e and at distal organs. Both perhaps occur simultaneously.

1.5. Statins in Sepsis

1.5.1. The Pharmacology of Statins

HMG-CoA reductase inhibitors (commonly referred to as statins) are drugs primarily used in the lowering of blood cholesterol by inhibiting the enzyme 3-hydroxy-3-methylglutaryl-coenzyme (HMG-CoA) reductase. This enzyme converts HMG-CoA to mevanolate and is an early rate–limiting step in the synthesis of cholesterol in the liver. Statins are effective in the primary and secondary prevention of cardiovascular disease and are widely prescribed to about 7 million people per year in the UK (228-232).

At present six statins are used in the UK, lovastatin, pravastatin, and simvastatin are fungal derived, whilst atorvastatin, rosuvastatin and fluvastatin are synthetically derived. Simvastatin and atorvastatin account for 85% of statin usage in the UK. Statins are safe and well-tolerated drugs with relatively low levels of drug discontinuation (3.9%); however hepatic dysfunction (incidence 1.7%) and muscle toxicity are the most serious side effects. Muscle toxicity is often asymptomatic with reversible rises in Creatinine kinase (CK). The incidence of rhabdomylosis is hard to estimate, but a recent study quantified the incidence as 0.3-8 cases per 10000 person-years of statin exposure (230, 233-235).

1.5.2. Pleiotrophic effects of statins

Over the past decade several retrospective analysis of large cohorts of patients' admitted to hospital demonstrated that statins were associated with a lower mortality and morbidity from infection and sepsis. This was followed by prospective

observational studies, most of which suggested that treatment with statins therapy prior to infectious insults improved mortality. However, some of the studies were equivocal with only one suggesting that statin therapy was harmful (236-245).

These observational studies have led to further investigations into the mechanisms of immune modulation by statins and the development of statins as potential adjuvant therapy in sepsis.

The inhibition of HMG-CoA reductase also prevents the synthesis of the important isoprenoid intermediaries, isopentenyl pyrophospate (IPP), farnesylpyrophosphate (FPP) and geranylgeranylpyrophosphate (GPP). These molecules play important roles in downstream protein prenylation via the small family of GTPases, Rho and Rac, and it has been postulated that the inhibition of these molecules are responsible for the pleiotrophic effects of statins (228, 229, 246-249).

The inhibition of Rho and Rac GTPases results in significant downstream effects that may modulate multiple pathways in sepsis pathophysiology. It has been proposed that statins inhibit neutrophil migration into the lungs and endothelial adhesion via reductions in Selectins, LFA-1 and Mac-1 expression, whilst TLR-4 and 2 expression is down regulated through effects on monocytes. This may reduces inappropriate neutrophil migration and adhesion, whilst enhancing targeted migration into infected tissues (150, 248, 250-254).

Statins have been shown to reduce the release of pro-inflammatory mediators, such as CRP (released from hepatocytes in response to IL-6), TNF-α, IL-6 and CXCL-8. The reduction in CRP by statins has received great attention, as it widely measured and is a generic marker of inflammation. Evidence from randomised controlled trials show that lowering CRP is associated with improved outcome in cardiovascular disease and in bacterial pneumonia (249, 255-263).

The coagulation cascade plays a crucial role in the pathogenesis of sepsis. Statins may prevent the pro-coagulant effects of sepsis by improving platelet function, reducing thrombin and fibrinogen formation. They also promote fibrinolysis via tissue plasminogen activator (tPA), platelet-activation inhibitor-1 (PAI-1) and by increasing endogenous protein C levels (264-270).

Finally, statins may stabilise endothelial cell function by attenuating the response to inflammatory cytokines, which promotes leucocyte adhesion and iNOS activity. Reduced vasomotor tone is caused by reduced eNOS activity and an increase in iNOS resulting in hypotension. Statins reversed this by increasing endothelial derived NO by increasing eNOS activity (238, 246, 247, 271-273).

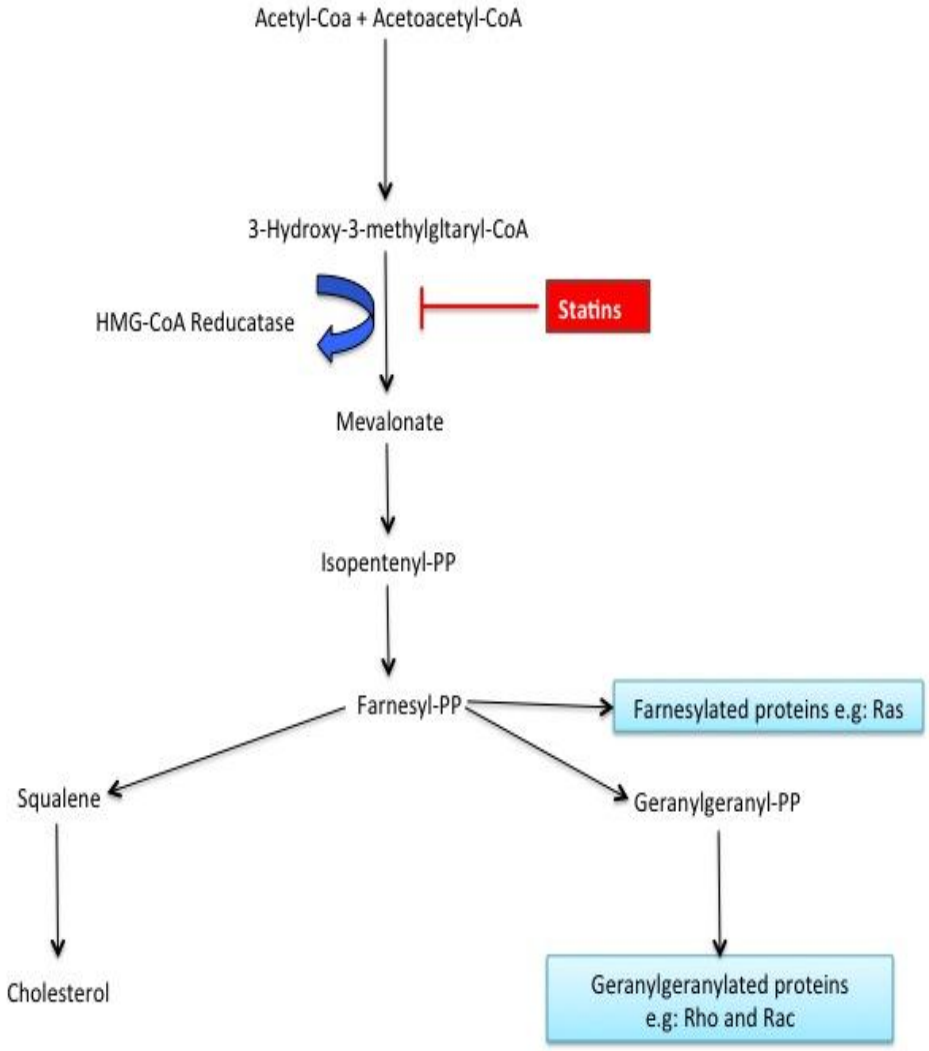


Figure 1.14: The pharmacology of statins/HMG-CoA reductase inhibitors.

An illustration of the stages in cholesterol synthesis from Acetyl-CoA, the site of action of statins and the isoprenoid intermediaries that are responsible for the pleiotrophic effects of statins. PP= pyrophostate..

1.5.3. Evidence for statin therapy in sepsis

The first published evidence of improved outcomes from sepsis in patients receiving statins was in 2001, when Liappis et al demonstrated a 22% reduction in mortality from bacteraemia in patients receiving a statin. Almog et al followed this in 2004 with a prospective study showing that prior statin use significantly reduced the progression of bacterial infections to severe sepsis and reduced ICU admissions. Since, many retrospective and prospective studies have confirmed these findings and are supported by subsequent meta-analysis (236, 237, 239-242, 245, 274-279).

1.5.3.1. Statin use in animal models of sepsis

Ando et al in 2000 used an LPS model of sepsis to demonstrate that pre-treatment with cervistatin (20mg/kg) resulted in an attenuation of the proinflammatory response with a subsequent increase in 7-day survival. Merx et al followed this showing that mice pre-treated with 10μg/ml simvastatin had improved survival, preserved haemodynamics and reduced leucocyte adhesion compared to untreated mice in a CLP model of sepsis. A subsequent study demonstrated that simvastatin (40mg/kg) reduced mortality and attenuated sepsis induced acute kidney injury by preserving renal tubular function, reversing the renal microvascular hypo-perfusion and hypoxia associated with CLP mediated sepsis (280-282).

Although the above models demonstrate significant benefits of statin therapy in murine models of sepsis, the doses used exceeded physiological doses (20-80mg per day in an adult = 0.25-1mg/kg for an 80kg human) making applications to human sepsis difficult.

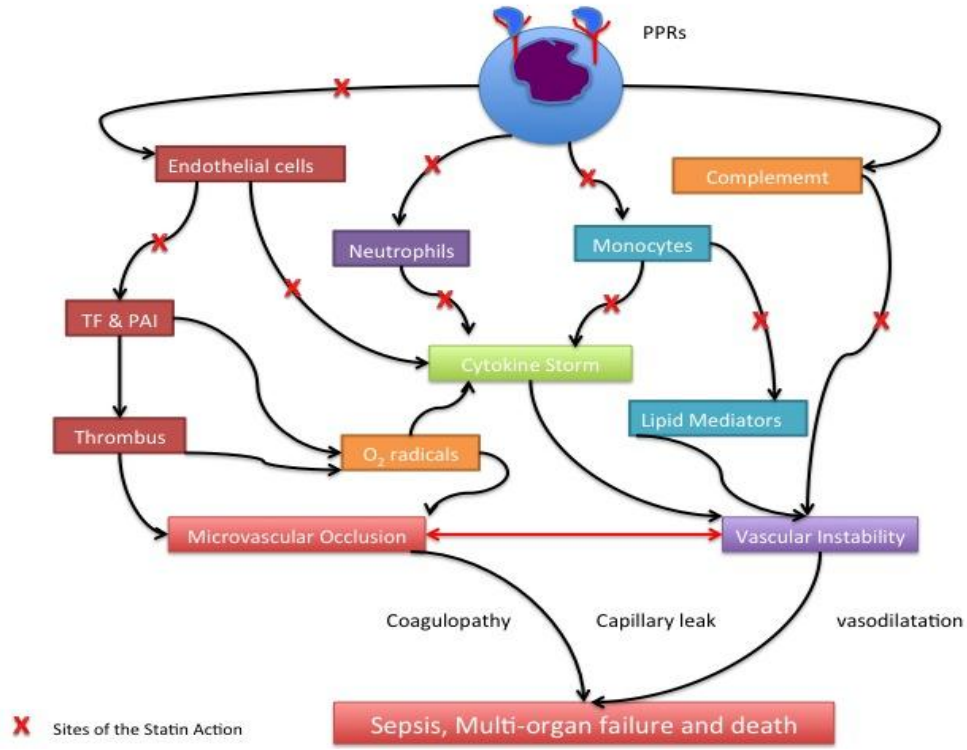


Figure 1.15. The proposed mechanism of statins in sepsis.

An illustration of the pathogenesis of sepsis leading to multi-organ failure with the pathways inhibited by statins shown by the red crosses. Statins are thought to exert effects beyond their lipid-lowering effects. These have been termed “pleiotrophic” effects and potentially modulate the inflammatory response of sepsis at multi-levels. Statins have effects upon the coagulation system inhibiting tissue factor (TF) and platelet activator inhibitor -1 (PAI-1), thereby preventing microvascular occlusion. Additionally via actions on innate immune cells and the complement system, the release of cytokines and chemokine expression is down regulated resulting in preservation of endothelial function. By suppressing these vital pathways in the development of multi-organ failure in sepsis, statins have been postulated as a potential immunomodulatory therapy in sepsis. Adapted form Cohen J et al, 2002 (126).

1.5.3.2. Statin use in human sepsis

Studies investigating the role of acute statin administration in human sepsis are inconclusive. Phase II studies suggest that the acute use of statins in sepsis attenuates the inflammatory response, with lower levels of IL-6, TNF-α and CRP observed and a reduction in pulmonary neutrophil infiltration (150, 152, 260, 283).

Craig et al demonstrated that the use of simvastatin 80mg improved respiratory mechanics, oxygen index and Sequential Organ Failure Scores (SOFA) as well as pulmonary CXCL-8 levels and systemic CRP levels. This proof of concept trial demonstrated that statins were well tolerated in critical illness and had potential in showing real benefit to patients (284, 285)

Kruger et al demonstrated in that low dose atorvastatin administered to patients with severe sepsis (randomised placebo-controlled trial) in the ICU did not improve mortality or length of stay, but subgroup analysis demonstrated that continuation of pre-admission statin therapy reduced mortality (286). Whilst another trial on statin use in the ICU was suspended early due to futility as no improvement in mortality was observed in patients acutely treated with statins for ventilator-associated pneumonia (VAP). In fact mortality in the statin group was higher but did not reach significance (p=0.054) (287).

Two further trials have questioned the rationale of acute statin administration in the context of acute illness. The first randomly assigned patients with ARDS to receive either rosuvastatin 20mg or placebo and was also terminated early due to futility with

no significant difference in 60-day mortality (p=0.21). However it suggested that rosuvastatin therapy was associated with a higher probability of developing hepatic and renal dysfunction and consequently statin use in ARDS could not be advocated (288).

The second assessed the use of simvastatin 40mg on the exacerbation rate of patients with a diagnosis of Chronic Obstructive Pulmonary Disease (COPD). This trial also demonstrated no benefit of simvastatin therapy on the rate of COPD exacerbations with no deleterious effects of statins observed (289).

1.5.4. Summary

Statins clearly have anti-inflammatory properties that are capable of modulating the inflammatory response at multi-levels, with evidence from retrospective and prospective cohort studies suggesting that statin use is associated with improved outcomes from sepsis. This is supported by human models of sepsis demonstrating an attenuation of the inflammatory release with statins (152, 230, 245, 289-291).

Murine models have shown that pre-treatment improves mortality by modulating several pathways in the pathogenesis of sepsis. However, this so far has not been translated into successful human randomised controlled trials, with some of the data suggesting that statins may actually be harmful if administered in severe sepsis (280, 281, 284, 286-289, 292).

1.6. Hypothesis

It was hypothesised that sepsis would exaggerate the decline in neutrophil function observed with age.

1.7. Aims of the thesis

This thesis is a report of investigations studying the potential impact of statin therapy in the treatment of infections and sepsis. The specific aims of these investigations are:

1. To investigate whether in-vivo treatment with statins corrects alterations in neutrophil function observed in healthy elderly participants.
2. Characterise neutrophil function in terms of migration, ROS production, phagocytosis, apoptosis and NETs in patients with sepsis.
3. Determine whether in-vitro treatment of neutrophils from sepsis patients with simvastatin improves any alterations observed in neutrophil functions.

Chapter 2: Methods

2.1: Ethical Approvals.

All patients and healthy volunteers in the studies reported provided informed written consent. In circumstances where patients were unable to provide consent a legal representative (personal or designated consultee) provided assent. Retrospective consent was sought in cases where possible, when patients regained the ability to consent. All studies received the appropriate ethical and local approvals (Regional Ethics Committee references: 11/SC/0356 & 11/YH/0270)

2.2: Recruitment of Healthy Controls

Healthy volunteers were required to act as controls for patients. Young controls (less than 45 years of age) were recruited from staff and students at the University of Birmingham via a poster advertisement. Elderly controls were recruited from the Birmingham 1000 Elders Cohort, a research cohort of healthy volunteers above the age of 60 willing to take part in medical research. To be considered healthy, participants were not suffering from serious debilitating acute or chronic illness. Well-controlled health problems, such as hypertension and asthma did not preclude enrolment.

2.3: Patient recruitment

Patients enrolled for the sepsis study were recruited from the Heart of England NHS Foundation Trust and the University Hospital Birmingham NHS Foundation Trust between September 2011 and October 2013. Patients with sepsis were recruited from the acute medical admissions units (AMU) and the ICUs at both sites.

The Heart of England NHS Foundation trust is a university affiliated teaching trust spread over three sites that geographically covers the north and east of Birmingham with a total of 1449 in-patient beds. Patients were recruited from the largest site, Birmingham Heartlands Hospital in the east of Birmingham. This site has a 44 bed AMU and a 19 bed general ICU. The new University Hospital Birmingham NHS Foundation Trust is the largest single site hospital in the UK with 1213 in-patient beds, an 84 bed AMU and a 100 bed ICU. It is the major tertiary referral centre for the region but also serves the population of central and southwest Birmingham.

2.3.1. Diagnosis of Sepsis

Sepsis, severe sepsis and septic shock were diagnosed according to the SSCG definitions (see table 1.4 and 1.5). Screening was performed daily on the AMUs and ICUs and patients who developed sepsis within 48 hours of admission were considered for enrolment. Exclusion criteria were pregnancy, age below 18 years of age, imminent withdrawal of treatment or refusal to gain consent.

2.3.2. Data Collected

Data collected from enrolled patients included demographic details, pre-existing co-morbidities, drug history, their observations on admission, baseline haematology and biochemistry results. These are shown in table 2.1. Data was also collected for the first seven days of their hospital stay with physiological, haematological and biochemical data recorded. Antibiotic therapy, positive microbiology and organ support requirements were also collected. In addition outcome data, such as admissions to ICU, length of hospital stay and mortality were collected.

2.3.3. Severity Scores

Data collected was used to calculate diseases severity using a variety of different schemes outlined below. They incorporate a variety of physiology and biochemical variables that impact on survival to enable risk stratification, prognostication and treatment effects.

2.3.3.1. APACHE II Score

The acute physiology and chronic health evaluation (APACHE) score is a severity scoring system applied to patients within 24hours of admission to ICU. The original score was developed in 1981 and since then has been modified several times. The APACHE-II score was first described in 1985 and is still the most widely used. It incorporates 12 routinely measured parameters allocating each one a score between 0 and 4 (see table 2.2). A total score between 0-71 is generated after accounting for age, past medical history and surgical conditions. A predicted mortality is also calculated. Higher scores correspond to increased severity and a higher probability of death. Although not all patients in the study were admitted to ICU, this scoring system was used to calculate disease severity (293, 294).

2.3.3.2. SAPS-II Score

The simplified acute physiology score (SAPS) II is a severity of diseases classification system and is also applied to patients admitted to the ICU within 24 hours. It uses 12 physiological parameters, age, type of admissions and previous co-morbidities to generate a score between 0-163 and also a predicted mortality score (see table 2.2). It is superior to the APACHE-II at comparing the disease severity in patients with different pathologies (295).

Table 2.1. Data collected on patients entered into the study

Data Collected on Entry into study	
Demographics	Age
	Sex
	Date of admission
	Ethnicity
	Pre-existing co-morbidities
	Drug History
	Source of sepsis
Baseline Physiology	Temperature
	Pulse (bpm)
	Blood pressure and MAP* (mmHg)
	Respiratory rate (breaths/minute)
	Saturations (%)
	Inspired Oxygen Fraction (%)
	Glasgow Coma Scale
Baseline Investigations	White Cell Count ($x10^9$/L)
	Platelets ($x10^9$/L)
	INR**
	Urea and Creatinine (µmol/L)
	Sodium (mmol/L)
	Potassium (mmol/L)
	Lactate (mmol/L)
	pH
	Standardised Base Excess
Classification of sepsis	Sepsis or Severe Sepsis

*

MAP = mean arterial pressure **INR = International normalised ratio. Sepsis and severe sepsis classified according to international guidelines from the parameters recorded

Table 2.2. Components of the Acute Physiology and Chronic Health Evaluation Score (APACHE II) and the Simplified Acute Physiology Score (SAPS II).

APACHE II	SAPS II
Temperature	Temperature
Heart rate	Heart rate
Mean arterial pressure	Systolic Blood Pressure
Respiratory rate **PaO_2/FiO_2 ratio**	PaO_2/FiO_2 ratio
Serum Creatinine	Serum Urea Urine Output
Serum sodium	Serum sodium
Serum potassium	Serum potassium
Arterial pH or serum bicarbonate	Serum bicarbonate
White Cell Count	White Cell Count
GCS	GCS
Haematocrit	Age
Age	Admission type
Chronic Health	Chronic diseases

PaO_2/FiO_2 ratio: partial pressure of oxygen/fractional inspired oxygen concentration. GCS= Glasgow Coma Scale.

2.3.3.3. SOFA Score

The sequential organ failure assessment (SOFA) score classifies disease severity based upon six individual organ dysfunctions, with each organ receiving a score between 0-4, with a maximum score of 24. Higher scores are predictive of poor outcomes from disease. The organs assessed are the respiratory, cardiovascular, renal, coagulation, hepatic and neurological systems (see table 2.3) (296).

The SOFA score is a dynamic score that be calculated serially, on admission to hospital or ICU and on subsequent days. A SOFA score of greater than 11 at any time correlates with poor outcomes, whilst change in SOFA score (delta SOFA score) have been shown to be a valuable predictor of mortality, with increases in the SOFA score by 4 or more points within 48hours of admission correlating with mortality. The SOFA score has been suggested as triage tool for assessment of survival and for rationing ICU resources in a pandemic situation. However, the H1N1 pandemic of 2009 showed that using the SOFA score triage tool to ration ICU services was a poor predictor of outcome, with many patients surviving despite initial worsening of organ failure (296-299).

The SOFA score is best used as a measure of treatment effect and to assess the resolution of disease.

Table 2.3: Components of the sequential organ failure assessment (SOFA) score.

Organ	Indicator	0	1	2	3	4
Respiratory	PaO_2: FiO_2 (mmHg)	>400	<400	<300	<200	<100
Cardiovascular	Hypotension (µg/kg/min)	None	MAP<70mmHg	Dob any dose	Nor<=0.1	Nor>0.1
Renal	Creatinine (µmol/L) (Urine output)	<110	110-170	171-299	300-440 (<500ml/day)	>440 (<200ml/day)
Liver	Bilirubin (µmol/L)	<20	20-32	33-101	102-204	>204
Coagulation	Platelets (10^9/L)	>150	<150	<100	<50	<20
Central Nervous System	GCS	15	13-14	10-12	6-9	<6

MAP= mean arterial pressure, Dob= dobutamine, Nor= Noradrenaline, GCS= Glasgow Coma Scale, PaO_2: FiO2= partial pressure of oxygen/fractional inspired oxygen concentration.

2.4. Sample Collection

Blood samples were collected at the time of recruitment (day 1), day 4 and day 7. Experienced medical practitioners took bloods peripherally or from in-situ arterial and central venous cannulae. A total of 36mls of blood was taken into 2 EDTA (4mls each) and 4 lithium heparin (7mls each) vacutainers (Becton-Dickinson, Oxford, UK). Samples were then placed on ice and transported to the laboratory for processing and analysis.

2.5. Sample Processing

2.5.1. Peripheral blood processing

Blood samples were processed within 45 minutes of collection.

2.5.2. Plasma sample

A plasma sample was obtained by centrifuging blood from Lithium Heparin vacutainers at 800G for 10minutes at 4ºC. Plasma was stored in 500µl aliquots at -80ºC until analysed.

2.5.3. Isolation of Neutrophils

Neutrophils were isolated on a Percoll (pH 8.5-9.5; Sigma-Aldrich, Dorset, UK) density gradient as previously described (87). Blood collected in Lithium Heparin and EDTA vacutainers (Becton Dickinson) were transferred into separate 50ml sterile Falcon^{TM} tubes (Becton Dickinson). 2% dextran (Sigma-Aldrich) was added (1ml for every 6mls of blood) and gently mixed prior to incubation for 30minutes at room temperature to sediment the erythrocytes. The leucocyte-rich plasma was carefully

layered on a Percoll (Sigma-Aldrich) density gradient consisting of 2.5mls of 80% Percoll (Sigma-Aldrich) and 5 mls of 56% Percoll (Sigma-Aldrich) (see figure 2.1).

To prepare the gradients, a working stock of Percoll was made by mixing 45 ml of Percoll (Sigma-Aldrich) with 5 ml of 9% (v/v) sodium chloride (Sigma-Aldrich). The 80% Percoll was prepared by diluting 40 ml of the Percoll stock with 10 ml 0.9% (v/v) sodium chloride (Baxter) whilst 56% Percoll comprised of 28 ml of the working Percoll stock and 22 ml of 0.9% (v/v) sodium chloride (Baxter). Gradients were then prepared by carefully layering 5 ml of 56% Percoll on top of 2.5 ml 80% Percoll in a 15ml sterile Falcon^{TM} tube (Becton Dickinson).

The gradients and leucocyte-rich plasma were centrifuged at 220G for 20 minutes with no acceleration and no brake at room temperature. Neutrophils were removed from the 80% and 56% gradient interface and re-suspended then washed in phosphate buffered saline (PBS; Gibco Invitrogen, Paisley, UK) at 440G for 10minutes at room temperature. Post-centrifugation the supernatant was discarded and the cells re-suspended in either RPMI 1640 (Sigma-Aldrich) or in Hanks Balanced Salt Solution supplemented with calcium and magnesium (HBSS: Gibco Invitrogen; 12.61mM $CaCl_2$, 4.93mM $MgCl_2$, 4.07mM $MgSO_4$) at the appropriate concentrations for the functional neutrophil assays. Purity was assessed by cytospin and staining with Giemsa stain (Diff-Qik; Gentaur Europe, Brussels, Belgium) routinely yielding neutrophil purity of greater than 95% and a viability of >98% as evaluated by tryptan blue exclusion

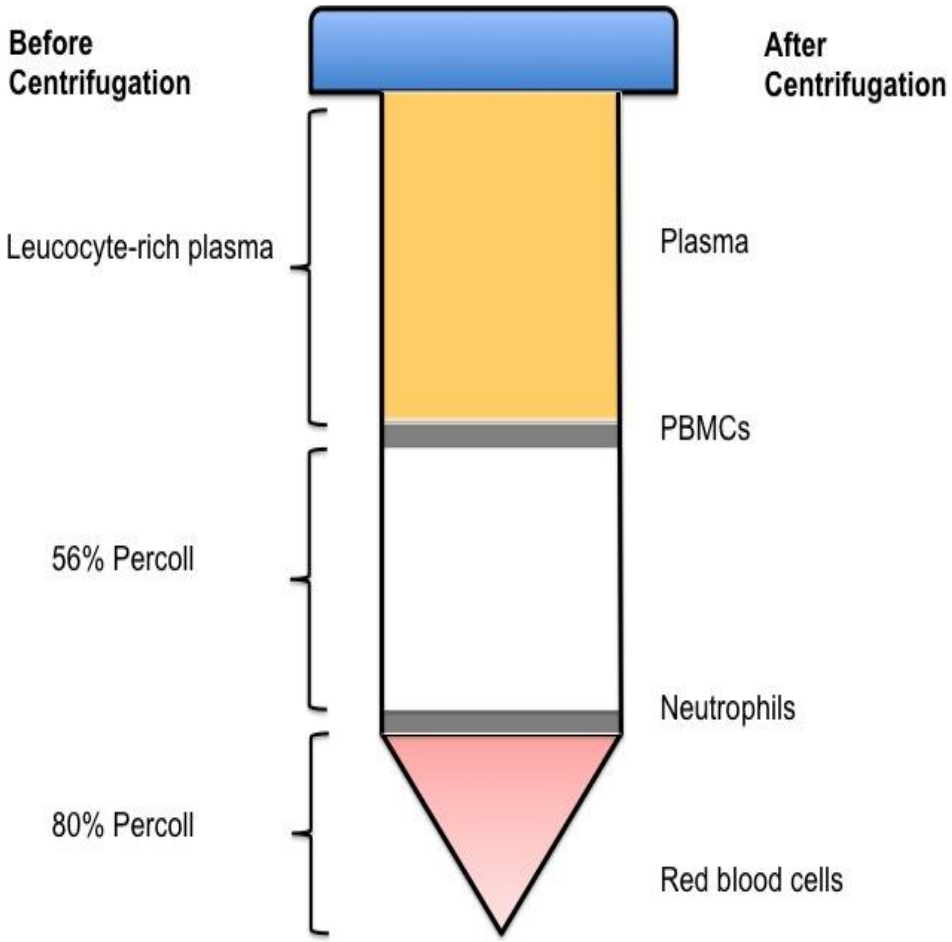


Figure 2.1. Isolation of neutrophils using a Percoll density gradient.

Illustration showing the differential Percoll gradients on the left and the subsequent layers following centrifugation at 220G for 20 minutes on the right. The neutrophils were extracted from the 80% and 56% Percoll (Sigma-Aldrich) interface.

2.6. Functional Neutrophil Assays

2.6.1. Neutrophil Migration

Neutrophil migration was assessed using an Insall Chamber (Weber Scientific International Ltd, Teddington). This improved chemotaxis chamber (see figure 2.2) allows the migratory dynamics of individual cells to be assessed and provides greater information than traditional methods that provide only quantitative measures of cell migration (300-302).

Isolated neutrophils from Lithium-Heparin vacutainers were re-suspended in RPMI 1640 (Sigma-Aldrich) at a concentration of 5 $x10^6$/ml. Bovine Albumin Fraction V (Sigma-Aldrich) was added to the neutrophils at a final concentration of 1.125% v/v. Neutrophils were then placed on a cleaned (with 0.4M H_2SO_4) albumin coated coverslip (22x22mm, Surgipath Medical Industries Inc. Europe) and allowed to adhere for 20 minutes at room temperature. Once adhered the coverslip was inverted and placed on a clean Insall Chamber pre-filled with sterile RPMI 1640 (Sigma-Aldrich). The RPMI 1640 (Sigma-Aldrich) was replaced with 100nM CXCL-8 (R&D systems, Abingdon, UK) or 10nM fMLP (Sigma-Aldrich) as chemoattractants. Gradients were allowed to develop for 5 minutes before assessment of migration. These doses were selected based on validation data performed by Hannah Greenwood (personal communication).

Real-time video microscopy using a Leica DMI6000B with DFC360FX camera was used to capture neutrophil migration. Images were captured every 20 seconds for 12minutes producing 36 frames. This time frame was considered optimal for

migration studies (personal communication with Dr. Elizabeth Sapey). Images were analysed using Image J software (Wayne Rasband, NIH, Bethesda) using vector analysis. Images were divided into ten individual segments and one cell randomly selected within each segment for analysis. Therefore ten randomly selected cells were analysed.

2.6.1.1. Parameters used to assess neutrophil migration

Three measures of migration were calculated based on the Image J software (Wayne Rasband). Firstly chemokinesis defined as the speed the neutrophil travels in any direction over time was calculated in μm/minute. Secondly directional migration or chemotaxis was measured in μm/minute. This was measured as the speed the neutrophil travelled in the direction of the chemotactic gradient. The measurements of distances was recorded in pixels and subsequently converted to μm following reference to the digital image and the magnification used. Thirdly the chemotactic index is calculated. This was an overall measure of the directional migration. This was calculated using the cosine of the angle between the cells direction and the orientation of the chemotactic gradient at each time frame forming a vector analysis of movement. A score of -1 to 1 was given to each cell, with a score of 1 representing perfect directional migration whilst -1 represents migration directly away from the chemo-attractant.

In addition to the three neutrophil migration indices calculated, the adherence of neutrophils to the coverslip and the number of adherent cells migrating was calculated. Adherence was calculated by counting the number of neutrophils in the viewing plane that were adherent to the coverslip. This is expressed as a percentage

of the total number of neutrophils that were visualised in the viewing plane. The number of migrating neutrophils was calculated by counting the number of neutrophils in the viewing plane that initiated and subsequently moved in any direction. This is also expressed as a percentage of the total number of neutrophils visualised.

2.6.2. Reactive Oxygen Species (ROS) measurement

A luminol-based chemiluminescence assay was used to measure ROS as it detected both intracellular and extracellular free radicals (303). Freshly isolated neutrophils from Lithium-Heparin vacutainers (Becton Dickinson) were reconstituted to $1x10^6$/ml in HBSS supplemented with calcium and magnesium (Gibco, Invitrogen). $1x10^5$ neutrophils were dispensed into wells of a 96-well white-bottomed flat plate (CoStar; Sigma-Aldrich) containing 25 µL of luminol (working concentration 100 µM; pH 7.3; Sigma-Aldrich) and 50 µL HBSS supplemented with calcium and magnesium (Gibco, Invitrogen). Cells were then treated with either 25 nM PMA (Sigma-Aldrich), 1.25µM CXCL-8 (R&D systems), 2.5µM fMLP (Sigma-Aldrich) or vehicle control and ROS generation assessed at 1 minute intervals for 60 minutes using a Berthold Centro LB 960 luminometer (Berthold Technologies, Hertfordshire, UK). Experiments were performed in quadruplicate, with ROS production calculated as area under the curve (AUC) (see figure 2.3). The increase in ROS to stimuli was represented as the percentage change from baseline.

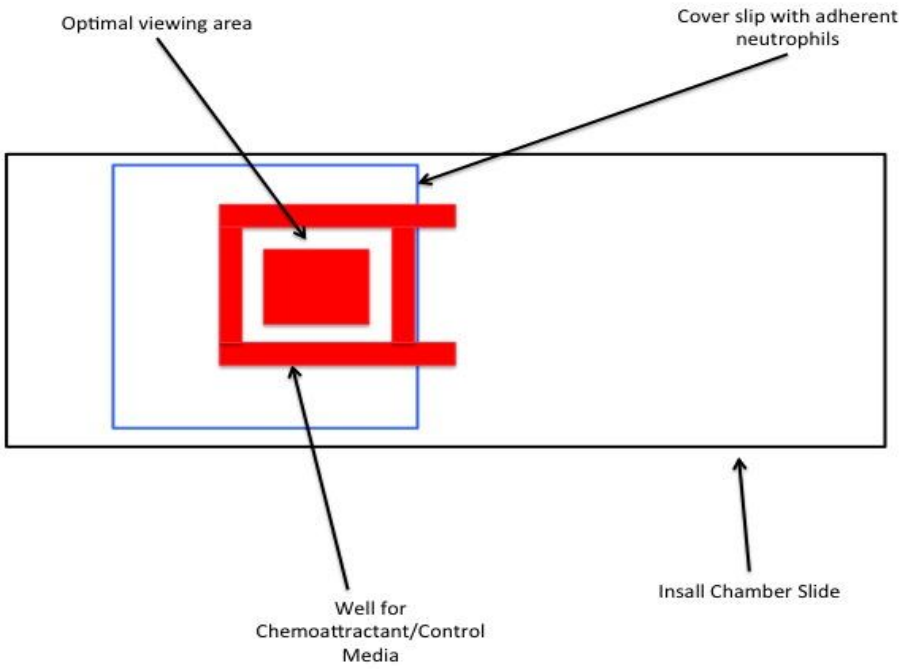


Figure 2.2. Diagram of the Insall Chemotaxis Chamber

Migration experiments were performed by adhering extracted neutrophils on to an albumin-coated coverslip. This was then inverted and carefully placed over the Insall Chamber of the chemotaxis slide. The chemoattractant/control media was placed in a well. Time-lapse video microscopy was and the optimal viewing area is shown.

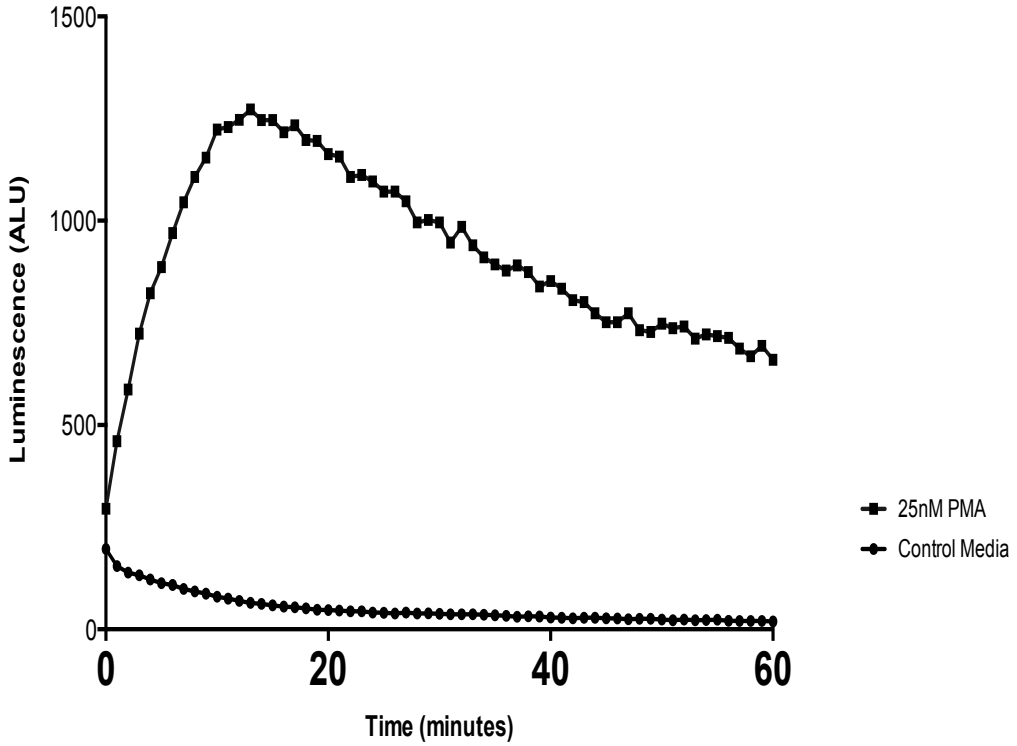


Figure 2.3. Reactive oxygen species production from neutrophils to the control media and 25nM PMA.

The graph shows an example of the curves produced when reactive oxygen species (ROS) production was measured in neutrophils. ROS was measured in control media (HBSS) and to 25nM phorbol-12-myristate-13-acetate (PMA) every 1minutes for 60minutes using a chemiluminescence technique. The area under the curve represented overall ROS production.

ALU = Arbitrary Light Units

2.6.3. Phagocytosis

Phagocytic function of neutrophils was assessed using a commercially available assay and was carried out as per the manufacturer's instructions. The assay used *E. coli* and *S. aureus* bioparticles conjugated to a fluorescent dye pHrodo (Invitrogen) that only fluoresced once in an acidic environment, such as that of the phagolysosome.

Freshly isolated neutrophils from Lithium-Heparin vacutainers (Becton Dickinson) were re-suspended at 1 $x10^6$/ml in RPMI 1640 (Sigma-Aldrich). 1 $x10^5$ cells were placed in a 96-well "U" bottomed plate (Becton Dickinson) and incubated at 37ºC with 5% CO_2 with 400µg/ml of the fluorescently labelled *E.Coli* (pHrodo; Invitrogen) or *S.Aureus* (pHrodo; Invitrogen) for 60minutes, 45 minutes or 30 minutes. A negative control with the equal volume of cells were also incubated with *E.Coli* or *S.Aureus* (pHrodo; Invitrogen) and placed on ice for 60minutes. Following incubation, the cells were placed on ice to halt phagocytosis, were washed twice (250G at 4ºC for 5 minutes) with 2% ice-cold PBS/BSA and transferred to flow cytometric tubes for analysis.

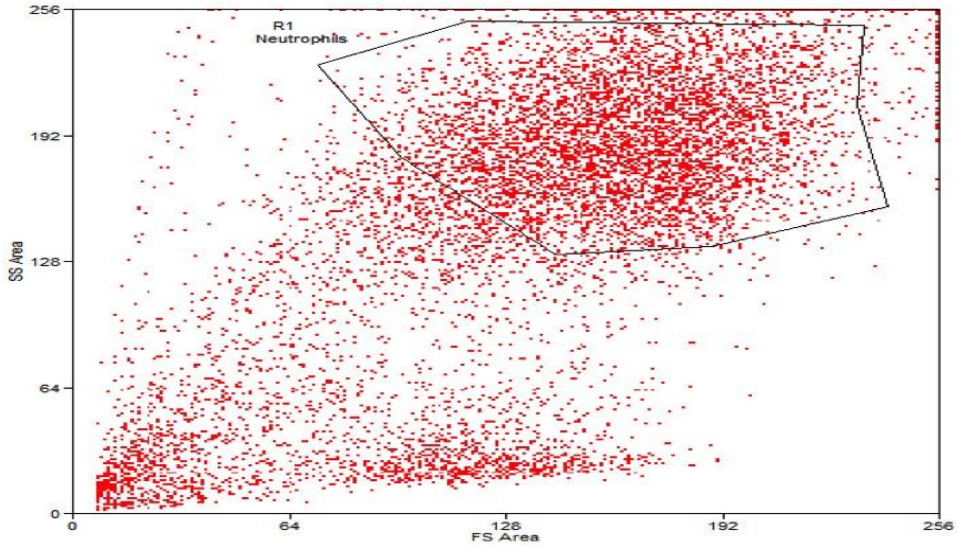
Flow cytometry was carried out on a $CyAN_{ADP}$ (Beckman Coulter) with forward scatter and side scatter gating used to identify the neutrophil population and eliminate cell debris. The pHrodo beads were conjugated to Phycoerythrin (PE). Data was analysed using Summit v.4.3. (Dako, Colorado, USA). Three measures were used to quantify phagocytic function. Firstly the percentage of pHrodo "bright" cells, secondly the median fluorescent intensity (MFI) indicated the number of bacteria

phagocytosed by the cells. Lastly a phagocytic index (PI) was calculated by multiplying the percentage of pHrodo “bright” cells with the MFI (see equation below). This provided a quantitative measure of neutrophil phagocytic function (figure 2.4).

Phagocytic Index = (Percentage of pHrodo “bright” cells/100) x MFI

Data for the three indices of phagocytosis was represented as AUC (see figure 2.5).

A



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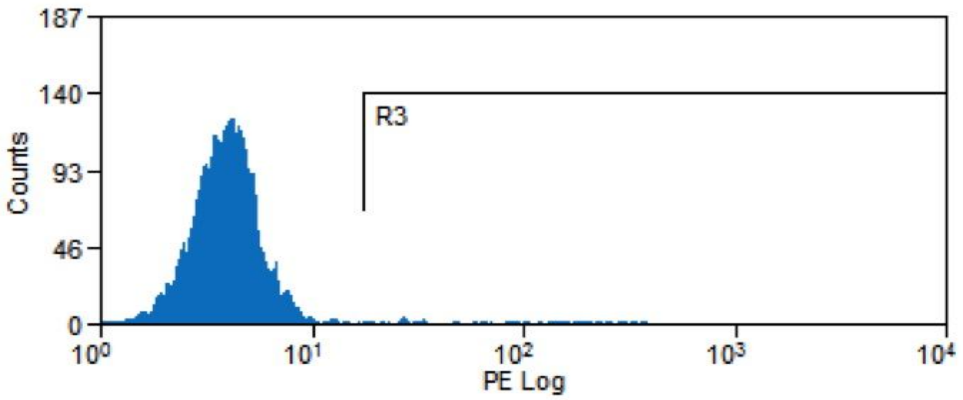


C

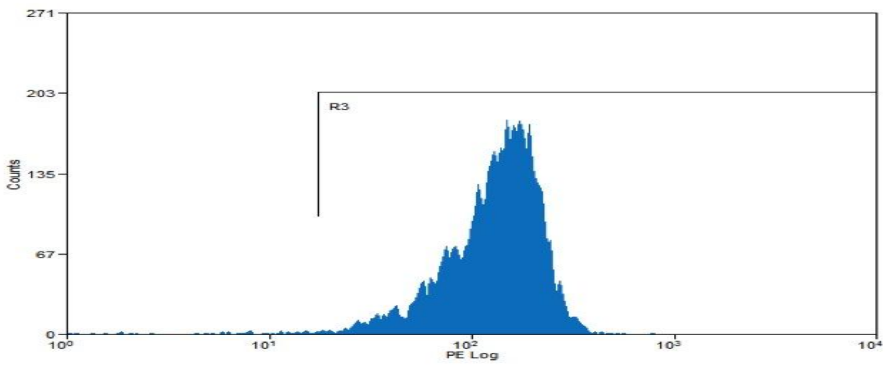


Figure 2.4. Typical plots acquired from flow cytometry using for phagocytosis. A represents the gating based on forward and side-scatter to identify the neutrophil population. B represents the typical plot for the negative control. C represents phagocytosis at 60 minutes.

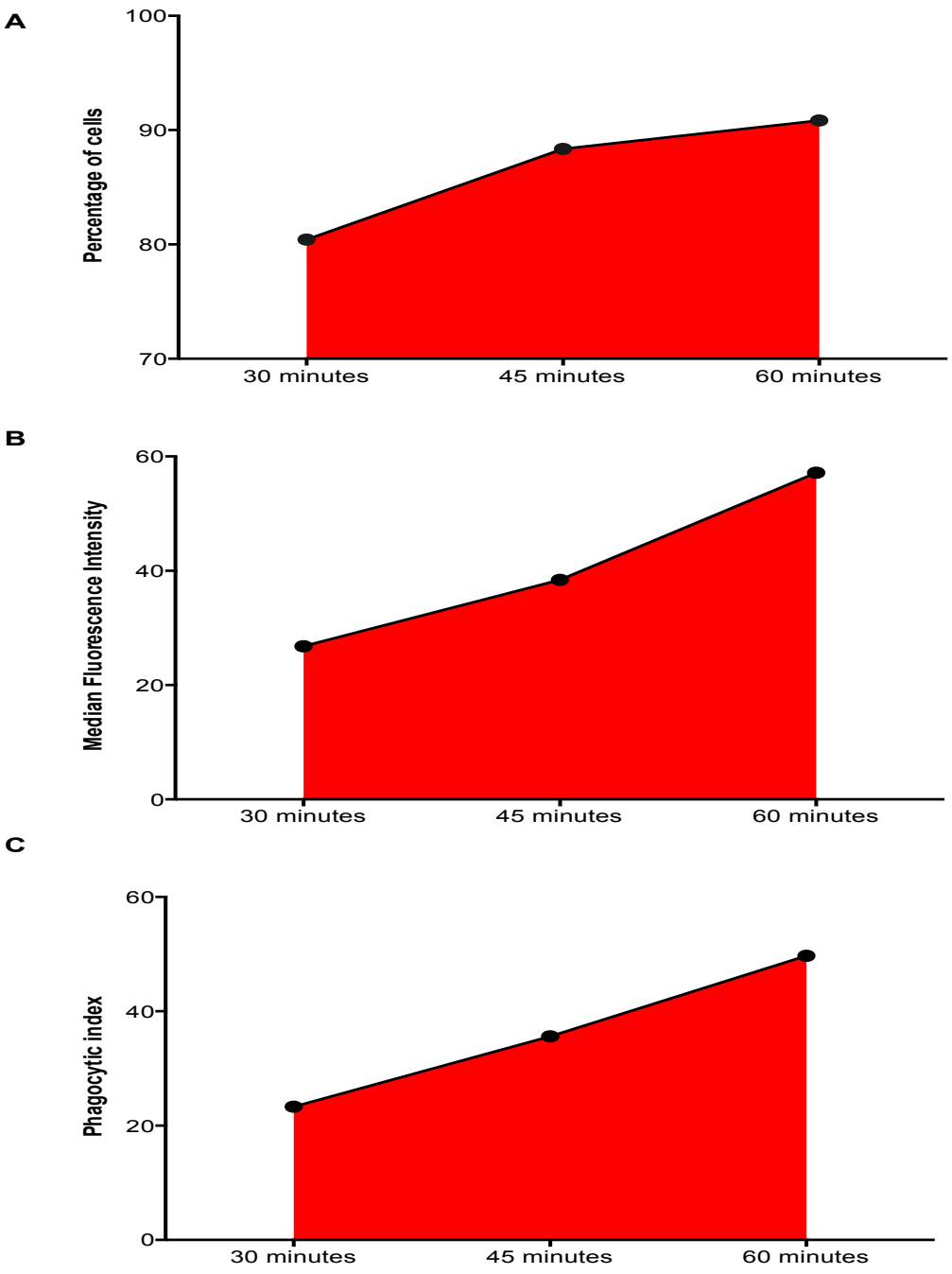


Figure 2.5. Area under the curve calculations for phagocytosis indices. Phagocytosis indices were calculated at three time points (30, 45 and 60 minutes). Data for phagocytosis was expressed as area under the curve (AUC) for each of the indices'. This is shown in the above graph with the red area representing the AUC.

2.6.4. Quantification of Neutrophil Extracellular Traps (NETs) Formation

Freshly isolated neutrophils from EDTA vacutainers (Becton Dickinson) were used in the quantification of NET formation. Neutrophils were re-suspended in RPMI 1640 (Sigma-Aldrich) supplemented with 2nM L-Glutamine, 100U/ml Streptomycin and 100ug/ml Penicillin (GPS; all purchased from Sigma-Aldrich), to ensure the culture remained sterile, at a concentration of 1 $x10^6$/ml. 1 $x10^5$ cells were placed in a 96-well flat bottomed plate (Becton Dickinson) with an additional 75µl of RPMI 1640 with GPS (Sigma-Aldrich). Cells were then stimulated with 25nM PMA (Sigma-Aldrich) or an additional 25µl of the media was added for the negative control and incubated for 3 hours at 37°C supplemented with 5% CO_2. Experiments were performed in quadruplicate.

Following incubation samples were treated with 200units of micrococcal nuclease (MNase; Sigma-Aldrich) and 1µM of SYTOX Green (Invitrogen) and incubated in the dark for 10 minutes at room temperature. This process stained and digested the extracellular DNA only. Samples were then transferred to into 500µl eppindorfs and pelleted at 5000rpm for 10minutes. The DNA containing supernatant was transferred (170µl) into a 96- well black flat-bottomed plate (CoStar; Sigma-Aldrich) and fluorescence measured in a BioTek Synergy 2 fluorometric plate reader (NorthStar Scientific Ltd, Leeds, UK) with a filter setting of 485nm excitation and 530nm emission. Background fluorescence from unstimulated negative controls was subtracted from the stimulated values. Figure 2.6 shows the production of NETs using 25nM PMA and a 3hour incubation period in healthy controls, whilst figure 2.7 shows microscopy images of NET production using the above assay (303).

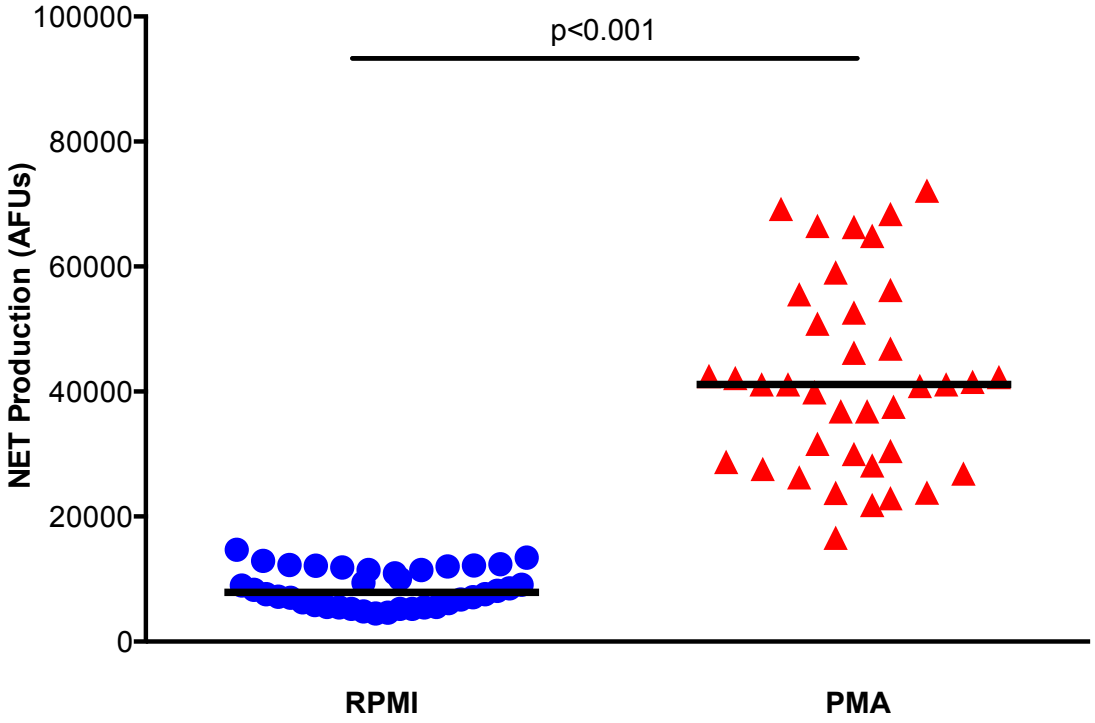


Figure 2.6: NET production in healthy volunteers.

NET production was evaluated to the control media (RPMI 1640, Sigma-Aldrich) and 25nM phorbol-12-myristate-13-acetate (PMA -Sigma Aldrich). The dot-plot represents individual values from 38 healthy donor neutrophils. Net production is represented on the y-axis and is expressed as AFUs. The greater the AFUs the greater the NETs production. The horizontal bar represents the median value. *p<0.001 in a Wilcoxon-signed rank test.

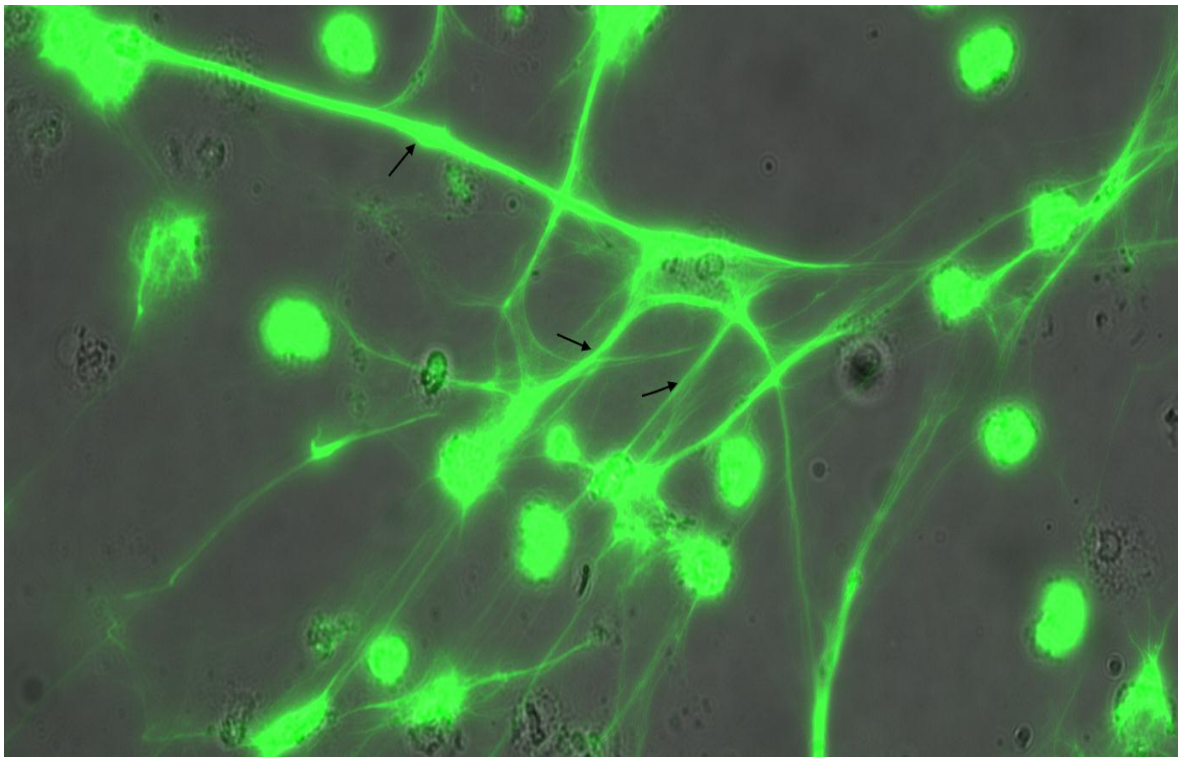


Figure 2.7. Fluorescent microscopy images of neutrophils extracellular trap formation in response to 25nM PMA.

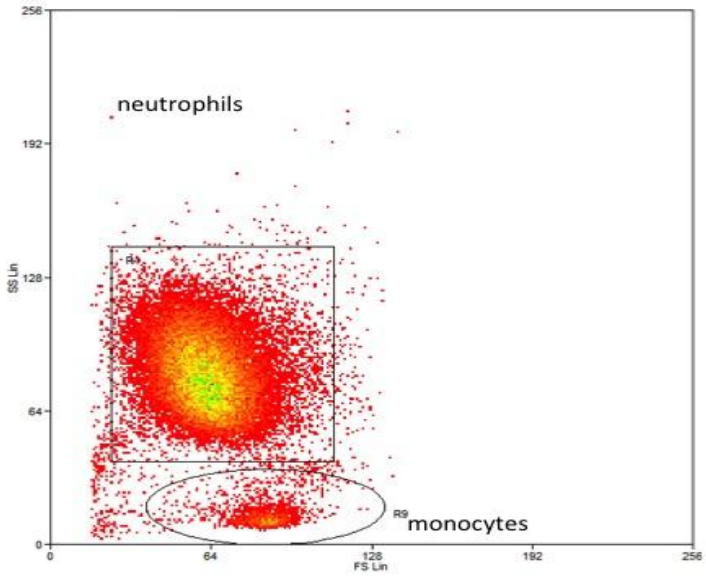
This shows a fluorescent image of neutrophils following 3hrs of incubation with 25nM phorbol-12-myristate-13-acetate. NETs are visible and are shown by the arrows within the image.

2.6.5. Neutrophil Apoptosis

Freshly isolated neutrophils from EDTA vacutainers (Becton Dickinson) were suspended in RPMI 1640 with GPS (Sigma-Aldrich) at a concentration of $1x10^6$/ml. $2x10^5$ cells were added to cytometric tubes and the cells pelleted by centrifugation at 600G for 4minutes at 4°C. The pellet was washed twice and re-suspended (600G for 4minutes at 4°C) in Annexin buffer (BD Biosciences, Oxford, UK).

Apoptosis was assessed by flow cytometer using a $CyAN_{ADP}$ (Beckman Coulter). The washed cells were stained with Fluorescein Isothiocyanate (FITC) conjugated Annexin V (dilution 1:100; BD Biosciences) and incubated for 20 minutes at 4°C. Just prior to flow cytometric analysis SYTOX Blue (conjugated to violet, dilution 1:2000; Invitrogen) was added to the samples. Cell debris was eliminated using appropriate side and forward scatter gating. Data was analysed using Summit v.4.3. (Dako). The software carried out colour compensation automatically. Areas of cells that were negative for both (alive) Annexin V only positive (early apoptotic), positive for both (late apoptotic) and SYTOX Blue only positive (necrotic) were individually calculated (see figure 2.8). Data is represented as the percentage of cells in various stages of apoptosis.

A



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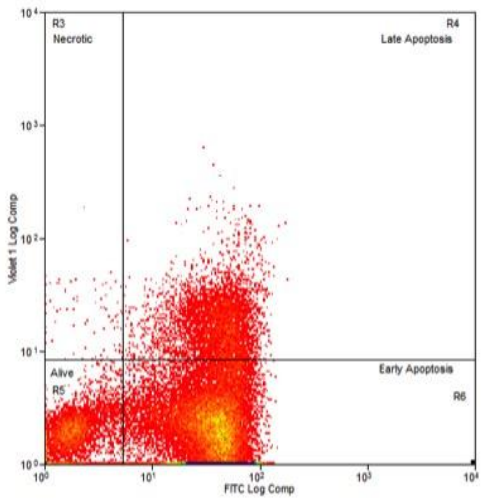


Figure 2.8. Example flow cytometry plots for analysis of neutrophil apoptosis.

A shows the typical gating strategy employed for using forward scatter and side scatter to identify the neutrophil population. B shows an example of the flow cytometry plot for neutrophil apoptosis with the various cell populations marked in the four quadrants. The percentage of cells within each quadrant was calculated and used for analysis

2.6.6. Neutrophil Receptor Expression

The expression of various cell surface receptors on neutrophils was measured using flow cytometry. 100µl of whole blood was aliqoted into flow cytometry tubes and washed twice in 2mls of 1%BSA/PBS (250G for 5minutes at 4°C). The supernatant was discarded and the antibodies and relevant isotypes were added to the blood according to the manufacturers guidance and incubated in the dark for 60minutes on ice. The antibodies used are shown in table 2.4. Following incubation the solution was washed twice in 2mls of 1% PBS/BSA (250G for 5minutes at 4°C) and the supernatant carefully discarded. 2mls of FACS lysing solution (eBiosciences, Hatfield, UK) was added to each tube and incubated for 15minutes in the dark to remove erythrocytes.

Following erythrocyte lysis the cells were pelleted (250G for 5minutes at 4°C) and re-suspended and washed twice in 2mls of 1% PBS/BSA (250G for 5minutes at 4°C). They were immediately run on the Accuri C6 flow- cytometer (BD Biosciences). Data was analysed using the Cflow Plus software (BD Biosciences). Colour compensation was carried out using a specific mathematical calculator provided by Accuri Cflow software (BD Biosciences).

Table 2.4. Primary antibodies and the isotype controls used to measure the neutrophil receptor expression.

Antibody	Isotype Control	Colour	Concentration	Clone
CD11a	IgG1	FITC	1µg/ml	HI 111
CD14	IgG1	APC	0.25 µg/ml	61D3
CD16	IgG1	FITC	1 µg/ml	ebioCB16
CD97	IgG1	APC	0.125 µg/ml	VIM3b
CD63	IgG1	PE	0.125 µg/ml	H5C6
CD45	IgG1	FITC	0.5 µg/ml	2D1
CD62L	IgG1	APC	0.5 µg/ml	DREG-56
CD18	IgG1	PE	0.25 µg/ml	MHR73-11

All products were bought from eBiosciences, Hatfield, UK.

FITC: Fluorescein Isothiocyanate, PE: Phycoerythrin, APC: Allophycocyanin.

TLR4: Toll-like receptor 4

2.7. In-vitro simvastatin experiments

2.7.1. Statin dose-response

A statin dose response was carried out using the neutrophil migration assays (see section 2.6.1) to select the most appropriate dose for the in-vitro experiments (see figure 2.9). These doses were chosen on the basis that 1μM is thought to reflect the approximate plasma concentration following 40-80mg oral simvastatin. This calculation is based on the premise that simvastatin has a high-first pass metabolism resulting in about a 5% bioavailability and is strongly protein bound. Therefore, the concentration of predicted is[1]:

(40mg x 0.05)/5000ml (average blood volume) = 0.4 *μ*g/ml,

Molecular weight of simvastatin is 418 *μ*g/ml,

Therefore this is approximately 1*μ*M.

The dose response experiments suggested that the optimal in-vitro dose that can modify neutrophil migration is 1μM. Additionally a study in patients with ARDS following an oral dose of 80mg simvastatin suggested that the median plasma concentration of simvastatin was approximately 1μM (IQR 0.46-3.5μM) however, there was great variability (personal communication with Professor Danny McAuley). Recent evidence suggests that the anti-inflammatory effects of simvastatin are dose –dependent with larger effects seen at 40mg and 80mg compared to lower doses. Therefore based on the above calculation and these experiments this concentration

[1] Ghosh C et al, Drug repurposing screen identifies Foxo1-dependent Angiopoietin-2 regulation in sepsis, critical care medicine, In Press

was chosen to investigate the in-vitro effects of simvastatin on neutrophil function in sepsis as it reflected an approximate dose of 40-80mg simvastatin (304-306).

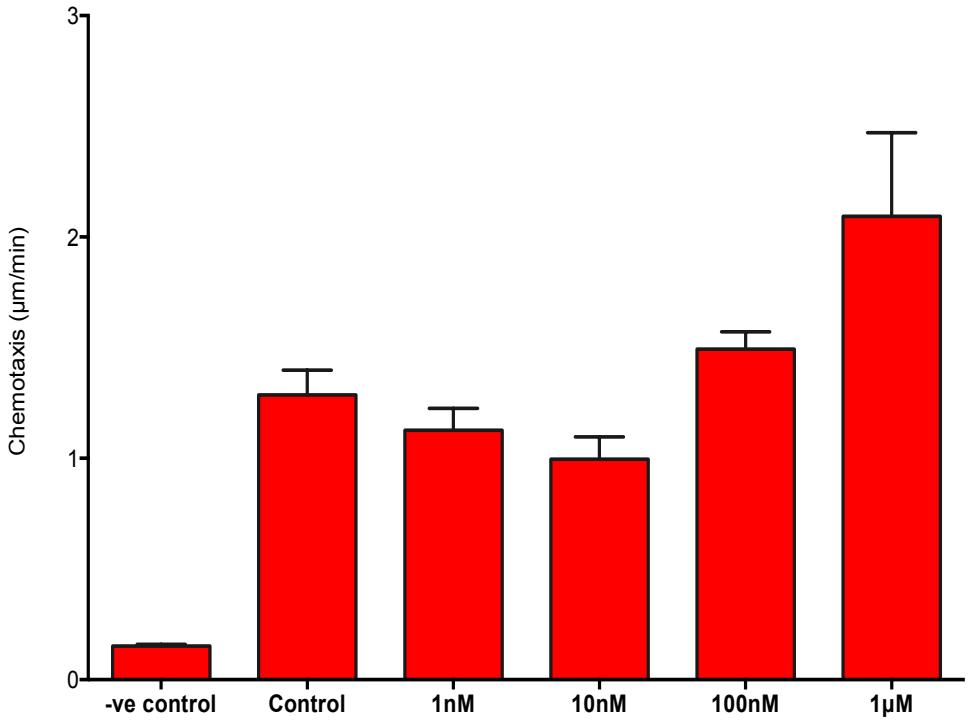


Figure 2.9.Simvastatin dose-response

The response of healthy control neutrophil chemotaxis to various doses of simvastatin (Sigma-Aldrich), negative (-ve) control and in the control media (RPMI 1640). N=11. Bars represent the mean and the error bars SEM.

2.7.2. Effect of vehicle control (DMSO) and 1μM simvastatin on un-stimulated NET production

Freshly isolated neutrophils were incubated with 1μM simvastatin (Sigma-Aldrich) for 40 minutes prior to assessment of neutrophil function. Assays were then carried out as detailed above (section 2.6.1).

The vehicle control for simvastatin (Sigma-Aldrich) was the solvent dimethyl sulfoxide (DMSO; Sigma-Aldrich). To test the potential confounding effect of DMSO on neutrophil function 10 separate NETs experiments were carried out (see section 2.6.4). Neutrophils were incubated in 1μM simvastatin (Sigma-Aldrich) and an equivalent dilution of DMSO (Sigma-Aldrich). NETs were quantified to RPMI 1640 with GPS (Sigma-Aldrich), DMSO (Sigma-Aldrich) and 1μM simvastatin (Sigma-Aldrich). The addition of DMSO (Sigma-Aldrich) or 1μM simvastatin (Sigma-Aldrich) to unstimulated neutrophils did not significantly alter NET production compared to NET production in just the control media ($p=0.436$, Freidman's Tests, $N=10$; see figure 2.10).

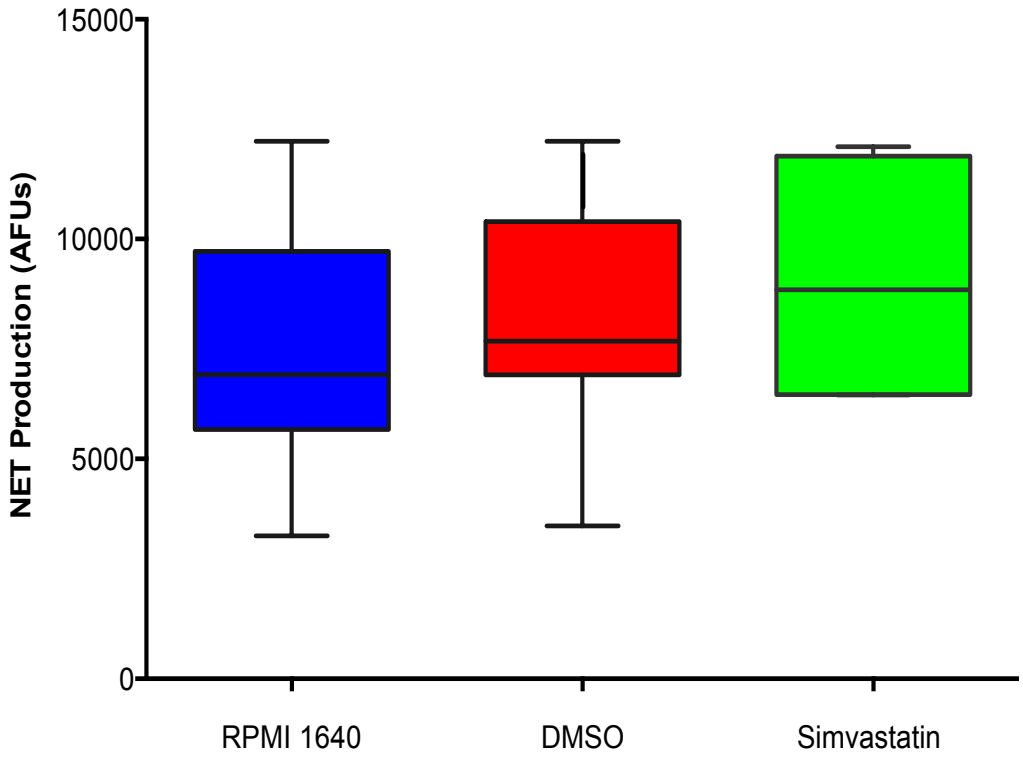


Figure 2.10 NET production in neutrophils to RPMI 1640 supplemented with GPS, DMSO and simvastatin 1μM.

NET production was evaluated in 10 patients with to RPMI 1640 (Sigma-Aldrich) and dimethyl sulfoxide (DMSO; vehicle for simvastatin; Sigma-Aldrich) and simvastatin 1µM (Sigma-Aldrich). Net production is represented on the y-axis as AFUs. The bars represent the median and the IQR and the error bars a Turkey distribution. Friedman's test, p=0.436, N=10.

2.8. Statistical Analysis.

Dr. Peter Nightingale, medical statistician at the University Hospital Birmingham NHS Foundation Trust, provided statistical advice for the data analysis. Statistical analysis was performed using GraphPad Prism Version 6 (La Jolla, California, USA) or PASW v.18.0 (Chicago, Illinois, USA).

Continuous data was tested for normality using a Shapiro-Wilk, however when sample numbers were low a Kolmogorov-Smirnov test was used. Parametric data was analysed using a *Student's* t-test for two independent samples or a paired t-test for matched samples. Where two or more groups were analysed a one-way analysis of variance (ANOVA) test was used for independent samples and a matched-pairs ANOVA for related samples. Non-parametric data were analysed with a Mann-Whitney U test (independent samples) or a Wilcoxon signed rank test (related samples) for two groups. Analysis with two or more groups was performed using a Kruskal-Wallis test (independent samples) or a Freidmann's test (related samples). Where two or more groups were analysed a relevant post-hoc test (ANOVA: Bonferroni test, Kruskal-Wallis test: Dunn's test) were performed between all variables to determine where the significant differences lay. A Pearson or Spearman correlation was used for parametric and non-parametric data respectively. Categorical data was analysed using a Fisher's exact test for two variables and a chi-sqaured (χ^2) used when greater than two groups were analysed. All tests were two-tailed with results considered significant if $p<0.05$.

Chapter 3: Simvastatin 80mg for the modulation of neutrophil migration in the healthy elderly.

3.1. Introduction

Elderly patients are at increased risk of developing severe infections and have a high morbidity and mortality from them. This has been attributed to a functional decline in innate and adaptive immune responses, termed immunosenescence, resulting in the poor containment and elimination of invading pathogens and an exaggerated host response. Neutrophils are an essential part of the innate immune response and alterations in their functions play a role in the pathogenesis of infection in the elderly. Functional neutrophil studies in the healthy elderly have demonstrated impaired chemotaxis, higher ROS production, reduced phagocytosis and NET production. Potential therapeutic options that improve neutrophil function in the elderly could potentially improve outcomes from infection in this susceptible group (72, 73, 75, 77, 78, 80, 82, 85-88, 307, 308).

HMG-CoA reductase inhibitors commonly referred to as statins, improve outcomes from infection in observational studies. Data from these studies suggest that statin users are older and have greater co-morbidities compared to non-statin users, but despite this they show a reduced mortality from severe infections suggesting that statins modulate the immune response (230, 236, 245, 309, 310).

The effects of statins on neutrophil function remain controversial, with studies in both healthy volunteers and in those with severe infection suggesting that in-vivo statin therapy reduces neutrophil chemotaxis. These authors have suggested that reducing the number of migrating neutrophils into infected tissues suppresses collateral damage caused by excessive ROS and NET production and reduces the release of

pro-inflammatory cytokines preventing the inflammatory cascade that leads to eventual organ failure (150, 152, 311).

However, recent data from our group, using time-lapse video microscopy demonstrated that neutrophils from healthy elderly donors retained their speed of migration (chemokinesis) but showed significant decreases in targeted migration (chemotaxis) compared to young controls. The in-vitro treatment of neutrophils with simvastatin restored the migratory dynamics of elderly donor neutrophils to values observed from young donors (86).

Based on this evidence a trial was designed to test whether the treatment of healthy elderly participants with 80mg of simvastatin for 14 days would improve the migratory responses of their neutrophils.

This trial was conducted in conjunction with Dr Hannah Greenwood, who assisted with the functional neutrophil assays and was overseen by Dr Elizabeth Sapey (principal investigator) and Professor David Thickett (chief investigator).

3.2. Methods

3.2.1. Study Design

This was a double-blind placebo controlled cross over randomised trial. All participants provided written consent prior to randomisation and the study received ethical approval (11/SC/0356).

3.2.2. Study Participants

Subjects were registered on the Birmingham1000 Elders database and were invited to take part in the study. No financial incentives were provided. Subjects were screened for eligibility by trained medical staff that took a medical history and examined them to rule out significant acute or chronic illnesses. Subjects had their body mass index (BMI) calculated, baseline blood tests taken for biochemistry and haematology and lung function tests performed. The inclusion and exclusion criteria are shown in table 3.1. Well-controlled hypertension, defined as participants with blood pressure recording within the normal range (systolic pressure less than 159mmHg and a diastolic pressure less than100mmHg) and receiving treatment with anti-hypertensive agents, were not excluded, nor were participants with asthma who were only on beta-agonist inhalers (e.g. salbutamol) as required.

Table 3.1. Inclusion and exclusion criteria for entry into the study

Inclusion	Exclusion
Age≥ 60 years	Unable to provide consent
Mentally competent to participate	History of significant chronic illnesses (e.g. diabetes, COPD, cancer, CVD, auto-immune diseases)
Able to provide written informed consent	On regular medications listed below*
MRC dyspnoea scale < 2	Clinical evidence of acute viral or bacterial infections
Normal spirometry (FEV1 and FVC > 70% predicted).	Statin intolerance or allergy
Normal peripheral oxygen saturations (≥92%)	Alcohol misuse
Free of medications listed below*	
No clinical evidence of acute infections or chronic illnesses	
Normal liver, kidney function and Creatinine kinase	

FEV1: Forced expiratory volume in 1 second. FVC: Forced vital capacity.
COPD: Chronic obstructive pulmonary disease.
CVD: Cardiovascular disease, defined as congestive cardiac failure (NYHA >II), angina (CCS>II), or previous documented ischaemic heart disease. Well-controlled hypertension was not an exclusion criterion.

* Medications that would prevent entry into trial

- HMGCoA reductase inhibitors
- Insulin and oral medications for diabetes
- Corticosteroids
- Non-steroid anti-inflammatory drugs
- Antibiotics
- Theophyllines
- PDE4 inhibitors
- Anti-platelets
- Anti-anginal drugs
- Ciclosporin
- Fibric acid derivates

3.2.3. Randomisation and Blinding

Study drugs were prepared, randomised and packaged identically by Bilcare Ltd (Powys, UK). Computer-based block randomisation was performed in a 1:1 method by Bilcare Ltd. (Powys, UK) and the pharmacy (University of Birmingham NHS Foundation Trust Pharmacy, Birmingham, UK) prescribed in a numerical order and held the randomisation code in a blinded fashion. Subjects eligible for entry were randomised into two groups. The first group were assigned to receive 80 mg daily of simvastatin for 14 days followed by the placebo for 14 days, whilst the second group received placebo (14 days) then the simvastatin (80mg daily for 14 days). The two treatment periods were separated by a 14-day wash out period, the time taken for the bone marrow to produce a new pool of circulating neutrophils (see figure 3.1). Subjects, medical staff, and laboratory staff were all blinded from treatment allocation and un-blinding performed only after all subjects had completed the trial and all data analysed.

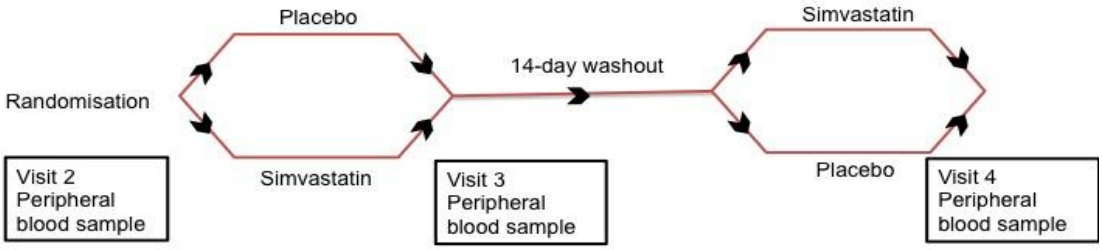


Figure 3.1. A flowchart showing the two-randomisation pathways in the crossover trial design.

3.2.4. Study Protocol

3.2.4.1: Participant Monitoring

Following enrolment and randomisation into the study, subjects started their first treatment course (14 days). A member of trial's team called them three times during the treatment to ensure compliance with medication and to monitor for serious adverse events. Subjects attended an appointment with the trial team after the completion of the first course of treatment. A medical history and examination was performed and bloods for safety and neutrophil experiments taken. Subjects were next contacted by telephone prior to the commencement of the second treatment course, as a reminder. The same procedure occurred during the second treatment course with three telephone calls and an appointment with the trial's team at the end.

3.2.4.2. Blood Collection

Bloods were collected from patients on entry into the trial and following the completion of each treatment course. Experienced nurses or doctors collected bloods from venous sites using a vacutainer system. Safety bloods were taken to monitor renal and liver function and CK. Lipid profiles were taken at each visit but were not reviewed by any member of the trial team until the end. This ensured that potential bias was eliminated and the trial remained blinded to investigators. Bloods for the functional neutrophil assays were taken simultaneously.

3.2.4.3. Functional Neutrophil Assays

Neutrophils were extracted from whole blood as described in section 2.5.3. Neutrophil migration was performed as described in section 2.6.1, ROS measurement as in section 2.6.2, phagocytosis as in section 2.6.3, and assessment

of NETS as in section 2.6.4. Experienced and trained staff performed all neutrophil assays in a blinded fashion.

3.2.5. Study outcomes

3.2.5.1 Primary outcome

The primary outcome of the study was an improvement from baseline in neutrophil chemotaxis in subjects treated with simvastatin 80mg once daily for 14-days. Additional migratory dynamics assessed were chemokinesis (speed), chemotactic index, neutrophil adherence, the percentage of adhered neutrophils that migrated and the time (in seconds) to commence migrating.

3.2.5.2 Secondary outcomes

Secondary outcomes were changes in phagocytosis, ROS and NETs in neutrophils from subjects treated with simvastatin. Additional outcomes were the safety and tolerability of high dose simvastatin.

3.2.6. Sample size calculation and statistical analysis.

Based on in-vitro data suggesting statins improve neutrophil migration, it was assumed that a similar magnitude of effect would occur in-vivo. Eighteen participants would be required to complete each arm to detect a significant improved ($p<0.05$) in neutrophil chemotaxis (mean difference 1.8 μm/min, SD 0.6 μm/min) with a power of 80%. To allow for dropouts, a target recruitment of 24 participants was selected for the study.

Dr. Peter Nightingale (Medical Statistician, University Hospital Birmingham) provided statistical advice. Data collected whilst participants were on simvastatin and placebo

were taken away from baseline values collected prior to treatment. The differences between pre and post treatment values for each group were used for data analysis. Continuous data was tested for normality using a Shapiro-Wilk's test. Parametric data was analysed using a paired t-Test and non-parametric data using a Wilcoxon's signed rank test. Categorical data were analysed using a Fisher's exact test or a χ^2-test.

3.3. Results

3.3.1. Subject Characteristics

Twenty-four subjects in total were consented and randomised into the study with 20 completing both treatment courses. Three subjects withdrew after initial randomisation and one after completion of the first treatment course, which was the placebo. A modified consort diagram (see figure 3.2) shows the flow of subjects in the study.

The demographic details of subjects are shown in table 3.2. The mean age was 71.9 years (range 60-94) with a similar number of men and women (9 vs. 11 p=0.752; χ^2 test).

Compliance with treatment was excellent with only one subject forgetting a single dose. All others reported completing the allocated treatment courses. The total cholesterol of patients was measured as a surrogate marker of compliance and of drug effect and was studied post trial completion. Placebo had no impact on serum cholesterol levels whilst simvastatin treatment significantly reduced cholesterol levels

compared to baseline (see table 3.3). This confirms that the drug was taken and had a therapeutic effect.

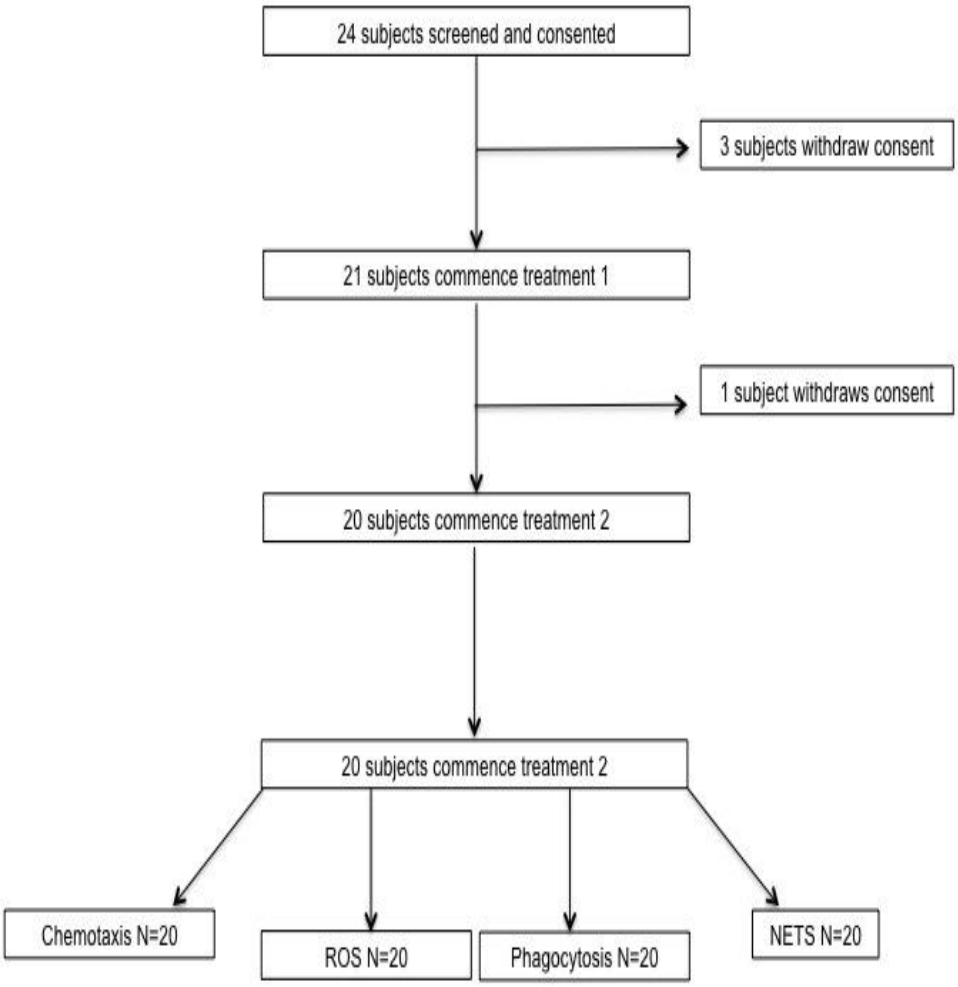


Figure 3.2: Modified Consort Diagram.

This modified consort diagram shows the screening and recruitment of subjects in the study. It also shows the number of subjects who had complete sets of functional neutrophil experiments carried out.

Table 3.2. The demographic, physiological and biochemical characteristics of subjects on recruitment into the trial.

Age, mean (range), years	71.9 (60-94)
Gender, n (%), males	9 (45%)
Smoker	
Never, n	12
Ex-, n (PYH)	6 (21.5)
Current, n (PYH)	2 (21.5)
Co-morbid conditions	
Hypertension	6
Medications	
Anti-hypertensive drugs	
ACEi	1
Diuretics	1
Ca^{2+} channel blocker + diuretic	3
Beta-blocker	1
Oxygen Saturations (%)	97 (96-98)
FEV1*, mean ± SD (% predicted)	2.67±0.7 (115.5±19.1)
FCV*, mean ± SD (% predicted)	3.57±0.8 (123.3±21.9)
BMI, mean ± SD	26.04±3.7
Blood Pressure (mmHg)	142 (132-149)
Creatinine (µmol/L)	79 (69.5-80.5)
ALT (µmol/L)	17 (15-22)
Bilirubin (µmol/L)	8 (7-10)
CK (IU/L)	80 (59.5-134.5)
TSH (mU/L)	1.9 (1.1-3.7)

All values represent the median (IQR), unless otherwise stated.

PYH: pack year history. FEV1: Forced expiratory volume in 1 second. FVC: Forced Vital Capacity. BMI: Body mass index. ALT: Alanine Transferase. CK: Creatinine Kinase. ACEi =Angiotensin Converting Enzyme inhibitor. Ca^{2+}: Calcium.

* FEV1 measured in litres/sec and FVC measured in litres.

Table 3.3. Total serum cholesterols at baseline and following treatment with placebo and statin.

	Baseline	Placebo	Simvastatin
Total Cholesterol (mmol/L)	5.7 (5.2-6.4)	5.8 (5.1-6.1)	3.8 (3.6-4.4)
p-value*		0.100	<0.001

*

p-value represents Wilcoxon-signed rank test between baseline and the treatment group. N=20 in each group.

3.3.2. Primary Outcome: Neutrophil Migration

3.3.2.1. Neutrophil chemotaxis

Chemotaxis (directional migration) was significantly improved in subjects following simvastatin treatment towards CXCL-8 (0.26μm/min {IQR 0.01-0.61μm/sec} vs. 0.03μm/sec {IQR -0.73 - 0.34μm/sec} p=0.042; Wilcoxon-signed rank test) and fMLP (0.34μm/sec {IQR 0.84-1.26μm/min} vs. 0.02μm/min {IQR -0.51-0.35μm/min} p=0.006 Wilcoxon-signed rank test). See figure 3.3.

3.3.2.2. Neutrophil chemokinesis

Neutrophil chemokinesis was unchanged towards CXCL-8 (0.33μm/sec {IQR -0.30 – 0.78μm/min} vs. 0.15μm/min {IQR -0.38-0.94μm/min} p=0.890 Wilcoxon-signed rank test) and fMLP (-0.394μm/sec {IQR -1.77-1.0μm/sec} vs. 0.16μm/sec {IQR -0.46-1.00μm/sec} p=0.349 Wilcoxon-signed rank test) following simvastatin or placebo treatment.

3.3.2.3. Neutrophil Chemotactic Index

The neutrophil chemotactic index was unchanged towards CXCL-8 (0.08 {IQR -0.02 – 0.17} vs. -0.04 {IQR -0.12 – 0.10} p=0.123 Wilcoxon-signed rank test) and fMLP (0.05 {IQR -0.05 – 0.17} vs. -0.03 {IQR -0.14 – 0.06} p=0.105 Wilcoxon-signed rank test) following simvastatin or placebo treatment.

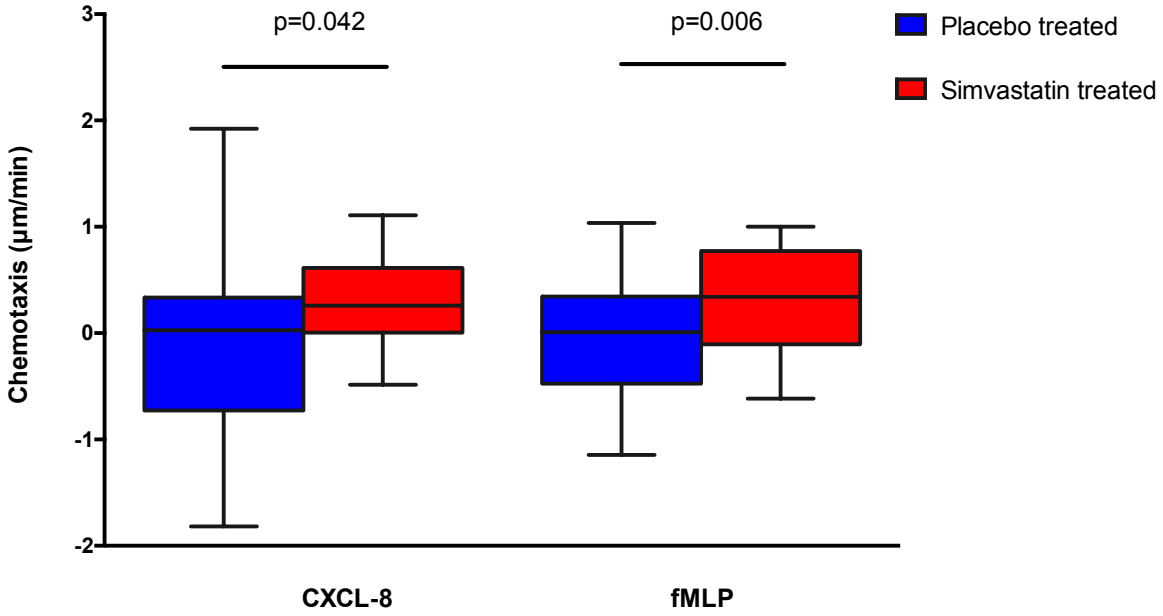


Figure 3.3. The effect of simvastatin and placebo treatment on neutrophil chemotaxis from healthy elderly subjects.

The chemotaxis of neutrophils donated from healthy elderly participants towards 100nM interleukin-8 (CXCL-8) and 10nM N-formylmethionyl-leucyl-phenylalanine (fMLP). Bars represent the median difference compared to baseline chemotaxis and IQR and the error bars a Tukey distribution. N=20 in each group.

3.3.2.4. Neutrophil adherence

Simvastatin treatment significantly reduced the adherence of neutrophils in the control media (-10% {IQR -24 - -1.5%} vs. -4% {IQR -8.8 - -1.3%} p=0.033; Wilcoxon signed rank test), and to CXCL-8 (-18% {IQR -25 - -9.0%} vs. -1.0 {IQR -7.0 – 4.0%} p<0.001; Wilcoxon signed rank test). A reduction was also seen with fMLP but this did not reach significance (-4.0% {-11.5 - 3%} vs. 1% {IQR -2.0 – 5.0%} p=0.096; Wilcoxon signed rank test).

3.3.2.5. Percentage of neutrophils migrating

The percentage cells migrating was unchanged to CXCL-8 (19.5% {IQR -0.50 – 33.8%} vs. 23% {IQR 7.5 – 47.3%} p=0.162 Wilcoxon-signed rank test) but reduced to fMLP (-7.0% {IQR -15.5 - -3.5%} vs. 1.0% {IQR -3.0 – 2.0%} p<0.001 Wilcoxon-signed rank test) following treatment with simvastatin.

3.3.2.6. Time to the Initiation of migration

The time to first migration was increased with simvastatin therapy towards CXCL-8 (20secs {IQR 0-35secs} vs. 0secs {IQR -20 -15sec} p<0.001; Wilcoxon signed rank test) and to fMLP (20secs {20-40secs} vs. 0secs {IQR -10-20sec} p<0.001 Wilcoxon signed rank test).

3.3.2.7. Summary

In-vivo simvastatin therapy for 14 significantly improved neutrophil chemotaxis towards both CXCL-8 and fMLP. The additional parameters assessed showed that simvastatin reduced neutrophil adherence and increased the time taken to initiate migration to both CXCL-8 and fMLP.

3.3.3. Secondary Outcomes

3.3.3.1. Phagocytosis

A total of 17 healthy elderly patients had successful phagocytosis experiments performed at baseline and following placebo and simvastatin therapy. The in-vivo treatment of healthy elderly subjects with simvastatin showed no effect of the phagocytosis of either *E.Coli* or *S.Aureus* in any of the three indices used to measure phagocytosis. This is shown in table 3.4

3.3.3.2. ROS production

No significant change was observed in basal neutrophil ROS production following simvastatin or placebo compared to baseline (285 ALU {-30377 -25922 ALU} vs. 1985 ALU {-34495 – 28881, p=0.898; Wilcoxon signed rank test) or when neutrophils were stimulated with PMA, fMLP and CXCL-8 (see table 3.5).

3.3.3.3. NET Production

No significant change to the production of NETs was observed following simvastatin or placebo compared to baseline values in the basal state or when neutrophils were stimulated with PMA, fMLP, CXCL-8, or LPS (see table 3.6).

Table 3.4: The effect of simvastatin treatment on the phagocytosis of pHrodo labelled *E.Coli* and *S.Aureus* in healthy elderly subjects.

	Placebo	Simvastatin	p-value
E.Coli			
pHrodo "bright" cells	67.5 (-80.8-329.5)	180.5 (-140-277.5}	0.980
MFI	749.0 (-48.0-1279)	564.0 (-439.0-1111.0)	0.064
PI	690.0 (-79.0-1207)	580.0 (-22.0-1347)	0.404
S.Aureus			
pHrodo "bright" cells	0.0 (-141.0-167.8)	-165.5 (-293.0-74.8)	0.108
MFI	951 (-1080.0 -1664.0)	831 (-254.1-951.5)	0.562
PI	1217 (-588.7-2048.0)	257 (-616.7-1951.0)	0.196

Measures used to quantify phagocytosis were the pHrodo "bright" cells, the median fluorescent intensity (MFI) and the phagocytic index (PI). All values represent the change in the area under the curve (AUC) compared to baseline values following treatment with placebo or simvastatin. Values represent the median difference compared to baseline (IQR) with p-values from a Wilcoxon signed rank test. N=17 in each group.

Table 3.5: The percentage change in Reactive Oxygen Species (ROS) production to various stimuli in neutrophils from subjects at baseline and following treatment with simvastatin.

	Placebo	Simvastatin	p-value*
PMA	-41.3 (-295.8 – 640.0)	284 (-197.3 - -781.8)	0.393
fMLP	-2.83 (-129.2 – 311.2)	63.0 (-207.5 - 290.1)	0.734
CXCL-8	-79.2 (-105.7- -40.1)	-89.4 (-113.6 - -32.5)	0.946

Values represent the percentage change in the area under the curve (AUC) compared to baseline values following treatment with placebo and simvastatin and are represented the median difference compared to baseline median (IQR), where N=20 in each group. AUC is a measure of ROS production over one hour. *Wilcoxon signed rank test.

PMA: Phorbol-12-myristate-13-acetate, 25nM.

fMLP: N-formylmethionyl-leucyl-phenylalanine, 2.5μM.

CXCL-8: Interleukin-8, 1.25μM.

Table 3.6: Neutrophil extracellular trap formation in subjects at baseline and following treatment with simvastatin.

	Placebo	Simvastatin	p-value
Unstimulated	1786 (-2039 – 6254)	1763 (-765.1 – 5521)	0.985
PMA	6964 (-17992 – 22048)	5985 (-24415 - 30189)	0.729
fMLP	1099 (-2818 – 2719)	1355 (-2113 – 6518)	0.216
CXCL-8	1270 (-1099 – 4473)	2439 (-2227 – 7062)	0.522
LPS	4048 (2833 – 6279)	7115 (-839.1 – 9851)	0.596

Values represent the change from baseline NET production following treatment with simvastatin and placebo (N=20 in each group). Values represent the median difference compared to baseline (IQR) with p-values from a Wilcoxon signed rank test. NET production is measured as Arbitrary Fluorescent Units (AFUs).

PMA: Phorbol-12-myristate-13-acetate, 25nM.

fMLP: N-formylmethionyl-leucyl-phenylalanine, 2.5μM.

CXCL-8: Interleukin-8, 1.25μM.

LPS: Lipopolysaccharide, 100ng/ml

3.3.3.4. Safety and Tolerability

The drug was well tolerated with no serious adverse events during the treatments. One subject had an asymptomatic rise in their CK whilst two reported mild muscle aches during simvastatin therapy. Neither of these two subjects had rises in CK levels and did not miss any treatment doses. One patient had an acute migraine whilst on simvastatin, however was known to suffer with these. One patient developed an upper respiratory tract infection whilst on placebo. No rises in hepatic transaminases or deterioration in renal function was observed (see table 3.7).

Table 3.7. The liver function, Creatinine Kinase and renal function of subjects at baseline and on simvastatin therapy.

	Placebo	Simvastatin	p-value
ALT (μmol/L)	17 (13-21)	17 (15-21)	0.942
Bilirubin (μmol/L)	7 (6.5-10)	7 (2.3-10)	1.0
Creatinine (μmol/L)	74.5 (64-81.3)	74.5 (68.5-80)	0.668
CK (mIU/L)	78 (56.5-104)	74.5 (64.8-115.3)	0.909

ALT: Alanine Transferase.

All values represent medians (IQR) and the p-value calculated using a Wilcoxon signed-rank test.

3.4. Discussion

This study is the first to our knowledge that has demonstrated improved neutrophil chemotaxis following treatment with simvastatin 80mg daily for two weeks in healthy elderly subjects. These modifications in neutrophil migration may help explain the improved outcomes seen in infection amongst patients on statin therapy. Importantly high dose simvastatin proved to be safe and well tolerated by subjects with no deleterious effects exerted upon the other neutrophil functions assessed (ROS production, phagocytosis and NETs).

The improvement seen in neutrophil migration with in-vivo simvastatin therapy replicates the effects observed with in-vitro therapy in neutrophils donated from healthy elderly subjects within our group. Closer analysis of migratory dynamics demonstrated that simvastatin reduced neutrophil adherence and slowed the initiation of migration (both to CXCL-8 and fMLP).

The albumin-coated cover slip used in the chemotaxis assay is a surrogate for ICAM-1, which binds to β2 integrins MAC-1 and LFA-1 and are essential for the adhesion of neutrophils to the endothelium. Migration of neutrophils in this assay relies on neutrophils crawling along the coverslip and is dependent on MAC-1 and LFA-1 expression. Statins have been shown in multiple studies to reduce binding of neutrophils to the endothelium by down regulating LFA-1 and MAC-1 expression. Additionally statins also reduce ICAM-1 expression on the endothelium itself to further reduce neutrophil adhesion (254, 312-314).

The reduction in adhesion results in fewer neutrophils transmigrating across the endothelium into the infected site. However, statins improve the chemotactic dynamics of the adhered neutrophils enabling them to take a direct route to the infected site and potentially resulting in their earlier arrival. The shorter route taken may also reduces collateral tissue damage, as neutrophils would release less MPO, NE and ROS to assist with migration through the endothelium and tissues to reach the sites of infection. This is supported by several studies that have shown that statins attenuate neutrophil chemotaxis into infected tissues. No study has assessed the individual migration of cells as in-vitro studies have used modified-Boyden chambers to determine the number of cells that migrate through an extracellular matrix, whilst in-vivo studies quantify neutrophil migration by counting the numbers of cells in aspirates from infected tissue, such as peritoneal fluid or BALF (150, 152, 256, 280, 315, 316).

A similar study in healthy male volunteers (mean age 31.6 years, age range 23-48 years) studied the effects of in-vivo atorvastatin 40mg daily, on neutrophil chemotaxis. Migration was assessed using human pulmonary artery endothelial cell (HPEAC) monolayer to replicate an extracellular matrix within a Boyden chamber. Chemotaxis was significantly reduced following statin therapy in response to LPS leading the authors to conclude that attenuating neutrophil chemotaxis contributes to the improved outcomes from sepsis in patients on pre-existing statin therapy. The two studies have several key methodological differences, firstly the populations were markedly different in gender and age, with our study representative of an at risk population. Secondly the methods used to assess chemotaxis were different. Finally

the statins used and the doses were different. The choice of statin for immune modulation remains unclear at present with evidence to suggest that the various statins differ in their ability to modulate the immune system. A recent study has suggested that rosuvastatin was better than simvastatin in modulating the adaptive immune system whilst another demonstrated improved T-cell responses with simvastatin compared to lovastatin and atorvastatin (261, 311, 317, 318).

Murine models of sepsis have demonstrated that pre-treatment with statins attenuates neutrophil recruitment to sites of sepsis and is associated with reduced disease severity and improved survival (150, 256, 315). This has been replicated in an LPS model of pulmonary inflammation in humans where 30 healthy individuals were randomly allocated to placebo or simvastatin for 4 days prior to inhalation of LPS. Subjects pre-treated with statins had fewer neutrophils in BALF samples and produced less MPO and MMP-7, 8 and 9 compared to placebo, suggesting that simvastatin not only attenuates neutrophil migration but also the inflammatory response that leads to ARDS (152).

Reduced adhesion seen with simvastatin treatment may also prevent the accumulation of neutrophils within vascular beds attenuating tissue damage and hypoxia that is caused by excessive neutrophil adherence to within micro-vascular beds. Neutrophils release ROS and NETs within these narrow vessels damaging the endothelium and occluding blood flow (3).

It had been proposed that the anti-inflammatory actions of statins would inhibit bacterial clearance and phagocytosis but in-vitro and in-vivo studies have failed to show this. The effect of various in-vitro statins upon leucocyte function in healthy individuals demonstrated that lovastatin, simvastatin and atorvastatin up-regulated phagocytosis in a dose dependent manner, whilst pravastatin had no effect. The mechanism is still unclear, but it has been suggested that reduction in cell membrane cholesterol with statin therapy increases its fluidity and thus assists with phagocytosis. Studies on macrophages suggest that statins enhance phagocytosis via the RhoGTPases, and inhibition of sterol metabolism. Although this study failed to replicate the previous beneficial effects of statins on phagocytosis, importantly no deterioration in phagocytosis was seen either (40, 150, 319-322).

Statins have been shown to cause a dose-dependent reduction in ROS production by inhibition of NADPH oxidase and through anti-oxidants properties. Although ROS is essential for pathogen killing, excessive production leads to collateral tissue injury. With age and infection ROS production in enhanced and can lead to unnecessary tissue damage. Reducing ROS production via statins may provide a mechanism to reduce their harmful effects. Although the study was not powered to detect a change in ROS production, it was expected that a decrease would be seen. A possible reason for this was the reliability of the assay with very large inter-assay variability and poor reproducibility (75, 76, 78, 150, 183).

The impact of simvastatin on NET formation has not been previously described in-vivo in healthy elderly patients. The mechanism of NETosis is closely linked with

ROS production. It was postulated that perhaps if a reduction in ROS was seen a parallel reduction in NET formation might also have been observed. In keeping with the ROS results no change in basal or stimulated (PMA, CXCL-8 or LPS) NET formation was observed following treatment with simvastatin compared to placebo.

The cross-over design of the study was chosen to allow participants to act as their own control, thereby reducing variability between the treatment groups. Additionally this methodology permitted a reduction in the overall numbers that needed to be recruited. One of the main disadvantages of this form of trial design is the potential for a “carry-over” effect, where the first treatment may alter the results of the second treatment. This was accounted for by the washout period of 14-days that was long enough for the bone marrow to generate a new pool of circulating neutrophils. Additionally, simvastatin has a short half-life (2-3hrs) and therefore theoretically no residual effects of the drug should have been seen following the second treatment (1, 323). Secondly temporal effects of the treatments (i.e. in which order the treatments were allocated) may have been a potential confounder. However, all participants and investigators, including those performing the neutrophil assays were blinded to treatment allocations. This, as well as the washout period, should have ensured that any temporal effects were accounted for.

In conclusion, this study has demonstrated that high dose simvastatin can improve neutrophil migration and potentially attenuate excessive neutrophil migration. Simvastatin therapy in-vivo reduces the number of adherent and migrating neutrophils, but improves the directional migration of these neutrophils ensuring they

arrive at the sites of infection faster and cause minimal collateral tissue damage by taking a direct route. Once at the site of infection anti-microbial toxicity is unaffected by simvastatin therapy. This suggests that the improved outcomes from sepsis in patients on statin therapy may in part due to improved neutrophil migration leading to the earlier containment and elimination of pathogens. Further research is required to confirm whether simvastatin can modulate the functions of neutrophils in patients who have an established infection.

The studies that follow will assess whether simvastatin therapy in-vitro can modulate neutrophil functions of patients admitted to hospital with sepsis.

Chapter 4: Neutrophil migration in sepsis and the effect of in-vitro simvastatin exposure on their migratory dynamics.

4.1. Introduction

The preceding chapter (Chapter 3) demonstrated that 80mg simvastatin improved neutrophil chemotaxis to CXCL-8 and fMLP in a cohort of healthy elderly participants and replicated the in-vitro effects previously observed.

Aberrant neutrophil chemotaxis has been well described in sepsis and is associated with poorer containment and elimination of pathogens allowing dissemination of infection, and exaggerating the host's response (2, 148, 154, 159).

The vital role of neutrophil migration in infection has lead to it being an attractive target for therapies in infections. It was proposed that the beneficial effects of statin therapy observed in patients with sepsis might in part be due to improved neutrophil migration and bacterial elimination. The experiments described in this chapter investigate neutrophil migration in sepsis using time-lapse video microscopy and the potential effects of in-vitro simvastatin therapy.

4.1.1. Hypothesis

Based on the literature and on previous results reported it was hypothesised:

1. Neutrophil migration would be significantly reduced in patients with sepsis.
2. In-vitro simvastatin therapy would improve the migratory dynamics of neutrophils from patients with sepsis.
3. Adhesion molecules would be up-regulated in sepsis potentially contributing to aberrant neutrophil migration.

4.1.2. Aims

The aims of the experiments were to:

1. Characterise neutrophil migration in sepsis
2. To investigate the effects of in-vitro simvastatin therapy on neutrophil migration in sepsis
3. Characterise the cell surface expression of adhesion receptors in neutrophils in healthy controls and in patients with sepsis (L-Selectin/CD62L, Beta-2-integrin/Cd11b-Cd18, and CD63).

4.2. Methods

4.2.1. Healthy control recruitment

Healthy controls were recruited into the study as described in section 2.2. The baseline data from healthy elderly subjects recruited into the statin trial described in chapter 3 were used as controls.

4.2.2. Patient recruitment

Patients with sepsis were entered into the trial as described in section 2.3. The SSCG definitions for sepsis were used to screen eligible patients (see tables 1.4 and 1.5). Exclusion criteria were as described in section 2.3.1.

4.2.3. Data collected

Data collected is described in section 2.3.2.

4.2.4. Sample collection

Blood samples for the chemotaxis experiments were taken in Lithium heparin vacutainers (Becton Dickinson) as described in section 2.4.

4.2.5. Neutrophil isolation

Neutrophils were extracted using a Percoll gradient as described in section 2.5.3. Once isolated neutrophils were suspended in RPMI 1640, which will be referred to as the “basal state” for the remainder of this chapter.

4.2.6. Neutrophil migration experiments

Neutrophil migration was assessed using an Insall Chamber (Weber Scientific) to the chemoattractants 100nM CXCL-8 (R&D systems) and 10nM fMLP (Sigma-Aldrich). A detailed description of the experiment methodology is provided in section 2.6.1. Migration was assessed using three parameters, chemokinesis (speed in any direction), chemotaxis (directional migration) and the chemotactic index (see section 2.6.1.1.). The number of neutrophils adhering to the coverslip was also calculated. Adherence was calculated by dividing the number of adherent cells by the total number of cells in the original viewing plane. Adherence was expressed as the percentage of neutrophils that adhered compared to the total neutrophil count in the viewing plane.

4.2.7. Simvastatin Experiments

Freshly isolated neutrophils were incubated with 1μM simvastatin (Sigma-Aldrich) for 40minutes prior to migration being assessed as described in section 2.7. All neutrophil assays were performed un-blinded.

4.2.8. Cell surface receptor expression experiments

The expression of the cell surface receptors CD18, CD11a, CD62L and CD63 were quantified from whole blood using flow cytometry analysis. The detail methods are described in section 2.6.6.

4.2.9 Statistical Analysis

Continuous data was tested for normality a Shapiro-Wilk's test, however if sample sizes were too small a Kolmogorov-Smirnov test. All continuous data are represented as median (IQR), with non-parametric statistical tests performed as described in section 2.9. Where two or more were analysed using a Kruskal-Wallis test, Dunn's post-hoc tests were performed to identify the nature of the statistical differences between the groups. Categorical data were analysed using a Fisher's exact test for two groups and a χ^2-test for more than two groups.

No current data exists regarding neutrophil migration in sepsis using the technique employed within this study. It was therefore difficult to accurately determine the number of patients that would be required to be recruited. However based on in-vitro data from healthy participants suggesting that ageing reduces chemotaxis, it was estimated that a total of 40 patients with sepsis would have to be recruited for any differences to be seen. Therefore to allow for a 10% experimental failure rate, it was proposed that 45 patients with sepsis be recruited. The data that would be generated would be used to power future neutrophil functional studies in patients with sepsis.

4.3. Results

4.3.1. Demographics of all participants recruited

In total thirty-seven healthy controls, consisting of the twenty healthy elderly participants described in chapter 3 and seventeen young controls and forty-five patients with sepsis were recruited into the studies described within the remainder of the thesis. The demographic characteristics of the two groups are shown in table 4.1.

The groups were well matched for age and gender, however the sepsis patients had a greater number of co-morbidities and were taking more medications than healthy controls. The functional neutrophil assays performed on the recruited sepsis cohort are shown in figure 4.1.

4.3.2. Healthy Elderly Controls

Twenty healthy elderly controls had their neutrophils assessed for migration. The demographic details for these controls have been described in section 3.3.1 and table 3.2. In summary the mean age was 71.9 years (range 60-94) with 9 (45%) males and 11 (55%) females.

Table 4.1. The demographic details of the healthy controls and sepsis patients.

	Controls N=37	Sepsis N=45	p-value
Age	62 (32.5-71.0)	66.5 (48.8-75.0)	0.125*
Sex, male (%)	17 (45.9%)	29 (64.4%)	0.119#
Number of Co-morbidities/participant, n			
0	31	16	
1	6	14	
2	0	12	
3+	0	3	<0.001Φ
Co-morbidities§, n			
None	31	16	
CVS disease	6	11	
Respiratory disease	0	3	
Chronic renal disease	0	2	
Diabetes	0	15	<0.001Φ
Medications§, n			
None	31	16	
Anti-hypertensive	6	13	
Beta-blocker	1	8	
Anti-platelet	0	6	
Oral hypoglycaemic	0	10	
Insulin	0	8	
Inhaled beta-agonist	0	3	
Statins	0	17	<0.001Φ
Severe sepsis		31 (68.9%)	
APACHE II score		11 (5.8-32.2)	
SAPS II score		33 (13-40)	
ICU Admission, N		14 (31.1%)	
Inotropes alone		4	
Respiratory support		2	
Multi-organ support		8	
WCC (109/L)		16.5 (13.3-21.8)	
CRP (mg/L)		152 (82-307)	
SOFA Score		4 (1-6.5)	
Length of Stay,		10 (6-23.5)	

All values represent the median (IQR), unless otherwise stated. *Represents a Mann-Whitney-U test, #represents a Fisher's exact test and Φ a χ^2-test. §Patient's may have more than one co-morbidity and may have been on more than one class of medication.

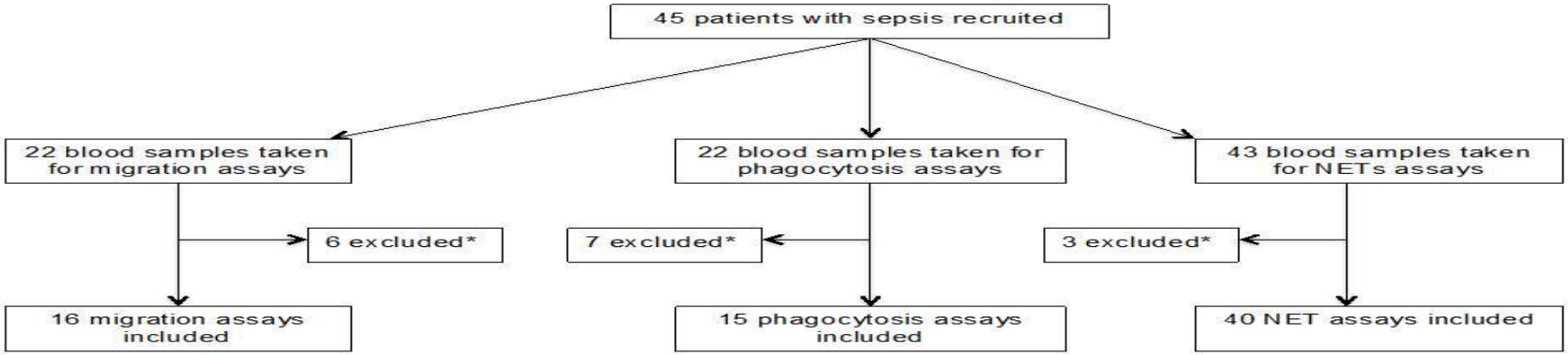


Figure 4.1. A modified consort diagram showing the recruitment of sepsis patients and the neutrophil function assays performed.

The * shows the number of patients who did not have particular assays performed. The reasons for failure of neutrophil assays were inadequate neutrophil isolation, prolonged bench time before assay initiation, experimental error and equipment failure.

4.3.2. Impact of sepsis on neutrophil migration

A total of 16 patients recruited with sepsis had migration experiments performed on their isolated neutrophils. The demographic details are shown in table 4.2. There was a similar gender split between the healthy controls and the sepsis patients recruited, however the healthy elderly controls were significantly older (p=0.002, Mann-Whitney U test). The majority of patients recruited had evidence of severe sepsis (N=11, 68.8%) with 6 (37.5%) requiring admission to ICU. Pneumonia was the commonest source of sepsis (9 patients, 56.2%) followed by UTI (4 patients, 25%). Mortality was 37.5% with a median length of stay in the survivors of 12 days (range 2-153 days).

In the basal state neutrophils do not migrate directionally and show limited random movement. Sepsis did not alter this, with no meaningful migration seen in neutrophils from sepsis patients and healthy controls (see table 4.3 and figure 4.2).

4.3.2.1. Chemokinesis

Chemokinesis (speed in any direction) towards CXCL-8 and fMLP was significantly reduced in neutrophils from sepsis patients (see table 4.3 and figure 4.2).

4.3.2.2. Chemotaxis

As with chemokinesis, neutrophil chemotaxis (directional migration) towards CXCL-8 and fMLP was significantly impaired in patients with sepsis compared to healthy controls (see table 4.3 and figure 4.2).

4.3.2.3. Chemotactic Index

The chemotactic index (overall measure of migration) was significantly impaired towards CXCL-8 and fMLP (see table 4.3 and figure 4.2).

Table 4.2. Characteristics of healthy controls and sepsis patients for the neutrophil migration experiments.

	Controls N=20	Sepsis N=16	p-value
Age, years	69 (67-78)	59 (35.8-71)	0.002*
Sex, male	9 (45%)	10 (62.5%)	0.335#
No. of Co-morbidities			
0	14	5	
1	6	9	
2	0	2	0.039Φ
Co-morbidities§, n			
None	14	6	
CVS disease	6	5	
Respiratory disease	0	3	
Chronic renal disease	0	1	
Diabetes	0	3	<0.001Φ
Medications§, n			
None	14	7	
Anti-hypertensive	6	5	
Beta-blocker	1	2	
Anti-platelet	0	2	
Oral hypoglycaemic	0	3	
Insulin	0	1	
Inhaled beta-agonist	0	1	
Statins	0	5	<0.001Φ
Severe Sepsis, n		11 (68.8%)	
APACHE II		14.1±2.2	
SAPS II		29.3±3.9	
ICU admission, n		6 (37.5%)	
Inotropes alone		1	
Respiratory support		1	
Multi-organ support		4	
WCC, median (IQR)		19.8 (14.8-22.7)	
CRP,		169.1±30.3	
SOFA Score, median (IQR)		3 (0-7)	
th of Stay, median (IQR)		12 (6.0-45.5)	
Mortality, n (%)		6 (37.5%)	

All values represent the median (IQR), unless otherwise stated. *Represents a Mann-Whitney-U test, #represents a Fisher's exact test and Φ a chi-sqaured test. §Patient's may have more than one co-morbidity and may have been on more than one class of medication.

Table 4.3. The chemokinesis and chemotaxis of neutrophils from healthy controls and sepsis patients.

	Healthy Controls N=20	Sepsis	p-value
Chemokinesis (μm/min)			
RPMI	2.89 (2.36-3.22)	2.57 (1.82-3.30) N=16	0.774
CXCL-8	3.71 (3.39-4.74)	3.09 (2.14-3.76) N=14	0.015
fMLP	3.97 (3.49-4.48)	2.28 (1.98-3.11) N=15	<0.001
Chemotaxis (μm/min)			
RPMI	-0.03 (-0.19-0.30)	0.01 (-0.13-0.19) N=16	0.778
CXCL-8	0.86 (0.40-1.82)	0.38 (0.08-0.70) N=14	0.031
fMLP	0.70 (0.21-1.35)	0.24 (-0.08-0.64) N=15	0.025
Chemotactic Index			
RPMI	0.00 (-0.05-0.05)	-0.05 (-0.21-0.01) N=16	0.08
CXCL-8	0.17 (0.10-0.30)	0.06 (0.03-0.10) N=14	0.005
fMLP	0.15 (0.03-0.25)	0.02 (-0.04-0.10) N=15	0.001

The table above illustrates the chemokinesis, chemotaxis and chemotaxis of neutrophils in healthy controls and sepsis patients in their basal state and towards 100nM Interleukin-8 (CXCL-8) and 10nM N-formylmethionine-leucyl-phenylalanine (fMLP). Values are shown as median (IQR) with p-values from a Mann- Whitney U test.

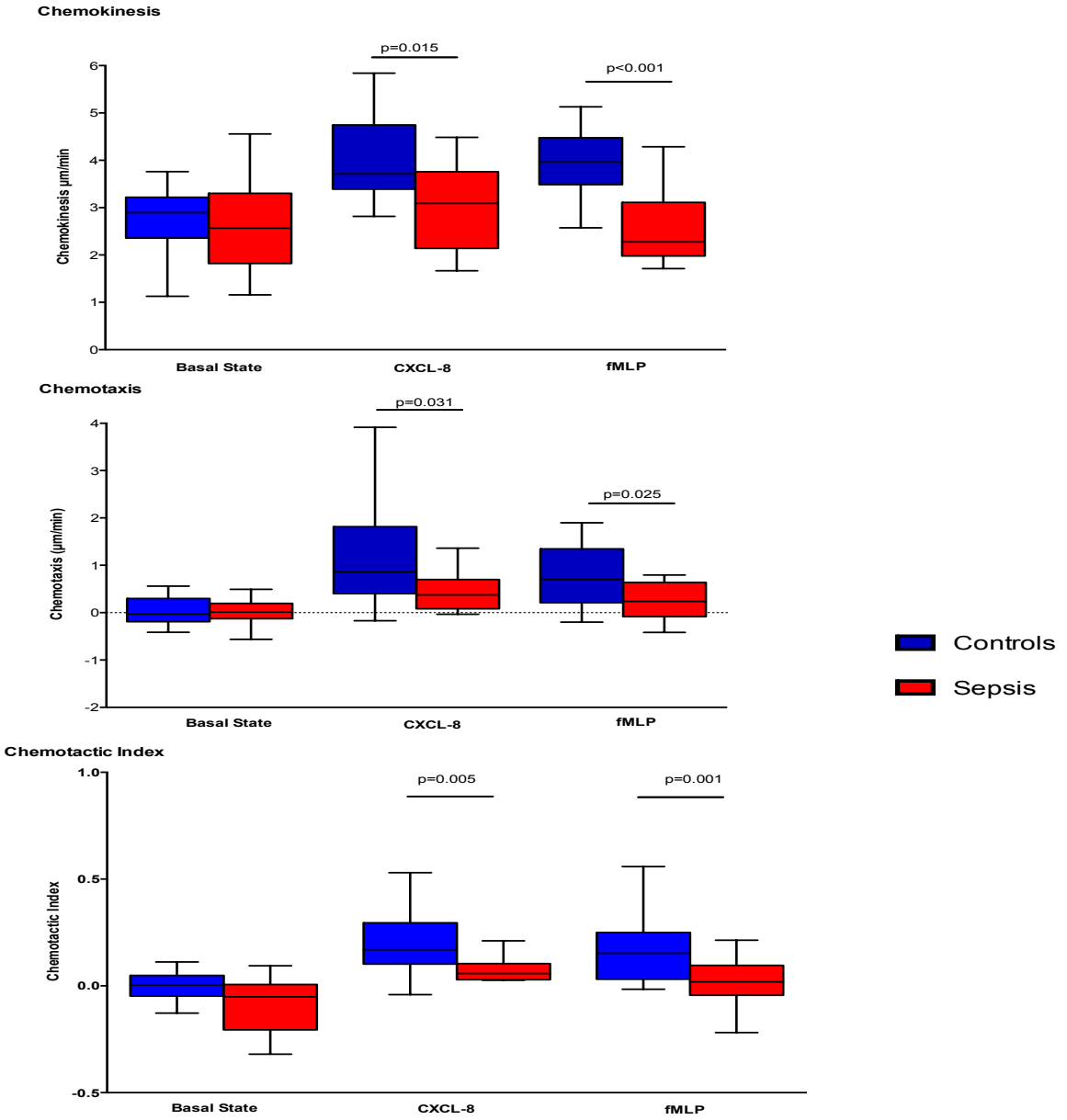


Figure 4.2. The chemokinesis, chemotaxis and chemotactic index of neutrophils in healthy controls and patients with sepsis.

The upper plot shows the chemokinesis of neutrophils in the basal state, to 100nM Interleukin-8 (CXCL-8) and 10nM N-formylmethionine-leucyl-phenylalanine (fMLP). The middle plot shows the chemotaxis of neutrophils whilst the lower plot shows the chemotactic index of neutrophils in the basal state and to CXCL-8 and fMLP. Bars represent the median and IQR with the whisker's a Tukey distribution. P-values are from a Mann-Whitney U test. N=20 for healthy controls. N=16 in the basal state, N=14 for CXCL-8 and N=15 for fMLP in the sepsis cohort.

4.3.3. Sequential changes in neutrophil migration in patients with sepsis.

The data above has shown that sepsis reduces the ability of neutrophils to migrate. Neutrophils from sepsis show reduced chemokinesis and chemotaxis resulting in a poorer chemotactic index suggesting that neutrophils are “paralysed” and are unable to effectively migrate towards sites of infection.

Of the 16 sepsis patients recruited, 13 had migration experiments performed on day 4 and 12 had experiments on day 7. Three patients showed worsening of sepsis related organ dysfunction with increases in their SOFA score by day 7 compared to their admission scores. The remainder displayed evidence of improved organ dysfunction where present at baseline (indicated by a SOFA score greater than 1). This suggests that the majority of patients (75%) were in resolving phase of sepsis by day 7. To assess whether sepsis resolution improves migration, neutrophils from sepsis patients were isolated on days 4 and 7 following admission and migration assays performed.

4.3.3.1. Chemokinesis

Compared to healthy controls the chemokinesis of neutrophils remained significantly suppressed on all three days to CXCL-8 (Kruskal-Wallis, $p=0.005$) with a Dunn’s multiple comparison post-hoc test demonstrating that a significant difference between healthy controls and the chemokinesis of neutrophils on day 4 was present ($p=0.013$). Neutrophil chemokinesis towards fMLP was also significantly reduced on sequential day analysis (Kruskal-Wallis, $p<0.001$) with a Dunn’s multiple comparison post-hoc tests demonstrating a significant difference between healthy controls and chemokinesis on day1 ($p<0.001$) and day4 ($p=0.015$). (See table 4.4).

4.3.3.2. Chemotaxis

Neutrophils from sepsis patients showed suppressed chemotaxis on sequential days compared to healthy controls towards CXCL-8 and fMLP, however this did not reach significance. See table 4.4.

4.3.3.3. Chemotactic Index

The chemotactic index was significantly reduced towards CXCL-8 (Kruskal-Wallis p=0.025) with a Dunn's multiple comparison post-hoc tests demonstrating a significant difference between healthy controls and the chemotactic index of neutrophils on day 7 (p=0.026). The chemotactic index of neutrophils towards fMLP was also significantly reduced (Kruskal-Wallis p=0.008) over the three days with a Dunn's multiple comparison post-hoc tests demonstrating a significant difference between healthy controls and the chemotactic index of sepsis neutrophils on day 1 (p=0.017). See table 4.4.

Table 4.4. Sequential changes in neutrophil chemokinesis and chemotaxis in patients with sepsis.

	Healthy Controls N=20	Day 1 Sepsis	Day 4 Sepsis	Day 7 Sepsis	p-value
CXCL-8		N=14	N=13	N=12	
Chemokinesis	3.71* (3.39-4.75)	3.09 (2.14-3.76)	2.72* (1.89-3.42)	2.96 (2.21-3.94)	0.006
Chemotaxis	0.86 (0.40-1.82)	0.38 (0.08-0.69)	0.86 (0.17-1.05)	0.31 (0.09-0.68)	0.099
Chemotactic Index	0.17* (0.10-0.30)	0.06 (0.03-0.10)	0.12 (-0.21-0.32)	-0.04* (-0.09-0.10	0.025
fMLP		N=15	N=5	N=10	
Chemokinesis	3.97* (3.49-4.47)	2.28* (1.98-3.11)	2.33* (1.97-2.96)	3.13 (2.52-3.94)	<0.001
Chemotaxis	0.70 (0.21-1.35)	0.24 (-0.08-0.64)	0.38 (0.07-0.48)	0.22 (-0.05-1.03)	0.137
Chemotactic Index	0.15* (0.03-0.25)	0.02* (-0.04-0.10)	-0.04 (-0.10-0.11)	0.04 (-0.03-0.26)	0.008

The chemokinesis, chemotaxis and chemotactic index of neutrophils towards 100nM Interleukin-8 (CXCL-8) and 10nM N-formylmethionine-leucyl-phenylalanine (fMLP) in healthy controls and in sepsis patients on days 1, 4 and 7. Chemokinesis and chemotaxis are measured in μm/min. Values represented as median (IQR) with p-values from a Kruskal-Wallis test comparing changes in the migration of sepsis patients with the healthy controls. The number of samples tested is shown (N). * values that were significant in a Dunn's post-hoc tests compared to healthy controls.

4.3.4.5.Patient migration based on sepsis resolution.

Patients with sepsis were classified as resolvers of sepsis and non-resolvers based on changes in their SOFA scores. The changes in chemotaxis towards CXCL-8 and fMLP between day 1 and 7 in patients who had sequential samples was analysed. In total 12 patients had sequential samples for migration towards CXCL-8, with 9 “resolvers” and 3 non-resolvers, whilst 10 patients had sequential samples for migration towards fMLP (3 “non-resolvers” and 7 “resolvers”).

These patients had their chemotaxis plotted for day1 and 7 to see whether a trend to improvement was seen in resolvers and a continued deterioration observed in the non-resolvers (see figure 4.3). This demonstrated that some of the resolvers show improvements in migration, whilst others do not change or in a few cases worsen. Non-resolvers, although limited by number (N=3) all showed continued deterioration in their chemotaxis at day 7 towards both CXCL-8 and fMLP.

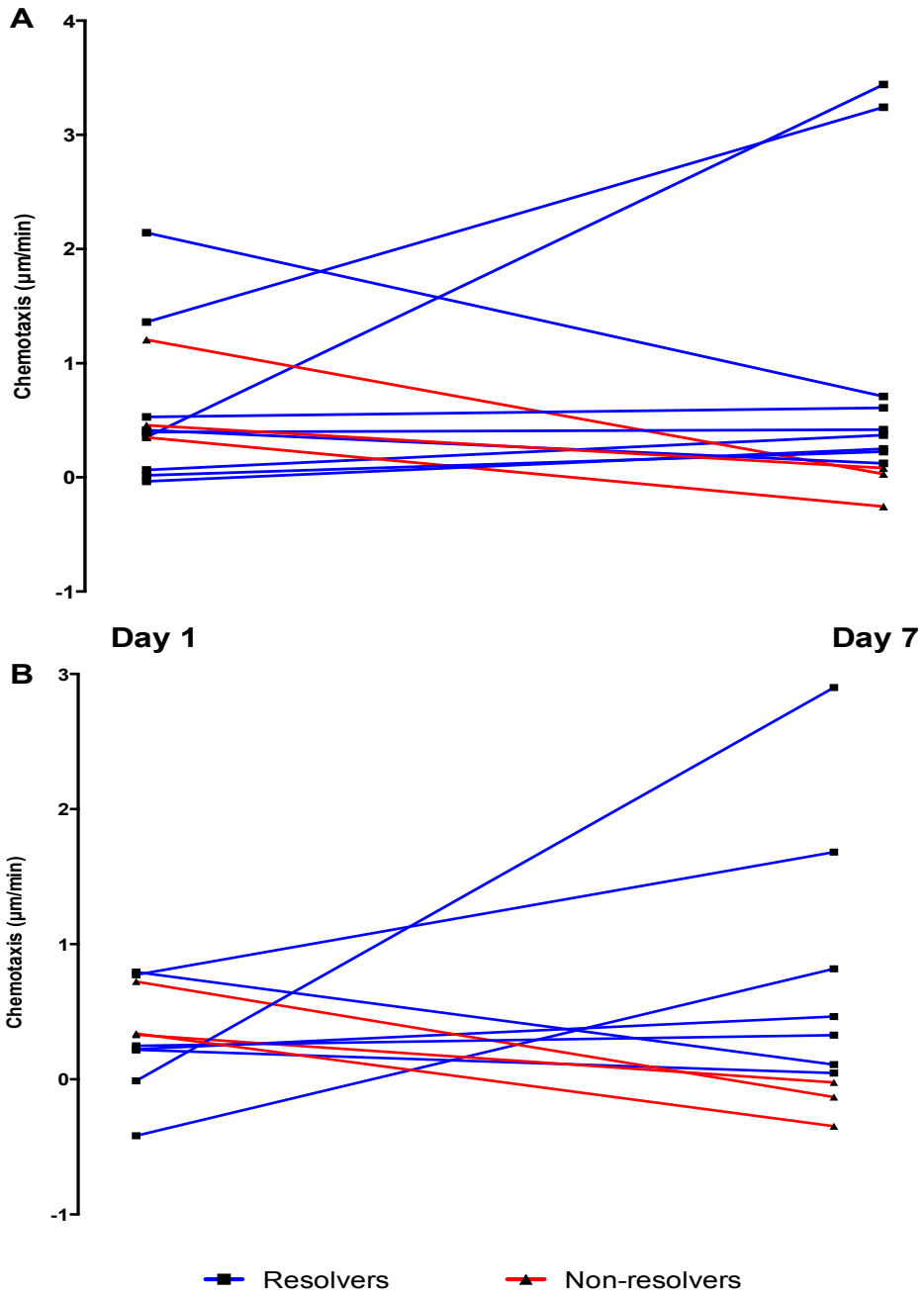


Figure 4.3. Chemotaxis in non-resolvers and resolvers of sepsis.

The line chart shows the individual chemotaxis of neutrophils from sepsis donors with day 1 shown on the left and day 7 shown on the right. The blue lines represent patients who demonstrated resolution of sepsis, whilst the red lines represents non-resolvers of sepsis based on changes in their SOFA scores. A, represents chemotaxis towards 100nM Interleukin-8 (CXCL-8; N=12) and B represents chemotaxis towards 10nM N-formylmethionine-leucyl-phenylalanine (fMLP; N=10).

4.3.5. The effect of in-vitro simvastatin treatment on neutrophil migration in sepsis.

In summary these experiments have shown that as sepsis resolves (from day 1 to day 7) neutrophil migration remained impaired, with non-resolvers from sepsis showing worsening of migration. The following experiments investigated whether in-vitro simvastatin therapy could potentially improve neutrophil migration.

Ten patients with sepsis had their neutrophils treated with 1 μM in-vitro simvastatin and assessed for migration towards the chemoattractants CXCL-8 and fMLP. No effect of in-vitro simvastatin treatment was observed when neutrophils migration was assessed to CXCL-8. See table 4.6. However, when neutrophils migration was assessed to fMLP, deterioration in chemotaxis (-0.09 {-0.19-0.18}, vs. 0.38 {0.11-0.74} p=0.045, Wilcoxon signed rank test) was seen. Although a significant reduction in the chemotactic index was observed (-0.09 {-0.27 - -0.02} vs.0.01 {-0.22-0.12} p=0.049, Wilcoxon signed rank test) the neutrophils in neither group were moving directionally and this probably does not reflect a true impact of simvastatin. No effect was observed for chemokinesis. See table 4.5 and figure 4.4.

Table 4.5. The effect of 1μM in-vitro simvastatin exposure on the migration of neutrophils from sepsis patients to CXCL-8 and fMLP.

	Vehicle Control N=10	Simvastatin Treated N-10	p-value
CXCL-8			
Chemokinesis (μm/min)	3.09 (2.61-3.61)	3.02 (2.57-3.79)	0.695
Chemotaxis (μm/min)	0.38 (0.27-0.70)	0.51 (0.07-0.78)	0.625
CI	0.06 (0.03-0.12)	0.06 (-0.05-0.16)	1.0
fMLP			
Chemokinesis (μm/min)	2.41 (1.94-3.28)	2.62 (2.44-3.44)	0.311
Chemotaxis (μm/min)	0.38 (0.11-0.74)	-0.09 (-0.19-0.18)	0.045
CI	0.01 (-0.22-0.12)	-0.09 (-0.27- -0.02)	0.049

The table illustrates the four migratory parameters assessed in neutrophils from 10 patients with sepsis to 100nM interleukin-8 (CXCL-8) and 10nM N-formylmethionine-leucyl-phenylalanine (fMLP) with and without incubation with 1μM simvastatin. The vehicle control was an equivalent concentration of dimethyl sulfoxide (DMSO). Data are represented as the median (IQR) with p-values from a Wilcoxon-signed rank test. CI= Chemotactic Index

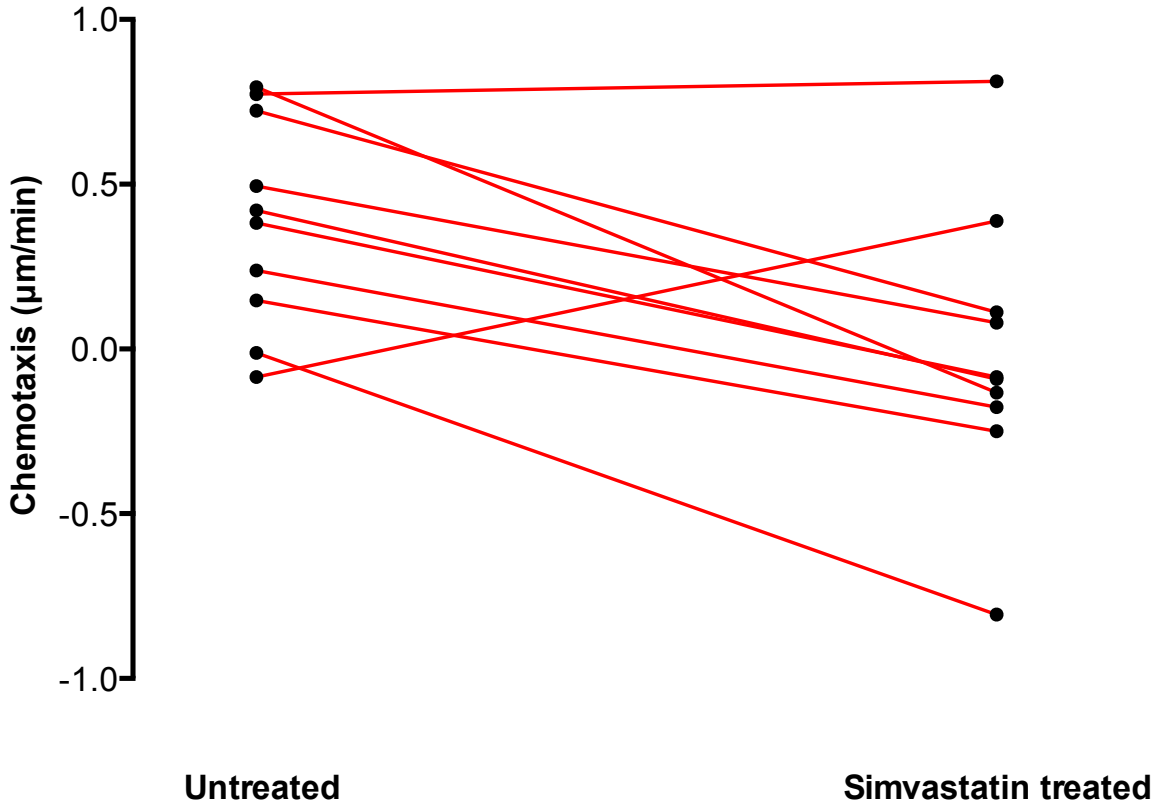


Figure 4.4 The effect of in-vitro simvastatin on the chemotaxis of neutrophils from sepsis patients towards fMLP.

The effect of in-vitro simvastatin therapy on neutrophils chemotaxis towards 10nM N-formylmethionine-leucyl-phenylalanine (fMLP) in 10 sepsis patients. Each line represents the chemotaxis of neutrophils from an individual patient in an untreated state and following incubation for 40minutes with simvastatin 1µM.

4.3.6. Neutrophil Adherence

The adherence of neutrophils from patients with sepsis was increased compared to healthy-aged controls in the neutrophils basal state (92% {IQR 89-100%} vs. 81% {IQR 74-83%) p<0.001 Mann-Whitney U test), and when CXCL-8 (94.1% {IQR 93-100%) vs. 92% {IQR 86-95%) p=0.022 Mann-Whitney U test) and fMLP (100% (IQR 97-100%) vs. 87% {IQR 82-92%} p<0.001 Mann Whitney U test) were used as chemoattractants (see figure 4.5).

In-vitro simvastatin incubation reduced the adhesion of neutrophils to the albumin-coated cover slip, replicating the in-vivo data from Chapter 3. Analysis of individual slides demonstrated reduced adhesion of neutrophils treated with simvastatin both when CXCL-8 (83.8% {IQR 72.8-91.7%} vs. 94.7% {IQR 92.1-98.6%} p=0.027 Wilcoxon signed rank test, N=10) and fMLP (91.7% {IQR 84.5-100%} vs. 100% {IQR 97.4-100%} p=0.016 Wilcoxon signed rank test, N=10) were used as chemoattractants (see figure 4.5).

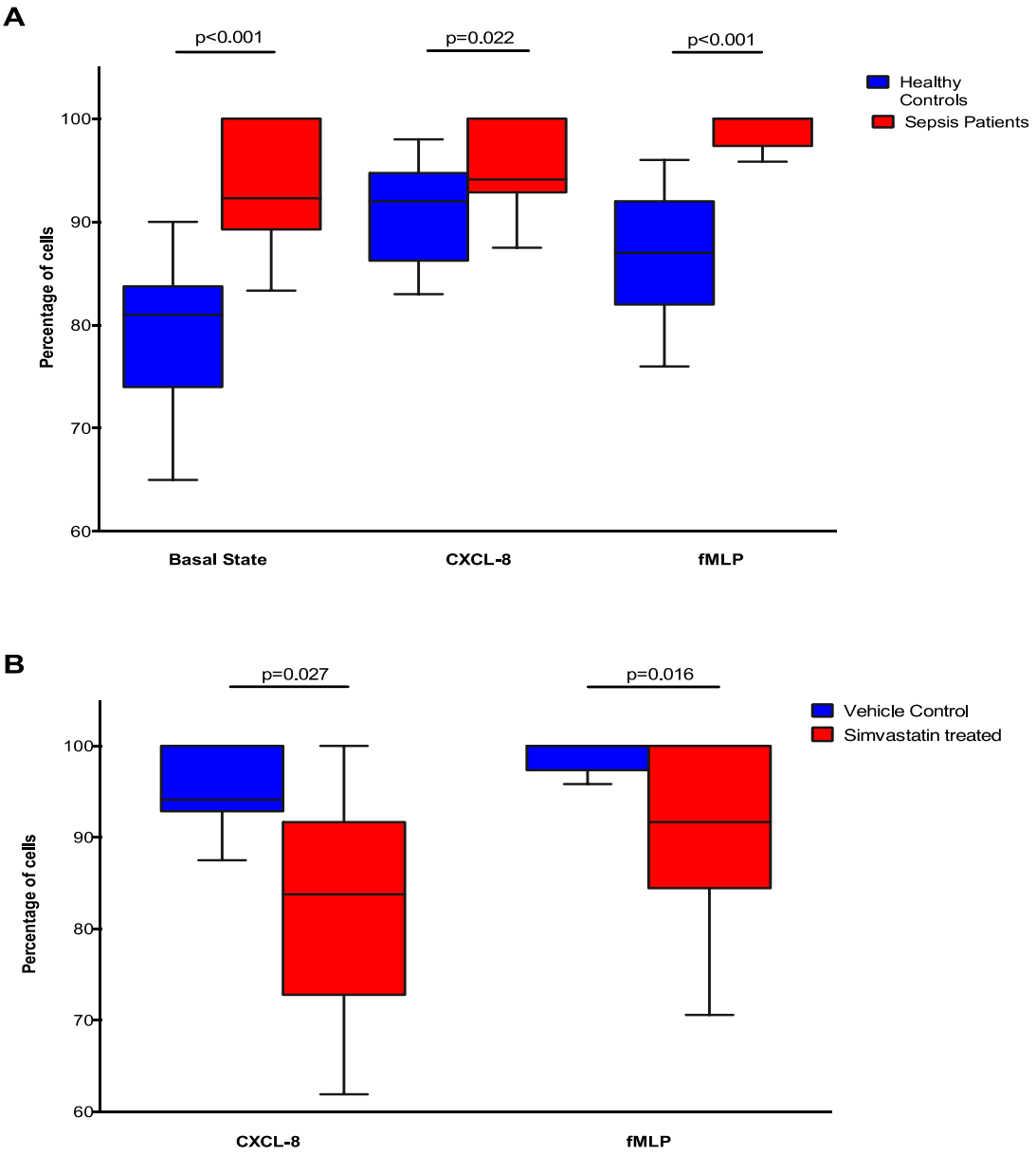


Figure 4.5. Neutrophil adherence in patients with sepsis.

A shows the percentage of neutrophils adherent to the albumin coated cover slip in 19 healthy controls and in patients with sepsis (sepsis N=16 in basal state, N=14 in CXCL-8 and N-15 in fMLP. B shows the effects of in-vitro simvastatin exposure on neutrophil adherence in 10 sepsis patients. The bars represent the median and IQR with the error bars from a Tukey distribution. p-values from a Mann-Whitney U test. CXCL-8: Interleukin-8, 100nM. fMLP = N-formylmethionine-leucyl-phenylalanine, 10nM.

4.3.7. Cell surface receptor expression in neutrophils

The data above have shown that in sepsis neutrophil migration towards common chemoattractants is significantly reduced and that as sepsis resolves no improvement in migration is observed. Statins did not improve migration towards CXCL-8 and may further impair directional migration in neutrophils towards fMLP. Adhesion of neutrophils was increased in sepsis with in-vitro simvastatin reducing overall adherence.

To explore the potential mechanisms underlying this impaired migration the cell surface receptor expression of neutrophils in sepsis was investigated. The expression of the adhesion molecules L-Selectin (CD62L), Beta-2-Integrin (CD11b/CD18) and the adhesion/activation receptor CD63 were investigated in 17 healthy controls and 14 patients with sepsis. (See figure 4.6).

4.3.7.1. L-Selectin expression in sepsis.

The number of neutrophils expressing L-Selectin was significantly reduced in sepsis patients compared to healthy controls (77.3% {IQR 55.5-93.3%} vs. 94.4% {IQR 87.1-98.3%} p=0.047, Mann-Whitney U test). The number of receptors being expressed by neutrophils (represented by the MFI) was lower in the sepsis cohort, however this failed to reach significance (19792 AFUs {IQR 9279-29913 AFUs} vs. 27731 AFUs {IQR 22893-39937AFUs} p=0.116, Mann-Whitney U test).

4.3.7.2. Beta-2-Integrin expression in sepsis

Beta-2-Integrin is composed of the receptors Cd11b and CD18. Neutrophils broadly express Cd11b/CD18 and this was unchanged in sepsis with virtually all neutrophils

expressing CD11b (97.6% {IQR 84.2-99.3%} vs. 96.9% {IQR 68.7-99.7%}, p=0.914, Mann-Whitney U Test) and CD18 (99.6% {98.9-100%} vs. 99.8 {99.6-99.9}, p=0.230, Mann-Whitney U Test). The MFI was significantly greater in sepsis for CD18 indicating greater receptors expression per cell in sepsis compared to healthy controls (36618 AFUs {IQR 21759-52923 AFUs} vs. 21492 AFUs {IQR 13344-24453 AFUs}, p=0.023, Mann-Whitney U test). The MFI for CD11b was unaffected by sepsis in relation to levels of expression in healthy controls (7868 AFUs {IQR 7274-8654 AFUs} vs. 8479 AFUs {IQR 7389-9438} p=0.374, Mann-Whitney U test).

4.3.7.3. Expression of the activation marker CD63.

The percentage of neutrophils expressing the activation and adhesion marker CD63 was greater in sepsis patients compared to the healthy controls (77.1% {IQR 53.9-94.2%} vs. 45.5% {IQR 29.1-68.5%} p=0.040, Mann Whitney U test). Despite a greater percentage of cells expressing CD63 a trend towards reduced receptor expression was observed in sepsis compared to the healthy controls (3869 AFUs {IQR 2111-4571} vs. 5109 AFUs {IQR 3403-5581}, p=0.052, Mann-Whitney U Test).

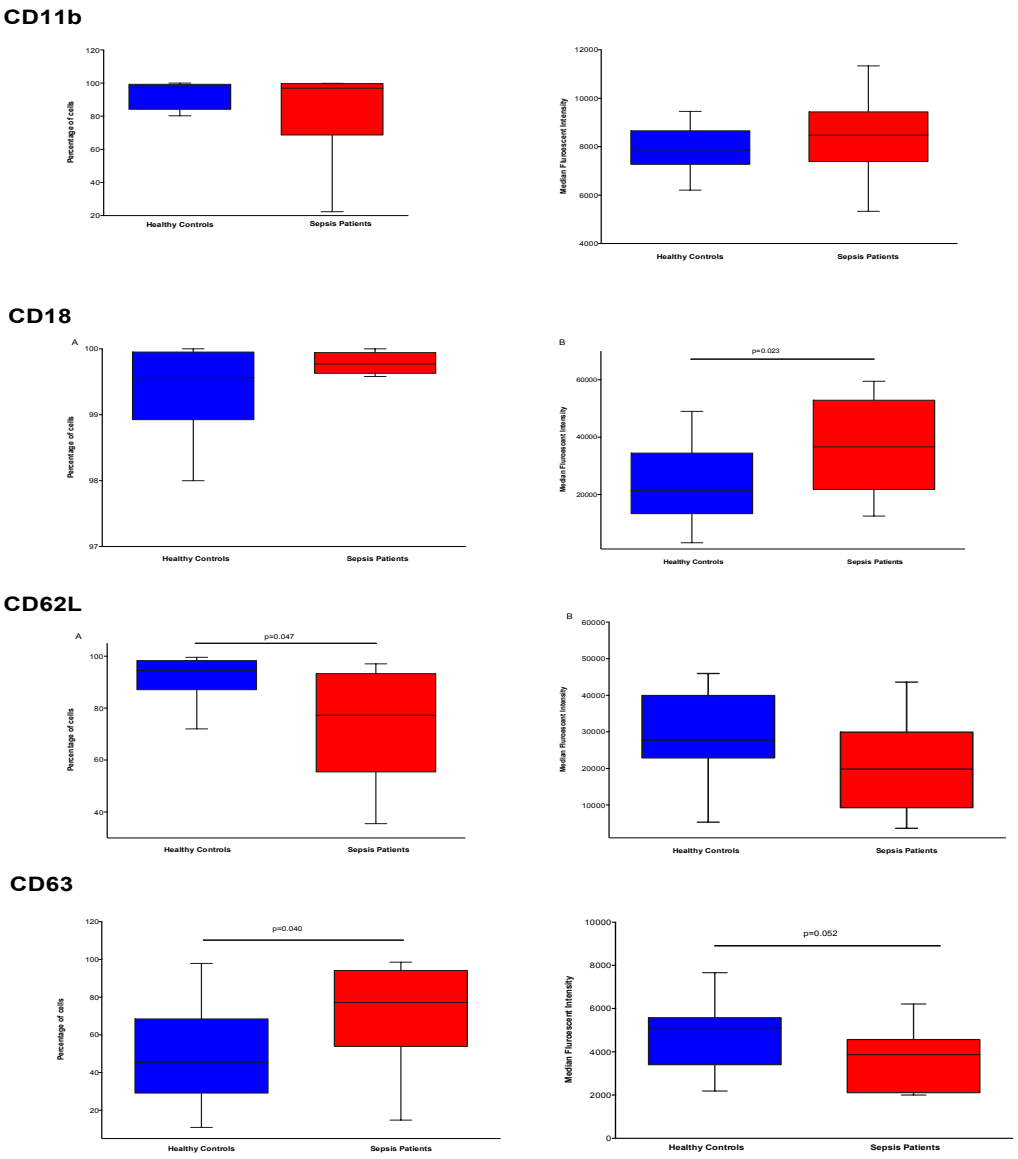


Figure 4.6. The cell surface expression of chemokine and adhesion receptors on neutrophils from healthy controls and sepsis patients.

A shows the percentage of neutrophils expressing the relevant named cell surface receptors and B shows the median fluorescent intensity of the expression and represents the overall number of receptors. The box-whisker plots represent the median and IQR with the whiskers a Tukey distribution with p-values from a Mann-Whitney U test. N=17 for the healthy controls and 14 in the sepsis cohort.

4.4. Discussion

The ability of neutrophils to migrate to sites of sepsis is of critical importance to enable the phagocytosis and killing of invading bacteria. The consequences of impaired neutrophil migration are thought to allow the rapid replication and dissemination of pathogens, promote an exaggerated host response, which can, eventually lead to the death of the host. Previously, Sapey el al have shown that healthy ageing is associated with a functional decline in the migratory dynamics of neutrophils suggesting poor neutrophil function contributes to the increased incidence of sepsis and the poorer outcomes in this group (3, 78, 148, 159).

Neutrophils from sepsis patients caused a further deterioration in neutrophil migration resulting in a state of neutrophil “paralysis” which persisted despite sepsis resolution. Neutrophil “paralysis” resulted from reduced chemokinesis (speed in any direction) and a reduction in directional migration (chemotaxis and chemotactic index). Based on results from Chapter 3, in-vitro simvastatin therapy was used to pharmacologically modulate the migratory dynamics of neutrophils in sepsis, however these experiments suggest that in-vitro simvastatin therapy during sepsis does not improve migration and may further impede it.

Neutrophil “paralysis” in sepsis has been previously reported with the findings here confirming them. However, this is the first study to replicate these using time-lapse video microscopy to determine the alterations that occur in the migratory dynamics of neutrophil in sepsis. The Insall Chamber used in these experiments allows individual neutrophils to be tracked over time providing greater information compared to the

modified Boyden chambers used in other studies that merely provides data on numbers of cells that cross an artificial endothelial barrier (2, 154-156, 301).

The sequential analysis of neutrophil migration was limited due to a small sample size, especially for migration towards fMLP. However the analysis indicated that despite physiological and biochemical improvements in patients observations neutrophil function remained impaired with similar migratory dynamics observed on admission and by day 7. In the small number of non-resolvers from sepsis (N=3) sequential analysis showed a worsening of neutrophil chemotaxis, which may be contributing to the failure of sepsis resolution. Placing these results together, it seems probable that in sepsis neutrophil migration is inhibited by the primary inflammatory response, but that this persists beyond the initial early recovery phase. The exact mechanism for this are unclear, however it could be that a new pool of "healthy" circulating neutrophils is required to be generated by the bone marrow, and the septic process impairs this. The initial bone marrow response to an inflammatory stimulus is the release of mature neutrophils into the circulation. However with persistent inflammation the effectiveness of the bone marrow to adequately meet demand is reduced, with immature cells being released. On going inflammation may cause bone marrow suppression and this may account for the poor resolution of neutrophil migratory dysfunction observed in these experiments. Perhaps an additional assessment of neutrophil migration in survivors following complete resolution of the inflammatory process would have demonstrated significant improvements in migration(109, 128, 324-326).

An alternative explanation could be that patients who develop sepsis may have pre-existing aberrant neutrophil migration predisposing them to developing severe infections and the changes may perhaps just reflect this, with poor migration at baseline and on sequential analysis.

To explore the mechanisms contributing to altered migration neutrophil adherence, the expression of the adhesion molecules CD18, CD11b and CD63 along with the L-selectin (CD62L) were quantified. Overall the data suggested increased neutrophil adhesion with corresponding increases in the expression of the CD18 and CD63. No change in CD11b was seen, with it being broadly expressed in neutrophils from healthy controls and in patients with sepsis.

Neutrophil adhesion is an integral step in the transmigration of neutrophils from blood into tissues. Adhesion is mediated by integrins that bind to ICAM-1 and 2 on the endothelium. Integrins consist of an alpha and beta chain with CD18 being the common beta chain that binds with the alpha chains CD11a to form the LFA-1 or to CD11b to form MAC-1. Both these integrins (LFA-1, MAC-1) bind ICAM-1 to facilitate neutrophil adhesion and subsequently mediate crawling. The migration model used in these experiments uses a human albumin coated coverslip that acts as a surrogate for ICAM-1, thus any changes seen in neutrophil migration and adhesion would reflect altered LFA-1 or MAC-1 expression by neutrophils (19, 22, 24, 25).

The significant rise in CD18 expression suggests an overall increases in adhesion mediated by LFA-1 and MAC-1. Recent studies propose that LFA-1 mediates firm

adhesion to the endothelium whilst MAC-1 mediates crawling and transmigration. Therefore the increased adhesion and the corresponding rise in CD18 expression, with no increase in Cd11b may indicate a preference for LFA-1 up-regulation in sepsis leading to increased neutrophil adhesion at the expense of MAC-1 mediated crawling and hence account for the reduction in migration observed in these experiments (20, 327).

Increased neutrophil adhesion combined with migratory failure could lead to an accumulation of activated neutrophils in micro-vascular beds. These aggregated neutrophils may occlude vascular beds potentially causing distal hypoxia, release ROS and granules leading to local and distal tissue damage precipitating organ dysfunction in sepsis. This is postulated as a mechanism whereby migratory failure of neutrophils contributes to the pathophysiology of sepsis and MOF (3).

L-Selectin is involved in the capturing free flowing neutrophils to the endothelium and their slowing prior to firm adhesion. The reduction in L-Selectin has been previously documented with experiments showing that sepsis causes shedding of L-selectin possibly mediated via increased TNF-alpha converting enzyme (TACE) activity. Experimental models of sepsis have demonstrated that increased sepsis severity is associated with increased L-selectin shedding, suggesting that this as a contributor factor in reduced migration due to failure of endothelial-neutrophil interactions. If taken in conjunction with increased adhesion and reduced chemokine receptor expression (CXCR-2), it can be postulated that neutrophils fail to adequately respond

to the chemoattractants leading to migratory failure and thus aggregate and occlude vascular beds due to their increased propensity to adhere (166, 328-334).

In-vitro simvastatin therapy did not improve the migratory dynamics of neutrophils as predicted but did reduce neutrophil adherence. Studies in patients with acute lung injury (ALI) and in CLP models of sepsis have demonstrated reduced neutrophil infiltration into pulmonary and peritoneal tissues with statin therapy. Reduced infiltration was associated with a reduction in the inflammatory response and an improved survival in the ALI cohort. Reduced neutrophil infiltration with statins has been attributed to reduction in LFA-1, MAC-1 and their endothelial ligand ICAM-1 and would explain the decrease in adherence seen in these experiments. Neutrophils from the sepsis patients can become overwhelmed by the “cytokine storm” resulting in a desensitisation to chemoattractants and the internalisation of chemokine receptors, perhaps accounting for the poor response of simvastatin therapy in established sepsis on neutrophil function. Emerging evidence suggests that the activation of the PPAR-gamma system is involved in inhibiting migration in sepsis. Statins up-regulate PPAR expression and this may partially explain abrogation of migration with statins seen in these experiments and those previously reported (150, 152, 253, 254, 280, 284, 312, 313, 315).

The incubation period of 40minutes was chosen based on healthy control data demonstrating this as the optimal time frame for chemotaxis to be improved. The lack of effect of in-vitro simvastatin in the sepsis cohort may be related to this, however

the reduction observed in adhesion would suggest that simvastatin was biological effective at the dose and incubation time chosen.

There is compelling evidence that effective neutrophil migration is required in infection and that sepsis impairs migratory function. The exact mechanism of sepsis-induced migratory failure is complex, with excess cytokine and NO production, altered expression of chemokine receptors, adhesion receptors and TLR, as well as changes in actin assembly all being implicated. Therapies aimed at improving chemotaxis in experimental sepsis have demonstrated increased influx of neutrophils to sites of infection, an exaggerated pro-inflammatory response and bacterial clearance within these sites and an attenuated systemic response, suggesting that improved migration should improve sepsis outcomes. In contrast many clinical studies suggest that reducing neutrophil influx is beneficial as this attenuates both local and systemic inflammatory responses and therefore improves survival. Perhaps, effective early migration to infected sites is optimal to allow effective elimination and containment whilst in sepsis reducing migration may prevent exacerbating tissue injury and thereby improve outcomes (2, 148, 154-156, 159, 160, 165-167).

In conclusion, the experiments above used time-lapse video-microscopy for the first time to demonstrate neutrophil “paralysis” in sepsis patients. This may be mediated by an increase in LFA-1 expression leading to increased cell adherence but reduced migration. The incubation of sepsis neutrophils with therapeutic concentrations of simvastatin did not improve the migration of “paralysed” neutrophils but did reduce

their adherence. This reduced adherence may lead to reduced micro-vascular occlusion by aggregated neutrophils and may explain the improvements in organ and endothelial function observed with statins in sepsis.

Chapter 5: Neutrophil phagocytosis in sepsis and the potential for in-vitro simvastatin exposure to modulate phagocytosis.

5.1. Introduction

Once neutrophils have migrated to the sites of migration they must eliminate the invading pathogens. This is achieved through the ingestion of pathogens via phagocytosis and intracellular killing by the release of granules into the phagolysosome. The mechanism of phagocytosis is described in detail in section 1.1.2.2 (38-40, 335).

The efficiency of phagocytosis is impaired with age, with neutrophils ingesting fewer bacteria and this impairment has been proposed as a mechanism leading to the poorer outcomes during infective episodes observed in the elderly (81, 87, 88, 336, 337). In sepsis phagocytosis is poorly characterised, with both suppressed phagocytosis and enhanced phagocytosis being reported. Like with many neutrophil functions a balanced response under tight homeostatic control would be ideal, as poor phagocytosis leads to the rapid dissemination of the infection. Increased phagocytosis, if contained within the cell with effective maturation of the phagolysosome should eliminate bacteria faster and theoretically lead to earlier resolution of infection. However cells can be activated but unable to ingest and intracellularly kill bacteria effectively (termed frustrated phagocytosis) leading to collateral tissue damage via neutrophil degranulation and ROS production (38, 169, 173, 179, 338-340).

Phagocytosis is dependent on the recognition of pathogens and related products by a variety of cell surface receptors. Some key receptors are CD16 (FcγRIIIb) and CD14 (LPS-receptor). Additional receptors that are closely linked with phagocytosis

are CD63, a marker of degranulation into the phagolysosome and CD11b (CR3), which binds C3a to induce phagocytosis (40, 341-343)

Observational data demonstrates that statins (HMG-CoA reductase inhibitors) improve outcomes in infection and sepsis, however recent randomised controlled trials have failed to demonstrate any clear benefit of simvastatin therapy in acutely ill patients. In-vivo 80mg simvastatin showed a trend towards improved phagocytosis of *E.Coli* in the healthy elderly with no changes in the phagocytosis of *S.Aureus* (Chapter 3). It is unclear whether statin therapy would modulate neutrophil phagocytosis in sepsis, which could lead to the improved outcomes observed seen in some studies (237, 245, 277, 309, 310).

5.1.1. Hypothesis

It was hypothesised that phagocytosis would be:

- Impaired with age
- That sepsis would cause a further decline in phagocytosis
- That in-vitro simvastatin therapy would enhance neutrophil phagocytosis.
- That neutrophils would show reduced expression of cell surface receptors indicating an immunosuppressed phenotype.

5.1.2. Aims

The aims of this study were to:

1. Characterise the effects of age on neutrophil phagocytosis.
2. Characterise neutrophil phagocytosis in a population with sepsis

3. Assess whether in-vitro simvastatin could modulate neutrophil phagocytosis in sepsis.
4. Phenotype neutrophils from healthy controls and sepsis patients by cell surface expression of CD16, CD11b, CD63 and CD14 on neutrophils to reveal potential mechanisms of altered phagocytosis in sepsis.

5.2. Methods

5.2.1. Healthy control recruitment

Healthy young controls were recruited into the study as described in section 2.2. The baseline data from healthy elderly subjects recruited into the statin trial described in chapter 3 were also used as controls.

5.2.2. Patient recruitment

Patients with sepsis were entered into the trial as described in section 2.3. The SSCG definitions for sepsis were used to screen eligible patients (see tables 1.4 and 1.5). Exclusion criteria were as described in section 2.3.1.

5.2.3. Data collected

Data collected is described in section 2.3.2.

5.2.4. Sample collection

Blood samples for the phagocytosis experiments were taken in Lithium heparin vacutainers (Becton Dickinson) as described in section 2.4.

5.2.5. Neutrophil isolation

Neutrophils were extracted using a Percoll gradient as described in section 2.5.3.

5.2.6. Phagocytosis experiments

Phagocytosis experiments were performed using a flow cytometer and fluorescent labelled *E.Coli* and *S.Aureus* (pHrodo; Invitrogen) according to the manufacturers instructions as detailed in section 2.6.3.

Phagocytosis was measured as the percentage of pHrodo "bright" neutrophils and the median fluorescent intensity (MFI) representing the number of bacteria phagocytosed. The phagocytic index (PI) was calculated by multiplying the percentage of fluorescent positive neutrophils by the MFI. This provided an overall quantification of phagocytic capacity. The AUC for each parameter was calculated as described in section 2.6.3.

5.2.7. Simvastatin Experiments

Freshly isolated neutrophils were incubated with 1μM simvastatin for 40minutes prior to phagocytosis being assessed as described in section 2.7. All neutrophil assays were performed un-blinded.

5.2.8. Cell surface receptor expression experiments

The expression of the cell surface receptors CD16, CD11a, CD14 and CD63 were quantified from whole blood using flow cytometry analysis. The detail methods are described in section 2.6.6.

5.2.9 Statistical Analysis

Continuous data were tested for normality using a Shapiro-Wilk's test, however if sample sizes were too small a Kolmogorov-Smirnov test. All continuous data are represented as median (IQR), with non-parametric statistical tests performed as

described in section 2.9. Where two or more were analysed using a Kruskal-Wallis test, Dunn's post-hoc tests were performed to identify the nature of the statistical differences between the groups. Categorical data were analysed using a Fisher's exact test for two groups and a χ^2-test for more than two groups. Correlations were performed using a Spearman correlation test, with results represented as the correlation co-efficient and the 95% confidence interval.

5.3. Results

5.3.1. Healthy Controls

Twenty-four healthy controls had their neutrophils characterised for phagocytosis to un-opsonised pHrodo labelled *E.Coli* and *S.Aure*us (Invitrogen). The median age of the healthy controls was 68 (range 22-94) with 12 males and 12 females.

5.3.2. Age and Phagocytosis

Within the healthy controls there was no correlation between the subjects' age and phagocytosis of *E.Coli* as measured by percentage of pHrodo "bright" cells, the MFI, or the PI (see table 5.1). Healthy controls were further divided into young (age <45years) and old (age >60) cohorts. No difference between young and elderly cohorts was observed for *E.Coli* phagocytosis for pHrodo "bright" neutrophils (AUC: 2665 {IQR 2472-2837} vs. 2533 {IQR 2256-2722} p=0.262 Mann Whitney U test), MFI (1265 {IQR 838-2032} vs. 1141 {IQR 614-2013} p=0.625, Mann Whitney U test) or PI (1172 {IQR 662-1911} vs. 928 {IQR 503-1852} p=0.473, Mann Whitney U test) (see figure 5.1).

When *S.Aureus* was used, there was an age-related decline in phagocytosis with a significant negative correlation for the MFI (Spearman's correlation, p=0.032) and PI (Spearman's correlation, p=0.020). No changes related with age were seen for the pHrodo "bright" neutrophils (see table 5.1).

When divided into young (age <45years) and elderly healthy cohorts (age >60), age was associated with a decline in phagocytosis. The MFI (1638 {IQR 900-3175} vs. 2892 {IQR 2421-4180} p=0.026, Mann Whitney U test) and the PI (1550 {IQR 723-2827} vs. 2729 {IQR 2189-3893} p=0.022, Mann-Whitney U) were significantly lower in the elderly. The number of pHrodo "bright" neutrophils was reduced in the elderly but this failed to reach significance (2662 {IQR 2431-2862} vs. 2798 {IQR 2701-2892} p=0.077, Mann Whitney U test. See figure 5.1.)

5.3.3. Gender and Phagocytosis

Gender impacts upon outcomes from sepsis, with females less likely to die compared to age-matched males. Therefore the effect of age upon neutrophil phagocytosis was investigated(106). No difference was seen between neutrophils from healthy male and female donors to *E.Coli* or *S.Aureus* (see table 5.2).

Table 5.1. Correlation between age and phagocytosis of pHrodo labelled (Invitrogen) *E.Coli* and *S.Aureus*

	R	95% CI	p-value*
E.Coli			
pHrodo "bright"	-0.329	-0.648-0.088	0.108
MFI	-0.169	-0.538-0.254	0.419
PI	-0.250	-0.596-0.173	0.227
S.Aureus			
pHrodo "bright"	-0.341	-0.662-0.085	0.103
MFI	-0.438	-0.721- -0.023	0.032
PI	-0.473	-0.742- -0.073	0.020

Correlations between age and the three measures of phagocytosis, the "pHrodo" bright neutrophils, median fluorescent intensity (MFI) and the phagocytic index (PI). The co-efficients (r) and p-values are from a Spearman's correlation. The 95% confidence interval (CI) is shown for each correlation.

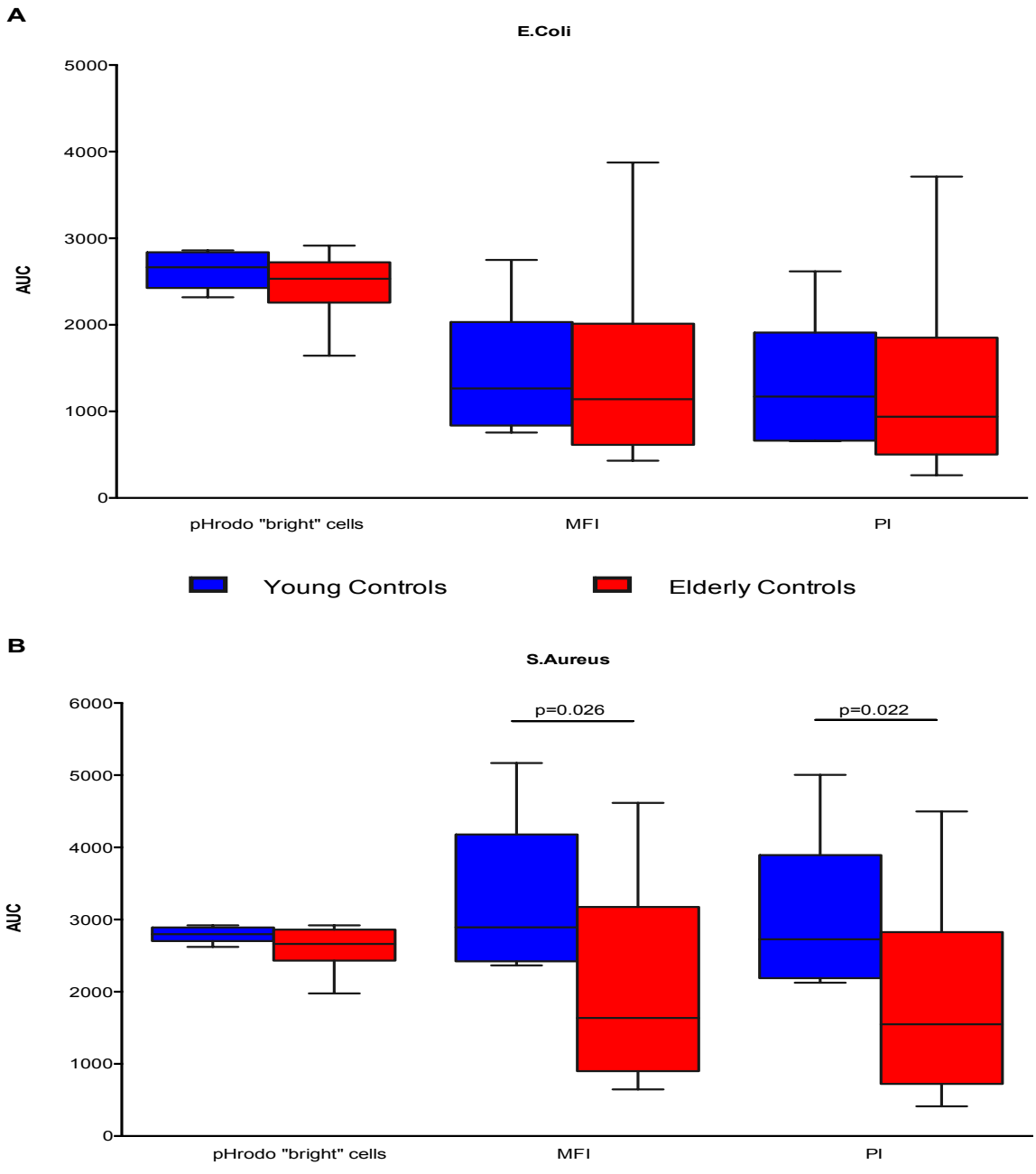


Figure 5.1. Differences in phagocytosis between young and elderly controls.

The graphs show the differences in the neutrophil phagocytosis of young controls (age less than 45 years, N=7) and elderly controls (age greater than 60, N=17). The three indices used are the pHrodo “bright” neutrophils, the median fluorescence intensity (MFI) and the phagocytic index (PI) and are represented as the area under the curve (AUC). A shows *E.Coli* phagocytosis and B *S.Aureus* phagocytosis. The bars represent the median and IQR with the whiskers representing a Tukey distribution. p-values from a Mann-Whitney U test.

Table 5.2. Neutrophil phagocytosis of healthy elderly controls split by gender to pHrodo (Invitrogen) labelled *E.Coli* and *S.Aureus*.

	Males N=12	Females N=12	p-value*
E.Coli			
pHrodo "bright"	2597 (2387-2768)	2542 (2445-2752)	0.875
MFI	1276 (710-2279)	1349 (816-2124)	0.458
PI	1045 (485-2126)	1171 (658-1897)	0.525
S.Aureus			
pHrodo "bright"	2713 (2610-2892)	2691 (2562-2872)	0.743
MFI	2692 (764-3401)	2295 (1622-3068)	0.786
PI	2380 (647-3278)	2019 (1356-2839)	0.718

Measures used to quantify phagocytosis were the pHrodo "bright" neutrophils, the median fluorescence intensity (MFI) and the Phagocytic Index (PI). All data is expressed as area under the curve (AUC) with values representing the median (IQR) and with p-values from a Mann-Whitney U test.

5.3.3. The impact of sepsis on phagocytosis

The experiments reported above suggest that increasing age is associated with a decline in the phagocytosis of un-opsonised *S.Aureus* by neutrophils. Gender did not impact upon the neutrophil phagocytosis of *E.Coli* or *S.Aureus*. The studies below investigated the effect of sepsis on the neutrophil phagocytosis of these bacteria.

Fifteen of the recruited sepsis patients had phagocytosis experiments carried out on their neutrophils. The demographics are shown in table 5.3. The sepsis and healthy controls were well matched for age and gender. The majority of patients recruited (12 {80%}) met the criteria for severe sepsis with pneumonia being the commonest source of sepsis (9 {60%} patients), followed by urinary tract infections (3 {20%} patients). Five patients (33%) were admitted to ICU. Mortality was 33.3% (5 deaths) with a median length of hospital stay of 9 days (range 5-57).

Table 5.3. Characteristics of healthy controls and sepsis patients that had neutrophil phagocytosis experiments performed.

	Controls N=24	Sepsis N=15	p-value
Age	68 (33-72)	57 (32-74)	0.502*
Sex, male (%)	12 (50%)	9 (60%)	0.742[#]
No of Co-morbidities			
0	18	5	
1	6	7	
2	0	3	0.012[Φ]
Co-morbidities[§], n			
None	18	6	
CVS disease	6	6	
Respiratory disease	0	1	
Chronic renal disease	0	0	
Diabetes	0	4	0.039[Φ]
Medications[§], n			
None	18	5	
Anti-hypertensive	6	6	
Beta-blocker	1	3	
Anti-platelet	0	1	
Oral hypoglycaemic	0	2	
Insulin	0	3	
Inhaled beta-agonist	0	1	
Statins	0	5	0.005[Φ]
APACHE II		11 (5.8-32.2)	
SAPS II		33 (13-40)	
ICU Admission, N		5 (33.3%)	
Inotropes alone		1	
Respiratory support		1	
Multi-organ support		3	
WCC		14.4 (12.3-22.6)	
CRP		222 (92-407)	
SOFA Score		6 (2-9)	
Length of Stay,		9 (6-25)	
Mortality, n (%)		5 (33.3%)	

Values represent the median (IQR) unless otherwise stated. * Mann-Whitney U test, [#]Fisher's exact test and [Φ] χ^2-test. [§] Patients may have more than one co-morbidity and be taking more than one medication.

5.3.3.1. Sepsis increases the phagocytosis of *E.Coli* by neutrophils

Neutrophils donated from patients with sepsis showed significant up-regulation of un-opsonised *E.Coli* phagocytosis compared to healthy elderly controls (see table 5.4 and figure 5.2). The percentage of pHrodo "bright" neutrophils was unchanged at either time point, however the number of bacteria phagocytosed per neutrophil (represented by the MFI) was significantly higher at all three times points, resulting in a greater PI (overall measure of phagocytosis) for *E.Coli* in sepsis patients.

5.3.3.2. Sepsis does not alter the neutrophil phagocytosis of *S.Aureus* bacteria.

Donor neutrophils from sepsis patients did not display any differences in the phagocytosis of un-opsonised *S.Aureus* compared to healthy controls in any of the three indices of phagocytosis (see table 5.4 and figure 5.2). This suggests that sepsis increases the capability of neutrophils to ingest un-opsonised gram-negative bacteria but not gram-positive bacteria.

5.3.3.3. The effect of age on neutrophil phagocytosis in sepsis.

An age-related decline was observed in healthy controls. To assess the impact of age on the phagocytosis of neutrophils in sepsis a correlation was performed between age and markers of neutrophil phagocytosis. No association was seen between age and the neutrophil phagocytosis of *E.Coli* or *S.Aureus*. (See table 5.5).

Table 5.4. Phagocytosis of pHrodo (Invitrogen) labelled *E.Coli* and *S.Aureus* by neutrophils from healthy controls and sepsis patients.

	Healthy N=24	Sepsis N=15	p-value
E.Coli			
pHrodo "bright" cells	2547 (2419-2747)	2439 (1943-2781)	0.554
MFI	1161 (693-2013)	2124 (1772-4412)	0.001
PI	1012 (607.4-1852)	1926 (1263-3619)	0.008
S.Aureus			
pHrodo "bright" cells	2693 (2611-2870)	2664 (1864-2825)	0.180
MFI	2365 (1171-3267)	3000 (1980-3629)	0.227
PI	2125 (958-3062)	2606 (1153-3284)	0.676

Measures used to quantify phagocytosis were the number of pHrodo "bright" neutrophils, the median fluorescence intensity (MFI) and the Phagocytic Index (PI) and all are expressed as area under the curve (AUC) with values representing the median (IQR) and with p-values from a Mann-Whitney U test.

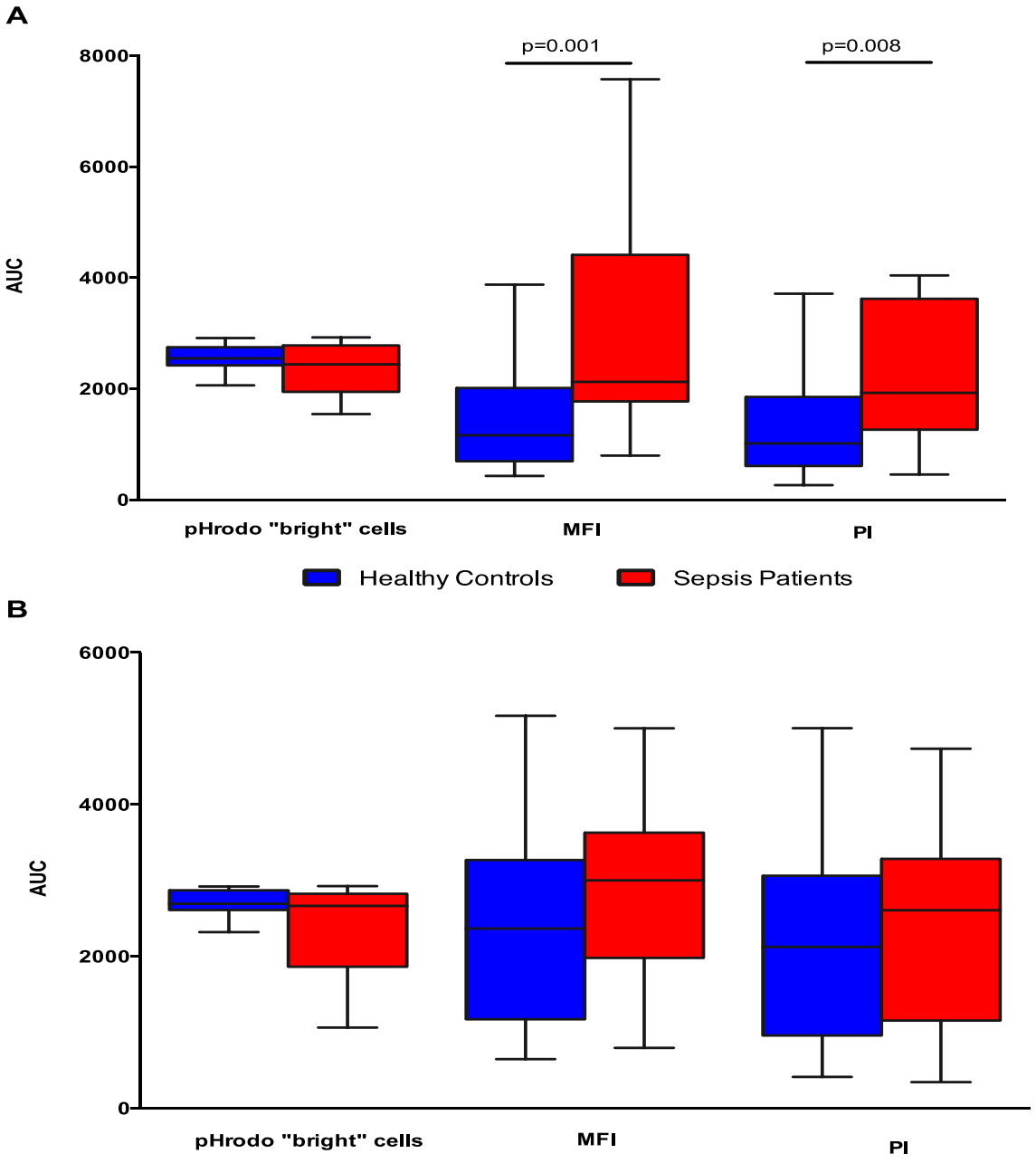


Figure 5.2. Phagocytosis to pHrodo labelled *E.Coli* and *S.Aureus* in healthy controls and in sepsis patients.

The graphs above show the differences in phagocytosis between healthy controls (N=24) and sepsis patients (N=15). The three indices used are the pHrodo "bright" neutrophils, the median fluorescence intensity (MFI) and the phagocytic index (PI) and are represented as the area under the curve (AUC). A shows *E.Coli* phagocytosis and B *S.Aureus* phagocytosis. The bars represent the median and IQR with the whiskers representing a Tukey distribution. p-values from a Mann-Whitney U test.

Table 5.5. Correlation between age and neutrophil phagocytosis in sepsis patients

	r	95% CI	p-value*
E.Coli			
pHrodo "bright" cells	-0.080	-0.616-0.507	0.788
MFI	-0.267	-0.722-0.349	0.370
PI	-0.275	-0.726- 0.342	0.356
S.Aureus			
pHrodo "bright" cells	-0.013	-0.552-0.534	0.960
MFI	-0.048	-0.576-0.508	0.864
PI	-0.048	-0.576-0.508	0.864

Correlations between age and the three measures of phagocytosis, pHrodo "bright" neutrophils, median fluorescent intensity (MFI) and the phagocytic index (PI). The co-efficients (r) are from a Spearman's correlation. The 95% confidence interval (CI) is shown for each correlation.

5.3.3.4. Neutrophil phagocytosis in patients with sepsis on sequential days.

The results describe enhanced neutrophil phagocytosis of *E.Coli* and unchanged *S.Aureus* phagocytosis in sepsis compared to healthy controls. To assess the changes that occur over time in sepsis, phagocytosis was measured on sequential days (day1, day 4 and day7) where possible.

Patients with sepsis showed improvements in their SOFA score (median SOFA score 6 {IQR 2-9} vs. 0 {IQR 0-4} p=0.045 Wilcoxon signed rank test) and a trend towards a reduction in CRP by day 7 (165 {IQR 60.7-165} vs. 29 {IQR 15.5-152.5} p=0.054 Wilcoxon signed rank test) compared to baseline indicating sepsis resolution. No change in WCC was seen (14.4 {IQR 10.9-22.2} vs. 12.6 {IQR 8.8-19.4} p=0.492 Wilcoxon signed rank test). No change in SOFA score, CRP or WCC was observed at day 4 compared to baseline (see table 5.6). Not all patients had sequential sampling due to patient discharges (N=3), deaths (N=2) and consent withdrawal for venesection (N=1).

No change in the overall number of neutrophils (pHrodo “bright) ingesting *E.Coli* was observed on sequential day analysis. The MFI was significantly greater on all three days a compared to healthy controls (Kruskal-Wallis p=0.005) resulting in a raised PI on all three days compared to healthy controls (Kruskal-Wallis p=0.029). Dunn’s post hoc tests demonstrated a significant difference between healthy controls and the MFI (p=0.006) and PI (p=0.046) on day 1 in sepsis patients. No changes in the phagocytosis of *S.Aureus* were observed with any of the measures of phagocytosis (see table 5.7).

Table 5.6. The SOFA scores, CRP and WCC of patients during the course of sepsis.

	Day 1 N=15	Day 4 N=10	Day 7 N=9	p-value Day 1 vs. 4	p-value Day 1 vs. Day 7
SOFA Score	6 (2-9)	5 (0-8)	0 (0-4)	0.606	0.045
CRP **(mg/l)**	165.0 (60.6-374.3)	130 (69.0-320)	29.0 (15.5-152.5)	0.705	0.054
WCC **(x10^9/l)**	14.4 (10.9-22.2)	17.6 (10.7-27.8)	12.6 (8.8-19.4)	0. 804	0.492

Changes in Sequential Organ Failure Assessment (SOFA) score, C-reactive protein (CRP) and White Cell Count (WCC) between day 1, 4 and 7 in patients with sepsis. Values are median (IQR) and p-values are from Wilcoxon ranked sum test.

Table 5.7. The phagocytosis of pHrodo labelled *E.Coli* and *S.Aureus* (Invitrogen) in healthy controls and in sepsis patients on sequential days.

	Healthy Controls N=24	Day 1 N=15	Day 4 N=11	Day 7 N=9	p-value
E.Coli					
pHrodo "bright" cells	2547 (2419-2747)	2439 (1943-2781)	2491 (2293-2828)	2363 (2203-2607)	0.606
MFI	1161* (693-2013)	2124* (1772-4412)	2011 (1189-3583)	1836 (1419-3503)	0.005
PI	1012* (607.4-1852)	1926* (1263-3619)	1538 (930-3424)	1650 (1178-3344)	0.029
S.Aureus					
pHrodo "bright" cells	2693 (2611-2870)	2664 (1864-2825)	2458 (1963-2712)	2549 (2308-2746)	0.140
MFI	2365 (1171-3267)	3000 (1980-3629)	2407 (1435-4474)	2546 (1582-4642)	0.514
PI	2125 (958-3062)	2606 (1153-3284)	2104 (1142-4054)	2372 (1352-4120)	0.878

Values represent the area under the curve (AUC) and are shown as median (IQR) with p-values from a Kruskal-Wallis. * represents Dunn's post hoc test where $p<0.05$.

5.3.4. In-vitro simvastatin therapy does not affect neutrophil phagocytosis.

Neutrophils from sepsis patients were incubated with 1μM simvastatin for 40minutes prior to the phagocytosis experiments to assess whether statins could modulate neutrophil phagocytosis in sepsis. Simvastatin did not alter any of the parameters used to quantify neutrophil phagocytosis to either *E.Coli* or *S.Aureus* at any time point assessed (see table 5.8).

5.3.5. Surface Expression of phagocyte receptors in healthy controls and sepsis patients.

The experiments above have shown that the phagocytosis of *E.Coli* is enhanced in sepsis and that in-vitro simvastatin therapy does not modulate phagocytosis in these patients. No change in the phagocytosis of *S.Aureus* by neutrophils from sepsis patients was observed. To investigate the potential mechanisms that may increase phagocytosis the surface expression of CD16, Cd11b, CD14 and CD63 in neutrophils from healthy controls (N=17) and sepsis patients (N=14) were evaluated. The healthy controls were significantly younger (33.0 years {IQR 28.0-40.5years} vs. 57.0 years {IQR 32.0-74.0years} p=0.039 Mann Whitney U) with no significant gender differences (9 males vs. 9 males p=1.00 Fisher's Exact test).

Table 5.8. The effect of in-vitro 1μM simvastatin exposure on the phagocytosis of *E.Coli* and *S.Aureus* (Invitrogen) by neutrophils from sepsis patients.

	Vehicle Control N=12	Simvastatin N=12	p-value
E.Coli			
pHrodo "bright" cells	2562 (2330-2813)	2625 (2534-2835)	0.622
MFI	3956 (2473-4661)	3834 (2510-4827)	0.970
PI	3741 (1917-4135)	3472 (1943-3898)	0.970
S.Aureus			
pHrodo "bright" cells	2730 (2345-2859)	2729 (2382-2875)	0.424
MFI	3371 (2403-4571)	3252 (2588-4452)	0.146
PI	2809 (2078-4127)	3042 (2226-3992)	0.151

Measures used to quantify phagocytosis were pHrodo "bright" cells, the median fluorescence intensity (MFI) and the Phagocytic Index (PI). All are expressed as area under the curve (AUC) with values representing the median (IQR) and with p-values from a Wilcoxon ranked sum test. The vehicle control was RPMI 1640 with the equivalent concentration of dimethyl sulfoxide (DMSO).

5.3.5.1. Sepsis reduces CD16 expression on neutrophils

CD16 is also known as FcγIIIB, a receptor that binds to IgG and is involved in the phagocytosis of bacteria by neutrophils. CD16 is expressed avidly by neutrophils and no difference was seen on the percentage of cells positive for CD16 between healthy controls and sepsis patients (95.5% {IQR 90.4-97.0%} vs. 96.1% {IQR 89.4-97.6%} p=0.883, Mann Whitney U test). However the median MFI (a measure of the level of expression per cell) was significantly lower in sepsis patients (77791 AFUs {IQR 68061-111644 AFUs} vs. 59683 AFUs {IQR 40799-73306 AFUs} p=0.014, Mann Whitney U) representing reduced overall expression of CD16 on neutrophils.

5.3.5.2. Sepsis does not alter Cd11b expression on neutrophils.

Cd11b is the receptor for C3a that coats invading pathogens. The binding of C3a to CD11b triggers phagocytosis. The expression of CD11b was unchanged in sepsis patients compared to healthy controls. Almost all neutrophils expressed Cd11b in both the controls and the sepsis patients (97.6% {IQR 84.2-99.3%} vs. 96.9% {IQR 68.7-99.7%} p=0.914 Mann Whitney U) with similar median MFI values (7868 AFUs {IQR 7274-8654 AFUs} vs. 8494 {IQR 7389-9438 AFUs} p=0.374, Mann Whitney U).

5.3.5.3. Sepsis up-regulates CD14 expression in neutrophils

CD14 is the LPS receptor and has been implicated in the phagocytosis of un-opsonised gram-negative bacteria. The number of neutrophils expressing CD14 in sepsis patients is significantly higher than in healthy controls (35.1% {IQR 17.8-49.3%} vs. 4.6% {IQR 3.5-12.3%} p<0.001 Mann Whitney U). No difference was seen in the number of receptors per cell represented by the MFI (2134 AFUs {IQR 1358-15572 AFUs} vs. 2069 {IQR 1696-3247 AFUs} p=0.940, Mann Whitney U).

5.3.5.4. Sepsis up-regulates CD63 expression in neutrophils

CD63 is a marker of granule release and is a marker of neutrophil degranulation into a phagosome. Its expression has been closely linked with phagocytosis. More neutrophils from sepsis patients expressed CD63 compared to healthy controls (77.1% {IQR 53.9-94.2%} vs. 45.5% {IQR 29.1-68.5%} p=0.040, Mann Whitney U), with a trend towards a decrease in the MFI observed between the groups (3869 AFUs {IQR 2111-4571 AFUs} vs. 5109 {IQR 3403-5581 AFUs} p=0.052, Mann Whitney U).

5.4. Discussion

Neutrophil phagocytosis is vital for the effective containment and elimination of bacteria. Advancing age is associated with an increased incidence of sepsis with the elderly having poorer sepsis-related outcomes. It has been proposed that an age-associated decline in phagocytic function contributes to the dissemination of infection and the development of sepsis and MOF (344).

Several studies have demonstrated that although neutrophils retain their ability to phagocytose bacteria, their efficiency is significantly reduced with advancing age. The experiments reported replicate the age-associated decline in phagocytosis with un-opsonised *S.Aureus* but not to un-opsonised *E.Coli*. It is difficult to account for the lack of reduction in phagocytosis with age in *E.Coli* as previous studies have suggested that impairment is seen with both species. Additionally both *E.Coli* and *S.Aureus* infections are associated with a high mortality in the elderly, and it has been suggested that the poor phagocytosis of both species may contribute to this (87, 88, 179, 307, 336, 337, 345-347).

The changes in phagocytosis in patients with established sepsis remain controversial. The data here suggests that in sepsis neutrophils increase their phagocytic capacity towards the gram-negative anaerobe *E.Coli* but not to *S.Aureus* (gram-positive aerobe). Martins et al who demonstrated that patients with severe sepsis and septic shock up-regulate phagocytosis by increasing the number of bacteria ingested per cell support this. There is also evidence to suggest bacterial species may affect phagocytosis dynamics following inflammation with enhanced

E.Coli phagocytosis seen in patients following trauma. In these patients the phagocytosis of *S.Aureus* was unaltered, as in these experiments, but was down regulated to *Klebsiella pneumoniae*. It is suggested that the priming of neutrophils in sepsis by circulating cytokines (e.g. LPS) are responsible for the enhanced phagocytosis in circulating neutrophils, with blockade of these priming mechanisms leading to down-regulation of the phagocytosis (169-171, 173, 348-350).

Other authors have found that phagocytosis is reduced in sepsis suggesting that impairments lead to the dissemination of bacteria and death of the host. Wenisch et al demonstrating that in patients with gram-negative severe sepsis neutrophils showed reduced phagocytosis of *E.Coli* compared to matched healthy controls. Danikas et al also showed reduced neutrophil phagocytosis and dysfunctional monocyte function in sepsis and associated this with an increased mortality from sepsis (338, 344).

Phagocytosis is triggered by the recognition of pathogens by specific receptors. Two important receptors are CD11b and CD16, which recognise bacteria coated with C3 and IgG respectively. In agreement with other studies no difference in CD11b (C3R) was seen between healthy controls and sepsis patients suggesting that altered recognition of complement opsonised bacteria is unaffected in sepsis (87, 351).

As previously demonstrated, the experiments here showed a reduction in CD16 expression in patients with sepsis compared to healthy controls. CD16 expression has been linked to the age-related decline in *E.Coli* phagocytosis. However the

precise role of reduced CD16 expression in inflammation is complex. It is clear that CD16 is intimately involved in phagocytosis with neutrophil activation leading to increased expression of internalised CD16 receptors to the cell surface. This may be counteracted however, by increased shedding of the receptor once it has bound and ingested the pathogens. In addition the neutrophilla induced by sepsis causes the mass release of neutrophils from the bone marrow. These neutrophils are immature and have been characterised to express lower levels of CD16 than fully matured neutrophils. These immature cells are capable of phagocytosis and intracellular pathogen killing, but are less efficient than their mature counterparts. These findings suggest that perhaps the lower CD16 levels seen are due to receptor shedding by phagocytosing neutrophils and the reduced expression seen in immature neutrophils (87, 175, 351).

The LPS receptor, CD14 is an important mediator of the cellular response to sepsis interacting with TLR-4 and 2 to initiate the intracellular signalling response that triggers cytokine release from leucocytes. Recent evidence suggests that CD14 is important in the phagocytosis of un-opsonised gram-negative bacteria. In this study neutrophils from sepsis patients had increased expression of CD14. This may explain the differences observed between *E.Coli* and *S.Aureus* seen in the results, with increased phagocytosis of *E.Coli* being mediated via a CD14 pathway. The relevance of adequate CD14 expression remains under investigation with conflicting results. A murine model of sepsis demonstrated that CD14 knockout mice had an increased susceptibility to gram-negative sepsis and a reduced phagocytosis of *E.Coli* by neutrophils. Blockade of CD14 has also been suggested as a potential therapeutic

target for sepsis, with murine models suggesting an improved survival and attenuated host inflammatory response to caecal ligation in CD14 knockout mice. This study did not investigate phagocytosis but evaluated the host's inflammatory response. Blockade of CD14 may be useful in established septic shock where reducing the host response may be beneficial, but in the early stages of sepsis potential blockade risks bypassing an important mechanism of phagocytosis (342, 343, 352-357).

The mechanism involved in the increased phagocytosis of *E.Coli* by neutrophils in sepsis is likely to be multi-factorial. Taking all the data in context with the current literature, the experiments here suggest that circulating neutrophils adopt a pro-inflammatory phenotype, evidenced by raised CD14 and CD63 receptor expression and this may account for the increased phagocytosis of *E.Coli* (53, 335).

Although levels of phagocytosis of *E.Coli* remained elevated throughout the course of sepsis at days 4 and 7, this was not significant in the post-hoc analysis, suggesting that neutrophils show a trend towards resolution as sepsis resolves. Neutrophils have a short circulating life span and despite the prolonged survival initiated by the initial response to sepsis it is probable a new, younger pool of neutrophils are predominant. It is possible, that due to decreased levels of inflammation these left-shifted show lower levels of activation and thus there phagocytic capacity is comparable to healthy controls.

The in-vivo study on the effects of simvastatin therapy upon healthy elderly neutrophil function reported in Chapter 3 showed a trend towards increased neutrophil phagocytosis of *E.Coli* following simvastatin treatment in the healthy elderly. The in-vitro exposure of neutrophils from patients with sepsis to therapeutically relevant concentrations of simvastatin therapy did not alter neutrophil phagocytosis. The time frame of incubation used was 40minutes and should have been long enough to cause a biological effect, as previous in-vitro studies with simvastatin have demonstrated improvements in neutrophil function within this time. Neutrophil phagocytosis in sepsis seems to be normal if not up regulated in sepsis and therefore additional modulation with statins may not be possible in these circumstances. Importantly, in-vitro simvastatin did not have any deleterious effects upon phagocytosis,

There were several limitations within the experiments carried out here. Firstly un-opsonised bacteria were used to determine the phagocytic efficiency of neutrophils. This does not truly reflect a physiological model as the vast majority of organisms are opsonised by complement or immunoglobulins prior to ingestion. These opsonised mediated phagocytic pathways result in rapid and effective phagocytosis. The mechanisms of un-opsonised phagocytosis in relation to *E.Coli* have been partially explained by a CD14 expression, however the mechanisms involved with *S.Aureus* remain poorly understood. This may explain why no differences in either direction were observed. Secondly, the cell-surface expression of neutrophils was not performed on the same cohort of healthy controls, with the controls being significantly

younger than the sepsis cohort. In addition, neutrophil cell surface expression was not performed sequentially.

In conclusion, the experiments suggest that increasing age was associated with a decline in the phagocytosis of *S.Aureus* and may explain why the elderly are prone to developing serious sepsis from it. In the context of sepsis, the phagocytosis of *E.Coli* by neutrophils was up regulated and this is potentially mediated by the adoption of a pro-inflammatory phenotype via an increase in CD14 and CD63 expression. The use of in-vitro simvastatin did not modulate neutrophil phagocytosis in sepsis.

Chapter 6:Neutrophil extracellular traps in patients with sepsis and the effect of in-vitro simvastatin on their production.

6.1 Introduction

Neutrophils are essential to host defence against invading organisms. They migrate to sites of infection, where they phagocytose bacteria and other pathogens to kill them. The two preceding chapters investigated neutrophil migration and phagocytosis in patients with sepsis, demonstrating aberrant neutrophil migration and a retained phagocytic ability and possible up-regulation of *E.Coli* phagocytosis.

The resolution of inflammation relies on the eventual death of neutrophils and other innate immune cells. Neutrophil extracellular traps (NETs) have recently being described as a novel form of cell death distinct from apoptosis, whereby the extracellular release of nuclear DNA laden with histones and granule products enables neutrophils to continue their antimicrobial functions after their own death (16, 59).

The mechanisms of NETosis have been described in detail in section 1.1.2.4. NETs can be generated by both oxidant-dependent mechanisms that rely on ROS production via NADPH and via oxidant independent pathways. Additionally NETosis is a distinct from apoptotic cell death that needs to be distinguished in experimental studies on NETs. Therefore investigations of NETosis require the evaluation of ROS production, and apoptosis. To clearly investigate potential mechanisms involved the quantification of certain activation markers on neutrophils would be valuable. The expression of CD63, CD45 and CD14 are closely linked with neutrophil activation, degranulation and ROS, whilst CD16 can be used to identify neutrophil maturity (175, 358-360)

The exact role of NETs in health and disease remains under investigation. There is evidence to suggest that an inability to generate NETs results in an increased vulnerability of infection and an exacerbation of sepsis, whilst others suggest that NETosis causes tissue damage and worsens organ dysfunction in inflammation. NETs may also possess some anti-inflammatory properties by neutralising circulating cytokines and chemokines. Emerging evidence in NETs biology has suggested that NET production is impaired with age and may contribute to the increased incidence of sepsis in this group. Whilst in sepsis, the inflammatory mediators in infection are thought to increase the production of NETs both locally and systemically (211, 214, 303, 361-363).

As previously stated, the use of statins prior to the development of infections is associated with improved outcomes. Recent RCTs have failed to prove that the acute administration of statins can improve outcomes in the critically ill. In chapter 3, the oral administration of simvastatin 80mg daily for 14 days did not alter NET production. However, Chow et al's initial data demonstrated that statins increase NET production and they postulated that this may contribute to the mechanism by which statins modulate inflammation (364).

6.1.1. Hypothesis

Based on the current evidence it was hypothesised that:

1. Neutrophils from healthy elderly donor would produce fewer NETs compared to healthy young donors.

2. Neutrophils from sepsis patients would produce greater NETs compared to controls and that resolution of sepsis will result in NET production returning to levels seen in controls.
3. In-vitro treatment with simvastatin would up-regulate NET formation further.
4. ROS production would be up regulated in sepsis and this would correlate with increased NETosis.

6.1.2. Aims

The studies in this chapter aimed to:

1. Establish NET production in health and the impact of healthy aging on NET production.
2. Determine NET production in sepsis patients.
3. Determine any sequential changes in NET production with sepsis resolution.
4. Determine the effect of in-vitro simvastatin therapy upon NET production in sepsis.

To explore the mechanism of any changes observed:

1. ROS production in healthy controls and in patients with sepsis was measured.
2. The effect of in-vitro simvastatin therapy upon ROS production was determined.
3. Apoptosis was measured in healthy controls and in patients with sepsis
4. The expression of CD16, CD45, CD14 and CD63 was measured.

6.2. Specific Methods

6.2.1. Healthy control recruitment

Healthy young controls were recruited into the study as described in section 2.2. The baseline data from healthy elderly subjects recruited into the statin trial described in chapter 3 were also used as controls.

6.2.2. Patient recruitment

Patients with sepsis were entered into the trial as described in section 2.3. The SSCG definitions for sepsis were used to screen eligible patients (see tables 1.4 and 1.5). Exclusion criteria were as described in section 2.3.1.

6.2.3. Data collected

Data collected is described in section 2.3.2.

6.2.4. Sample collection

Blood samples for the NETs and apoptosis experiments were taken in EDTA vacutainers (Becton Dickinson), whilst the experiments for ROS were collected in Lithium Heparin vacutainers (Becton Dickinson) as described in section 2.4.

6.2.5. Neutrophil isolation

Neutrophils were extracted using a Percoll gradient as described in section 2.5.3.

6.2.6. Neutrophil Extracellular Traps Experiments

Freshly isolated neutrophils were stimulated to generate NETs by incubating them within the control media (RPMI 1640 Sigma Aldrich) or in the positive control 25nM PMA (Sigma-Aldrich) as described in section 2.6.4. Following incubation for 3 hours NET production was quantified by the degree of fluorescence measured in a BioTek

Synergy 2 fluorometric plate reader (NorthStar Scientific Ltd, Leeds, UK) with a filter setting of 485nm excitation and 530nm emission. Background fluorescence from unstimulated negative controls was subtracted from the stimulated values.

NET production was measured as arbitrary fluorescent units (AFUs). The greater the degree of fluorescence the higher the AFU value which in turn corresponded with greater NET production.

6.2.7. Simvastatin Experiments

Freshly isolated neutrophils were incubated with 1μM simvastatin (Sigma-Aldrich) at 37℃ with 5% CO_2 for 40minutes (see section 2.7) prior to the NETs experiments as described above (section 6.2.6). All neutrophil assays were performed un-blinded.

6.2.8. Apoptosis Experiments

Apoptosis was measured in freshly isolated neutrophils immediately following isolation, 4 hours and 24hours later. Apoptosis was measured using flow cytometry and staining of the neutrophils with Annexin V (BD) and Sytox Blue (Invitrogen) as detailed in section 2.6.5. The percentage of cells that were alive (negative for Annexin V and Sytox Blue), in early apoptosis (Annexin V positive only), late apoptosis (Annexin V and Sytox Blue positive) and necrotic (Sytox Blue positive only) were recorded.

6.2.9. Statistical Analysis

Continuous data were tested for normality using a Shapiro-Wilk's test, however if sample sizes were too small a Kolmogorov-Smirnov test. All continuous data are represented as median (IQR), with non-parametric statistical tests performed as

described in section 2.9. Where two or more were analysed using a Kruskal-Wallis test, Dunn's post-hoc tests were performed to identify the nature of the statistical differences between the groups. Categorical data were analysed using a Fisher's exact test for two groups and a χ^2-test for more than two groups. Correlations were performed using a Spearman correlation test, with results represented as the correlation co-efficient and the 95% confidence interval.

6.3. Results

6.3.1. NET production in healthy volunteers

NET production was assessed in 37 healthy individuals who acted as the healthy controls for the sepsis patients. The participants had a median age of 62 years (age range 20-94years), with 17 males and 20 females (p=0.642, Fisher's exact test) making up the cohort. Health was defined as individuals that were systemically well and not suffering from any significant chronic or acute illness. Only two of the healthy controls were smokers.

6.3.1.1 Ageing and NET Production

Immunosenescence causes alteration in neutrophil function and is proposed as one of the mechanisms leading to the increased rates of sepsis with advancing age. There is data to suggest NET production is reduced with healthy ageing (303). We assessed NET production in the young and elderly to assess whether immunosenescence altered NET production in our participants, to replicate previously published data.

6.3.1.2. Baseline Characteristics

Twenty healthy elderly (age>60) and seventeen young participants (age<45) had NETs assays performed on extracted neutrophils. The participants were well matched for gender (8 males vs. 10 males, p=0.330, Fisher's exact test). A deliberate difference in age (32 years {28.5-35.5} vs. 70 years {66.3-77} p<0.001; Mann-Whitney U Test) was present.

6.3.1.3. NET formation between young & elderly healthy donors

Neutrophils extracted from healthy elderly donors produce more NETs when stimulated with 25nM PMA (Sigma-Aldrich) compared to healthy young donor neutrophils (50411 AFUs {IQR 41407-65974 AFUs} vs. 37562 AFUs {IQR 29386-41748 AFUs}, p=0.003, Mann-Whitney-U test). Although baseline NET formation to the control media was greater in the elderly controls, this did not reach statistical significance (8679 AFUs {IQR 6312-12114 AFUs} vs. 7085 AFUs {IQR 5341-9712 AFUs}, p=0.217, Mann-Whitney U test).

To assess whether age was a predictor of increased NET production, a Spearman's correlation was performed. This demonstrated no correlation between age and the ability of neutrophils to generate NETs in the control media (r=0.099 95% CI -0.242-0.418, p=0.560) or in response to stimulation with 25nM PMA (r=0.283 95% CI -0.05-0.563, p=0.090).

6.3.1.4. Gender and NET production

Gender has been shown to impact upon survival from sepsis, with females having better outcomes compared to age-matched males. These has been attributed to the protective roles of oestrogen, which enhance innate immunity and the deleterious

role of male androgens in suppressing cell-mediated immunity (106). Therefore the role of gender was explored in regards to NETs from healthy donors. The same cohorts of healthy volunteers described above were used and separate according to gender, with 17 males and 20 females (p=0.642, Fisher's exact test). The two groups were well matched for age (64 years (IQR 33-70years} vs. 66 years {IQR: 32-71 years}, p=0.884, Mann-Whitney U test). No differences in NET production in relation to gender were observed in the control media (7595 AFUs {IQR 5603 vs. 11732 AFUs vs. 8382 AFUs {IQR 5219 vs. 12092 AFUs} p=0.807, Mann-Whitney U test) or to 25nM PMA (41666 AFUs {IQR 30139-56068} vs. 41695 {IQR 32923-52206}, p=0.832, Mann-Whitney U).

6.3.2. The impact of sepsis on NET production

The previous experiments demonstrate no age or gender related effects upon NET formation. The studies below investigate the impact of sepsis upon NET formation using a cohort of twenty-nine age-matched healthy controls. Sepsis patients recruited were well matched for age, however a significantly greater number of males were recruited into the sepsis population (72.5% vs. 44.8% p=0.026, Fisher's exact test). See table 6.1.

Patients with sepsis showed similar baseline NET production compared to healthy controls (7633 AFUs {6149-7633 AFUs} vs. 7587 AFUs {5675-12058 AFUs} p=0.728, Mann-Whitney U). On stimulation with 25nM PMA (Sigma-Aldrich) neutrophils from patients with sepsis produce less NETs compared with healthy controls (28994 {IQR 17613 – 41237} vs. 42475 {IQR 38728-57645} p<0.001, Mann-Whitney U test) on their admission to hospital (see figure 6.1.).

Table 6.1. NET production in age-matched healthy controls and sepsis patients

	Healthy Controls	Sepsis	p-value
N	29	40	
Age	67 (37-73.5)	65 (43.5-74.5)	0.880*
Sex, male (%)	13 (44.8)	29 (72.5)	0.026#
No. of Co-morbidities			
0	23	17	
1	6	14	
2	0	6	
3+	0	3	0.009Φ
Co-morbidities§, n			
None	23	17	
CVS disease	6	13	
Respiratory disease	0	3	
Chronic renal disease	0	2	
Diabetes	0	11	0.002Φ
Medications§, n			
None	23	17	
Anti-hypertensive	6	11	
Beta-blocker	1	6	
Anti-platelet	0	5	
Oral hypoglycaemic	0	7	
Insulin	0	4	
Inhaled beta-agonist	0	3	
Statins	0	15	<0.001Φ
APACHE II		15 (8.3-19.8)	
SAPS II		32 (18.8-42)	
ICU Admission, N		13 (32.5%)	
Inotropes alone		2	
Respiratory support		3	
Multi-organ support		8	
WCC (10^9/L)		16.8 (13.1-22.6)	
CRP (mg/L)		139.5 (81-302.5)	
SOFA Score		4 (1-7)	
Length of Stay, days		10 (6-19.5)	
Mortality, n (%)		11 (27.5%)	

Values represent the median (IQR) unless otherwise stated. * Mann-Whitney U test, #Fisher's exact test and Φ χ^2-test. § Patients may have more than one co-morbidity and be taking more than one medication

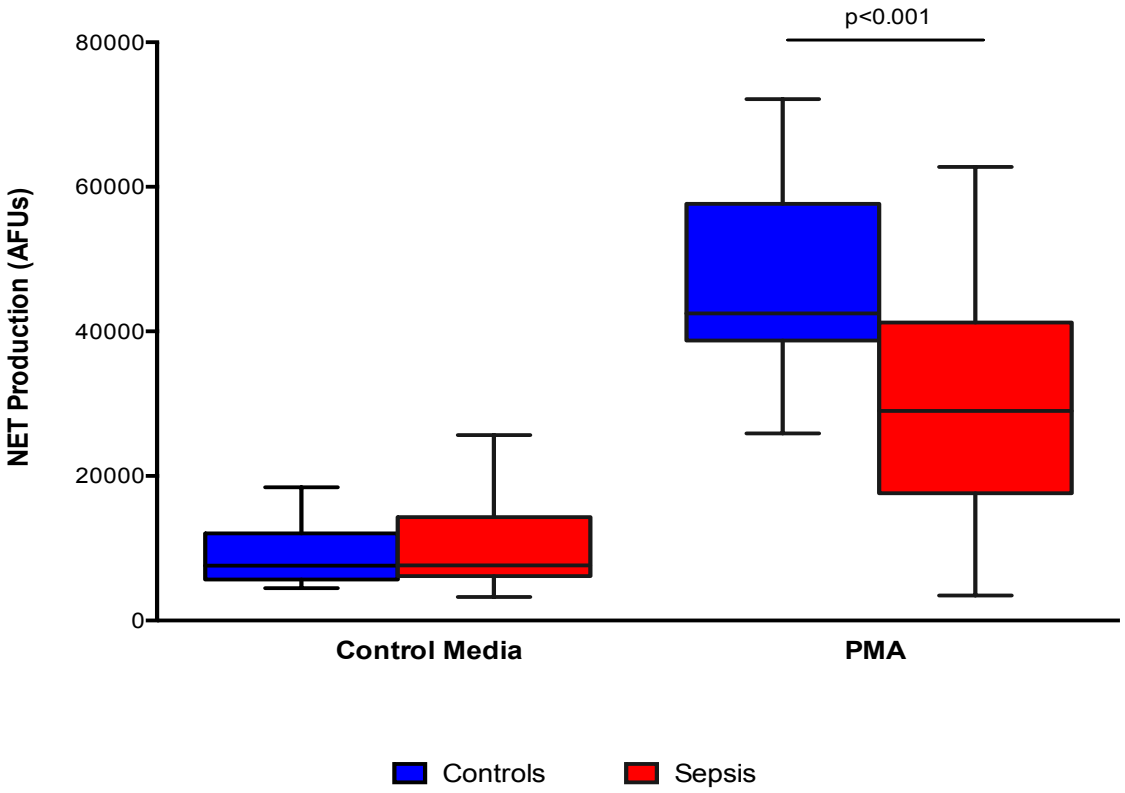


Figure 6.1. NET production from healthy controls and sepsis patients.

NET production was evaluated to the control media (RPMI 1640 Sigma-Aldrich) and 25nM phorbol-12-myristate-13-acetate (PMA, Sigma-Aldrich) in 29 healthy age-matched controls and 40 patients with sepsis. Net production is represented as arbitrary fluorescent units (AFUs). Boxes represent the median and IQR with the whiskers representing a Tukey distribution. p-value from a Mann-Whitney U test.

6.3.2.1 Sequential Day NET Production in Patient's with Sepsis.

In order to investigate the dynamic changes that may occur with NET production over the course of the sepsis episode, NETs were analysed on sequential days. NET production was assessed at baseline (day1) and subsequently on day 4 and 7 where possible. Due to discharges home, in-hospital deaths, refusal to consent to venesection and inadequate neutrophils not all eligible patients had NETs performed at each time point. This is shown as a modified flow diagram in figure 6.2.

Compared to admission, by day 7 patients overall were showing improvements in biochemical markers of sepsis (CRP: 139.5 {IQR 81-302.5} vs.104 {IQR 23-130} p=0.003 Mann-Whitney U test) and in their physiological scores (SOFA: 4 {IQR 1-7} vs. 0 {IQR 0-5} p=0.016 Mann-Whitney U test). Only 7 patients had persistent organ dysfunction by day 7 as measured by either increases or in SOFA scores or less than 3-point reduction in SOFA.

Overall a significant difference between PMA-derived NET production in sepsis patients on sequential days (day1, 4 and 7) and healthy controls (Kruskal-Wallis $p<0.001$) was observed. A Dunn's post-hoc multiple comparison test, demonstrated that NET production (PMA-derived) on day 1 ($p<0.001$) and day 4 ($p=0.004$) was significantly suppressed compared to age-matched controls. Although NETs on day 7 were lower and had not returned to baseline levels, this did not reach statistically significance ($p=0.124$). No difference in NET production in the control media was observed. This is illustrated in table 6.2 and in figure 6.3.

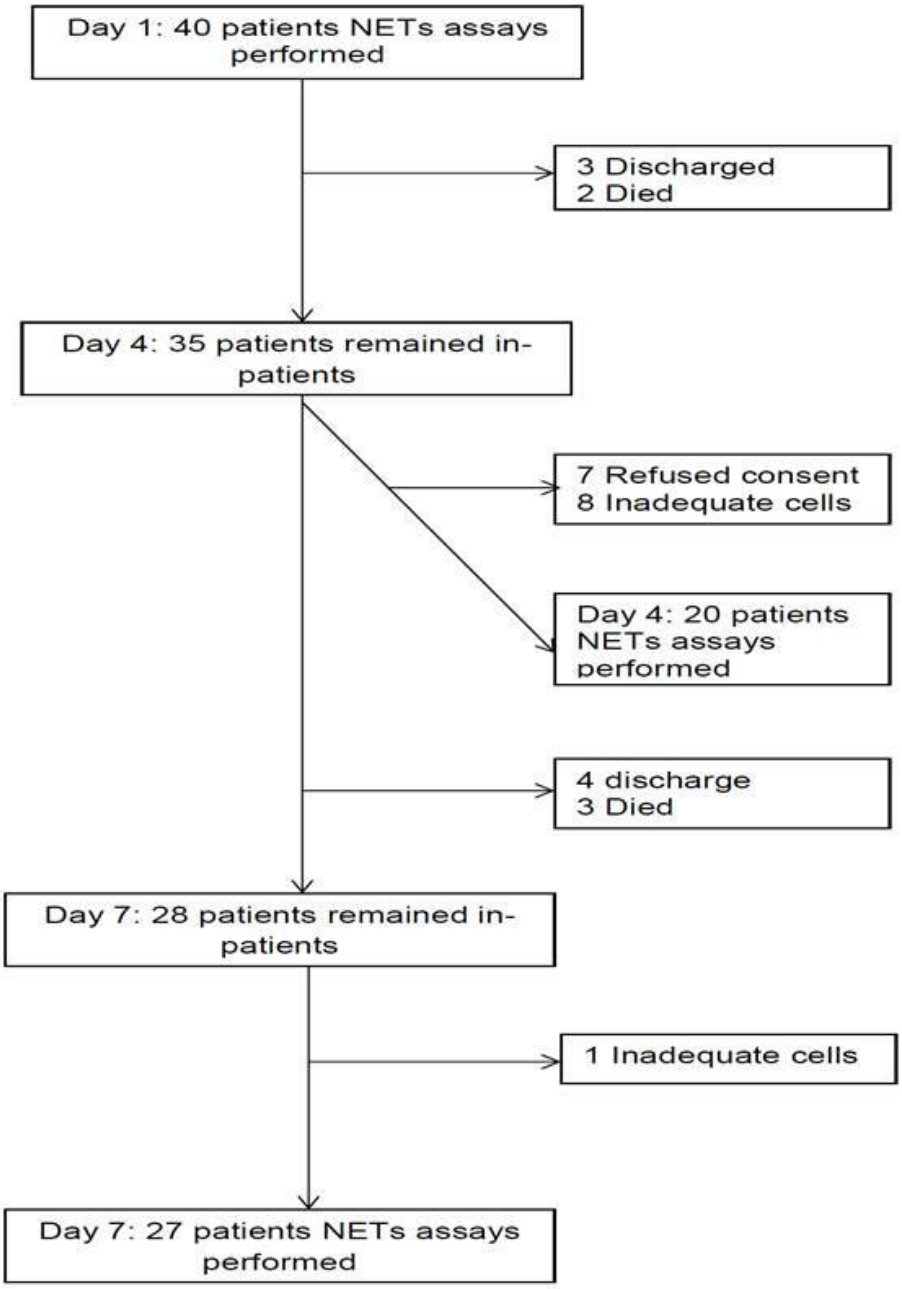


Figure 6.2. A modified consort diagram showing the neutrophil extracellular trap (NETs) experiments performed on patients with sepsis.

Table 6.2. NET production in healthy controls and in patients with sepsis on sequential days.

	Controls N=29	Day1 N=40	Day4 N=20	Day 7 N=27	p-value
Control Media	7598	7633	7585	8982	0.972
Median (IQR)	(4489-12058)	(6149-14313)	(6217-14881)	(5893-13701)	
PMA	42475	28994	27914	35485	<0.001
Median (IQR)	(38728-57645)	(17613–41237)	(19939-42446)	(22436-45222)	
		p<0.001*	p=0.004*	p=0.124*	

NET production was evaluated to the control media (RPMI 1640 Sigma-Aldrich) and 25nM phorbol-12-myristate-13-acetate (PMA, Sigma-Aldrich) in controls and in sepsis patients on days 1, 4 and 7. NET production expressed as arbitrary fluorescence units (AFUs), with data represented as median (IQR) and p-values from a Kruskal-Wallis test. *The p-value from a Dunn multiple comparison post-hoc test, where the significant difference is between the healthy controls and the sepsis patients.

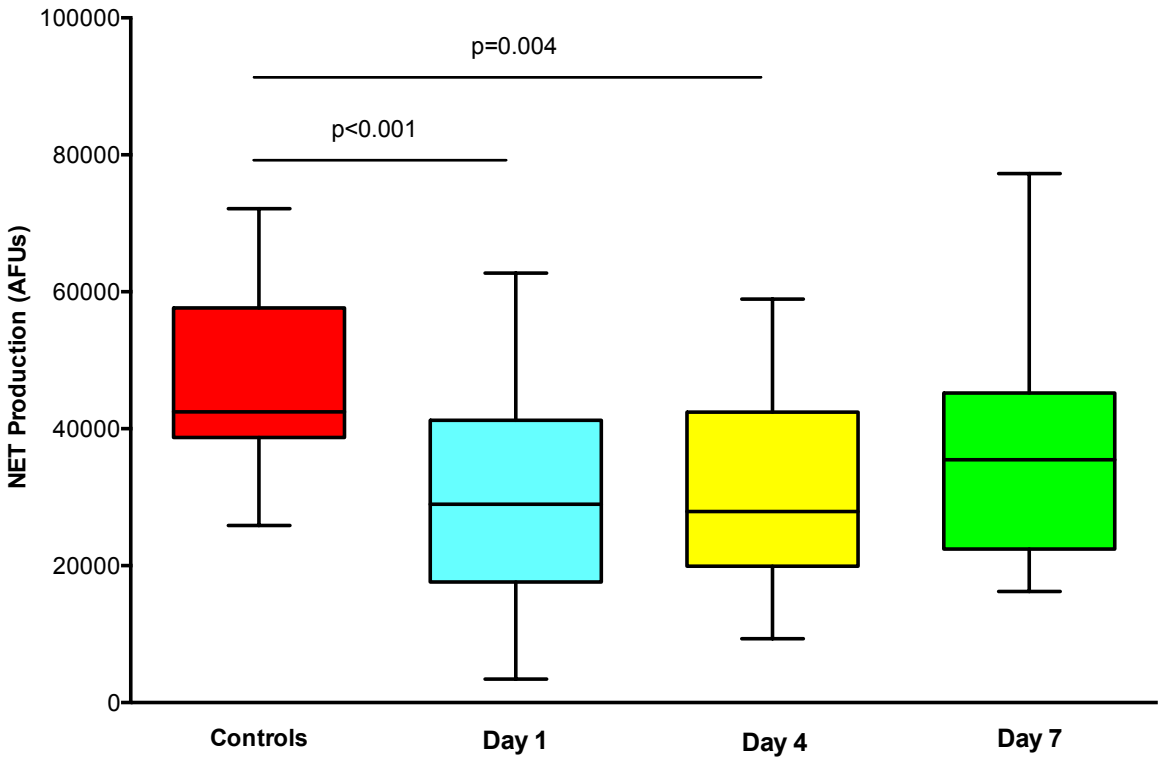


Figure 6.3: NET production in healthy controls and in patients with sepsis on sequential days.

NET production was evaluated in 29 age-matched healthy controls and in sepsis patients on day 1 (n=40), day 4 (n=20) and day 7 (n=27). NET production is in response to 25nM phorbol-12-myristate-13-acetate (PMA, Sigma Aldrich) and is represented as arbitrary fluorescent units (AFUs). Bars represent the median (IQR) and the whiskers a Tukey distribution. A Kruskal-Wallis test demonstrated a significant difference ($p<0.001$). The p-values shown in the graph represent the results of the Dunn's post-hoc test.

The results above demonstrate a reduction in NETosis in sepsis and shows that despite resolution of sepsis NET production at day 7 remains lower than levels observed in healthy controls. As sepsis encompasses a spectrum of illness, with a heterogeneous affected population and with variability in the severity of the insult a univariate analysis was carried out to see whether some of the principle factors that affect sepsis outcomes correlated with NETs in sepsis.

Univariate analysis was performed with NET production to 25nM PMA (Sigma-Aldrich) as the dependent variable and independent variables were age, sex, severity of sepsis (based on the SSCG definitions (96)), APACHE II and SAPS II Score, length of hospital stay, ICU admission and mortality. This suggested that severity of sepsis (Spearman's r= -0.336, p=0.024), APACHE II (Spearman's r=-0.333, p=0.035) and SAPS II (Spearman's r= -0.408, p=0.011) correlated with suppression of NET formation. All these three factors are surrogates for sepsis severity, suggesting that the increased severity of sepsis reduces NET formation. Therefore patients were categorised according to sepsis severity (SSCG definition (96)) and NET formation further analysed.

6.3.2.2. Severity of Sepsis and NET Formation

The demographics, physiological severity scores and outcomes for patients admitted with sepsis and severe sepsis are shown in table 6.3.

Patients with sepsis without organ dysfunction showed no change in 25nM PMA (Sigma-Aldrich) induced NET production compared with healthy controls on sequential day analysis ($p=0.360$, Kruskal-Wallis). However patients with severe sepsis showed a significant and persistent reduction in PMA induced NET production ($p<0.001$, Kruskal-Wallis), with a Dunn's post hoc analysis demonstrating that NETs are reduced on day1 ($p<0.001$) and day4 ($p<0.001$) compared to healthy controls.

When NET production between healthy controls, sepsis patients and severe sepsis were compared, a significant difference was seen on each day (Kruskal-Wallis, day 1: $p<0.001$, day 4: $p<0.001$ and day 7:$p=0.036$). Post-hoc analysis revealed similar results with the difference on each sequential day being between the healthy controls and severe sepsis patients (day 1: $p<0.001$, day 4: $p<0.001$ and day 7: $p=0.036$). Additionally the post-hoc analysis on day 1 between the groups demonstrated that patient with severe sepsis have reduced NET production compared with sepsis patients ($p=0.022$). This is illustrated in table 6.4 and in figure 6.4.

Further analysis of patients with sepsis, severe sepsis and those with septic shock was carried out to assess whether increased severity of sepsis was associated with greater NET suppression. The demographics, physiological severity scores and outcomes for patients with severe sepsis and septic shock are shown in table 6.5.

A significant difference was observed between healthy controls and patients with sepsis, severe sepsis and septic shock on day 1 and 7 (Kruskal-Wallis, day 1: $p<0.001$ and day 7: $p=0.002$). Post-hoc analysis using a Dunn's test demonstrated that both severe sepsis and septic shock were associated with a reduction in NETs compared to healthy controls on day 1. On day 7, post-hoc analysis revealed that only severe sepsis was associated with a reduction in NETs. No difference between severe sepsis and septic shock were seen. See table 6.6 and figure 6.5.

These results suggest that the severity of septic insult alters NET production, with no effect on NETs observed in patients with sepsis compared to healthy controls. However in patients with severe sepsis and septic shock NET production in response to PMA (Sigma-Aldrich) is significantly suppressed. Recovery from sepsis was not associated with the return of stimulated NET formation to levels observed in healthy controls in patients with severe sepsis.

Table 6.3. The characteristics of patients with sepsis and severe sepsis.

	Sepsis N=12	Severe N=28	p-value
Age	61 (35.8-71.5)	68 (51-75)	0.309
Sex, male (%)	8 (57.1%)	23 (74.2%)	0.307*
APACHE II	8.00 (5.3-10.3)	18 (10-20)	<0.001
SAPS II	18.5 (10.5-24.3)	37 (25-46)	<0.001
WCC ($x10^9/L$)	16 (15-21.1)	16.6 (12.5-22.8)	0.641
CRP (mg/L)	217.5 (89.5 -338.3)	146 (57-304)	0.529
SOFA Score	0 (0-1)	6 (4-9)	<0.001
Length of Stay (days)	8.0 (6.0-13.0)	10.0 (6.0-28.5)	0.320
Mortality, n (%)	1 (7%)	10 (32.3%)	<0.001*

Demographic data, severity scores and biochemical test results for patients with sepsis and severe sepsis. Values are represented as the median (IQR) unless otherwise stated with p-values from a Mann-Whitney U test, *represents a Fischer's exact test.

Table 6.4. NETs values in controls and patients with sepsis and severe sepsis on sequential days

	Healthy Controls	Sepsis	Severe Sepsis	p-value*
Day 1/Baseline	42475**§ (38728-57645) N=29	41286# (26142-57917) N=12	23773**#§ (15781-35388) N=28	<0.001
Day 4		42144 (23378-46624) N=7	26253**§ (17037-32258) N=13	<0.001
Day 7		35751 (20828-74914) N=9	34589** (19332-41534) N=18	0.036
p-value Φ		0.360	<0.001	

NET production was evaluated to 25nM phrobol-12-myristate-13-acetate (PMA, Sigma-Aldrich) in controls and in patients with sepsis and severe sepsis on days 1, 4 and 7. NET production expressed as arbitrary fluorescence units (AFUs) with values represented as median (IQR) with numbers of samples tested. *Corresponds to a Kruskal-Wallis between the controls, sepsis patients and severe sepsis patients on each separate day. **Corresponds to the significant variable in to a Dunn's post hoc test showing significance against the healthy controls on each day, whilst the #corresponds to significant differences between variables. Φ correspond to Kruskal-Wallis between the NET values for each group (sepsis and severe sepsis) and the healthy controls, with the § representing significant variables in to a Dunn's post hoc test.

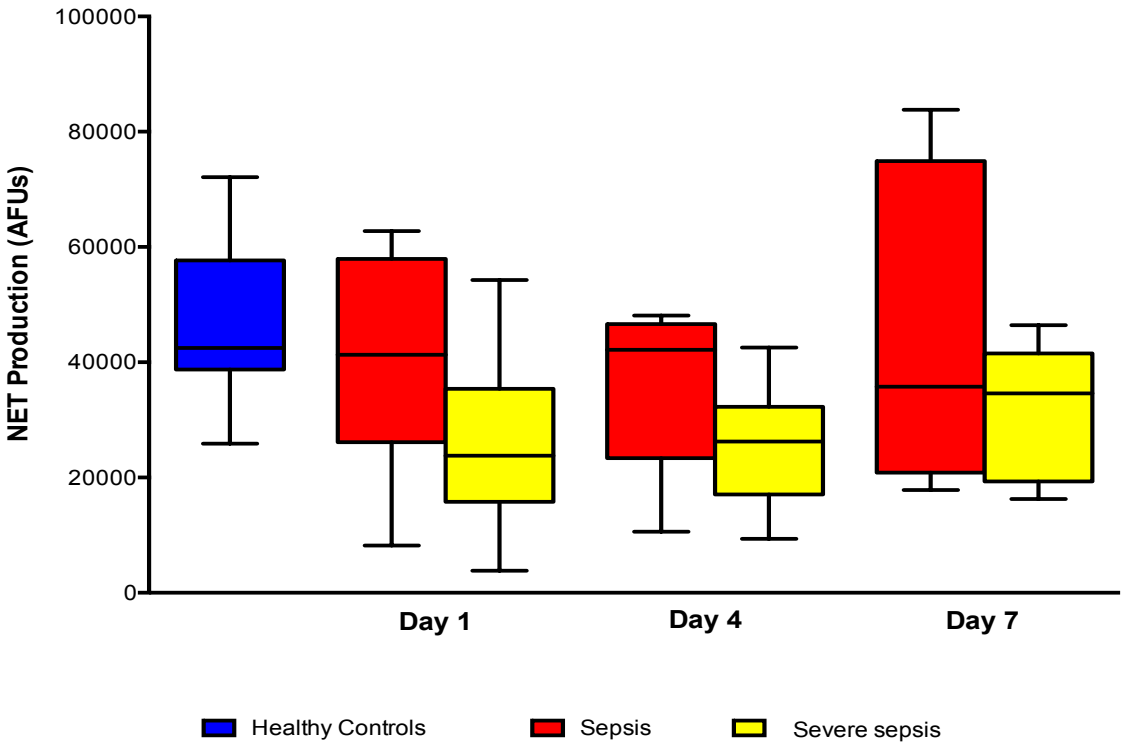


Figure 6.4. NET production in patients with sepsis and severe sepsis on sequential days.

NET production was evaluated to 25nM phorbol-12-myristate-13acetate (PMA, Sigma-Aldrich) in 29 age-matched healthy controls and in patients with sepsis and severe sepsis on day 1 (n=12 vs. n=28), day 4 (n=7 vs. n=13) and day 7 (n=9 vs. n=18). NET production is represented as arbitrary fluorescent units (AFUs). The bars represent the median and IQR and the whiskers a Tukey distribution.

Table 6.5. Demographic and baseline physiological severity scores and biochemical tests for patients with severe sepsis and septic shock.

	Severe Sepsis N=15	Septic Shock N=13	p-value
Age	61.5 (52.5-78.5)	71 (48-74)	>0.999
Sex, male (%)	10 (62.5%)	13 (86.67%)	0.220*
APACHE II	17 (8.3-19)	19 (14-23)	0.136
SAPS II	34.5 (19.8-42)	40 (30-53)	0.091
WCC ($x10^9/L$)	14.20 (12.60-20.03)	20.4 (10.20-26.30)	0.213
CRP (mg/L)	197.0 (23 -320)	132.5 (95-291.3)	0.938
SOFA Score	4 (0-1)	9 (4-9)	0.063
Length of Stay (days)	8 (6-26)	27 (8-34)	0.131
Mortality, n (%)	2 (12.5%)	8 (53.33%)	<0.001*

Demographic data, severity scores and biochemical test results for patients with severe sepsis and septic shock. Values are represented as the median (IQR) unless otherwise stated with p-values from a Mann-Whitney U test. *represents a Fischer's exact test.

Table 6.6. NET values in controls and patients with severe sepsis and septic shock.

	Healthy Controls	Sepsis	Severe Sepsis	Septic Shock	p-value*
Day 1/Baseline	42475**§ (38728-57645) N=29	41286 (26142-57917) N=12	33480**§ (6352-41966) N=15	23278**§ (18188-30000) N=13	<0.001
Day 7		35751 (20828-74914) N=9	34589**§ (19223-41056) N=9	39895 (30343-46419) N=9	0.002
p-value#		0.456	<0.001	<0.001	

NET production was evaluated to 25nM phorbol myristate acetate (PMA, Sigma-Aldrich) in healthy controls and in patients with sepsis, severe sepsis and septic shock on days 1 and 7. NET production expressed as arbitrary fluorescence units (AFUs) with values represented as median (IQR) with numbers of samples tested. *p-value corresponds to Kruskal-Wallis between the controls, sepsis, severe sepsis and septic shock patients on each separate day. **Corresponds to the significant variable in a Dunn's post hoc test showing significance against the healthy controls on each day. #represent a Kruskal-Wallis between the NET values for each group (sepsis, severe sepsis and septic shock) and the healthy controls. §represents the significant variables in a Dunn's post hoc analysis against the healthy control value.

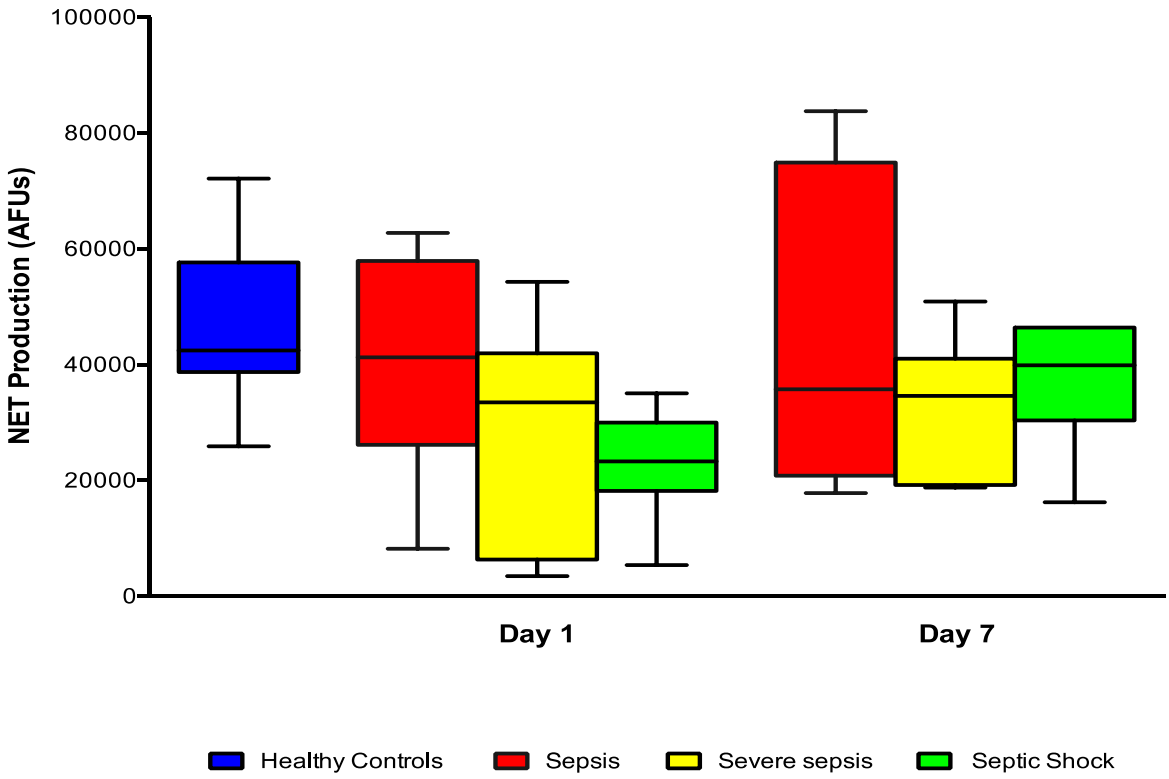


Figure 6.5. NET production in patients with sepsis, severe sepsis and septic shock on days 1 and 7.

NET production was evaluated to 25nM phorbol-12-myristate-13-acetate (PMA, Sigma-Aldrich) in 29 healthy age-matched controls and in patients with sepsis, severe sepsis and septic shock on day 1 (n=12 vs. n=15 vs. n=13) and day 7 (n=9 vs. n=9 vs. n=9). NET production is represented as arbitrary fluorescent units (AFUs). The bars represent the median and IQR and the whiskers a Tukey distribution.

6.3.3. The effect of simvastatin 1μM on NET production in neutrophils from sepsis patients.

Neutrophils from sepsis patients incubated with 1μM simvastatin showed an increase in basal NET production to the control media on day 1, but this failed to reach significance (p=0.060, Wilcoxon signed rank test). However, by day 7, a small but significant increase in basal NET production was seen in cells treated with simvastatin (p=0.013, Wilcoxon signed rank test). No differences were observed on day 4 (p=0.893 Wilcoxon signed rank test). Simvastatin 1μM significantly reduced PMA induced NET production in neutrophils from sepsis patients on days 1 (Mann-Whitney U, p=0.024) and 4 (Mann-Whitney U, p=0.001) and 7 (Mann-Whitney U, p=0.034). This is shown in table 6.7 and figure 6.6. This suggests that simvastatin therapy potentially increases basal NET production but further suppresses PMA induced NET production in sepsis patients on days 1, 4 and 7.

In summary NETs are reduced in patients with increasing severity of sepsis and this suppression persists despite physiological resolution of sepsis. Simvastatin therapy in-vitro further attenuates PMA-induced NET formation in these patients, but in the basal state seems to slightly increase NET production.

NETs production in the assay used is dependent on activity of NADPH oxidase to generate ROS and on the induction of neutrophil death. Alterations in either of these factors would affect NET production seen in these experiments. The subsequent experiments were performed to assess whether changes in ROS or apoptosis of neutrophils could explain the changes seen in sepsis patients.

Table 6.7. NET production in neutrophils from sepsis patients in response to in-vitro treatment with 1μM simvastatin.

	Vehicle Control	Simvastatin Treated	p-value
Unstimulated			
Day 1 N=21	7227 (6117- 9903)	9955 (6615-12509)	0.060
Day 4 N=16	7341 (6219-10542)	7653 (6276-9352)	0.893
Day 7 N=12	6459 (5488-8972)	8452 (7677-10088)	0.014
25nm PMA Stimulated			
Day 1 N=21	32489 (21887-47290)	31322 (14329-39289)	0.024
Day 4 N=16	29091 (19939-42446)	19789 (10808-29402)	0.001
Day 7 N=12	38592 (33345-67159)	32398 (21206-49799)	0.034

Neutrophils from sepsis patients on day 1, 4 and 7 were treated with or without simvastatin 1μM simvastatin and NET production evaluated in the control media and to 25nM phorbol-12-myristate-13-acetate (PMA, Sigma-Aldrich). NET production expressed as arbitrary fluorescence units (AFUs) with values represented as median (IQR) with the numbers of samples tested. p-values from a Wilcoxon signed rank test. The vehicle control was RPMI 1640 supplemented with an equivalent concentration of dimethyl sulfoxide (DMSO).

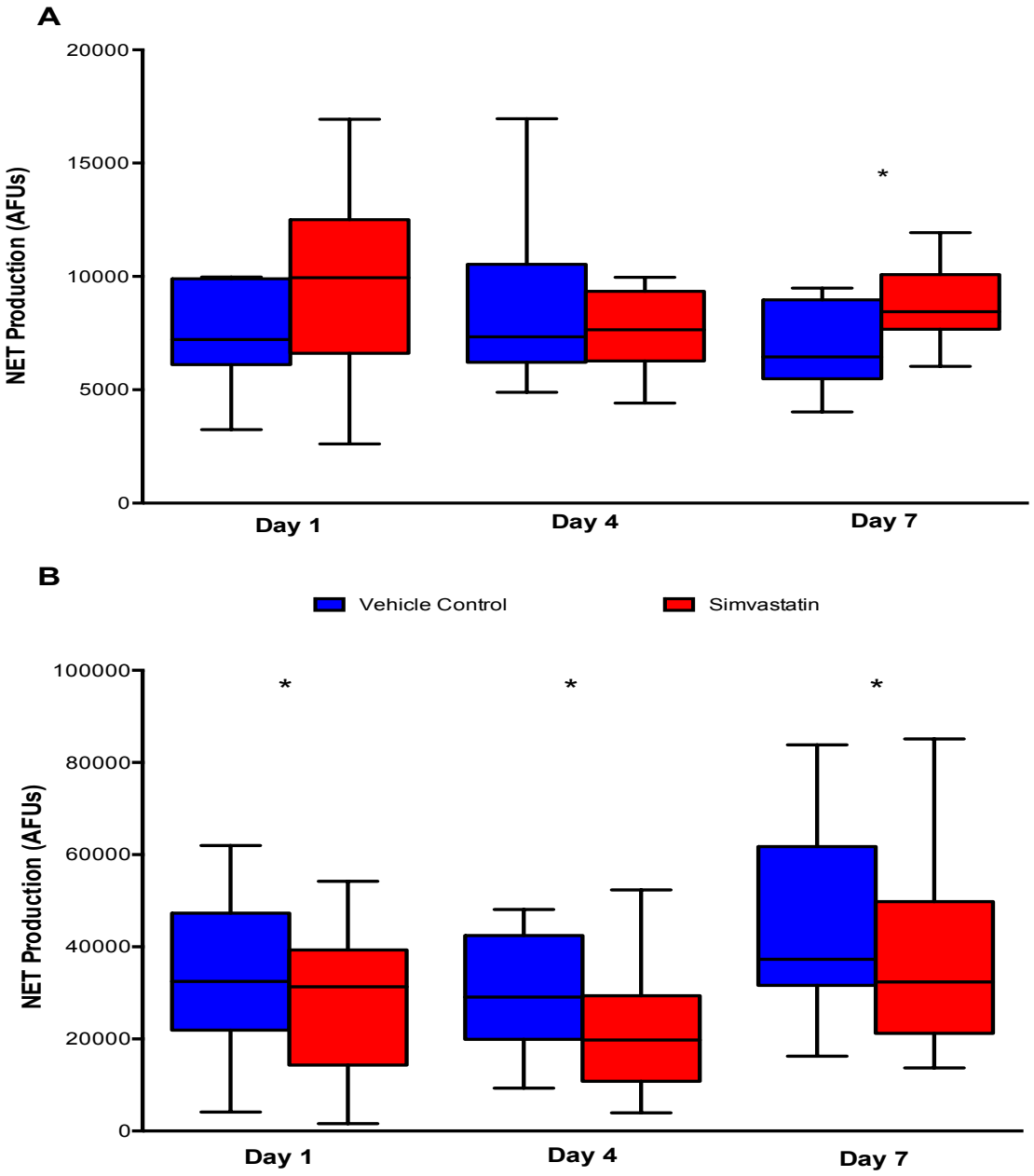


Figure 6.6: NET production in response to in-vitro treatment with 1 *μ*M simvastatin.

Neutrophils from sepsis patients on day 1 (N=21), 4 (N=16) and 7 (N=12) were treated in the vehicle control (RPMI 1640 with DMSO) or with simvastatin 1μM simvastatin. NET production is represented as arbitrary fluorescent units (AFUs). **A** represents NET production in the vehicle control (RPMI 1640, Sigma-Aldrich). **B** represents NET production in response 25nM phorbol-12-myristate-13-acetate (PMA, Sigma-Aldrich). Bars represent the median and IQR with the whiskers a Tukey distribution. * represents a $p<0.05$ in a Wilcoxon signed rank test.

6.3.4. Reactive Oxygen Species (ROS) Production of Neutrophils

6.3.4.1. ROS production in the healthy elderly controls and sepsis patients

ROS production was measured in 21 healthy elderly controls and 20 patients with sepsis. Their demographics are shown in table 6.8. ROS production in neutrophils from sepsis patients in the control media showed lower levels of ROS compared to those from the healthy elderly controls (AUC: 9415 {IQR 5451-18696} vs. 45275 {IQR 20865-54064} $p<0.001$, Mann-Whitney U test). However when neutrophils were stimulated with 25nM PMA (Sigma-Aldrich) and fMLP (Sigma-Aldrich) sepsis patients showed significant increases in ROS production compared to healthy controls. See table 6.8.

6.3.4.2. Sequential changes in ROS production during sepsis

NET production was persistently lower in sepsis patients on sequential days despite evidence of physiological resolution of sepsis. ROS was measured sequentially to assess whether elevated ROS production persisted. A significant difference was seen in the ROS production of patients with sepsis on sequential days compared to healthy controls (Kruskal-Wallis, $p<0.001$) in response to 25nM PMA (Sigma-Aldrich). A Dunn's post-hoc test revealed that ROS production was significantly elevated in sepsis patients on day1 ($p<0.001$), day 4 ($p<0.001$) and day 7 ($p<0.001$). No difference was seen in response to fMLP (Sigma-Aldrich). See table 6.9.

Table 6.8. The demographics and the ROS production in healthy controls and sepsis patients

	Healthy Controls	Sepsis	p-value
N	21	20	
Age, median (Range)	69.0 (60-94)	60.5 (18-89)	0.015*
Sex, male (%)	9 (43)	10 (50)	0.758**
PMA (% change) Median (IQR)	576.3 (482-756.3)	3684 (1269-10972)	<0.001*
fMLP (% change) Median (IQR)	339.9±53.5	1084.0±283.8	0.020*

Demographic details of the healthy controls and sepsis patients that donated neutrophils and had reactive oxygen species (ROS) measured. ROS production to 25nM phorbol-12-myristate-13-acetate (PMA, Sigma-Aldrich) and 2.5μM fMLP are shown. ROS values are displayed as the percentage increase in the area under the curve (AUC) from baseline. *represents a Mann-Whitney U test, **represents a Fisher's exact test.

Table 6.9. ROS production on sequential days to PMA in patients with sepsis.

	Controls N=21	Day1 N=20	Day4 N=10	Day 7 N=9	*p-value
PMA (% change)	576.3 (482.0-756.9)	3684[#] (1269-10972)	3020[#] (1557-3708)	5150[#] (568.4-11271)	<0.001
fMLP (% change)	267.3 (161.4-547.0)	810.7 (67.7-1730)	409.0 (112.0-1507)	772.7 (354.2-1514)	0.313

Reactive oxygen species production is expressed as the percentage increase in the area under curve (AUC) from baseline values to 25nM phorbol-12-myristate-13-acetate (PMA) and 2.5μM fMLP. Measurements were taken in healthy controls and in sepsis patients on day1, day4 and day7. Values represent the median (IQR) and the *p-value if from a Kruskal-Wallis test. [#]represents significance in a Dunn's post-hoc multiple comparison test compared to the healthy controls.

6.3.4.3. Correlation between ROS production and NET formation.

As described earlier, this NETs assay is dependent on ROS production. Therefore a correlation between these two variables in healthy controls and in sepsis patients was performed. A negative correlation between ROS and NETs in both healthy controls (Spearman's r=-0.477, 95% CI: -0.743 to -0.001; p=0.042) and in sepsis patients (Spearman's r=-0.618, 95% CI: -0.837 to -0.228; p=0.004) was observed, suggesting neutrophils that produce lower ROS are able to generate more NETs (see figure 6.7).

6.3.4.4. The effect of simvastatin on ROS production in sepsis neutrophils

Simvastatin potentially reduces ROS production by direct inhibition of NADPH oxidase and via anti-oxidant properties. The incubation of neutrophils with simvastatin 1μM significantly reduced baseline ROS production (AUC: 14030 {IQR 5565-20529} vs. 9126 {IQR 4026-20900}, p=0.023, Wilcoxon signed rank test, N=20) and ROS production following PMA stimulation (% change from baseline: 3625% {IQR 1670-9193%} vs. 2010% {IQR 1275-4108%} p=0.011 Wilcoxon signed rank test). See figure 6.8.

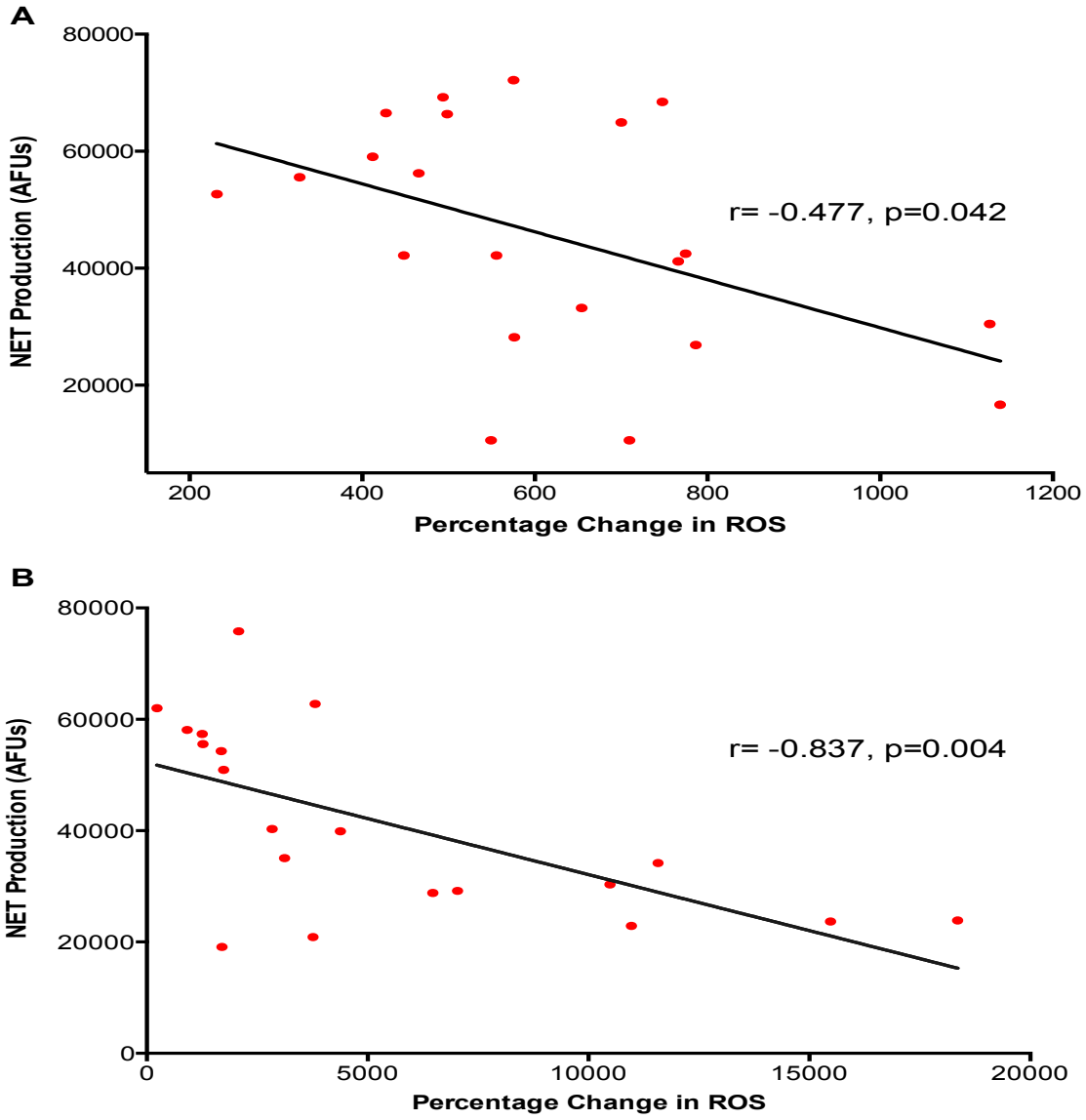


Figure 6.7 Correlation between ROS production and NETs in healthy controls and in patients with sepsis.

A shows the Spearman's correlation between ROS production and NETs in 21 healthy controls. B shows the Spearman's correlation between ROS production and NETs in 20 sepsis patients.

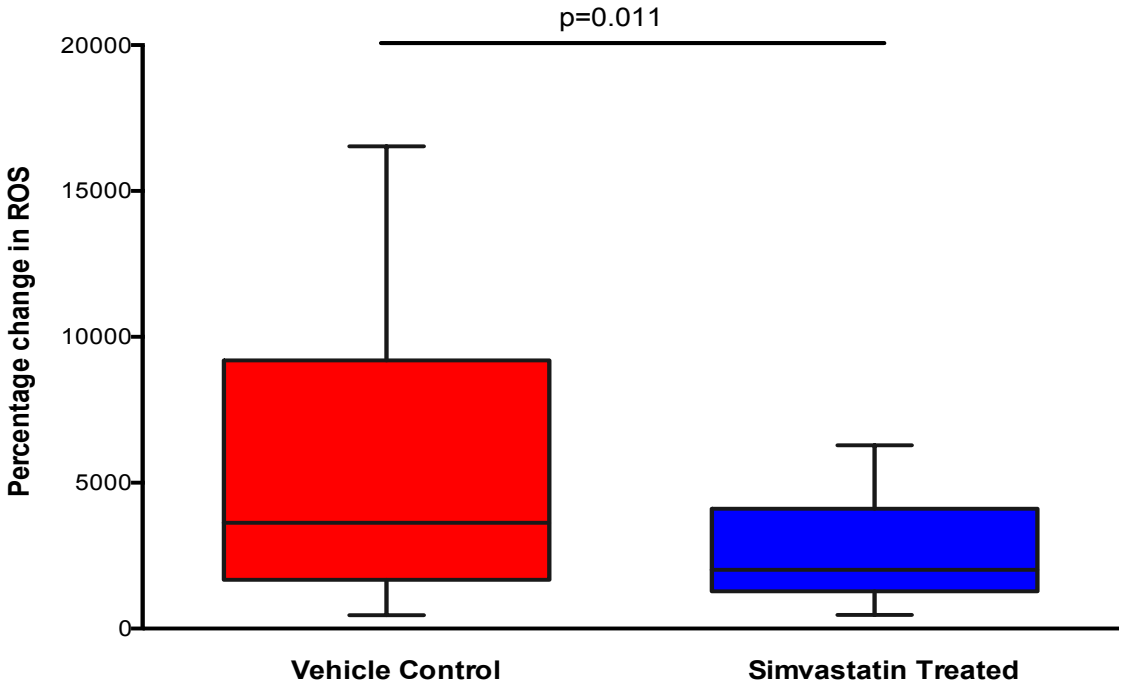


Figure 6.8. The effect of in-vitro 1μM simvastatin on ROS production in neutrophils from sepsis patients stimulated with 25nM PMA.

The effect of incubation of sepsis neutrophils with 1μM simvastatin for 40 minutes on reactive oxygen species (ROS) production in response to stimulation with 25nM phorbol-12-myristate-13-acetate (PMA, Sigma-Aldrich). ROS is measured as the percentage change in the area under the curve from baseline. The bars represent the median and IQR and the whiskers a Tukey distribution. p-value is from a Wilcoxon-signed rank test, where N=20.

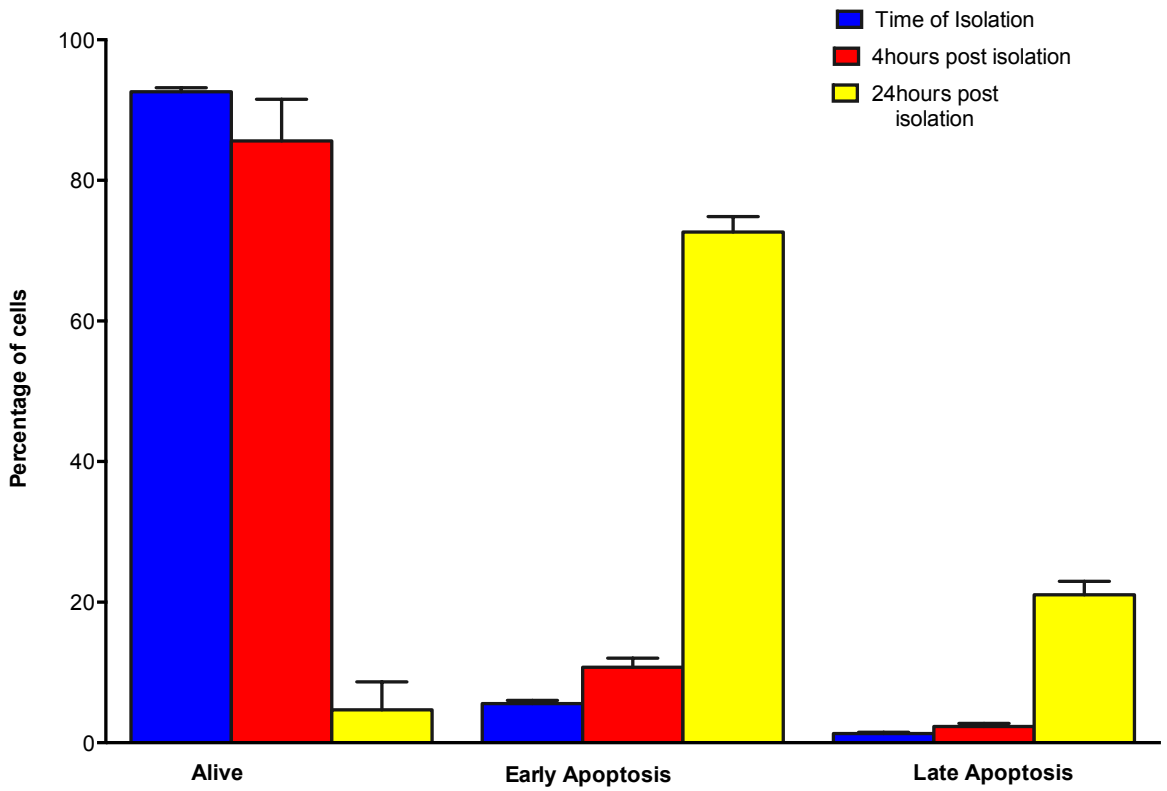
6.3.5. Apoptosis of Neutrophils

Apoptosis was measured in neutrophils from healthy and sepsis donor on immediate isolation from whole blood (approximately 90 minutes). Apoptosis experiments were repeated at 4 hours, as this directly corresponded to the time at which NET formation was assessed. A 24-hour apoptosis measurement was also performed to determine the impact of sepsis on neutrophil apoptosis.

6.3.5.1. Apoptosis in Healthy Donor Neutrophils

Neutrophils from 19 healthy donors had apoptosis experiments performed. The mean age of these healthy donors was 43.4 years (range 21-71 years) with 9 males and 10 females. At baseline almost all the neutrophils extracted were alive (92.6%) with a 5.5% shift of neutrophils into early apoptosis (Annexin V positive) by 4 hours. At 24 hours the vast majority of neutrophils were in early or late apoptosis (72.7% and 21.1% respectively) with only 4.7% of neutrophils still alive. Very few cells underwent necrosis in healthy donors (0.5% vs. 1.4% vs. 1.6%). See figure 6.9. No differences were observed in apoptosis when corrected for age (young vs. old) and for sex (male vs. females).

Figure 6.9. Neutrophil apoptosis in healthy donors.



Neutrophil apoptosis in 19 healthy controls measured at the time of isolation, at 4 hours and 24 hours after isolation by flow cytometry. The y-axis represents the percentage of cells alive (negative for Annexin V and Sytox Blue), in early apoptosis (positive for Annexin V) and those in late apoptosis (positive for Annexin V and Sytox Blue). The bars represent the median with the error bars the IQR.

6.3.5.2. Impact of sepsis on neutrophil apoptosis

Apoptosis from 17 patients with sepsis was measured and compared to the results from healthy controls. At the time of neutrophil isolation, sepsis patients had fewer "alive" cells (79.5% {IQR 70.7-83.3%} vs. 92.3% {IQR 90.4-92.3%} p<0.001, Mann-Whitney U test) compared to healthy controls. A greater numbers of the neutrophils were in early (18.8% {IQR 12-23.2%} vs. 5.3% {IQR 3.9-6.7%} p<0.001, Mann-Whitney U test) and late apoptosis (2.8% {IQR 1.0-3.6%} vs. 1.2% {IQR 0.5-2.1%}. p=0.03, Mann-Whitney U). See figure 6.10.

The rate of apoptosis was calculated by taking the percentage of Annexin V positive neutrophils at selected time point after isolation away from the percentage of Annexin V positive neutrophils at the time of isolation. This demonstrated no significant change in the rate of apoptosis in sepsis patients compared to healthy controls at 4hours (3.4% vs. 5.2% p=0.230 Mann-Whitney U).

At 24hours a significantly greater number of neutrophils from sepsis patients were alive (38.2% {IQR 19.2-48.5%} vs. 3.2% {IQR 1.5-7.3%} p<0.001, Mann-Whitney U). Patients with sepsis had fewer neutrophils in early (53.0% {IQR 33.2-62.6} vs. 73.6% {IQR 65.7-88%} p=0.002 Mann-Whitney U) and late apoptosis (10.1% (IQR 6.9-19.4%} vs. 21.6% {IQR 15.7-26.4%} p=0.027, Mann-Whitney U).

These results suggest that in patients with sepsis a pool of apoptotic neutrophils are circulating within the systemic circulation. However the viable neutrophils seem to be programmed to resist apoptosis and have prolonged ex-vivo survival. NETs are

produced from viable cells, and the assay used with PMA induces NETosis. It was hypothesised that the reduction in NETs may be related to the increased rate of apoptosis on isolation.

A correlation between the number of “alive” cells at 4 hours and the NET production was performed to test this. The 4 hours apoptosis time point was selected to reflect the point at which NETs were measured following isolation. A positive correlation between the number of neutrophils of "alive" cells at 4hours and NETs (Spearman’s r=0.500, 95% CI= 0.01-0.796, p=0.043) was found suggesting that the reduced NETosis in sepsis patients may be related to a reduced number of circulating "alive" cells. See figure 6.11

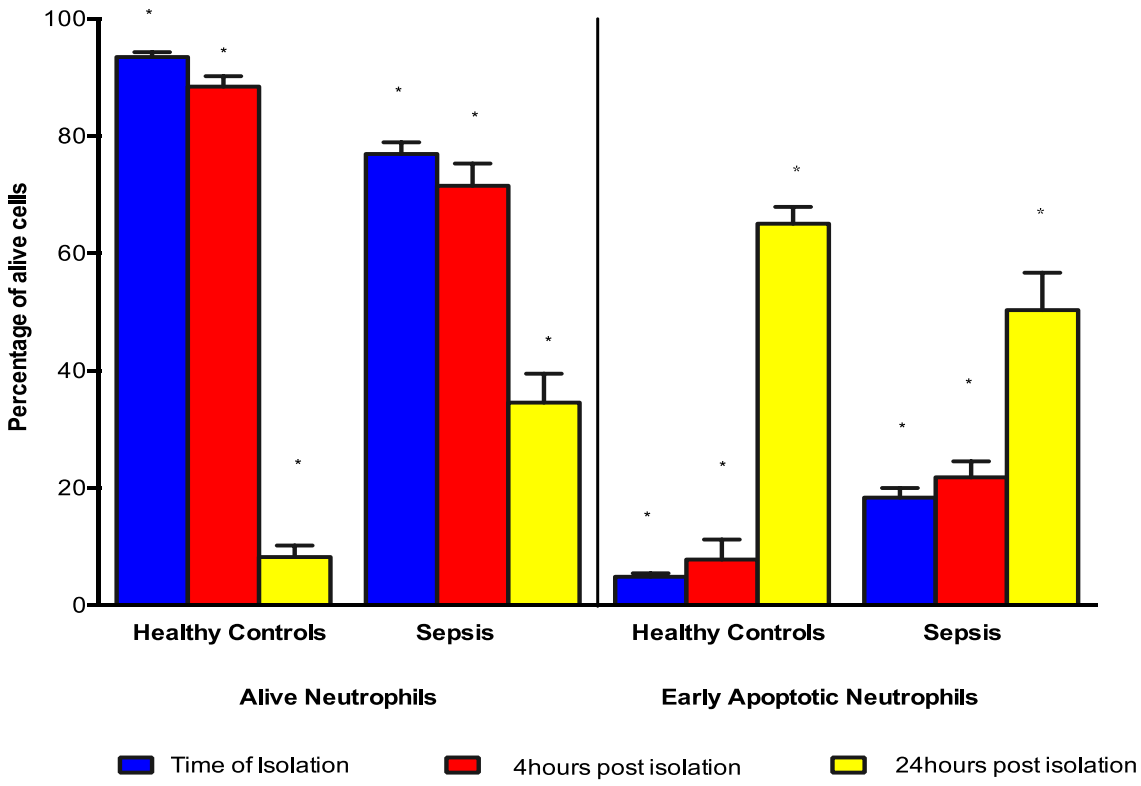


Figure 6.10. Neutrophil apoptosis in healthy donors and sepsis patients.

Neutrophil apoptosis in 19 healthy controls and 17 sepsis patients measured at the time of isolation, at 4 hours and 24 hours after isolation by flow cytometry. The y-axis represents the percentage of cells alive and in early apoptosis (positive for Annexin V). The bars represent the median with the error bars the IQR. *represent a $p<0.05$ between the corresponding time points for the healthy controls and sepsis patients.

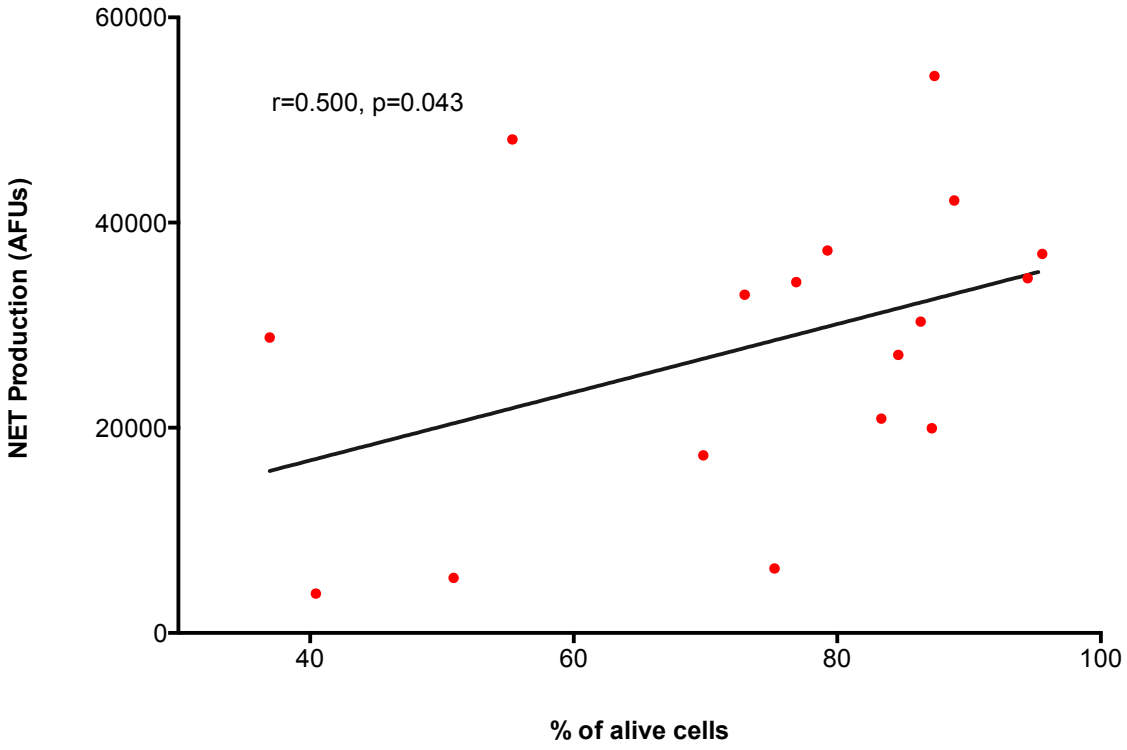


Figure 6.11. Correlation between "alive" neutrophils and NET production in sepsis patients.

Scatter graph showing the relationship between NET production in 17 sepsis patients and the percentage of "alive" neutrophils at 4 hours after isolation. A linear trend line is shown, with a “r” and p-value represented by a Spearman’s correlation co-efficient.

6.3.6. Cell surface receptor expression.

The cell surface expression of CD16 and the activation markers CD45, CD14, CD63 and the expression were measured in healthy controls and sepsis patients.

6.3.6.1. CD16 expression is reduced in sepsis

CD16 binds IgG coated bacteria and is in involved in the phagocytosis of bacteria. It can also be used as a marker of neutrophil age and apoptosis with recently mobilised neutrophils and apoptotic cells expressing low levels of CD16. As previously reported in section 5.3.5.1, the overall number of cells expressing CD16 was unchanged, however overall receptor expression, represented by the MFI, was significantly reduced (59682 AFUs {IQR 40799-73306 AFUs} vs. 77791 AFUs {IQR 68081-111644 AFUs} p=0.014 Mann-Whitney U test) in sepsis patients.

6.3.6.2. Neutrophils in sepsis showed increased expression of CD45, CD14 and CD63.

The expression of key activation markers was significantly increased in sepsis patients compared to healthy controls. The number of neutrophils expressing CD45 in sepsis patients was significantly increased (99.9% {IQR 99.7-100.0%} vs. 99.6% {IQR 95.4-99.9%} p=0.011, Mann Whitney U) as was the median MFI (32808 AFUs {IQR 20685 vs. 45370 AFUs} vs. 21988 AFUs {IQR 14161 vs. 30313 AFUs} p=0.038 Mann Whitney U) representing the number of receptors per cell. As reported in section 5.3.5.3 and 5.3.5.4 the percentage of cells expressing CD14 (35.1% {IQR 17.8-49.3} vs. 4.6% {IQR 3.5-12.3%}. p<0.001 Mann Whitney U) and CD63 (77.1% {IQR 53.9-94.2%} vs. 45.5% {IQR 29.1-68.5%} p=0.040, Mann Whitney U) was significantly increased in sepsis but no significant changes in the MFI were observed.

6.4. Discussion

The chapter describes NET production in healthy donor neutrophils using PMA and demonstrated that increasing age is associated with increased NET formation. Sepsis resulted in a reduction in NET production with persistent suppression of NETs despite sepsis resolution. The severity of the sepsis insult was found to have significant relevance, with severe sepsis and septic shock resulting in reduced NET production compared to both healthy controls and those patients with “mild” sepsis. Finally the in-vitro simvastatin therapy increased basal NET production, but further reduced NET production in response to 25nM PMA in sepsis patients. These finding are the opposite to the original hypothesis that predicted an increase in NET production in response to sepsis and that in-vitro simvastatin therapy would cause a further rise in NET production.

NET production results in the controlled release of chromatin and histones from neutrophils. NET release can occur through oxidant-dependent mechanisms that are closely linked with ROS production and eventual cell death (NETosis). However, oxidant-independent mechanisms of NET release have been demonstrated that do not result in cell death and allow the neutrophils to continue with their other anti-microbial functions. The assay used in these experiments to quantify NET production is dependent upon NADPH ROS production resulting in the eventual death of the cell via NETosis. Therefore any changes seen in NETs in healthy controls and sepsis patients must be due to either a reduction in the number of alive cells, alterations in ROS and/or changes in mechanisms regulating cell death via NETosis. Therefore

ROS generation and apoptosis in neutrophils was investigated to identify potential mechanisms involved in the changes seen in NETs (47, 59, 211, 365).

It is proposed that the increased NET production observed in the elderly controls may lead to exaggerated endothelial damage, precipitate organ dysfunction and partially explain the increased severity of sepsis observed in the elderly. Current evidence links ageing with a progressive increase in ROS, with excessive ROS being linked to many age-related diseases, such as neurodegenerative diseases, cardiomyopathy and cancers. Although these experiments did not assess ROS production in young controls, it would suggest that the increase in NETs with ageing might be associated with increased ROS generation (366, 367).

These results and those from previous studies of ROS production in the elderly are in contrast to Hazeldine et al who observed decreases in both NETs and ROS with age to CXCL-8 and LPS following neutrophil priming with TNF-α. They proposed the opposite, suggesting reduced ROS in the elderly leads to reduced NETosis and thus may account for poorer bacterial clearance and the increased rates of infection in the elderly. The differences could be related to the small sample size in Hazeldine et al's study (18 in total) compared with 37 participants used in these experiments. Additionally, the use of TNF-α to prime neutrophils may have had significant influences on NET production as it activates PAD4 citrullination, a key step in the eventual release of NETs. The age-related differences were observed in response to PMA stimulation in these experiments, an effect not observed in Hazeldine's study. The fluorometric methods used in these experiments and by Hazeldine et al for NET

generation have been validated using primarily PMA, with CXCL-8 and LPS derived NETs requiring microscopic techniques to detect the low levels of NETs generated (67, 75, 76, 362).

The ability of neutrophils to generate NETs at the sites of infection attenuates the spread of infection by containing and elimination of bacteria, fungi and viruses. Murine models of infection using caecal ligation have demonstrated that mice treatment with recombinant DNAse to deplete NETs have an increased mortality and increased the severity of sepsis. However widespread systemic NET release may trap bacteria in the microvasculature but at the expense of endothelial injury. The role of NETosis in sepsis remains poorly characterised with this study being the first show a persistent reduction in NETosis with sepsis (16, 210, 214, 215, 368).

A study in trauma patients revealed conflicting results to those described here, with inflammation induced by trauma causing an increased levels of NETs compared to healthy controls as opposed to the suppression seen in these experiments. They also showed NET production was increased in trauma patients who subsequently develop sepsis. Although trauma induces an inflammatory response similar to sepsis, it is difficult to translate results in trauma to sepsis, as the populations affected are significantly different, with trauma patients being younger and healthier. In addition the timing of the insult is much clearer compared with infections, which have a more insidious onset (212, 369).

The proposed mechanism based on the mechanistic studies of ROS, apoptosis and cell surface marking suggest that the reduction in NETosis is multifactorial with altered apoptosis, increased neutrophil activation (increased CD45, CD14 and CD63), ROS and immature neutrophils all contributing to the effects seen. The apoptosis experiments demonstrated reduced viability of freshly isolated neutrophils in patients with sepsis with a greater proportion of cells in the early stages of apoptosis. Sequential analysis of apoptosis following isolation showed prolonged neutrophil survival ex-vivo perhaps due the presence of pro-survival signals and the immaturity of the isolated neutrophils. The reduced expression of CD16 seen in neutrophils from sepsis may provide evidence to support the immature pool of neutrophils as this marker is reduced in these cells (175, 192, 365, 370, 371).

Neutrophils from sepsis patients showed greater ROS production with cell surface markers indicating increased activation. It would have been expected that an increase in NETosis would be observed as the two processes are interlinked. However, a negative correlation was observed between NET release and ROS production in the healthy elderly controls and in sepsis patients suggesting that circulating neutrophils generating high levels of ROS in response to PMA produce fewer NETs. This may suggest that activated neutrophils, such as those isolated in sepsis, maintain their ability to phagocytose bacteria and generate ROS but resist NETosis and apoptosis, which ultimately results in their death.

There is emerging evidence that not all circulating neutrophils have the capability to produce NETs with some authors suggesting that only 25% of the circulating

neutrophil pool are programmed to be NET producers, termed suicidal neutrophils. The remaining neutrophils are primarily programmed to carry out the traditional neutrophil functions but may produce NETs via an oxidant independent mechanism. This may also explain the reduction in NET formation observed in sepsis patients' with the isolated pool programmed to live longer and generate NETs independent of NADPH oxidase. The suicidal neutrophils may well have already delivered their antimicrobial load and died by the time patients have presented to hospital with sepsis (370).

The treatment of neutrophils from sepsis patients with 1μM simvastatin attenuated NET production further with this effect persisting to day 7 in response to PMA. The reduction seen in NETs was coupled with a reduction observed in ROS production with simvastatin treatment. Simvastatin reduces oxidative stress and inhibits NADPH oxidase thereby reducing ROS. This provides a mechanism for the reduced NETosis observed with simvastatin and is supported by the literature that suggests inhibition of ROS and NADPH oxidase leads to a reduction in NET formation. (363).

The incubation of unstimulated neutrophils with 1μM simvastatin raised basal NET production. This may support theories of oxidant independent NET release, as cells in the basal state did not produce large amount of ROS, yet NET release was increased. Statins have been shown to increase NETosis via an oxidant independent mechanism. These mechanisms were not investigated here and thus the potential of statins to actually increase NETosis to other stimuli cannot be commented upon (364).

This is in contrary to the results of Chow et al who demonstrated that 10μM simvastatin induced greater NET formation in in-vitro, ex-vivo and in-vivo models of sepsis. They suggested that statins were able to induce NET generation in an NADPH-independent manner and that enhanced NET production by statins was associated with reduced inflammation at sites of sepsis and improved bacterial clearance (364).

However several key differences arise between the two studies. Firstly, the populations were markedly differently as our study used patients with established sepsis and age-matched controls making the sample representative. Secondly, the dose of simvastatin used was therapeutically relevant in this study with the 1μM dose corresponding to 40-80mg simvastatin, the upper dosage limit of the drug. This dose has been shown in clinical trial of sepsis to be most efficacious and was the dose used in the HARP-2 trial (285).

Thirdly, the methods used by Chow to generate NETs involved culturing neutrophils overnight in the presence of statin and PMA. No apoptosis experiments were reported and therefore changes in NETosis could not be excluded due to cellular necrosis or dysregulated apoptosis.

Finally, the murine of model of sepsis, pre-treated mice with a diet of statin prior to inducing pneumonia with *S.Aureus*. This reflects previous research that demonstrates that pre-treatment with statins modulate the inflammatory response and leads to better outcomes, but does not reflect the situation of whether statins can

be used acutely to treat patients with established sepsis. The *S.Aureus* model of pneumonia is also not reflective of the common pathogens that cause pneumonia in humans and S.Aureus is known to induce rapid NETs through an oxidant independent manner (364).

Taking this study and Chow's study together it can be postulated that the early rapid release of NETs promoted by statins may be beneficial in containing infections at their source and preventing their dissemination. However as infection progresses to sepsis and MODS a reduction in systemic NET production would be beneficial to prevent further organ damage. Thus statins may act by more than one mechanism to modulate NET release in sepsis and thereby reduce the morbidity and mortality associated with it.

A key limitation in the methods used to assess NETosis was the use of a fluorometric technique. This assay allows the measurement of "cell-free" DNA, which is thought to relate directly to NETs, as the cells used are freshly isolated and have a high purity. Therefore the presumption is that any "cell-free" DNA should arise only from its release by neutrophils. This assay has been validated in conjunction with microscopy to confirm that DNA release is related to NET formation. However future experiments should incorporate both methods to add validity to any findings (16, 303, 372, 373)

In conclusion this study shows that immunosenescence is associated with increased NET formation compared to healthy young people and may be exacerbate organ dysfunction in sepsis and contribute to the increased incidence of sepsis in the

elderly. Sepsis reduces NET production with resolution of sepsis not associated with recovery of NET formation. The suppression of NETosis in sepsis is perhaps related to a pro-inflammatory phenotype adopted by neutrophils, with enhanced ROS production, prolonged survival and a mobilisation of neutrophils from the bone marrow resulting in an immature circulating pool.

Statins increase basal NET production possible via an oxidant independent mechanism. However, they further attenuate NET production to PMA in sepsis via inhibition of NADPH oxidase and consequently ROS and by doing so may prevent micro-vascular damage.

Chapter 7. General Discussion and Thesis Conclusions

Sepsis is an important global health issue and is one of the leading of cause of death. The incidence of sepsis continues to increase with an ageing population being partially attributed to the rise. Age is considered an independent risk factor for the development of sepsis and outcomes with inflama-ageing, immunosenescence and the reduced functional status of the elderly being implicated (70, 80, 91, 97, 104, 374).

Infection triggers a host response designed to contain and eliminate pathogens whilst minimising host damage. In sepsis an exaggerated SIRS and CARS responses occurs leading to the development of organ dysfunction and the eventual death of the host. Targeting the immune dysfunction in sepsis remains a focus for medical research with no successful immune-modulatory treatments currently available (91, 96, 109, 111, 128).

At the time this thesis was developed there was interest in statins as immune modulators in sepsis, with studies suggesting that statin therapy prior to the development of sepsis improves outcomes. The precise mechanism underpinning these beneficial effects are still unknown, however statins exert potent anti-inflammatory actions in addition to their anti-cholesterol effects. Statins are thought to attenuate neutrophil responses, by reducing migration into inflamed tissues, decreasing the production of pro-inflammatory cytokines and reducing neutrophil activation and ROS production (152, 237, 241, 245).

Research from within our group has demonstrated an age-related decline in neutrophil migration that could be improved with in-vitro simvastatin exposure. In addition the ASEPSIS trial (atorvastatin for the prevention of sepsis progression) showed that treatment with 40mg atorvastatin to statin-naïve patients with sepsis (no organ dysfunction) reduced the development of organ failure. This trial however failed to demonstrate any significant improvements in markers of inflammation (CRP, IL-6, TNF-α) with statin therapy, suggesting perhaps other mechanism were involved. It was on the back of these finding that the experiments within this thesis were designed to investigate the in-vivo and in-vitro effects of simvastatin of neutrophil function in healthy ageing and in sepsis (86, 375).

7.1. Neutrophil dysfunction in sepsis

Neutrophils are key mediators of sepsis intimately involved in containing and eliminating pathogens as well as resolving inflammation. However a balanced response is required with poor migration and ineffective anti-microbial actions causing dissemination of the bacteria, whilst excessive neutrophil recruitment to sites of infection may exacerbate host damage(1, 3).

Neutrophil functions are thought to decline with increasing age, with poor migration, reduced phagocytosis, increased degranulation and ROS production and decreased NET release documented. It was hypothesised that sepsis would exaggerate age-related innate immune dysfunctions of neutrophils. To assess this, the neutrophil functions of a cohort of healthy elderly subjects were compared with patients with sepsis, thus the control group, represented an “at risk” cohort allowing improved comparisons with the sepsis patients (78, 82, 86, 87, 307).

In summary, sepsis exaggerated immunosenescence by creating a state of neutrophil paralysis with reduced neutrophil migration. In contrast to the original hypothesis, neutrophils from sepsis patients generated greater ROS, demonstrated enhanced phagocytosis of *E.Coli* (no changes in *S.Aureus*) and released fewer NETs in response to PMA stimulation compared to healthy controls. Apoptosis experiments suggested that neutrophils in sepsis patients have delayed late apoptosis.

Potential explanations to these findings are detailed below:

Systemic inflammation is associated with prolonged neutrophil survival mediated by high concentrations of circulating G-CSF, GM-CSF, TNF-α and LPS. The potential delayed apoptosis results in a pool of "older" activated neutrophils circulating for 2-5 days (192, 198, 199). Ideally these neutrophils should have migrated to the sites of infection, however the prolonged exposure to cytokines and bacterial products is thought to cause neutrophil "paralysis" and migratory failure via an increased TLR-4 and -2 expression and the internalisation of key chemokine receptors (CXCR-1 and CXCR-2) (165, 166). Although these cells are unable to migrate they are activated, with migration experiments demonstrating increased neutrophil adhesion perhaps via an up-regulation of CD18 (Mac-1), whilst expression of activation markers was also significantly raised in sepsis (CD63, CD14 and CD45). In these experiments the increased expression in sepsis of CD63 in systemic circulating neutrophils is of particular significance as it suggests that degranulation of neutrophils is occurring in the systemic circulation without further stimulation. The overall consequences of this may be that activated neutrophils with prolonged life spans (delayed apoptosis) are

“trapped” within the systemic circulation and aggregate in the small blood vessels. These neutrophils continue to release MPO and NE (de-granulation), extracellular ROS and NETS in an uncontrolled manner resulting in collateral tissue damage and distal tissue hypoxia with resultant organ dysfunction that is the hallmark of sepsis (3, 156, 165, 166, 206, 214, 376).

Another potential explanation is a pool of immature band cells. An infectious insult results in rapid migration of neutrophils out of the systemic circulation into the infected sites. In response, the bone marrow releases large numbers of neutrophils into the systemic circulation, many of which have not fully matured. These immature bands cells are a well-recognised phenotype in sepsis and inflammation and can account for up to 50% of circulating neutrophils during sepsis (175, 360).

Although band forms were not directly measured in these experiments the reduced expression of CD16 (a surrogate marker for neutrophil age) suggested the presence of an immature phenotype. Although reduced expression may also be an indication of neutrophil activation with lower expression related to receptor shedding. Immature neutrophils demonstrate reduced efficacy for chemotaxis and phagocytosis compared to mature neutrophils. The data in these experiments suggest maintained, if not up-regulated phagocytosis (to *E.Coli*) in the context of impaired migration. The preserved phagocytosis may be related to heightened activation of neutrophils (as described above) that may overcome the effects of maturity on neutrophil phagocytic function (175, 377-379).

There is emerging evidence that neutrophils in infection and inflammation are capable of migrating from infected/inflamed tissues back into the systemic circulation in a process known as reverse transmigration. Although the potential role for a reverse transmigrated phenotype as a driver of neutrophil dysfunction in sepsis was not investigated in these experiments, the phenomenon may partially explain the increased activation (ROS and phagocytosis) observed in neutrophils from sepsis patients, as neutrophils that reverse transmigrate have prolonged life spans and are pro-inflammatory. They have been suggested as drivers of distal organ damage in sepsis as they circulate releasing ROS, de-granulating and phagocytosing foreign particles, whilst disseminating inflammation. This may further exacerbate the problem of “paralysed” and activated neutrophils in the systemic circulation (37, 227).

The phagocytosis experiments performed generated interesting results, which were bacterial species specific. Similar to the results demonstrated in the sepsis cohort, phagocytic function in neutrophils following traumatic SIRS has been shown to be affect by bacterial species, with enhanced phagocytosis of *E.Coli* and maintained *S.Aureus* previously shown. Sepsis neutrophils express greater CD14 and are thought to be “primed” by the inflammatory process and may account for the increased phagocytosis of gram-negative bacteria in infections (169, 171, 173, 344, 350, 354, 380).

Experiments involving phagocytosis have demonstrated both increased and decreased bacterial ingestion in sepsis (169, 344, 381). Phagocytic ability may be increased due to neutrophil activation via the up-regulation of phagocytic receptor

expression. However what is less clear in these previous studies is the efficacy of bacterial killing following ingestion. These experiments suggest that intra-cellular mechanisms of bacterial killing are preserved in sepsis, as the bacteria used in the pHrodo (Invitrogen) assay only fluorescence once acidification of the phagolysosome occurs. This is a key process in the intracellular mechanisms leading to pathogen elimination (169, 344).

In contrast to the original hypothesis neutrophils from the healthy elderly showed greater NETs compared to young controls in response to PMA. In sepsis, NET release to PMA was attenuated with the severity of sepsis corresponding to greater suppression. The process of NETosis has been closely linked with ROS production in some studies, however the experiments here suggested that despite increased ROS a decrease in NETs was observed. NETosis is a terminal event for the neutrophil and perhaps the reduction in NETs could be explained by the activated pro-inflammatory phenotype (increased ROS and phagocytosis) that neutrophils adopt with a prolonged survival. However, the model for NETosis doesn't reflect conditions in-vivo, with the biology of NET formation found to be complex with cytokines, platelets and bacteria all activating neutrophils to produce NETs. It seems probable that systemic NET release in the microvasculature occurs in response to various stimuli in an oxidant-dependent and independent manner. This may provide a mechanism of killing bacteria in the systemic circulation, however if these occur in areas where neutrophils have become "trapped" the NETs may contribute to the endothelial damage that results (8, 16, 59, 182, 192, 214, 215, 224, 363, 365, 368, 382).

7.2. The use of simvastatin to modulate neutrophil function.

The experiments detailed within the thesis suggested that in the healthy elderly aberrant migration could be improved by in-vivo 80mg simvastatin. Additionally, simvastatin did not have deleterious effects on other neutrophil functions and was well tolerated. These beneficial effects of simvastatin were not replicated in the in-vitro experiments in established sepsis however, as neutrophil migration and phagocytosis was not modulated by simvastatin, whilst ROS production and NETosis was attenuated.

The precise mechanism for the improved migration in the elderly was not investigated, however in-vitro work in the healthy elderly suggests that increased PI3K expression is associated with aberrant migration and the selective inhibition of P13Kγ and P13Kδ improved the accuracy of migration to levels similar to those observed in the young. This may be due to increased actin formation within the leading edge of the pseudopod and hence improve migratory accuracy. Simvastatin potentially inhibits PI3K activity and this may account for the improvements observed in neutrophil migration in the healthy elderly study (86, 383, 384).

In sepsis, neutrophil migration may be unresponsive to modulation due to the desensitisation and internalisation of chemokine receptors from the cytokine storm. These experiments suggested that simvastatin reduced neutrophil adhesion (in-vivo and in-vitro), ROS production and NET formation (in-vitro only). These effects of simvastatin may well lead to decreased aggregation of neutrophils within vascular beds leading to improved tissue perfusion and reduced endothelial damage.

Additionally the beneficial effects of statins on other pathways in sepsis, such as TLR function, reduction in iNOS and cytokine release may stabilise the endothelium to reduce tissue hypoxia, thereby improving some of the key pathways associated with sepsis-related organ dysfunction (154, 156, 230, 273, 312, 385).

Since the conception of the thesis, the use of simvastatin to improve outcomes in sepsis has been investigated in a number of clinical trials. All, with the exception of the ASEPSIS trial, have failed to show any benefit of acute statin therapy in the critically ill. Kruger et al used 20mg of atorvastatin to treat patients with severe sepsis admitted to ICU in a double-blinded randomised multi-centred Australian study. They found no benefit in the acute administration of atorvastatin in critically ill patients with sepsis. However pre-defined subgroup analysis of pre-existing statin users suggested that continuation of therapy during critical illness was associated with a reduced 28-day mortality, adding weight to evidence that statin administration prior to critical illness confers protection from death (286, 287, 292, 375, 386, 387).

Papazian et al investigated the use of 60mg simvastatin in reducing mortality from ventilator-associated pneumonia (STATIN-VAP trial). The trial was terminated early due to a higher mortality in the simvastatin group, although this was not significant. Despite the slight increase in mortality, simvastatin was still well tolerated by patients, with no rise in transaminases or CK noted between placebo and simvastatin groups. The HARP-2 study and SAILS studies which investigate the use of 80mg simvastatin and 20mg rosuvastatin in ARDS respectively suggested that statin therapy did not improve outcomes. In fact SAILS reported an increase in the incidence of hepatic

and renal dysfunction amongst statin users, whilst HARP-2 reported no adverse side effects with high dose simvastatin (287, 386, 387).

These large RCTs in patients with high disease severity suggest that in established severe sepsis statins are not beneficial. However the results from the healthy elderly trial reported here (Chapter 3) and the ASEPSIS trial suggest that perhaps early, or even pre-emptive therapy with simvastatin in high-risk groups, such as the elderly, may be beneficial in improving outcomes from sepsis. The vital issue regarding timing of statin therapy in infections is further highlighted by in-vitro data from patients with pneumonia (without sepsis) that suggests simvastatin improves migratory accuracy of neutrophils in these patients (personal communication with Dr Elizabeth Sapey), whilst in patients with established sepsis simvastatin had no effect (286, 287, 375, 386, 387).

An emerging theory in the treatment of sepsis is the notion of individualised immune modulating therapy based on the phase of inflammation patients' are experiencing. Classically sepsis can lead to exaggerated SIRS responses characterised by high levels of pro-inflammatory cytokines that lead to vasodilatory shock, MOF and death. The resolution of inflammation is dependent on a subsequent CARS occurring to re-establish immune homeostasis. However, there is now increasing evidence that patients hospitalised with sepsis suffer from hypo-immune response (exaggerated CARS) and it is often significant immunosuppression that leads to eventual death from sepsis. The exact mechanisms that determine individual responses in uncertain with potential genetic influences being implicated (91, 108, 128, 144, 388, 389).

As sepsis encompasses a heterogeneous population, the immune response is likely to be significantly different. This may account for some of the failures in recent ICU based trials targeting immune modulation. Patients who could be identified as suffering from exaggerated SIRS responses may benefit from immune-suppressive therapies, such as statins, whilst those with exaggerated CARS response may benefit from immune-stimulatory therapies (e.g. G-CSF) (109, 388, 390-393).

7.3. Limitations

The functional neutrophil assays and the experimental designs included within this thesis had several limitations, which are described below and may limit the generalisability of the findings.

The definitions of sepsis and severe sepsis remain non-specific (estimated at 58-61%), resulting in many hospitalised patients being classified as suffering from sepsis, as many will have a degree of inflammation. This in combination with poor biomarkers for sepsis and low yields from microbiological cultures may have resulted in some of the recruited patients not suffering from a primary infectious insult (100, 394, 395).

The non-specific nature of the definitions was reflected in the high incidence of severe sepsis in the recruited (68.9%) patients. Despite this high incidence, rates of admission to ICU were low (31.1%) with few patients requiring organ support (median SOFA score 4) and subsequently, mortality was low at 24.4%. Patients were classified into sepsis severity on the basis of their worst physiological and

biochemical parameters within 48hours of their admission, resulting in many being classified into the severe sepsis category based on individual parameters (e.g. INR >1.5, lactate >2mmol/L, systolic blood pressure <90). These parameters rapidly resolved with appropriate treatment but also did not generate a SOFA score. This may accounts for the high incidence of severe sepsis with a low mortality in these experiments. Future studies should try and define severe sepsis more precisely and perhaps, the use of a persistent or worsening organ dysfunction (measured by the delta SOFA) could be employed (396, 397).

Patients with sepsis reflected the heterogeneous population that is hospitalised with acute infections. The patients with sepsis were well matched for age and gender compared to the controls, however had a greater number of pre-existing health conditions and subsequently were taking a greater number of concomitant medications. The commonest conditions were diabetes mellitus and hypertension, with patients receiving a variety of oral hypogylcaemics, insulin and anti-hypertensive agents to manage their conditions. What is unclear is whether these patients were predisposed to sepsis due to constitutive neutrophil defects, or whether their sepsis caused the effects observed. Additionally many drugs used to treat diabetes, such as Metformin and the PPAR-γ inhibitors, and those used to treat hypertension (e.g. aspirin) may also neutrophil functions and may modulate pathophysiology of sepsis. Future neutrophil functional studies in patients with sepsis should be designed to analyse patients' neutrophil function following complete recovery from their illness. This would overcome the potential confounding effects of co-morbid conditions and medications, allowing patients to act as their own controls.

Finally the neutrophil function assays performed on the sepsis cohort were all performed un-blinded. This has the potential to introduce both experimenter and observer bias to the results obtained. Due to the nature of these assays, it would be difficult to completely eliminate these. However, future studies should try and blind the investigators performing the assays as much as possible to avoid these biases potentially affecting the results generated.

7.4. Future Work

This thesis characterised many of the features of neutrophil function in sepsis and explored the role of statins in modulating them. However many further studies are required to investigate underlying mechanism for the changes observed in sepsis and to further assess the potential of statins in sepsis.

1. Mechanism that up-regulate phagocytosis in sepsis should be explored by investigating the role of intra-cellular signalling mechanisms. Potential mechanistic pathways would be the potential role of PI3K signalling in phagocytosis in both health and in sepsis. Other bacterial species that are common sources of both community (*Streptococcus pneumonia, H. Influenzae*) and nosocomial (*Klebsiella pneumonia*) infections should be investigated to assess whether bacterial species contribute to alterations in neutrophil phagocytosis.
2. The reduction in NETosis in sepsis requires further work to determine underlying mechanisms and their potential implication in bacterial clearance and tissue damage. The differentiation between oxidant-dependent and independent mechanisms of NET release in sepsis should be identified. The potential of time-lapse video microscopy in directly visualising rapid NET production to live bacteria

and other inflammatory stimuli should be explored to determine oxidant-independent NETosis. The use of DPI and other NADPH-oxidase inhibitors should be investigated to determine their role in oxidant dependent NETosis. Additionally the activity of PAD4 in sepsis should be investigated and correlated with NET release, as this is a key mechanistic step in citrullation and subsequent NET release. NETosis should be quantified using both a fluorometric technique and light microscopy to visualise NETs. The stimulants used should include physiologically relevant stimuli, such as TNF-α, CXCL-6 and -8, and live gram-positive and gram-negative bacteria.

3. Patients with pneumonia should have functional neutrophil assays performed from neutrophils from blood and BAL to characterise the function of transmigrated neutrophils in comparison to circulating neutrophils.
4. The function of healthy neutrophils from old and young donors should be assessed following incubation in plasma from sepsis patients. These experiments may help determine the influence of the inflammatory environment on neutrophil functions.
5. The use of 80mg simvastatin should be investigated in the context of infections in the elderly. A study recruiting elderly patients admitted to hospital with an infection without significant organ dysfunction should help answer whether statins have a role in treating sepsis.
6. A future sepsis study should be designed to determine whether it is possible to determine which phase of sepsis patients are in, i.e.: SIRS or CARS. Initially experiments should commence with cytokine profiling to assess whether classification of patients into immune response sub-types is possible. These

studies may eventually lead to the development of individualised sepsis treatments in the future.

7.4. Conclusion

The experiments here suggest that neutrophils in sepsis display a specific phenotype, with a failure of migration observed in combination with a retained phagocytic capacity and a propensity to de-granulate and generate high ROS, whilst NETosis and apoptosis are both suppressed. This suggests that neutrophils contribute to the pathogenesis of organ dysfunction in sepsis by being “stranded” in the systemic circulation unable to reach sites the sites of infection, thereby allowing potential pathogen dissemination, whilst simultaneously increased systemic activation leads to collateral tissue damage.

For the first time, the age-related decline in neutrophil migration was corrected by in-vivo treatment with simvastatin in the healthy elderly. Applying this theory in-vitro, to neutrophils from sepsis patients showed no improvement in migration, but did demonstrate that simvastatin was able to attenuate both ROS production and NET release in response to PMA. The precise nature of the immune responses that alter neutrophil functions and their implications in sepsis remains uncertain with further research to investigate these indicated. Although high profile trials regarding statin therapy in critical illness have failed to prove any benefit, it is proposed, based on the positive findings of simvastatin treatment in the healthy elderly and suggestions that earlier statin therapy may be beneficial in sepsis, that treatment with simvastatin in elderly patients with early on-set sepsis be investigated.

Chapter 8: References

8.0 References

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Appendix I

Publications, presentations and prizes arising from this PhD thesis

Publications

H Greenwood, J Patel, R Mahida, Q Wang, D Parekh, R CA Dancer, H Khiroya, E Sapey, D R Thickett. **Simvastatin to modify neutrophil function in older patients with septic pneumonia (SNOOPI): study protocol for a randomised placebo-controlled trial.** Trials 2014, 15 (1), 332.

J M Patel, D R Thickett, F Gao, E Sapey. **Statins for Sepsis: Distinguishing Signal from the Noise When Designing Clinical Trials.** AMJRCCM 2013 188 (7), 874.

J M Patel, C Snaith, D R Thickett, Lucie Linhartova, T Melody, P Hawkey, A H Barnett, A Jones, T Hong, M W Cooke, G D Perkins and F Gao. **Randomized double-blind placebo-controlled trial of 40 mg/day of atorvastatin in reducing the severity of sepsis in ward patients (ASEPSIS Trial).** Critical Care 2012, 16:R231

Abstracts

JM Patel, H Greenwood, G Walton, J Lord, D Thickett, E Sapey

Pre-emptive or early adjuvant simvastatin therapy in elderly patients with infection and sepsis. The Lancet 2014 383, S79.

HL Greenwood, J Patel, GM Walton, D Griffiths, F Gao-Smith, JM Lord, D Thickett, E Sapey. **Simvastatin Improves Outcomes In Pneumonia By Modulating Neutrophil Function, But In-Vitro And In-Vivo Studies Suggest Pre-Emptive/early Therapy In The Elderly.** AMJRCCM 2014,189, A3978

Presentations

JM Patel, H Greenwood, G Walton, F Gao, JM Lord, E Sapey, DR Thickett. **Simvastatin as an adjuvant therapy for infection and sepsis–in-vitro and in-vivo studies suggest pre-emptive/early therapy in the elderly. British Thoracic Society Winter Meeting. December 2013.**

Published abstract: Thorax 68 (Suppl 3), A51-A52)

JM Patel **Neutrophil dysfunction in sepsis and modification with simvastatin treatment.** UKALI Seminar, Birmingham 2013**.**

Prizes

European Society of Anaesthesiology (ESA) Drager Prize for the best-published research paper. ESA Annual Congress, Barcelona 2013. 1st Prize 10,000 Euros for the ASEPSIS publication (see above).

Publications Planned

Chapters 3 & 4 are being combined to produce a publication on the effect of simvastatin on the migration of neutrophils. Publications regarding the up regulation of phagocytosis and the attenuation of NET production in sepsis are intended.