Perioperative neutrophil immune function

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

Chapter 4

Patient data collected and recorded by research nurses and doctors working for the PostOperative Morbidity (POM) study (Sister Sadaf Iqbal, Sister Laura Gallego-Paredes)

Murine bone marrow neutrophil samples were prepared by Dr Gareth Ackland

Chapter 5

HL-60 cell line was differentiated and maintained by Dr Ana Gutierrez del Arroyo

Signed:

Abstract

Within the UK severe sepsis is responsible for 29% of intensive care admissions and carries a mortality of 44.7%. Decades of research have failed to deliver a single clinically successful immune modulating therapeutic agent. These failings may be explained by fundamental methodological challenges of sepsis laboratory investigations, namely diagnostic uncertainty, an indeterminate onset and the identification of an immunologically similar control population.

The underlying hypothesis for this thesis is that the translational investigation of surgical patients may overcome many of these methodological challenges, since surgical trauma generates a homogenous inflammatory insult at a planned time to a carefully phenotyped human population. The biological basis for modelling sepsis with traumatic injury is discussed.

Firstly, I reviewed the current literature to demonstrate the methodological advantages of studying surgical patients as a surrogate for sepsis. Next, I performed an observational study of neutrophil immune function following major elective surgery which identified a reduced neutrophil respiratory burst and changes in cell surface immune receptor expression.

This impairment of activated neutrophil immune function was associated with resting neutrophil mitochondrial dysfunction, namely a raised mitochondrial membrane potential and increased production of reactive oxygen species. Using two different models of mitochondrial dysfunction I demonstrated that neutrophil respiratory burst may be regulated by altered mitochondrial functionality.

Finally, I provide evidence that the cytoplasmic target for this mitochondrial signal is the enzyme pyruvate kinase M2, which through oxidative inhibition reduces the production of the respiratory burst substrate NADPH by limiting flow of glucose through the hexose monophosphate shunt.

In summary, major elective surgery provides a translational model of human sepsis. Using this model, I demonstrate impairment of the neutrophil respiratory burst, and provide evidence that this is mediated through neutrophil mitochondrial dysfunction which promotes oxidative inhibition of the glycolytic regulatory enzyme pyruvate kinase.

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Abbreviations

2-NBDG	2-(*N*-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose
ADP	Adenosine Diphosphate
ACCP	American College of Chest Physicians
AMP	Adenosine Monophosphate
AMPK	Adenosine Monophosphate Kinase
ANOVA	Analysis of Variance
APACHE	Acute Physiology And Chronic Health Evaluation Score
Apaf	Apoptotic Protease Activating Factor
APC	Allophycocyanin
ARDS	Acute Respiratory Distress Syndrome
ASA	American Society of Anaesthesiology
ATP	Adenosine Triphosphate
BAL	Broncho Alveolar Lavage
Bax	Bcl-2-associated X protein
Bcl2	B-Cell Lymphoma 2 Apoptosis Regulator Proteins
Bid	BH3 Interacting-domain Death agonist
BSA	Bovine Serum Albumin
CD	Cluster of Differentiation Antigen
CDC	Centre for Disease Control
cDNA	Complimentary Deoxyribonucleic Acid
CGD	Chronic Granulomatous Disease
CO	Carbon Monoxide
CO_2	Carbon Dioxide
COPD	Chronic Obstructive Pulmonary Disease
CoQ	Co-Enzyme Q
CRP	C-Reactive Protein
CSF	Cerebrospinal Fluid
CXCR2	Chemokine-X-C Receptor 2 (IL-8 receptor)

CytC	Cytochrome-C Oxidase
DAMPs	Damage-Associated Molecular Patterns
DASA	Diarylsulfonamide
DCFH-DA	2',7'-dichlorodihydrofluorescein diacetate
DHE	Dihydroethidium
DHEA	Dehydroepiandrosterone
DHR	Dihydrorhodamine
$DiOC_6$	3,3'-Dihexyloxacarbocyanine Iodide
DMEM	Dulbecco Modified Eagles Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
dTTP	Thymidine triphosphate
dUTP	Deoxyuridine Triphosphate
ECG	Electrocardiogram
EDTA	Ethylenediaminetetraacetic Acid
EGFR	Epidermal Growth Factor Receptor
ELISA	Enzyme Linked Immunosorbent Assay
ERK	Extracellular signal-Regulated Kinase
ESR	Erythrocyte Sedimentation Rate
FACS	Fluorescence Activated Cell Sorting
Fc	(Immunoglobulin) Fixed Chain
FCCP	Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone
FITC	Fluorescein isothiocyanate
fMLP	N-Formylmethionine-leucyl-phenylalanine
FN	Fibronectin
FSC	Forward Scatter
G6P	Glucose-6-Phosphate
G6PDH	Glucose-6-Phosphate Dehydrogenase
GA3P	Glyceraldehyde-3-Phosphate
GFR	Glomerular Filtration Rate
GLUT	Glucose Transporter
GM-CSF	Granulocyte Macrophage – Colony Stimulating Factor

GTP	Guanosine Triphosphate
H_2O_2	Hydrogen Peroxide
HBSS	Hank Balanced Salt Solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIF	Hypoxia Inducible Factor
HIV	Human Immunodeficiency Virus
HL-60	Human Leukocyte 60 Cell Line
HLA	Human Leukocyte Antigen
HMGB1	High Mobility Group Box Protein 1
HPO	Horseradish Peroxidase
HV	Healthy Volunteer
ICU	Intensive Care Unit
Ig	Immunoglobulin
IKB	Inhibitory Kappa Beta
IL-1β	Interleukin-1 Beta
IQR	Interquartile Range
IRAK	Interleukin-1 Receptor Associated Kinase
ISS	Injury Severity Score
JAK	Janus Kinase
JC-1	Tetraethylbenzimidazolylcarbocyanine iodide
JNK	c-Jun N-terminal kinase
LED	Light Emitting Diode
LOS	Length Of Stay
LPS	Lipopolysaccharide
mAb	Monoclonal Antibody
MAPK	Mitogen Activated Protein Kinase
Mcl1	Induced Myeloid Leukaemia cell differentiation protein
METs	Metabolic Equivalents
MFI	Median Fluorescence Intensity
MMP	Mitochondrial Membrane Potential
MNF	Mononuclear Fraction
MnSOD	Manganese Superoxide Dismutase

MODs	Multi Organ Dysfunction Syndrome
MREC	Medical Research and Ethics Committee
mRNA	Messenger Ribonucleic Acid
mtDNA	Mitochondria Deoxyribonucleic Acid
mTOR	Mammalian Target Of Rapamycin
NAD	Nicotinamide Adenosine Dinucleotide
NADH	Reduced Nicotinamide Adenosine Dinucleotide
NDMA	N-methyl-D-aspartate
NETs	Neutrophil Extracellular Traps
NF-kB	Nuclear Factor - Kappa Beta transcription factor
NLRP3	NACHT, LRR and PYD domains-containing protein 3
NO	Nitric Oxide
NOD	Nod-like Receptor
NOX	NADPH Oxidase
NRF2	Nuclear factor (erythroid-derived 2)-like 2
O_2	Oxygen
O_2^-	Superoxide
OCR	Oxygen Consumption Rate
P_2Y_2	Purinergic Receptor Y2
PAMPs	Pathogen-Associated Molecular Patterns
PBL	Peripheral Blood Leukocyte
PBS	Phosphate Buffered Saline
PE	Phycoerythrin
PEG	Polyethylene Glycol
PEP	Phosphophenylpyruvate
PFA	Paraformaldehyde
PHOX	Phagocyte NADPH Oxidase (NOX2)
Pi	Inorganic Phosphate
PI	Propidium Iodide
PI3K	Phosphoinositide 3-kinase
PINK-1	PTEN-Induced putative Kinase
PIP_3	Phosphatidylinositol (3,4,5)-trisphosphate

PK	Pyruvate Kinase
PKC	Protein Kinase C
PKM2	Pyruvate Kinase M2 isoform
PMA	Phorbol Myristate Acetate
PMT	Photomultiplier Tube
POD	Post-Operative Day
POMs	Post-Operative Morbidity Score
POSSUM	Physiological and Operative Surgery Severity Score for enumeration of Mortality and Morbidity
PRRs	Pattern Recognition Receptors
PTEN	Phosphatase and Tensin homolog
qRT-PCR	(Semi) quantitative Reverse Transcription Polymerase Chain Reaction
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RPMI-1640	Roswell Park Memorial Institute 1640 solution
SAPS	Simplified Acute Physiology Score
SCCM	Society of Critical Care Medicine
SD	Standard Deviation
SDS PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SIRS	Systemic Inflammatory Response Syndrome
SOD	Superoxide Dismutase
SSC	Side Scatter
STAT	Signal Transducer and Activator of Transcription
TIRAP	Toll-Interleukin 1 Receptor Adapter Protein
TLR	Toll-like Receptor
TMRM	Tetramethylrhodamine, Methyl Ester, Perchlorate
TNF-α	Tumour Necrosis Factor Alpha
TNM	Tumour Nodes Metastasis classification system
TREM-1	Triggering Receptor Expressed on Myeloid cells 1
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labelling
UDG	Uracil-DNA Glycosylase

VAP	Ventilator Associated Pneumonia
VDAC	Voltage-Dependent Anion Channel
VSAQ	Veterans Specific Activity Questionnaire
WCC	White Cell Count

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Chapter 1 – Introduction

1.1 The challenges of studying sepsis

Sepsis is a clinical syndrome. It was newly defined in February 2016 as organ dysfunction caused by a dysregulated host response to suspected infection, and carries a mortality of 10% (Singer et al., 2016). The previous definition of sepsis, used by much of the published literature, described a systemic inflammatory response (SIRS) in the context of presumed infection, with sub-classifications of severe sepsis and septic shock describing coexistent organ dysfunction and treatment resistant hypotension respectively (Dellinger et al., 2013).

Within the United Kingdom severe sepsis accounts for 28.7% of intensive care admissions and confers a mortality of 44.7% (Harrison et al., 2006). The international incidence of sepsis may be rising (Dombrovskiy et al., 2007; Martin et al., 2003). To date, enormous investment and decades of research have failed to deliver a single immune-modulating agent that has been reliably shown to improve outcome from human sepsis (Alejandria et al., 2013; Angus et al., 2000; Lehmann et al., 2014; Ranieri et al., 2012; Sprung et al., 2008). This thesis will explore how the methodological challenges presented by study of human sepsis may be responsible for these limitations, and if an alternative model of human inflammation – using blood samples taken from major elective surgery patients – can overcome these challenges.

The methodological challenges of human sepsis research begin with the very diagnosis of sepsis itself. Sepsis definitions were designed to guide empirical bedside management of sepsis (Dellinger et al., 2013; Singer et al., 2016), not to facilitate the scientific interrogation of the pathophysiological mechanisms responsible. As such they rely upon non-specific physiological and laboratory parameters to identify inflammation and clinical suspicion to identify infection. Although the diagnostic accuracy maybe improved by the inclusion of latterly returned microbiological data, these data are often non-specific and the 'dose' of infection can never be known. It follows that the relative contribution of infective burden, maladaptive inflammatory responses and therapeutic plus iatrogenic interventions to disease progression within individual patients cannot be determined.

Identification of an appropriate control population presents the second key methodological challenge to human sepsis studies. Cohort or longitudinal study design is methodologically advantageous because septic samples may be compared to an individual's pre-illness control samples, improving the likelihood that the development of sepsis is responsible for observed changes to immune readouts. However, such studies are neither economically feasible nor

practicable for a disease which is relatively infrequent and has such a capricious onset as sepsis. Therefore, case-control investigations are often performed, where a separate control population – such as healthy volunteers - is identified. However, septic patients may possess a range of immunologically relevant confounding factors that are not represented within the non-septic control population. Specific examples include:

- Age - Sepsis is more common in the elderly (Henriksen et al., 2015). Advancing age is associated with reduced lymphocyte number and functionality (Montecino-Rodriguez et al., 2013), and diminished granulocyte effector activities such as respiratory burst (Valente et al., 2009).
- Gender - Sepsis is more common in men (McGowan et al., 1975; Offner et al., 1999). Enhanced protection during the proestrus period and following oestrogen replacement in postmenopausal women suggests oestrogen may confer direct protective effects from sepsis (Weniger et al., 2015), possibly through oestrogen mediated attenuation of proinflammatory cytokine secretion (Angele et al., 2014).
- Comorbid disease – Patients with overt immunosuppressive diseases such as Human Immunodeficiency Virus (HIV) are more likely to develop sepsis (Henriksen et al., 2015). Cancer is associated with an increased incidence of infection and a host of immunological changes, such as altered distribution of leukocytes across the gut and lung, natural killer cell depletion and lymphopenia (Ray-coquard et al., 2009; Snyder and Greenberg, 2010). Other diseases and syndromes are also associated with changes in resting markers of inflammation such as erythrocyte sedimentation rate (ESR) and C-reactive peptide (CRP), e.g. chronic heart failure (Fildes et al., 2009). Many of these confounding diseases and conditions may be undiagnosed at the time of sepsis presentation and remain so throughout the patient's intensive care unit (ICU) admission.
- Therapeutic interventions. Commonly administered therapies such as antibiotics (Kalghatgi et al., 2013; Kanoh, 2010), sedatives (Mikawa et al., 1998) and steroids (Clarke et al., 1993) have off-target immunological effects. These include inhibition of NF-kB secretion (Kanoh, 2010), enhanced production of reactive oxygen species (Kalghatgi et al., 2013), impaired neutrophil respiratory burst activity (Mikawa et al., 1998), and lymphocyte apoptosis (Clarke et al., 1993).

It follows that the overwhelming experimental noise present within clinical investigations may prevent real and relevant experimental signals from being identified. Traditionally, translational research has employed a range of laboratory models that aim to overcome this experimental noise and in which preliminary observations may be made. For example, the use of animal models, and healthy volunteer endotoxin/cytokine exposure are well established within sepsis research. With these models, the timed administration of an inflammatory insult, coupled with carefully controlled laboratory conditions avoid many of the methodological challenges of real-world sepsis research. However, these methodological advantages may be offset by their limited biological relevance. The biology of animal model sepsis is different to that in humans, while healthy volunteer studies cannot reproduce the severity of real-world disease. In principle, the use of patients undergoing major elective surgery provides many of the methodological benefits of animal model research coupled with the administration of a major inflammatory stimulus within human beings.

1.2 Models of human sepsis

1.2.1 Animal models

Modern transgenic and knock-down technology allow control over the location and timing of gene expression. Humanised models, where human myeloid cells are grafted into immunocompetent mice, reproduce specific features of human disease with greater accuracy. Despite these developments there are inherent physiological, pathological and phenotypic differences that limit their applicability. Murine metabolism (Radermacher, 2013), cardiovascular regulation (Doevendans et al., 1998) and gut flora (Salzman et al., 2002) are very different to humans. Wide variations in survival of different mouse strains has been reported (De Maio et al., 2005; Stewart et al., 2002). Pattern recognition receptor activation and signalling is fundamentally different (Lund et al., 2003; Rehli, 2002). For example, 10,000-fold higher doses of lipopolysaccharide required to recreate sepsis syndrome in rats (Shultz et al., 2007). In contrast to humans, circulating murine lymphocytes greatly outnumber circulating granulocytes. While human leukocyte messenger

ribonucleic acid (mRNA) expression is highly conserved following trauma, burns and endotoxin, murine homologs show little correlation and much wider variation to these different stimuli (Xiao et al., 2011). Immune effector functions differ, as do distribution of leukocyte subsets across gastrointestinal and pulmonary surfaces (Doeing et al., 2003; Rehli, 2002). The modality of septic death is also fundamentally different. Model animals typically die early, as a direct consequence of the initial pathophysiological insult (Warren, 2009), whereas human death usually follows a delayed multisystem dysfunction some time after initial resuscitation and therapeutic support (Martin et al., 2003).

Large animal models have also been developed. For ethical reasons these models are rarely used for survival studies (Fink, 2014). Similar to humans, sheep display marked changes in pulmonary artery pressure, cardiac output and lung microvascular permeability with nanomolar/kg doses of endotoxin (Traber et al., 1988) while pseudomonas aeruginosa infusions have been used to replicate sepsis itself (Booke et al., 2000). Non-human primates, such as Papio anubis are incredibly resistant to endotoxin and bacterial infusion (Hinshaw et al., 1981), and the use of such models to test pharmacological agents have not correlated with outcomes in human sepsis trials (Fink, 2014).

1.2.2 Healthy volunteer endotoxin and cytokine models

The administration of endotoxin (1-4ng/kg iv) or specific cytokines to healthy volunteers mimics certain aspects of sepsis such as the loss of granulocyte tumour necrosis factor receptor (TNF-R) (Calvano and Coyle, 2012; van der Poll et al., 1995) and the early hyperdynamic circulatory response phase of sepsis (Andreasen et al., 2008; Bahador and Cross, 2007; Lowry, 2005). However other aspects, such as the dynamic pattern of cytokine expression, differ markedly to patterns observed in established sepsis (Damas et al., 1992; Krabbe et al., 2002). For obvious ethical reasons the magnitude and duration of organ dysfunction, therapeutic interventions and comorbidities which characterise human sepsis cannot be replicated within these models (Andreasen et al., 2008).

1.2.3 Major elective surgery

Major elective surgery provides two distinct opportunities for cohort investigations of human sepsis. Firstly, the inflammatory insult of surgery itself may serve as a model of infection, and secondly, postoperative sepsis is relatively common. In both instances, the planned nature of elective surgery overcomes the methodological challenges of studying humans with established sepsis.

The planned nature of elective surgery permits cohort study design, enabling comparison of accurately timed post-insult samples with individualised control samples. By specifying operation types and surgical teams the 'dose' of surgery is relatively controlled. Such patients typically undergo extensive preoperative investigation, and are therefore carefully phenotyped (Stringer, 2010). Large numbers of patients may be recruited, with an estimated 234 million surgical procedures performed each year (Weiser et al., 2008). Surgical procedures themselves allow the acquisition of body tissue samples (Brealey et al., 2002; Henriksen et al., 2003; Kobayashi et al., 2008) the placement of indwelling catheters for future sampling (Moore et al., 1991) and access to non-invasive imaging modalities (Izquierdo et al., 2013; Purdon et al., 2009), while routine perioperative blood sampling allows serial collection of data without added risk. When these aspects are considered, major elective surgery may be considered to bring the methodological precision of laboratory research to real-world human inflammatory disease.

1.2.3.1 Modelling human sepsis with a surgical insult.

Experimental observations from postoperative patients are directly relevant to those with sepsis because human responses to infective and traumatic injury are highly conserved at molecular, genomic and clinical levels.

The molecular similarities begin with the very first checkpoint of inflammation, the release and detection of danger signals (Nathan, 2006). Endogenous danger signals are termed damage-associated molecular patterns (DAMPs), and consist of a diverse range of compartmentalised molecules/compounds whose presence out-with their usual location signals cellular injury. Exogenous danger signals are termed pathogen associated molecular patterns (PAMPs). DAMPs and PAMPs bind to membrane and cytoplasmic receptors termed pattern recognition receptors (PRR). Ultimately, both DAMPs and PAMPs promote

the assembly of shared signalling platforms, such as the inflammasome and apoptosome, and the activation of identical transcription factors, such as Nuclear Factor -kB (NF-kB). Shared responses to these infective and traumatic injury signals are apparent at many levels of the inflammatory cascade (Fig 1.1). Inflammation is not an all or nothing response, and exists on a homeostatic continuum. Some DAMPs, such as endotoxin are continuously released and the complex receptor signalling systems more similar to endogenous hormone-receptor interactions than those of bacterial toxins (Marshall, 2005).

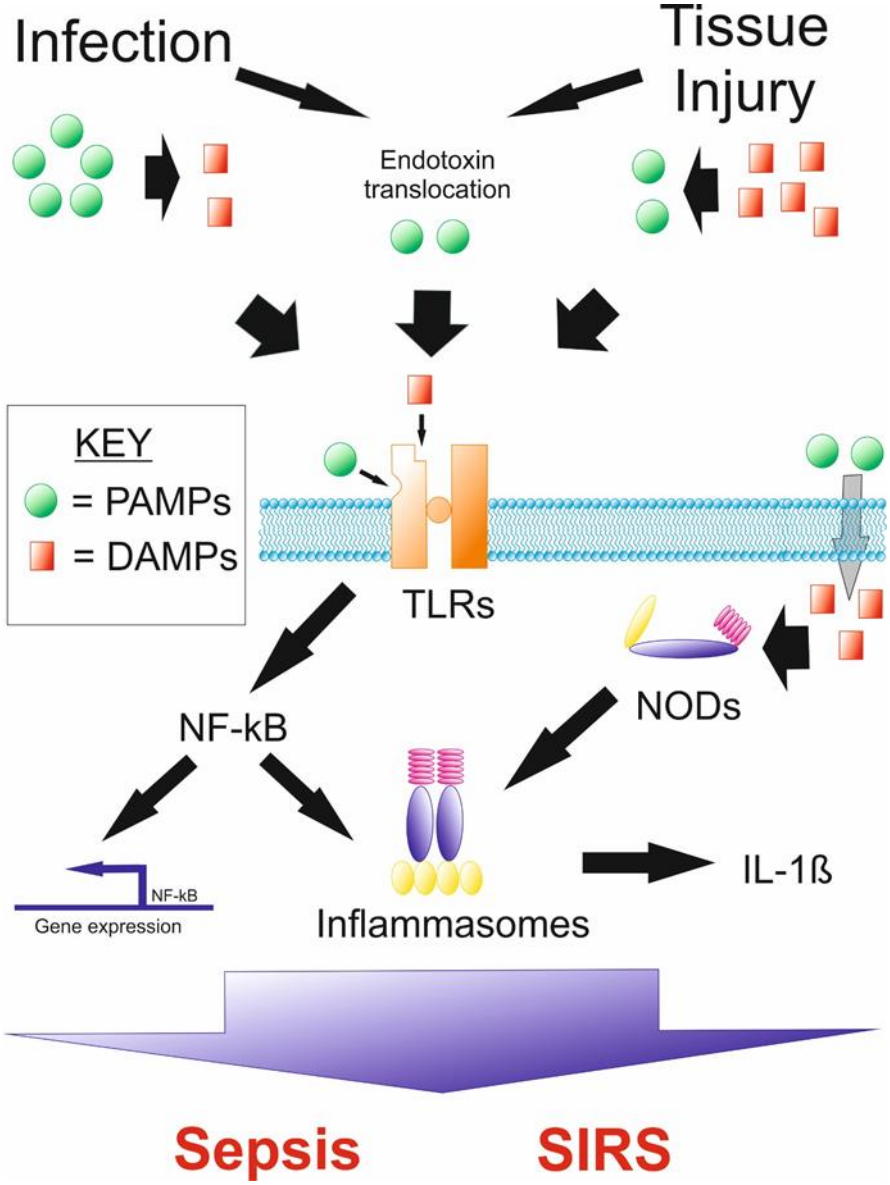

Figure 1.1 Molecular recognition of infective and traumatic injury.

Adapted from (Mollen et al., 2006)

Similarities between the molecular recognition of infective and traumatic injury include:

- **Shared activation of the same PRR by multiple DAMPs and PAMPs.** Some PRRs respond to both DAMP and PAMP ligands (Matzinger, 1994; Medzhitov and Janeway, 2002). This may be achieved through multiple ligand domains on the receptor itself, conserved molecular motifs on ligands, such as uncovered hydrophobic portions (Seong and Matzinger, 2004), the use of adapter proteins plus co-receptors and co-activation at lipid rafts (Pfeiffer et al., 2001; Triantafilou and Triantafilou, 2002). Toll-like receptor 4 (TLR4) is the most heavily investigated PRR, and is critical to the host response across a multitude of experimental paradigms (Ayala et al., 2002; Barsness et al., 2004). The TLR4 ligand repertoire is vast (Table 1.1).

Exogenous	Endogenous
Lipopolysaccharide	High Mobility Group Box I
Lipid A	Heparin sulphate
	Extra domain A of fibronectin
	Heat proteins 60, 70, Gp96
	Surfactant Protein A
	B-Defensin 2
	Fibrinogen
	Biglycan
	Hyaluronan oligomers

Table 1.1 Exogenous and endogenous Toll-like Receptor 4 agonists.

Adapted from (Mollen et al., 2006)

- **Shared signal transduction machinery by different PRR.** Toll like receptors and other PRR activate a highly conserved number of intracellular adapter proteins, such as MyD88 and TIRAP (Henneke and Golenbock, 2001; Tamassia et al., 2007). MyD88 recruits the IRAK family kinases, and subsequently MAPK and NF-kB. The transcription factor NF-kB is a key activator of innate immunity, and is a complex of Rela (p65)/p50 heterodimers which remain in the cytoplasm bound to inhibitory kB (IkB). Upon activation, IkB dissociates and NF-kB translocate to the nucleus, binding to gene promotors (Ghosh and Karin,

2002). Some of these down-stream pathways, such as Hypoxia Inducible Factor (HIF) are also directly activated by commonly used anaesthetic agents (Cramer et al., 2003; Peyssonnaux et al., 2005).

- **Co-release of DAMPs and PAMPs.** Tissue injury provides a portal for infection which will cause further tissue injury. Therefore, the release of DAMPs will be accompanied by PAMPs and vice versa.
- **Translocation of endotoxin.** The physiological redistribution of blood flow away from the gut which accompanies severe systemic inflammation may lead to translocation of PAMPs (such as endotoxin) to the systemic circulation (Ackland et al., 2000; Van Leeuwen et al., 1994).
- **Mitochondrial DAMPs are structurally similar to bacterial PAMPs.** Mitochondria contain molecular signatures of their protobacterial origins, such as circular Deoxyribonucleic Acid (DNA) with CpG repeats and formyl-peptides which activate TLR9 and formyl-peptide receptors respectively, and exemplify the evolutionary relationship between DAMPs and PAMPs (Bianchetti et al., 1977; Taanman, 1999). Plasma levels of mitochondrial DAMPs maybe elevated a thousand-fold higher in trauma patients (Zhang et al. 2010; Haimovich et al. 2014) and correlate with mortality (Simmons et al., 2013). The infusion of mitochondrial DAMPs into rats leads to the accumulation of fluid, albumin and neutrophils within the lungs, which maybe prevented by the co-administration of blocking antibodies, while the *in vitro* incubation of neutrophils with mitochondrial DAMPs leads to an activated neutrophil phenotype, with Ca influx, p38 MAP-kinase phosphorylation and IL-8 release (Q. Zhang et al., 2010).

The molecular similarities between infective and non-infective inflammation are supported by genomic investigations of circulating leukocyte mRNA expression. Highly conserved responses within the circulating leukocyte transcriptome have been identified following trauma, burns and endotoxin administration [(Xiao et al., 2011), with endotoxin-exposed volunteers and trauma patients demonstrating an 88% concordance in expression. Such observations provide genomic support to the clinical similarities between major surgery and sepsis patients.

These investigations have identified genome wide changes in expression – one investigation found that 16,820 of 20,720 reported genes changed expression (Xiao et al., 2011), while studies of more limited expression have also identified changes in many non-immune genes, such as glycolytic and circadian clock modules (Haimovich et al., 2014). Genomic studies of circulating leukocyte mRNA expression have shown strong concordance despite using different technologies (Fessler et al., 2002; Malcolm et al., 2003; Zhang et al., 2004).

The correlation between transcriptome and proteome expression is less clear. de Godoy et al found a strong relationship when investigating yeast replication (de Godoy et al., 2008), however a similar investigation of inflammatory gene/protein expression found little correlation (Fessler et al., 2002). The alternative approach to these 'bottom-up' technologies is to measure specific cellular functions that may be expected to alter clinical outcomes, such as assays of immune cell effector activities. For example, the investigation of sepsis induced lymphocyte apoptosis (Boomer et al., 2011; Hotchkiss et al., 1997).

These molecular and genomic data may therefore explain why patients with primarily traumatic or infective injury appear clinically similar (Matzinger, 1994; Seong and Matzinger, 2004). In contrast to the robust evidence base supporting the biological basis for modelling human sepsis with traumatic injury, it is unclear whether the theoretical methodological advantages of studying surgical patients are realised within the translational literature.

1.2.3.2 Modelling sepsis with postoperative infectious complications.

Postoperative sepsis is sufficiently common to make cohort investigations feasible. Even in prospective randomized controlled trials investigating surgical site infections, the incidence often exceeds 15% (Belda et al., 2005; Melling et al., 2001).

Postoperative sepsis is more common in patients with immunosuppressive disease (Charlson et al., 1994) cancer (Bateman et al., 2010; Charlson et al., 1994; Copeland et al., 1991), advanced age (Charlson et al., 1994; Copeland et al., 1991; Gupta et al., 2013; Manilich et al., 2013), malnutrition (Warnold and Lundholm, 1984; Windsor and Hill, 1988) and congestive cardiac failure (Charlson et al. 1994; T. H. Lee et al. 1999; Bateman et al. 2010). All of these conditions are common in non-surgical patients who develop sepsis (Angus et al., 2001).

1.3 Neutrophils as key players in sepsis and organ injury

Neutrophils are the predominant cell type of the early SIRS response, readily entering inflamed tissues and possessing a powerful antimicrobial arsenal (Smith, 1994). Inherited deficiencies of neutrophil function, such as chronic granulomatous disease and leukocyte adhesion deficiency, predispose to life-threatening bacterial infection. While an appropriate response to infection is necessary for survival, such powerful antimicrobial functions may also cause harm.

The experimental and clinical literature supports the autotoxic potential of neutrophils. Experimental data from animal models suggest that the timing of neutrophil activity in response to an index insult is critical in determining outcome, however this cannot be reliably tested within human sepsis patients because the time at which sepsis began is not known.

Autotoxic neutrophil behaviour may be associated with every stage of neutrophil activation and activity. Accumulation of neutrophils within the vasculature has been demonstrated during systemic illness (Brown et al., 2006). Migration of neutrophils through venules may directly damage endothelium, and promote further tissue injury (Abe et al., 1990). The passage of neutrophils into distant tissues, such as the lung (Mallick et al., 1989) and liver (Holman and Saba, 1988), may promote distant organ dysfunction. The release of toxic granules within infected tissues may damage local tissues. Through these mechanisms neutrophils may promote continued release of DAMPs, sustaining the inflammatory response and leading to a state of chronic non-resolving inflammation (Nathan, 2006). Due to the large numbers of neutrophils released each day, small changes in the rate of apoptosis may increase both circulating and tissue neutrophil numbers dramatically.

Neutrophils are also key regulators of the adaptive immune response. They may recruit classical antigen presenting cells through outside-in integrin signalling (Pillay et al., 2012), the release of granule contents (Chertov et al., 2000) and the secretion of cytokines/chemokines (Pelletier et al., 2010). Neutrophils may possibly present antigen directly, through cell surface Human Leukocyte Antigen-DR (HLA-DR) (Gosselin et al., 1993). Finally, neutrophils may polarise macrophage differentiation into pro and anti-inflammatory subtypes (Nathan, 2006), dependent upon prevalent conditions.

These potentially autotoxic behaviours may be offset by anti-inflammatory processes. Neutrophil extracellular traps are networks of extruded DNA that concentrate killing power, and in doing so, prevent the systemic spread of granule contents. The generation of an early robust neutrophil response may be necessary for the transition to the timely resolution of inflammation. Patients with chronic granulomatous disease, an inherited defect of the neutrophil respiratory burst, have foci of sterile inflammation and a predisposition to soft tissue abscesses. This observation is supported by mouse models that show unrestrained γΔ lymphocyte activity, enhanced IL-17 production and defective regulatory T-cell production, plus and a predisposition to autoimmune disease (Romani et al., 2008).

Within animal models neutrophil function has been dynamically manipulated through the timed depletion of the circulating pool with cytotoxic vinblastine or anti–GR1 antibodies. Within a model of ischaemia-reperfusion injury, pre-insult vinblastine increased the systemic translocation of gut bacteria, while post-insult depletion reduced distant organ injury (Deitch et al., 1990; Hoesel et al., 2005). Delayed neutrophil depletion may confer protection by reducing delayed injury to distant organs such as the lung and liver (Poggetti et al., 1992; Raghavendran et al., 2005). Within murine models of pancreatitis (Chen et al., 2015) and hepatitis (Liu et al., 2006), GR-1 antibody-mediated neutrophil depletion consistently reduces local tissue injury and systemic markers of inflammation. Similarly, pharmacological inhibition of neutrophil activation confers protection within murine models of endotoxic shock (Lowell and Berton, 1998; Nathens et al., 1997).

Definitive conclusions regarding the role of neutrophils in human sepsis have been frustrated by variability in experimental results. Bronchoalveolar lavage samples from patients with established lung injury reveal a correlation between alveolar neutrophil numbers and clinical outcome (Chollet-Martin et al., 1996; Miller et al., 1992; Steinberg et al., 1994), and similarly the presence of neutrophil precursors on admission to critical care is associated with both the presence of sepsis and worse clinical outcomes (Mare et al., 2015). Tests of actual neutrophil function, such as the neutrophil respiratory burst may be increased (Bass et al., 1986; Martins et al., 2003) or decreased (Wenisch and Graninger, 1995) in septic patients. Furthermore, the functional significance of the widespread reprogramming of the circulating leukocyte transcriptome, which includes metabolic as well as classical immune genes, remains unknown. Therefore, the surgical model of inflammation provides an opportunity to readdress these studies, using precisely timed clinical samples to

investigate the proposed dynamic interaction between neutrophil function in relation to an index inflammatory insult.

1.4 The metabolic nature of sepsis

The sepsis literature describes a hypometabolic and oxidative stress phenotype, present throughout bodily organs and circulating cells. Septic patient tissues demonstrate diminished ATP production with preserved dissolved oxygen tensions, (Carré and Singer, 2008; Fink, 2001; Padfield et al., 2005; Svistunenko et al., 2006), implying impaired cellular/mitochondrial utilisation of oxygen - a state termed cytopathic dysoxia. Patients with severe sepsis consume less oxygen than those with uncomplicated sepsis (Kreymann et al., 1993). Ex vivo interrogation of septic mitochondria has identified a broad range of functional changes across the full range of established mitochondrial activities.

The hypometabolic septic phenotype is supported by clinical trial data from studies of goal directed therapy, where the manipulation of oxygen delivery to peripheral tissues in sepsis does not appear to improve clinical outcome, suggesting that dysoxia is an important cellular feature of sepsis/tissue injury (Gattinoni et al., 1995; Hayes et al., 1993; Mouncey et al., 2015; Peake et al., 2014; Yealy et al., 2014)

The interpretation of these sepsis metabolic studies is limited by the absence of reliable control data. Like immune function, age, comorbid disease and therapeutic interventions may all alter metabolic function. Off target mitochondrial effects have been demonstrated with volatile anaesthetic agents (Cohen, 1973; Eckenhoff and Johansson, 1997; Hanley et al., 2002; Mazzanti et al., 1979), lignocaine (Werdehausen et al., 2007), antibiotics (Gootz et al., 1990; Pochini et al., 2008) and propofol (Marian et al., 1997; Schenkman and Yan, 2000; Stevanato et al., 2002). Anaesthetic agents also inhibit central nervous system glycolytic enzymes (Webb and Elliott, 1951). The surgical model of inflammation may therefore support the investigation of these (likely) dynamic metabolic changes by providing accurately timed patient samples.

How the hypometabolic phenotype affects circulating immune cells is less clear. Within lymphocytes a hypometabolic phenotype has been previously shown to be associated with both diminished circulating numbers and multiple deficiencies of lymphocyte function, such

as cellular proliferation, cytokine production and a tendency to apoptosis (Edwards et al., 2015). This phenotype was associated with excess postoperative morbidity.

A hypometabolic phenotype would be directly relevant to neutrophils. Activated neutrophils are critically dependent upon glycolysis and the hexose monophosphate shunt to provide bioenergetic adenine triphosphate (ATP) (chemotaxis/phagocytosis) and nicotinamide adenine dinucleotide phosphate (NADPH) (the respiratory burst). ATP is also required for gene regulation and protein synthesis within activated neutrophils (Kobayashi et al., 2003; McDonald et al., 1997).

Transcriptional and metabolic profiling of peripheral blood mononuclear cells has identified a shift from oxidative phosphorylation to aerobic glycolysis following addition of lipopolysaccharide (LPS) and C.albicans (Cheng et al., 2016) i.e. the same metabolic phenotype observed in cancer cells, termed the Warburg effect.

1.4.1 Mitochondria and sepsis

Mitochondrial dysfunction underlies the hypometabolism and oxidative stress of sepsis, as well as the increasing likelihood of cellular apoptosis. Mitochondria produce ATP through oxidative phosphorylation, where electrons are transferred from high energy substrates across four respiratory complexes to the terminal acceptor oxygen (Fig 1.2). The passage of electrons liberates protons that are pumped across the inner mitochondrial membranes, thereby creating a mitochondrial membrane potential (MMP). These protons pass back into the mitochondrial matrix through F1F0 ATP synthase, synthesising ATP from ADP.

Beyond production of bioenergetic ATP, the MMP also supports the transfer of other ions (such as calcium) and compounds into and out of mitochondria. The MMP is necessary for osmotic regulation and therefore mitochondrial viability, which in turn activates cellular apoptosis. Control of oxidative phosphorylation is mediated through substrate level allosteric control – i.e. ATP/Pi, NAD:NADH and O_2 - but remains incompletely understood (Balaban et al., 2005; Brown, 1992; Hüttemann et al., 2008).

Within the inner membrane individual respiratory complexes are grouped into larger super complexes which facilitate efficient electron transfer. Despite this, between 0.1% and 5% of electrons escape and react with local oxygen to form reactive oxygen species (ROS) (Boveris et al., 1972; Fridovich, 2004). Mitochondrial ROS are principally generated as superoxide during oxidative phosphorylation, mostly at respiratory complex III (Vincent et

al., 2004), but also from complex I (Lambert and Brand, 2004; Lenaz et al., 2006) and II (Dikalov, 2011).

Several processes have been identified which regulate and maintain the MMP. Obstructions to electron flow, including hypoxia and pharmacological inhibition of complexes, will increase ROS production and dissipate the membrane potential (Madesh and Hajnóczky, 2001; Zoratti and Szabb, 1995). This process may be actively regulated by promotion of reverse electron flow through the respiratory complexes (Lambert and Brand, 2004) and mitochondrial ATP levels (Costa and Garlid, 2008; Queliconi et al., 2011). Similarly, if mitochondrial respiration is compromised the F1F0 ATP-synthase may maintain the MMP through ATP consumption and reverse transport of hydrogen ions (Campanella et al., 2008).

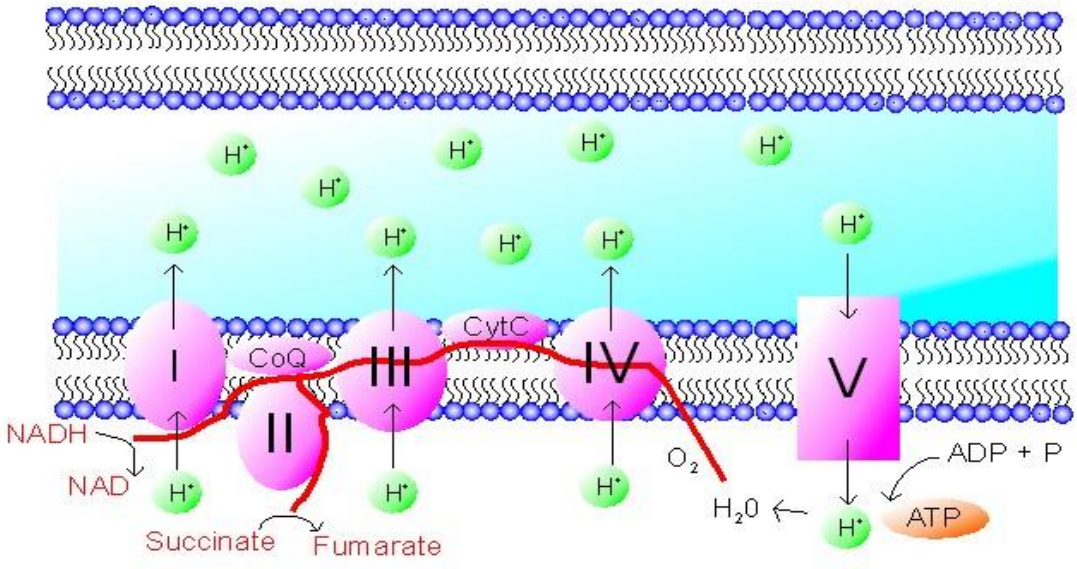

Figure 1.2 Mitochondrial Oxidative Phosphorylation.

The red line represents the route of electron flow. Blue shaded area depicts the intermembrane space.

Dysfunction of mitochondrial respiratory complexes has been demonstrated within muscle samples from septic patients (Brealey et al., 2002). This dysfunction may be reversibly regulated by small molecular mediators such as ROS, nitric oxide (NO) and carbon monoxide (CO) (Brunori et al., 1999).

Specific pharmacological inhibitors of respiratory complexes are commercially available, and maybe used as to create laboratory models of mitochondrial dysfunction. These inhibitors

demonstrate dose dependency, with lower doses causing hyperpolarisation and ROS production and higher doses leading to lipid peroxidation and super complex disaggregation (Lenaz et al., 2006). Hyperpolarisation of the MMP may lead to ROS production at complexes proximal to the inhibited complex (Chen et al., 2003). The addition of the respiratory complex I inhibitor metformin to human peripheral blood mononuclear cells leads to decreased cytokine production in presence of C.albicans, and decreased survival within a murine model of bacterial sepsis (Cheng et al., 2016).

The principal mitochondrial ROS product is O_2^-, which is charged and may only leave the mitochondrion following dismutation to lipid permeable H_2O_2 by mitochondrial manganese superoxide dismutase (MnSOD/SOD2). As well as affecting mitochondrial energy production, mitochondrial ROS may also affect cellular redox balance (Ballinger, 2005).

In addition to ROS production, the proton gradient across the inner mitochondrial membrane may be dissipated through membrane uncoupling proteins, which permit the facilitated transport of hydrogen ions along concentration gradients and essentially 'uncouple' proton movement from ATP production (Boss et al., 1998; Clapham et al., 2000). They may provide an alternative route to dissipating the proton gradient and limiting hyperpolarisation mediated ROS production (Brownlee, 2001; Kim-Han et al., 2001).

Within neutrophils, mitochondria have an established role in the regulation of neutrophil lifespan through a process termed constitutive apoptosis. The presence of neutrophil mitochondrial dysfunction may therefore plausibly alter their immune functionality.

1.5 Reactive oxygen species – a cellular signal

Reactive oxygen species (ROS) produced during these metabolic reactions may activate several cellular signalling processes. ROS reversibly oxidize specific amino acid residues (Dröge, 2002; Janssen-Heininger et al., 2008; Jones, 2008) at concentrations which occur during physiological metabolism, for example cysteine group oxidation by H_2O_2 (McDonagh et al., 2009). Any protein/enzyme system with exposed cysteine residues at the receptor or allosteric site may be subject to oxidative regulation; sequential reducing/non-reducing SDS PAGE western blotting has identified many cellular proteins that are sensitive to disulphide oxidation (Cumming et al., 2004).

The specific nature of these molecular interactions means ROS species should be considered individually, since their affinities for different targets will vary at these low doses. This molecular specificity – rather than ligand/receptor specificity of classical signalling pathways – may permit ROS to simultaneously alter multiple signalling pathways, transmitting a signal both widely and quickly.

The amplitude of ROS signals depends upon the rate of ROS detoxification as well as ROS production. These detoxification pathways are cellular compartment and species specific. The dismutation of O_2^- is catalysed by copper and zinc superoxide dismutase in the cytoplasm (McCord and Fridovich, 1969), and by manganese superoxide dismutase in mitochondria (Fosslien, 2003; Lebovitz et al., 1996). H_2O_2 is catalysed by catalase (Klichko et al., 2004), peroxiredoxins (Rhee et al., 2005) and glutathione peroxidase (Hellmich et al., 2005). Within an in-vitro endothelial model of sepsis, the peroxiredoxin/thioredoxin pathway appears more important than mitochondrial GSH for preventing mitochondrial dysfunction (Lowes and Galley, 2011). The glutathione pathway requires NADPH, and is essential for cellular ROS regulation (Pandolfi et al., 1995). NADPH synthesis is upregulated during oxidative stress, possibly through NADPH:NAD mediated control of glucose-6-phosphate dehydrogenase (G6PDH) activity (Filosa et al., 2003).

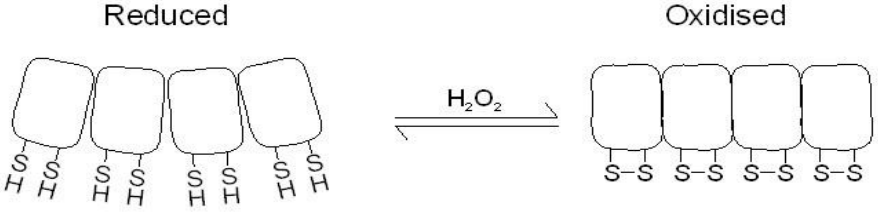

Figure 1.3 Redox regulation of enzyme function.

1.6 Neutrophil metabolism in health and sepsis

The intense metabolic requirements of activated neutrophils suggest that changes in supply of ATP and NADPH may alter neutrophil immune function, life span and, ultimately, sepsis outcome. The presence of such changes is supported by genomic investigations of the circulating leukocyte transcriptome, which describe a general increase in glycolytic gene expression and depression of mitochondrial gene expression. However, the functional consequence of these changes remains relatively underexplored. Many investigations have examined resting and ex-vivo activated neutrophil metabolism, and the genomic changes to metabolic genes, however few have addressed how the neutrophil metabolic profile alters during an inflammatory insult.

1.6.1 Neutrophil glycolysis and the hexose monophosphate shunt

Resting neutrophil ATP is principally supplied by glycolysis (Fig 1.4), and is readily demonstrated through use of metabolic inhibitors and radio-labelled carbon studies (Borregaard and Herlin, 1982; Cohn and Morse, 1959; Kakinuma, 1970; Reed and Tepperman, 1969; Sbarra and Karnovsky, 1959). Fractional CO_2 production is 5% in isolated human leukocytes (Beck, 1958), 8% in resting guinea pig leukocytes (Sbarra and Karnovsky, 1959) and 2% in resting DMSO differentiated HL60 cells (Ahmed et al., 1993). There is evidence to support reciprocal regulation between glycolysis and oxidative phosphorylation, with high levels of glucose reducing CO_2 formation, while high levels of oxygen impede glycolysis (Sbarra and Karnovsky, 1959).

Whether activated neutrophils utilise cytoplasmic ATP reserves (Borregaard and Herlin, 1982) or increase glycolytic ATP production is unclear (Sbarra and Karnovsky, 1959). Similarly, depending upon experimental conditions, glycolytic glucose may be obtained from endogenous glycogen stores or passively transported from the extracellular space via the Glucose transporter 1 (GLUT1) (Tan et al., 1998). Different sources of glucose may be used for different functions, for example extracellular glucose for chemotaxis and intracellular glycogen for phagocytosis (Weisdorf et al., 1982).

Neutrophils possess a propensity for the hexose monophosphate shunt pathway, with low fructose 1,6, bis-phosphate activity and six-fold higher G6PDH activity (Beck, 1958). Inhibition of G6PDH reduces activated neutrophil ROS production, but does not affect

other functions (Bender and Van Epps, 1985). The rate-limiting step of glycolysis is the conversion of phosphophenylpyruvate to pyruvate, by the enzyme pyruvate kinase (Minakami, 1968). Therefore, regulation of pyruvate kinase activity could exert substrate level control over the neutrophil respiratory burst (Olsen et al., 2003; Petty, 2001).

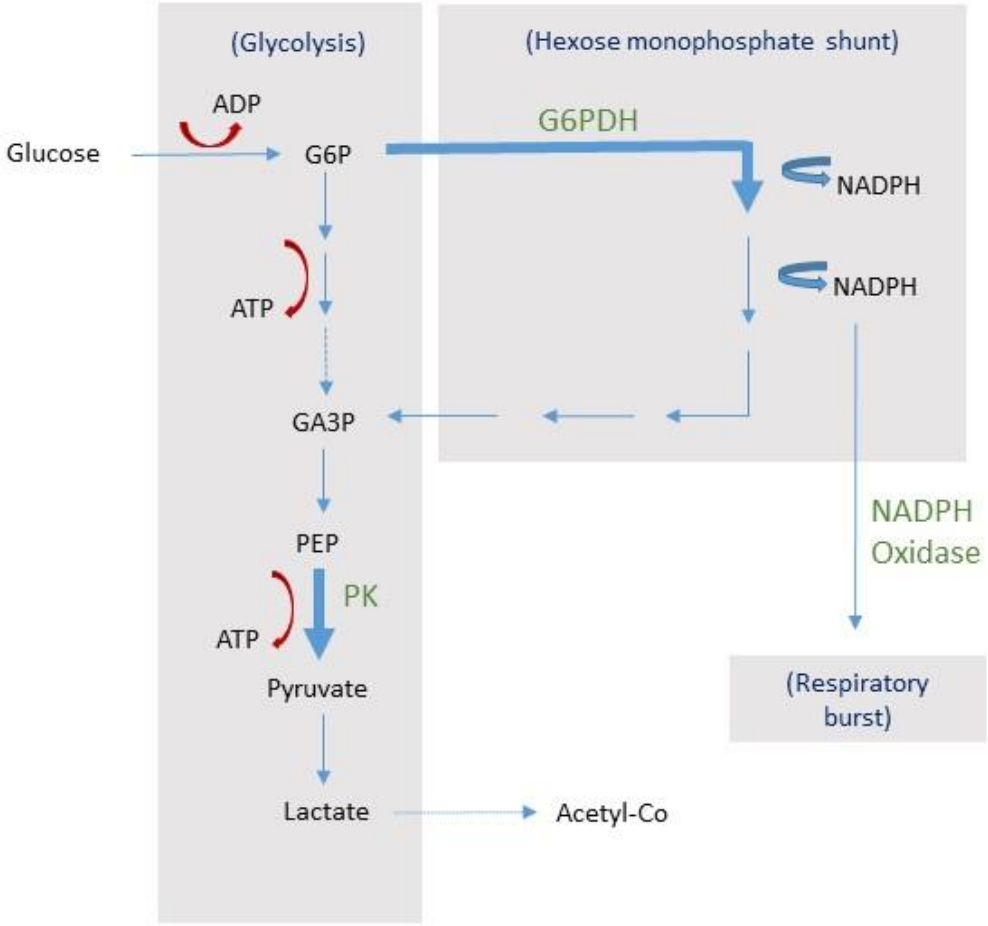

Figure 1.4. Neutrophil metabolism: glycolysis, the hexose monophosphate shunt and the respiratory burst.

Size of arrows represents enzyme activities (Fauth et al., 1993). *Thick arrows = 400-2000 mU/mg; dashed arrows <100mU/mg. G6P=glucose-6-phosphate; G6PDH=glucose-6-phosphate dehydrogenase; GA3P=glyceraldehyde-3-phosphate; PEP=phosphoenolpyruvate; PK=pyruvate kinase. Adapted from Oehler et al 2000.*

1.6.2 The role of neutrophil mitochondria

Although neutrophils are reliant upon glycolytic ATP, they do contain functional mitochondria. The addition of fluorescent mitochondrial probes, such as JC-1, Mitotracker and dihydrorhodamine-123 (DHR-123), reveals a complex mitochondrial network. These mitochondria can be depolarised by the uncoupling agent FCCP (Fossati et al., 2003; Maianski et al., 2004).

Neutrophil mitochondria demonstrate important differences to those from cells which rely mainly upon mitochondria for bioenergetic ATP production. Cytochrome c is poorly expressed (Maianski et al., 2004), while supracomplex organisation is absent in differentiated neutrophils and DMSO-differentiated HL-60 cells (van Raam et al., 2008). This may explain the relatively high levels of mitochondrial O_2^- (Maianski et al., 2004; Murphy et al., 2003). Inhibition of ATP transport into mitochondria and of glycolysis does not affect neutrophil mitochondrial MMP, suggesting it is maintained through de novo oxidative phosphorylation.

Ablation of the MMP with FCCP immediately inhibits neutrophil chemotaxis, but not the respiratory burst (Fossati et al., 2003). By contrast, PMA stimulation of the respiratory burst was inhibited following a two-hour incubation with the F1F0 ATP synthase inhibitor oligomycin. Although the loss of MMP is an early feature of apoptosis, addition of FCCP and oligomycin were not associated with an increased rate of *in vitro* apoptosis (Fossati et al., 2003). Neutrophils isolated from patients 12 hours after major surgery demonstrate MMP hyperpolarisation, increased mitochondrial ROS and an increased rate of apoptosis (using 7-AAD staining) which normalised at 24 hours (Delogu et al., 2001).

Inhibition of neutrophil mitochondrial complex I (rotenone, metformin) and complex III (antimycin-A, myxothiazol) are both associated with increased cytoplasmic H_2O_2, reduced activation of NF-kB and inflammatory cytokine production – as for exogenous H_2O_2 – which is reversed by polyethylene glycol-catalase. The systemic administration of these inhibitors conferred survival benefit within a murine LPS model, although no effector assays of neutrophil function were performed (Zmijewski et al., 2009, 2008)

Neutrophil mitochondria have an established role in mediating neutrophil apoptosis. Given the extended and interlinked roles of mitochondria within other cell types, mitochondria may therefore play a wider role within the regulation of neutrophil function. For example, within other cells mitochondria mediate ROS signalling, O_2 sensing (Brunelle et al., 2005),

calcium homeostasis, anion flux through the voltage-dependent anion channel (VDAC) family (Colombini, 2004), integrin signal transduction and the target of many drugs. VDAC activity and calcium exchange are critical for ROS production, with inhibition of VDAC reducing mitochondrial ROS (Zhou et al., 2011). These non-energetic processes may utilise respiratory complexes independently of oxidative phosphorylation, such as oxygen tension sensing at mitochondrial complex III (Brunelle et al., 2005).

1.6.3 Neutrophil metabolism during sepsis

Studies of neutrophil metabolism in septic patients have provided inconsistent results (Martins et al., 1999; Sheth et al., 2001; Watts et al., 2004). This may be partly explained by the many confounding factors of sepsis research. By contrast, the studies from trauma patients and healthy volunteers given endotoxin support highly conserved changes within the circulating leukocyte transcriptome to inflammatory stimuli. These extend far beyond those genes classically involved with immunity, such as those involved in metabolic function.

Exposure of healthy volunteers to low dose endotoxin increases circulating leukocyte transcription of glycolytic genes and decreases the expression of mitochondrial genes encoding for proteins within respiratory complexes I-V and pyruvate dehydrogenase (Calvano et al., 2005; Talwar et al., 2006). Changes in glycolytic genes are accompanied by increased expression of genes coding for components of the respiratory burst pathway.

These changes are associated with a fall in leukocyte ATP, a signal for activation of AMP activated protein kinase (AMPK), an increase in Hypoxia inducible factor alpha (HIF-1α) and prolongation of autophagy (Haimovich et al., 2014). AMPK and HIF-1 alpha have distinct, yet synergistic effects upon cellular metabolism. AMPK regulates mitochondrial biogenesis (Jäger et al., 2007; Kim et al., 2006) and is activated by the build-up of AMP, a signal for diminished energetic substrate availability. It acts in synergy with the growth signal integrating mammalian target of rapamycin (mTOR) (Papandreou et al., 2006; Semenza et al., 1994; Zhang et al., 2007). HIF-1 regulates cellular oxygen consumption by promoting glycolysis and inhibition of mitochondrial biogenesis and function (Papandreou et al., 2006), as well as by activating neutrophil immune functions (Peyssonnaux et al., 2005) .

Rather than a secondary phenomenon, these metabolic changes may even precede the classical immunological changes of inflammation. Healthy volunteers exposed to low doses of endotoxin demonstrate an altered metabolic transcriptome in the absence of measurable increases in systemic cytokine expression (Haimovich et al., 2014). Collectively these alternative models of human sepsis describe how inflammation, immunity and metabolism are intrinsically linked aspects of the cellular response to damage, and that the metabolic changes within circulating leukocytes may parallel those within solid organs/tissues during established critical illness.

The genomic evidence for upregulation of glycolytic activity is supported by a study of trauma patient neutrophil metabolic function (Oehler et al., 2000). The authors demonstrated an increase in pyruvate kinase expression – a rate-limiting step in resting neutrophil metabolism - associated with a 600-fold increase in enzyme activity on post injury days 5-7 post injury which was supported by increased glucose utilisation. Neutrophils isolated at 48 hours and incubated in presence of glucose had twice the respiratory burst capacity. This was abolished by addition of the G6PDH inhibitor dehydroepiandrosterone (DHEA) (Oehler et al., 2000). No other studies have addressed the impact of metabolic changes upon neutrophil immune functionality.

1.7 Neutrophil immune function

Neutrophil activation leads to the coordinated upregulation of several cellular immune processes, including phagocytic ingestion of bacteria, rapid production of ROS through the respiratory burst and the mobilisation of granule contents. These processes rely upon cellular remodelling, which, in turn, depends upon bioenergetic ATP. Tests of these effector processes are not routinely available in clinical practice, instead neutrophil function is commonly equated to circulating neutrophil numbers (Melvan et al., 2010).

1.7.1 Neutrophil surface receptors

1.7.1.1 Adhesion receptors

CD62L (L-selectin), and the CD11/CD18 family of integrins are the principal receptors responsible for neutrophil adhesion to endothelium, and the initiation of transmigration. CD62L is a single chain transmembrane glycoprotein responsible for low-affinity carbohydrate interactions (McEver and Zhu, 2010). Following receptor cleavage, signalling occurs via the retained membrane fragment through protein kinase C (PKC) and p38 MAPK (Smolen et al., 2000).

Integrins are heterodimeric transmembrane glycoproteins. Of the four CD11 subtypes, CD11b/CD18 (MAC-1 complement receptor) has the broadest functional role, mediating adhesion to endothelium (Smith et al., 1989) plus fibrinogen (Pluskota et al., 2004), Fc receptor mediated cytotoxicity (Van Spriel et al., 2001) and initiating a cascade of intracellular signalling processes (Plow et al., 2000; Schymeinsky et al., 2007; Walzog et al., 1995), including activation of the Src-family kinases. CD11b/CD18 signalling is ligand dependent. While ICAM-1, fibrinogen and myeloperoxidase may suppress apoptosis through CD11b/CD18, MAC-1 mediated phagocytosis hastens apoptosis (El Kebir and Filep, 2013).

Congenital deficiency of CD18 causes leukocyte adhesion deficiency syndrome, where patients have grossly reduced tissue neutrophil numbers (Anderson et al., 1985), which may be recreated by the addition of blocking antibodies to cells from healthy volunteers. Within

experimental models blockade of CD11/18 abolishes tissue plasma leakage and oedema (Lindbom, 2003; Wedmore and Williams, 1981). CD11b/CD18 has been identified as the target for the pro-resolving mediators lipoxin A4 and E1(El Kebir and Filep, 2013).

Dynamic changes in neutrophil cell surface CD11b and CD62L are well characterised. Both cytokine and PMA stimulation leads to a rapid (20 minutes) cleavage or "shedding" of L-selectin (Killock and Ivetić, 2010), with associated rise in soluble L-selectin (McGill et al., 1996; Smalley and Ley) and a more delayed increase in cell surface CD11b.

Following trauma the magnitude of CD11b rise and CD62 fall is correlated with the Injury Severity Score and the likelihood of postoperative complications (Rinder, 2006; Seekamp et al., 2001). These changes may persist for up to one week, although some authors have demonstrated a decrease (Chishti et al., 2004; McGill et al., 1996). Following vascular (Foulds et al., 2001) and orthopaedic surgery (Hughes et al., 2010) CD11b increase and CD62 decrease may persist for up to 3 days.

1.7.1.2 G protein coupled receptors

CXC chemokine receptor 2 (CXCR2) is a G-protein coupled IL-8 (CXCL8) receptor (Stadtmann and Zarbock, 2012) and has a demonstrable role within many inflammatory conditions. It is the most functionally diverse of all the neutrophil chemokine receptors (Chapman et al., 2009; Kurdowska et al., 2001). Activation of the receptor triggers dissociation into Gα and Gβɣ subunits which regulate adenylyl cyclase and phospholipase Cβ (PLCβ) plus phosphatidylinositol-3-kinases respectively (PI3K) (Stadtmann and Zarbock, 2012). PLCβ is believed to initiate a biphasic change in intracellular Ca^{2+}, and PI3K leads to PIP_3 and PKB/Akt activation (Futosi et al., 2013). These messengers then target membranous integrins (Stadtmann and Zarbock, 2012), Extracellular signal-regulated kinase (ERK) plus p38 MAP kinases (Mócsai et al., 2000), and the Src family of tyrosine kinases.

Within experimental models blockade of CXCR2 substantially reduces leukocyte recruitment, tissue damage and mortality (Stadtmann and Zarbock, 2012), possibly through reduction in CXCL-1 mediated CXCR2 signalling, following the release of IL-1α from necrotic cells (Chen and Nuñez, 2010). In established sepsis neutrophil cell surface CXCR2 expression is reduced by 50% (Cummings et al., 1999), and these cells displayed diminished responses to established CXCR2 ligands. Following blunt chest trauma CXCR2 is reduced at 24hrs (Visser et al., 2011)

1.7.1.3 Pattern recognition receptors (PRR)

The majority of PRR characterisation has been performed in cell types other than neutrophils and there is evidence for cell specific aspects of apparently identical receptors (Sabroe et al., 2002). Neutrophil TLR stimulation activates neutrophils, causing cytokine/chemokine production and delayed apoptosis (Hayashi et al., 2003). Neutrophil TLR4 is downregulated following cardiopulmonary bypass, and returns to baseline at 24hrs (Hadley et al., 2007) and may be upregulated in sepsis (Härter et al., 2004). Lipopolysaccharide detection by TLR is dependent upon the CD14 co-receptor (Chow et al., 1999; Qureshi et al., 1999), which in turn requires plasma lipopolysaccharide binding protein for efficient binding (Wright et al., 1991). CD14 may also enhance TLR2 signals. In vitro addition of cytokines causes a two-fold increase in neutrophil cell surface CD14 over twenty minutes (Wright et al., 1991). Following cardiopulmonary bypass neutrophil CD14 transiently increases, before returning to baseline values at 24 hours (Kawasaki et al., 2001). While the down-regulation of monocyte CD14 is well established, the relevance in neutrophils has not been determined (Hadley et al., 2007).

The triggering receptor expressed on myeloid cells (TREM-1) is expressed on blood neutrophils, and through the adapter protein DAP12, serves to amplify TLR signalling pathways, with both TREM-1 and TLR4 being recruited to the same lipid rafts (Baruah et al., 2015; El Mezayen et al., 2007). Lipid rafts are dynamic, nanoscale, organised areas of cell membrane focussed around sphingolipids and cholesterol (Simons and Gerl, 2010). Signalling receptors may be concentrated within lipid rafts, which facilitates inter-receptor interactions.

Surface TREM-1 activation promotes neutrophil degranulation, the release of IL-8 plus TNF-α and activation of the phagocytosis/respiratory burst processes (Baruah et al., 2015; Bouchon et al., 2000). Conversely, soluble TREM-1 is released from neutrophil granules and appears to have counterregulatory properties (Baruah et al., 2015). Soluble TREM-1 is increased following LPS exposure in humans, however neutrophil surface TREM1 may be either increased or remain unchanged (Bouchon et al., 2000; Mahdy et al., 2006). Within septic patients samples both soluble and neutrophil surface TREM-1are raised (Gibot et al., 2005). TREM-1 blockade is protective within murine models of lipopolysaccharide administration and abdominal sepsis (Bouchon et al., 2001).

1.7.1.4 Fcɣ receptors.

These are responsible for phagocytosis of immunoglobulin-G opsonised pathogens and immune complexes. They belong to the immunoglobulin superfamily, and are sub-classified by ligand affinity. The low affinity receptors, which include CD16 (FcɣRIII) and CD32 (FcɣRII) are most important to neutrophil phagocytosis. Young neutrophils, with banded nuclei, express lower levels of cell surface CD16, termed $CD16^{dim}$, when compared to mature counterparts (Brown et al., 1989). Some authors have suggested $CD16^{dim}$ is a reliable marker of neutrophil immaturity, and recent bone marrow release (Orr et al., 2005). Following healthy volunteer endotoxin exposure, the circulating $CD16^{dim}$ population is increased, and these cells demonstrate impaired functionality when compared to pre-endotoxin control cells. Blood neutrophil CD16 cell surface receptor density is reduced following major trauma and maybe reduced (Fung et al., 2008; Holzer et al., 2010) or unchanged after major elective surgery (Wakefield et al., 1995). Preoperative and postoperative CD16 levels are higher in patients who develop postoperative sepsis than those who do not (Wakefield et al., 1995). Neutrophil apoptosis is accompanied by a 90% down-regulation of cell surface CD16, with no other cell surface markers showing such dramatic change (Dransfield et al., 1994).

1.7.1.5 Cytokine receptors

Cytokine receptors are grouped according to structure and ligand specificity into Type I, Type II, IL-1 and TNF families (Futosi et al., 2013). Type I and II receptors activate the constitutively associated JAK-STAT pathway (Ghoreschi et al., 2009) plus the Src-family, PI3-kinase-Akt and ERK/p38 MAP kinase. The activation of ERK promotes neutrophil survival in response to oxidative challenges (Midwinter et al., 2001). Phosphorylated STAT shuttles to the nucleus and regulates gene transcription. The IL-1 receptor family detect the early pro-inflammatory mediators IL-1 and IL-18. Within neutrophils IL-1 in neutrophils principally extends life span (Colotta et al., 1993). IL-18 has wider range of effects, including autocrine neutrophil activation (Fortin et al., 2009). TNF-α is a major cytokine triggering neutrophil activation (Nathan, 1987) through Janus kinase (JNK) and NF-kB activation (MacEwan, 2002). The TNF family includes the Fas receptors, which receive extracellular pro-apoptotic signalling through Fas-ligand.

1.7.2 Phagocytosis

Following engagement of Fc and complement receptors the plasma membrane extends around particulate matter, creating a phagocytic vacuole that then fuses with cellular granules. Phagocytosis requires extensive cytoskeletal rearrangement, activates the respiratory burst and ultimately commits the neutrophil to apoptosis (Rotstein et al., 2000; Watson et al., 1996). Neutrophils from a NADPH oxidase murine knockout ($PHOX^{-/-}$) demonstrate impaired salmonella phagocytosis (Huang et al., 2009).

Neutrophils may engulf IgG coated latex beads within 20 seconds (Segal et al., 1980), and phagocytose up to 50 individual bacteria in total. Circulating neutrophil phagocytosis is impaired following major abdominal surgery (Kawasaki et al., 2007) and lung resection surgery (Jones et al., 2014), but much more so in patients with established sepsis (Jones et al., 2014). Within critically ill patients diminished neutrophil phagocytosis is an independent risk factor for the acquisition of nosocomial infection (Morris et al., 2011).

1.7.3 Respiratory burst

The respiratory burst describes production of O_2^- by cell membrane and vesicle-bound NADPH oxidase (PHOX, or NOX2) (Fig 1.5). It is so called because of the rapid consumption of oxygen by activated neutrophils. $p47^{phox}$ translocation from cytoplasm to membrane causes a 100-fold increase in oxygen consumption (Guzik and Griendling, 2009). These intense metabolic demands suggest that the respiratory burst maybe substrate limited (Kindzelskii and Petty, 2002; Petty, 2001). Within macrophages, hexokinase localises with PHOX during the respiratory burst (Pedley et al., 1993). Genomic data from healthy volunteers exposed to LPS (Calvano et al., 2005) and proteomic analysis of trauma patients (Oehler et al., 2000) have identified an increase in the expression and activity of neutrophil glycolytic enzymes during human inflammatory responses.

Neutrophil ROS support more complex immunological processes than just the direct oxidation of pathogenic organisms. The potential difference across the vacuole membrane created by movement of O_2^- is compensated by movement of intracellular ions which create the osmotic and electrochemical conditions that favour activation of many enzymes and proteins contained within neutrophil granules (Segal, 2005), and formation of neutrophil extracellular traps (NETs) (Fuchs et al., 2007).

NETs are extracellular DNA structures released from viable neutrophils that serve to concentrate and localise neutrophil killing power. By localising and degrading cytokines NETs may also serve to limit inflammation (Schauer et al., 2014). The respiratory burst may therefore be considered a 'master switch' of neutrophil killing. Genomic investigations from healthy volunteer endotoxin studies support an early increase in burst machinery expression (Calvano et al., 2005).

The signal transduction pathways leading to PHOX activation is stimulus dependent. For example p38 MAPK is essential for TNF-α induced activation and for approximately 70% of the fMLP response, but is unnecessary following activation by complement–opsonised zymosan (Brown et al., 2004). Specific agents may therefore be used to investigate changes in O_2^- production, such as the protein kinase c activator phorbol myristate acetate (PMA) which maximally stimulates the sub-membrane burst machinery (Tyagi et al., 1988) by directly phosphorylating $p47^{phox}$ (Wyche et al., 2004) in the presence of Ca^{2+} (El Jamali et al., 2010). H_2O_2 stimulation of PHOX is likely upstream of PKC, acting through Ca^{2+} influx and c-Abl tyrosine kinase signalling (El Jamali et al., 2010). Initiation of NADPH oxidase activity has a lag phase of approximately 20 seconds (Briggs et al., 1986; Segal et al., 1980)

Excessive neutrophil ROS is associated with organ dysfunction [(Brown et al., 2006; Chabot et al., 1998; Crimi et al., 2006; Kolls, 2006; Strassheim et al., 2002). Paradoxically, studies of patients with chronic granulomatous disease - who possess functional mutations in PHOX subunits - and associated animal models have identified important anti-inflammatory roles for neutrophil ROS. As well as being predisposed to infection, chronic granulomatous disease patients also demonstrate sterile inflammatory lesions (Morgenstern et al., 1997) and an increased incidence of inflammatory bowel disease, granulomatous genitourinary obstruction and wound dehiscence (Almyroudis et al., 2013). Neutrophils isolated from these patients reveal enhanced secretion of IL-8 (Hatanaka et al., 2004), prolonged survival (Kasahara et al., 1997) and reduced uptake by macrophages (Hampton et al., 2002).

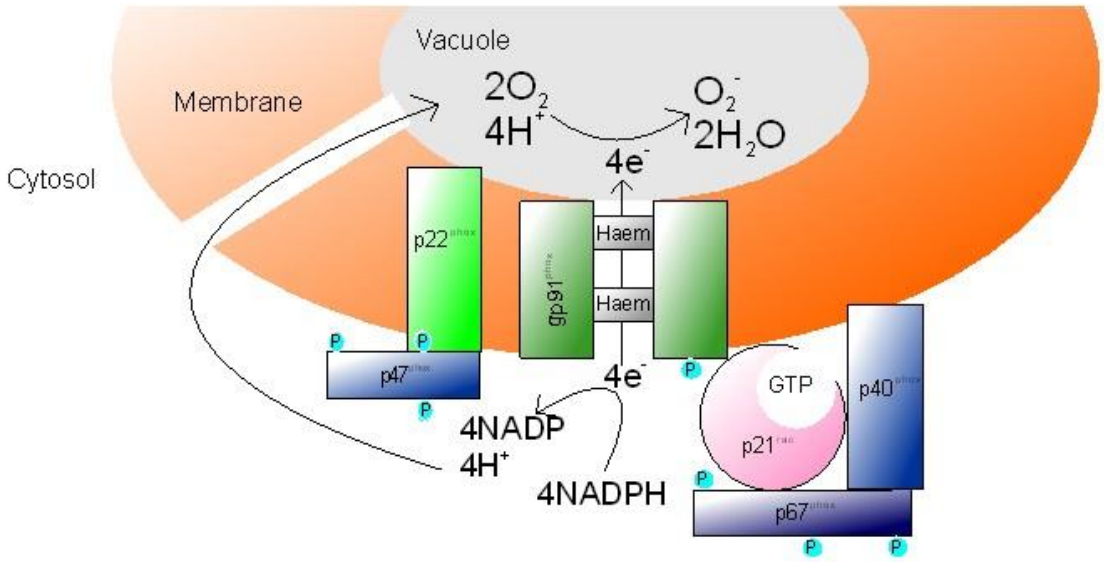

Figure 1.5 The neutrophil respiratory burst.

Adapted from (Segal, 2005) PHOX is activated by phosphorylation and translocation of cytoplasmic subunits p47^{phox}, p67^{phox}, p40^{phox} and p21^{rac} to membranous electron transporting flavocytochrome b558 (gp91^{phox} heterodimer and p22^{phox}) (Lambeth, 2004), as well as PKC and hexokinase (Nixon and McPhail, 1999). Hexomonophosphate shunt derived NADPH provides electrons that pass through flavocytochrome b558 to combine with vacuolar oxygen, generating O_2^- (Cross 2004). A proton pump completes the circuit (Henderson and Chappell, 1992; Henderson and Meech, 1999).

Murine models of chronic granulomatous disease consistently demonstrate sustained and exaggerated lung inflammation (Davidson et al., 2013; Deng et al., 2012; Schauer et al., 2014; Segal, 2007; Segal et al., 2010), and predisposition to autoimmune arthritis that may be prevented through reactivation of PHOX (Hultqvist et al., 2004). These observations may be explained by the associated impairment of phagocytosis and NET formation (Darrah and Andrade, 2012). These roles appear to require neutrophil activation, since baseline energy metabolism and glycolytic rate are unaffected in chronic granulomatous disease neutrophils (Borregaard and Herlin, 1982).

PHOX is now appreciated to be part of a wider NOX family. The unifying feature of NOX function is the transfer of electrons from NADPH to oxygen. NOX isoforms are expressed out-with professional phagocytes, have distinct subcellular location, regulatory factors and synthesise distinct ROS species, and have established roles in signalling and ion homeostasis (Lambeth, 2004; Steinberg and Grinstein, 2007). These observations suggest that PHOX may also possess an extended signalling role, through production of cellular ROS.

1.8 Neutrophil activation, effector function and life span.

Neutrophil life span is intrinsically linked to functional activity. In the absence of pro-inflammatory signals, unstimulated neutrophils readily enter constitutive apoptosis (Savill et al., 2002)], with an estimated life span of less than 1 day. Similarly, successful phagocytosis also signals apoptosis (Gilroy et al., 2004; Nathan and Ding, 2010; Savill et al., 2002). Between these two extremes of stimulation, 'activated' neutrophils may display increased longevity. Functional heterogeneity may exist within the wider circulating and sequestered neutrophil pool, as identified by variation within the circulatory and tissue pools of cell surface receptor expression, effector activities and life span.

Neutrophil lifespan is estimated to be 8-20 hours (Maianski et al. 2004; Luo & Loison 2008; Pillay et al. 2010). Without a survival signal neutrophils enter an automatic, or constitutive, death process that leads to reticuloendothelial (Savill et al., 2002, 1989) and tissue macrophage mediated removal. This short life span means changes in survival/death patterns will rapidly alter blood and tissue numbers. While prolonged neutrophil longevity may assist with pathogen removal (Nathan, 2006; Savill et al., 2002; Watson et al., 1997), inappropriate prolongation or failure to isolate dying neutrophils may perpetuate local tissue damage and prevent effective resolution of inflammation (Nathan, 2006; Nathan and Ding, 2010; Serhan and Petasis, 2011).

Established survival signals include many of the classical pro-inflammatory mediators governing activation and tissue migration, which act through a range of cell surface and cytoplasmic receptors. In vitro culture of neutrophils from healthy volunteers leads to rapid death (Akgul et al., 2001; Moulding et al., 2001). However the addition of inflammatory mediators or serum from septic/trauma patients may increase survival to 24-36 hours (Ertel et al., 1998; Haslett, 1992; Lee et al., 1993). These observations make in vitro neutrophil

investigations challenging, and the comparison of data from different laboratories difficult (Luo and Loison, 2008) with 6 hour apoptosis rates varying between 10% and 75% (Blomgran et al., 2007; Cowburn et al., 2005).

Most clinical studies from patients with active inflammation have identified prolonged neutrophil survival when compared to healthy volunteers. This includes sepsis (Ertel et al., 1998; Jimenez et al., 1997; Keel et al., 1997; Taneja et al., 2004), non-infective SIRS (Melley et al., 2005), burns (Chitnis et al., 1996) and Acute respiratory distress syndrome (ARDS) (Matute-Bello et al., 1997), as well as chronic inflammatory diseases such as Chronic obstructive pulmonary disease (Brown et al., 2009) and coronary artery disease (Garlichs et al., 2004).

In ARDS, worsening disease severity is associated with increased neutrophil survival (Feterowski et al., 2001; Matute-Bello et al., 1997). Data from trauma patients suggest apoptosis may be suppressed for up to three weeks (Ogura et al., 1999). Results from elective surgery are less clear, with apoptosis shown to be increased at 12 hours (Delogu et al., 2001) and decreased at 24 hours (Fanning et al., 1999).

Despite this inflammation driven prolongation of neutrophil lifespan, the very process of phagocytosis initiates apoptosis. Other morphological patterns of death include NETosis (Brinkmann et al., 2004; Fuchs et al., 2007), necrosis (Simon, 2003; von Gunten and Simon, 2006) and autophagy (Remijsen et al., 2011).

Neutrophil apoptosis promotes several processes that facilitate the timely resolution of inflammation. Apoptotic neutrophils become unresponsive to agonists, stop producing inflammatory mediators (Kobayashi et al., 2003) and may act as cytokine sinks where cell surface receptors persist in the absence of functional transduction machinery (Ariel et al., 2006). Macrophages which ingest apoptotic neutrophils switch from a pro-inflammatory to a pro-resolution phenotype (Fadok et al., 1998), releasing FasL which may further promote neutrophil apoptosis (Brown and Savill, 1999). Within a mouse model of endotoxic shock the infusion of apoptotic neutrophils improved survival, while infusion of necrotic neutrophils increased mortality (Ren et al., 2008).

Both intrinsic apoptosis (mitochondrial mediated, caspase-9 dependent) and extrinsic apoptosis (TNF, Fas-L dependent activation of caspase-8) occur within neutrophils. Constitutive neutrophil death represents a specialised form of intrinsic apoptosis. There is significant overlap between these two apoptotic modes within neutrophils; addition of Fas/Fas-L antagonists partially inhibited constitutive neutrophil death, while preservation of mitochondrial membrane potential inhibited Fas-L mediated death (Brown and Savill, 1999; Liles et al., 1996; Renshaw et al., 2000).

1.8.1 Constitutive neutrophil death

The morphological appearance of constitutive neutrophil death resembles classical intrinsic apoptosis in other cell types, with exteriorization of phosphatidylserine, formation of cytoplasmic vacuoles, and nuclear condensation (Payne et al., 1994; Savill et al., 1989). While caspase inhibition is widely reported to inhibit neutrophil apoptosis, it is unclear if caspase activation is absolutely necessary for constitutive death (Luo and Loison, 2008).

Neutrophil mitochondria appear essential for constitutive death. In other cell types localisation of the pro-apoptotic Bcl-2 homologs Bid and Bax to the outer mitochondrial membrane permits release of cytochrome-c through the mitochondrial permeability transition pore, leading to activation of caspase 9. However neutrophils express very low levels of cytochrome c (Liu et al., 2003; Maianski et al., 2004; Murphy et al., 2003; Pryde et al., 2000), and may compensate instead through the release of Apaf-1. Wide variations in neutrophil Bcl-2 expression has been reported (Moulding et al., 2001). The principal neutrophil anti-apoptotic Bcl-2 homolog, Mcl-1, has a very short half-life, and is regulated at transcriptional and translational levels by NF-kB and PI3K (Brenner and Mak, 2009; Edwards et al., 2004; Reed, 2006). Mcl-1 may therefore provide a common explanation to early constitutive death, and its inhibition during inflammation.

Neutrophil ROS contribute to apoptosis regulation. Neutrophils from patients with chronic granulomatous disease (Kasahara et al., 1997; Yamamoto et al., 2002), and those from healthy volunteers exposed to PHOX inhibitors (Coxon et al., 1996; Lundqvist-Gustafsson and Bengtsson, 1999; Perskvist et al., 2002) and ROS scavengers (Kasahara et al., 1997) demonstrate reduced rates of apoptosis in vitro. PHOX-independent ROS production may also occur (Fay et al., 2006) through hyperpolarisation of neutrophil mitochondria (Vincent

et al., 2004). While intracellular non-phagosomal ROS trigger apoptosis, post-phagocytosis intraphagosomal ROS does not (Blomgran et al., 2007, 2004). Possible ROS-dependent apoptotic targets include DNA damage/p54 (X. Zhang et al., 2003), MAPK/NF-kB (Fortenberry et al., 2001) and Fas-L independent Fas receptor clustering in lipid rafts.

1.8.2 Survival signals and activation induced death

Neutrophil functionality and survival are intrinsically linked, because most activation signals prolong lifespan. Pro-survival signals include CD11b/CD18 (Ross et al., 2006), Granulocyte macrophage colony stimulating factor (GM-CSF) (Lee et al., 1993), Interferon gamma (IFN-ɣ), leukotriene B4 (a C. Lee et al., 1999), C5a (Lee et al., 1993), CRP (Khreiss et al., 2002), serum amyloid (El Kebir and Filep, 2013), LPS (Colotta et al., 1992) and bacterial DNA (József et al., 2004). Many of these signals are situation dependent. For example, while integrin-mediated adhesion prolongs neutrophil lifespan, so does the addition of CD11b/CD18 blocking antibodies to extravasated neutrophils (Coxon et al., 1996). Intracellular signalling pathways overlap but show considerable ligand specificity, including PKB/Akt, p38 MAP-kinase, and NF-kB (Francois et al., 2005). Caspase-1 dependent cleavage of IL-1β may act as both a pro and anti-apoptotic signal in neutrophils (William et al., 1999).

In contrast to the effects of pro-survival signals inhibiting apoptosis, phagocytosis of a range of microorganisms is a powerful apoptotic signal (Perskvist et al., 2002; Rotstein et al., 2000; Watson et al., 1996). Phagocytosis driven apoptosis is prevented by antagonism of CD11b/CD18 (Coxon et al., 1996), and proceeds through PHOX and caspase-8 dependent pathways (Yamamoto et al., 2002; B. Zhang et al., 2003), possibly dependent upon ROS mediated lysosomal membrane permeabilisation (Blomgran et al., 2007).

Neutrophil apoptosis research has predominantly focussed upon the fate of blood-derived cells. It is less clear how tissue neutrophil survival is regulated. Tissue neutrophils may migrate back to the circulation (Buckley et al., 2006; Hughes et al., 1997) or through the lymphatics (Schwab et al., 2007).

1.8.3 Functional phenotypes and heterogeneity

The current literature describes a range of subtly different functional neutrophil phenotypes. It is unclear whether these exist within a spectrum, whether neutrophils move between them, and to what extent they may represent the product of experimental artefact. Subpopulations of differing functionality may exist within the wider neutrophil pool, although comparisons between studies are complicated by the different techniques and protocols used. The molecular basis for these functional changes is unclear. Possible explanations include variations in priming and tolerance phenomena, neutrophil maturity, and co-stimuli determined by neutrophil location acting through signal transduction modulation, preassembly of components such as PHOX, and genomic-driven changes in protein content.

While routine laboratory tests provide some information about neutrophil age through morphological quantification of nuclear segmentation, tests of actual neutrophil function are not routinely available. Flow cytometric quantification of cell surface receptors is widely used to define lymphocyte subpopulations, however it is currently unclear whether neutrophil surface receptors equate to similar functional phenotypes (Fontes et al., 1995; Seely et al., 2003; Stocks et al., 1996). Development of analogous neutrophil surface markers of function, or readily accessible functional assays may dramatically improve our understanding of an individual's inflammatory response and permit delivery of immune tailored medications.

1.8.4 Neutrophil priming and tolerance

The serial application of DAMP/PAMP signals may promote enhanced (priming) or diminished (tolerance) inflammatory responses. At a cellular level these responses may be partly explained by changes in neutrophil functionality (Condliffe et al., 1998). Priming could explain the predisposition of trauma patients who receive apparently minor secondary insults to develop ARDS and multiorgan failure (Moore et al., 2005). Similarly, a combined murine model of sequential gut ischaemic-reperfusion injury and endotoxin administration is consistently lethal, even though each insult alone is not (Koike et al., 1992). Tolerance is exemplified by the anergic responses to coliform colonisation of the respiratory tract within the critically ill, and in a murine model of septic peritonitis, where prior administration of LPS improved survival (Feterowski et al., 2001).

In vitro investigations of priming with pure ligands have identified ligand specific signalling cascades. For example, functional p38 MAPK is essential for TNF-α priming and responsible for 70% of the fMLP response but unnecessary for complement-opsonised zymosan stimulation (Brown et al., 2004). It is also implicated in LPS and GM-CSF priming (Johnson and Gomez-Cambronero, 1995; McLeish et al., 1998; Nahas et al., 1996; Waterman and Sha'afi, 1995). Lipopolysaccharide priming proceeds through TLR4 and CD14, with HMGB1 also priming PHOX function through TLR4 (Fan et al., 2007). At a cellular level LPS causes down-regulation of TLR4 within 30 mins, and this is magnified by a smaller priming dose of LPS (Parker et al., 2005).

In vivo data are more complicated. Within a single insult model of haemorrhagic shock, lung injury is TLR4 dependent (Ayala et al., 2002; Barsness et al., 2004). However when a sequential LPS injury is applied whole lung TLR4 expression is maintained (Fan et al., 2002). Tolerance, as defined by diminished cytokine production, has been described at 24 hours following endotoxin exposure (van der Poll et al., 1996), and up to four weeks in those with established critical illness (McCall et al., 1993).

The sub-cellular changes ultimately responsible for priming and tolerance are uncertain. While PHOX components are redistributed and partially phosphorylated following LPS and TNF-α application (DeLeo et al., 1998; Ward et al., 2000), preassembly of PHOX subunits does not seem to be responsible (Brown et al., 2004). The hexose monophosphate shunt may be upregulated within primed cells (Bass et al., 1986). In combination with wide spread changes in 'metabolic genes', it is plausible that metabolic control of substrate supply may represent an essential but relatively underexplored regulatory mechanism

1.9 Conclusion

The investigation of human immune function presents significant challenges. Specifically, the study of septic patients is complicated by uncertainty surrounding the sepsis diagnosis, the time at which sepsis began, premorbid status and off-site effects of therapeutic interventions. Healthy volunteer studies can never replicate the severity of real-world disease. In contrast, the study of the surgical patient appears to offer a methodological rigor that these other studies lack. Surgical patients are heavily phenotyped and receive a relatively homogenous insult at a known time, facilitating the acquisition of pre-insult control samples. Biological plausibility for modelling sepsis with surgical patients is provided by genotyping investigations that describe a highly conserved genomic response of circulating leukocytes to both trauma and infection, and clinical similarities of these two populations.

An appropriate inflammatory response to infection and trauma is essential so that the magnitude and duration of injury may be minimised, and autotoxic harm limited. Neutrophils are the most numerous cell type during the early, innate inflammatory response and possess a range of potent effector mechanisms. The respiratory burst appears to act as a master regulator by controlling the activation of these mechanisms and simultaneously serving to limit longer-term activity. Therefore, the surgical model presents an opportunity to investigate how neutrophil function changes following a major inflammatory insult, and for the characterisation and description of the cellular mechanisms that may underlie these changes.

1.10 Hypothesis

Patients undergoing major elective surgery demonstrate dynamic changes in neutrophil immune function, which may be mediated through alterations in neutrophil metabolic processes.

1.11 Aims

1. To determine whether the theoretical methodological advantages which the study of human surgical patients offers over the study of septic and trauma patients are realised in the translational surgical literature
2. To characterise the temporal response of neutrophil effector functions following major elective surgery.
3. To explore the metabolic processes which may underlie these changes in effector function.

Chapter 2 – General Methods

Patient samples were used throughout chapters 4, 5 and 6. Chapters 4 and 5 present observational data, that describes the perioperative neutrophil phenotype. All postoperative samples were compared to individualised preoperative controls. Chapter 5 and 6 provide mechanistic data, where chemical modulators of metabolic function were added to *preoperative* samples with the intention of mimicking the postoperative neutrophil phenotype.

2.1 Clinical data collection

Patient samples were collected from two clinical studies, following written informed consent, as detailed in Table 2.1

2.1.1 Study conduct

Patients were recruited from University College Hospital and The Royal Free Hospital between February 2011 and February 2014 from POMO and VISION-UK trials (Table 2.1) All postoperative samples were compared to their individual, matched preoperative control samples. Preoperative and postoperative care was conducted as per local guidelines. Both institutions have nurse-led preassessment clinics, with consultant support available as required. Surgery and anaesthesia were either delivered or immediately supervised by consultants. Local protocols regarding antibiotic and thrombo-prophylaxis, oral intake and physiotherapy plus occupational therapy were followed.

Postoperative Morbidity and Oxygen (POMO)	**VISION-UK**
MREC 09/H0805/58	MREC 10/WNo03/25
Collection timing: February 2011 to March 2012	**Collection timing**: March 2012 to August 2014
Inclusion criteria - Major elective surgery (abdominal/oesophageal/hepatic resection/gynaecological/urological reconstructive surgery and who meet following criteria: 1. ASA 3-4 2. Age >50 years 3. >2 risk factors defined by Revised Cardiac Risk Index	**Inclusion criteria** - Non-cardiac surgery and received general anaesthetic - At least overnight admission to hospital - >45 years of age
Exclusion Criteria - Refusal of consent - Concurrent lithium therapy - Acute myocardial ischaemia - Acute arrhythmias - Pregnancy - Palliative treatment only	**Exclusion Criteria** - Refusal of consent
Laboratory sampling time points Preoperative (Preassessment clinic), POD 3, POD 5. **Immune assay sampling time points** Preoperative (day of surgery), POD 1, POD 3, POD 5.	 **Immune assay sampling time points** Preoperative (day of surgery), POD1, POD2, POD3.

Table 2.1 Patient sampling criteria / protocol.

POD=Postoperative day

2.1.2 Data collection

Clinical and demographic data were collected prospectively as part of the POMO study protocol by trained research staff through analysis of written notes, electronic patient records and visits. Perioperative risk scores were calculated using the American Society of Anaesthesiology Physical Status Score (ASA, 2014), Physiological and Operative Severity Score for the enumeration of Morbidity and Mortality (POSSUM) (Copeland et al., 1991), Duke Activity Status Index (Hlatky et al., 1989) and Veterans Specific Activity Questionnaire Metabolic Equivalents (Myers et al., 2001). Full blood count samples were analysed by the hospital haematology laboratory using a Sysmex XE2100 analyser (Sysmex, Milton Keynes UK). The VISION patient database is currently locked to analysis, therefore only age, sex and operation type are presented for these patients.

2.1.3 Statistics

Continuous data are presented as mean with 95% confidence intervals for normally distributed data, otherwise median with interquartile range. For clarity, graphical data for all paired preoperative and postoperative are presented as paired line charts where $n<10$, and as box and whisker plots (median/IQR/min-max) where $n>10$. Where $n<6$ statistics are not provided. If $n \geq 6$ Wilcoxon matched pairs test was used, unless $n>10$ and box whisker plots suggested data was normally distributed, in which case Student's paired t-test was used. Categorical data are presented as absolute plus percentage values, and compared using Pearson's Chi^2 or Fisher's exact test as appropriate. All statistics were analysed using Prism 5.00 (Graphpad, USA).

2.2 Laboratory materials

2.2.1 Equipment

Equipment	Supplier	Address
Biological safety cabinet (Class II): Holten LaminAir Model 1,2	Thermo Scientific	Waltham, USA
Centrifuge: ALC PK120	ALC	Cologno Monzese, Italy
Centrifuge: Haraeus Megafuge 1.0R	Kendro Laboratory products	Langenselbold, Germany
Cryogenic vials: 2ml	Corning International	NY, USA
Falcon polypropylene tubes: 15ml, 50ml, 250ml	BD Biosciences	Oxford, UK
Filter unit: Nalgene 90mm (0.2µm filter)	Nalge Nunc International	NY, USA
Flow Cytometer off-line analysis: Kaluza 1.3	Beckman Coulter Inc	High Wycombe, UK
Flow Cytometer: Cyan ADP 9 colour (Summit 4.3.1)	Beckman Coulter Inc	High Wycombe, UK
Flow cytometry tubes: 5ml polystyrene round bottomed		
Haemocytometer (Neubauer)	Assistent	Germany
Heated water bath: Grant JB4	Grant Instruments	Cambridge, UK
Incubator for cell culture Galaxy R 170litre	RS Biotech Laboratory Ltd	Irving, UK
Microcentrifuge tubes: 1.5ml	StarLab GmbH	Ahrensburg, Germany
Microcentrifuge: Eppendorf 5415 C	Eppendorf	Hamburg, Germany
Microplate reader: Synergy Mx monochromator-based multi-mode	BioTek	Winooski, USA
Microscope: Zeiss Axiovert 25	Zeiss	Oberkochen, Germany
Pasteur pipettes (sterile): 3ml	Ramboldi Ltd	Limassol, Cyprus
Pipette tips (Diamond, sterilised) tips: 10, 200 and 1000uL	Gilson Inc	Middleton, USA
Radiometer ABL800 Flex blood gas analyser	Radiometer	Bronshoj, Denmark.
Real Time PCR: Rotor-Gene 6000	Corbett Life Sciences	Cambridgeshire, UK
Respirometer: Seahorse XF System	Seahorse	Copenhagen, Denmark
Serological pipettes (Costar, disposable): 10, 25, 50ml	Corning International	NY, USA

Table 2.2 Laboratory equipment

2.2.2 Chemicals and reagents

Reagent (Batch number)	**Supplier*** (Product number)
2-NBDG-FITC (Lot 1204217) *Working solution 10mg/ml DMSO, stored at -20°C*	Life technologies N13195
Annexin Buffer, made from: 100mM HEPES,140mM NaCl, 25mM $CaCl_2$, in distilled water *Sterile filtered as above, stored at 4°C.*	Sigma Aldrich
Antimycin A from *Streptomyces* (lot 061M4063V) *Stock solution 10mM in ethanol, stored at -20°C*	Sigma Aldrich (A8674)
Blood Lysis Buffer, made from: 0.84g $NaHCO_3$ 7.7g NH_4CL 1000ml distilled water *Sterilized by passing through 0.22µm filter, stored at 4°C*	Sigma Aldrich
Bovine Serum Albumin (lot 099K1447) *Stock solution 2% (w/v) in PBS, sterile filtered and stored at 4°C*	Sigma Aldrich (B4287)
Carbonyl cyanide -4(trifluoromethoxy)phenyldydrazone (FCCP) *Stock solution 10mM in ethanol, stored at -20°C*	Sigma Aldrich
Cell Tak Stored at 4°C	Corning
D-Glucose (Lot K29908114)	CTL Scientific Supply Corp *(101174Y)*
Diarylsulfonamide (DASA) *(Lot d00138745)* *Working solution 50mg/ml in DMSO, stored at -20°C*	Calbiochem *(550602)*
Dihydroethidium (DHE) (1446356v) *Working solution 10mM in DMSO, stored at -20°C*	Sigma Aldrich (37291)
Dimethyl Sulphoxide (DMSO)# *Stored at room temperature*	Sigma Aldrich (154938)
Dulbecco's Modified Eagle Medium Glutamax, 1g glc + pyruvate. *Stored at 4°C*	Invitrogen (21885-025)
Dulbeccos's Phosphate-Buffered Saline, Calcium and Magnesium-free. (PBS) *Stored at 4°C*	Invitrogen Ltd (14190-094)
E. coli-fitc (k-12 strain) (lot 929563)	Invitrogen (e2861)
E. coli LPS 0111:b4 l2630 – 25mg	Invitrogen
Ethanol (09/479 a10)	Prolab diagnostics (L947907)
Ficoll-Paque PLUS (Ficoll sodium diatrizoate) *Stored at room temperature.*	GE Healthcare (17-1440-03)
Hanks Balanced Salt Solution (HBSS) + $CaCl_2$ +$MgCl_2$ *Stored at 4°C*	Invitrogen (14025-050)
Heparin sodium 1000iu.ml^{-1} (sterile)	Leo Laboratories Ltd

Stored at 4ºC	
HEPES buffer(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) 2% gluteraldehyde	Sigma Aldrich
JC-1 *Stock solution 200x in DMSO, stored at -20ºC* (lot #505)	Immunochemistry *(#6260)*
Intracellular staining kit *Stored at -4ºC*	Life Technologies.
Lipopolysaccharide, from Salmonella Typhosa (lot 113K4087) *Stock solution in PBS sterile filtered, stored at 4ºC*	Sigma Aldrich (L6386)
MitoSOX Red (Lot 449012) *Working solution 5µM in HBSS/Ca/Mg, stored at -20ºC*	Invitrogen (M36008)
MitoTEMPO (lot H2113) *Working solution 10mg/ml DMSO, stored at -20ºC*	*Santa cruz* (Sc-221945)
Myxothiazol *from Myxococcus* *Stock solution 10mM in Ethanol, stored at -20ºC*	Sigma Aldrich (T55580)
Oligomycin (lot 061M8613) *Stock solution 8mg.ml^{-1} in ethanol, stored at -20ºC*	Sigma Aldrich (04876)
Paraformaldehyde *2% (v/v) working solution in PBS, stored at 4ºC*	Sigma Aldrich (P6148)
Phagoburst kit (Lot no 1323313109)	Glycotope biotechnology
Phagotest kit (Lot no 13948)	Glycotope biotechnology
Phorbol 12-myristate 13-acetate (PMA) (108K1443) *Stock solution 100µM in ethanol, stored at -20ºC*	Sigma Aldrich (P1585)
Propidium Iodide (PI) *Stock solution 50µg.ml^{-1} in PBS, stored at 4˚C*	Abcam plc
Rotenone (lot 046K1189) *Stock solution 10mM in ethanol, stored at -20ºC*	Sigma Aldrich (R8875)
RPMI medium 1640 + 0.3g/L L-glutamine + 20% heat inactivated fetal calf serum *Stored at -4ºC*	Invitrogen (21875-034)
Seahorse assay medium DMEM without sodium bicarbonate or phenol red, + 5.6mM glucose, 5mM pyruvate, 2mM L-glutamine. pH adjusted to 7.4 at 37oC and sterile filtered.	Sigma Aldrich
Tetramethylrhodamine, Methyl Ester, Perchlorate (TMRM) *Stock solution 10mM in DMSO, stored at -20ºC*	Life technologies
Trypan Blue solution (0.4%) *Stored at room temperature*	Sigma Aldrich (T8154)

Table 2.3 Chemicals and reagents.

** Abcam PLC, Cambridge UK, GE Healthcare, Buckinghamshire UK; Corning, Bedford, MA; CTL Scientific Supply Corp, NY, USA; Sigma Aldrich, Dorset UK Glycotope biotechnology – Heidelberg Germany, Leo*

Laboratories, Berkshire, UK; Invitrogen Ltd, Paisley UK Life technologies Paisley UK, Immunochemistry Technologies, MN, USA; Prolab diagnostics, Wirral, UK. # The concentrations of DMSO were all less than 0.02%, well below those documented to have direct effects upon mitochondrial membrane potential and apoptosis (1.5 – 2% v/v) (Lin et al. 1995; Liu et al. 2001). In addition, appropriate volumes of DMSO were added to all control wells.

2.2.3 Monoclonal antibodies and microbeads

Antibody-fluorochrome	**Clone** (product / batch**)**	**Isotype (**product / batch)	**Source**	**Role**
Annexin-V-FITC	(120-002-355/51309270 69)	n/a	Miltenyi Biotec Ltd	Binds to externalised phosphatidylserine (in the presence of calcium).
CD11-APC	M1/70.15.11.5 (130-091-241/ 5110802100	Rat IgG2b Santa Cruz Sc-2895/ lot H2709	Miltenyi Biotec Ltd	As above
CD11b-FITC	M1/70.15.11.5 (1300x81201/5 110916041)	Rat IgG2b Santa Cruz Sc-2895/ lot H2709	Miltenyi Biotec Ltd	Integrin receptor for C3bi, fibrinogen and factor X. Strongly expressed on myeloid cells, scanty expression on some lymphocytes
CD14-APC	TUK4 (1300x91243/5 110929113)	Mouse IgG2a (130-091-837/ 5120614134	Miltenyi Biotec Ltd	As above
CD14-FITC	TUK4 (130-080-701/ 5110527016	Mouse IgG2a (130-091-837/ 5120614134	Miltenyi Biotec Ltd	Part of the LPS receptor complex. Strongly expressed on monocytes / macrophages and weakly expressed on some neutrophils
CD16-APC	VEP13 (130-091-246/ 5130718150)	Mouse IgM (130-093-176/ 512102119)	Miltenyi Biotec Ltd	As above
CD16-FITC	VEP13 (1300x91244/5 110308207)	Mouse IgM (1300x93178/5 101208144)	Miltenyi Biotec Ltd	Low affinity IgG receptor. Present on neutrophils, macrophages and NK cells.
CD62L-APC	145/15 (130-091-755 5110906351)	Mouse IgG1 (1300x92214/5 120706226)	MACS	Recruitment of leukocytes across endothelium to inflamed tissues. Most haematopoietic cells express CD62L.
CD95(Fas)-APC	DX2 (305612/B128	Mouse IgG1 (1300x92214/5	Miltenyi Biotec	Member of TNF receptor superfamily – mediates

	820)	120706226)	Ltd	apoptosis. Expressed upon granulocytes, monocytes and many lymphocytes.
CXCR2 (CD182)	5E8/CXCR2 320704/B1472 74	Mouse IgG1 (1300x92214/5 120706226)	Biolegend	IL-8 receptor, as homo or heterodimer with CXCR1. Expressed on granulocytes, and many other haematopoietic cells
FcR blocker	120-000-442 512128065		MACS	
Ly-6G MicroBeads	130-092-332			Positive selection of mouse neutrophils
TLR4-PE (CD284)	HTA125 (312805/B156 775)	Mouse IgG2a (130-091-837/ 5120614134	Biolegend	Pathogen recognition receptor. Expressed on monocytes/macrophages, endothelial cells and low levels on neutrophils and B cells.
TREM-1-PE	193015 (FAB1278P/LC 10411011	Mouse IgG1 (1300x92214/5 120706226)	RD Systems	Amplification/transduction of pattern recognition receptor signals. Expressed on neutrophils, monocytes/macrophages and dendritic cells.

Table 2.4 Monoclonal antibodies and beads.

2.2.4 Fluorescent cellular probes

Probe*	Conversion product (excitation/ Fluorescence nm)	Marker	Notes
Dihydrorhodamine-123 (Poot et al., 1996; Rothe et al., 1988; Rothe and Valet, 1990)	Rhodamine123 (511/534)	H_2O_2, (Royall and Ischiropoulos, 1993) HOCl	Used for clinical tests of neutrophil burst. Accumulates in mitochondria.
Dihydroethidium (Lehmann et al., 1998)	Ethidium (520/610)	O_2^- (Walrand et al., 2003)	Hyper-fluoresce when bound to DNA.
MitoSOX RED (Mukhopadhyay et al., 2007)	Ethidium (520/610)	O_2^- (Cationic)	Ethidium conjugated with cationic triphosphohenyllinium# to target mitochondria
TMRM (Ehrenberg et al., 1988; Feng et al., 2009).	TMRM (535/573)	Mitochondrial membrane potential (Cationic)	Accumulates unchanged within mitochondrial intermembrane space.
JC-1 (Minamikawa et al., 1999; Salvioli et al., 1997)	Green monomers (514/529nm) Red J-aggregates (514/590nm)	Mitochondrial membrane potential (Cationic)	Accumulates unchanged within mitochondrial intermembrane space. Density dependent aggregation and colour change.

Table 2.5 Fluorescent cellular probes

**Stock details listed under Table 2.3, Chemicals and Reagents. #The inner mitochondrial membrane is far more negative (-140mV) than other organelles within the cell, therefore lipophilic cations preferentially accumulate there,* (Murphy and Smith, 2000)

2.2.5 Neutrophil activation agents

Activator*	Target	Working concentration	Application / notes
Opsonised E. coli	Phagocytosis: Fcγ, PRR, complement receptors.	Typically, x10^9/ml	FITC labelled bacteria to quantify phagocytosis, unlabelled bacteria to measure respiratory burst
Lipopolysaccharide (LPS)	LPS receptor (TLR4/CD14)	Dependent upon bacterial strain and LPS subtype. (0.5 to 100ng/ml)	Requires lipopolysaccharide binding protein.
fMLP	fMLP receptor	1 to 50 ng/ml	A potent chemoattractant, also a weak stimulus of respiratory burst – commonly used to identify primed neutrophils.
PMA	Protein Kinase C (Higher concentrations may directly activate NF-kB).	100ng/ml to 1ug/ml.	Potent and persistent activator of PKC – endogenous signals are more transient. Bypasses cell surface signalling machinery.

Table 2.6 Neutrophil activating agents

**Stock details listed within Table 2.3, Chemicals and Reagents.*

2.3. Blood work

Laboratory isolation of neutrophils has been repeatedly demonstrated to alter neutrophil function (Kotz et al., 2010; Kuijpers et al., 1991; Pallister and Topley, 2004). However, assays within whole blood maybe affected by the presence of other cell types and variable concentrations of neutrophils (Wenisch et al., 2001). Therefore, experiments were repeated within whole blood and isolated neutrophils where possible. Some assays, such as JC-1, cannot be used in whole blood. Heparin anticoagulation better preserves cell surface antigen expression (Elghetany and Davis, 2005), while citrate depletion of calcium may impair signalling processes necessary for neutrophil activation.

20ml of heparinised whole blood (19ml blood plus 1ml [1000 units] heparin) were collected for immune study studies, along with full blood count and chemistry samples for hospital laboratory analysis. Heparinised whole blood samples were immediately placed on ice and processed with 2 hours of collection.

2.3.1 Granulocyte isolation from whole blood

Neutrophil isolation was by density gradient centrifugation. 15ml whole blood was diluted 1:1 with phosphate buffered saline, layered over 15ml Ficoll-Paque within a 50ml Falcon tube and centrifuged at 220x g for 30minutes with braking disabled. After removal of all layers above the neutrophil/red cell fraction using Pasteur pipettes, 30ml lysis buffer was added and samples were placed on ice for 20 minutes and regularly vortexed. These falcon tubes were then centrifuged at 220x g for 6 minutes and the supernatant discarded. A second lysis stage was performed, as described above. Samples were then washed in PBS, and a 100µL aliquot mixed with 100µL trypan blue, counted with a haemocytometer and resuspended at $1x10^6$ cells per ml in PBS with 10mg/ml sterile filtered glucose and placed on ice. Manual counting of leukocytes is considered a reference standard (Roussel et al., 2012). Trypan blue revealed >98% viability of cells.

The granulocyte population includes neutrophils, eosinophils and occasional basophils. Neutrophils typically outnumber eosinophils 300-fold, therefore large scale investigations have considered the granulocyte population to be equivalent to a pure neutrophil population (Kotz et al., 2010; Xiao et al., 2011). Further isolation procedures may increase iatrogenic injury and activate neutrophils. Eosinophil contamination rates of 3% are typically

considered to be acceptable (Kotz et al., 2010). In addition to physical separation, during flow cytometry experiments neutrophils were specifically identified by co-labelling with a conjugated CD16 monoclonal antibody. For the respiratory burst experiments 'backgating' was used to remove cells with very low burst responses (see 2.6.4).

2.3.2 Neutrophil isolation from murine bone marrow

The Miltenyi Biotec isolation kit was used to positively select murine neutrophils. Ly-6G antibody is highly specific for murine neutrophils. Mixed cellular preparations were incubated with Anti-Ly-6G Biotin labelled monoclonal antibodies, then subsequently with Anti-Biotin magnetic microbeads. The cells were then passed through a magnetic field which retains the co-labelled cells, but allows other cells to pass through.

The Separation Buffer stock was diluted 1:20 with PBS to create a working solution (pH 7.2, 0.5% bovine serum albumin, 2mM EDTA). Heparinised murine bone marrow was extracted as described below, passed through a 30µm nylon mesh to remove clumps, counted and re-suspended in 200uL buffer per 10^8 cells. 50µL Anti-Ly-6G-Biotin was added per 10^8 cells, samples mixed and incubated at 4°C for 10 minutes. A further 150µL of buffer was added per 10^8 cells, then 100uL Ani-Biotin Microbeads per 10^8 cells. Samples were mixed and incubated for further 15 minutes at 4°C, then a further 5ml of buffer added per 10^8 cells and centrifuged at 300g for 10 minutes. Supernatant was aspirated and cells re-suspended in 500µL of buffer per 10^8 cells.

The MACS MS separation column was rinsed with 500uL buffer and placed in the separator. 500µL aliquots of cell suspension were then added to the reservoir, and repeated once reservoir was empty. The column was then removed, placed over a collection tube and flushed with 1ml buffer.

2.4 Animal models

An animal model was used for bone marrow experiments within Chapter 4. 8-12 week old male mice (weight 20-25g) were selected. Animals were housed with food and water available *ad libitum* in the specific pathogen-free central animal facilities of University College London Medical School (London, UK). Experimental protocols were approved by the UCL Institutional Animal Care and Use Committee, and performed per UK Animals (Scientific Procedures) Act, 1986. Experiments were conducted in accordance with the ARRIVE guidelines (Kilkenny et al., 2013).

Experiments were performed within a laboratory, in a separate room from the home cages. Topical bupivacaine was used for analgesia. During experiments body temperature was maintained using a warming mat regulated by a rectal temperature probe. Animals were randomised through numbering and random (blinded) selection of numbers, with treatment and control animals selected in alternating manner. Animal welfare was overseen by UCL biological services department.

2.5 HL-60 cell line

HL60 cells were obtained from ATCC (ATCC CCL-240). Cultures were performed within RS Biotech Galaxy R 170litre incubator (37.0°C, pCO_2 5.0kPa). All instrumentation was performed within a cleaned culture hood with laminar air-flow. Experiments were performed upon cells between passage 10 and 40. The cell line was maintained and differentiated by Ana Gutierrez del Arroyo. Cells were seeded at $2.5x10^5$/ml in RPMI 1640 (with 0.3g/L L-glutamine and 20% heat inactivated fetal calf serum). Cells were differentiated into neutrophil-like cells by the addition of 1.25% DMSO for 6 days prior to experimentation (Collins et al., 1979). All cells were washed twice in plain RPMI before functional assays were performed. Cells were counted using trypan blue as described above, and resuspended at 0.5M/ml.

2.6 Flow cytometry

Flow cytometry uses lasers to describe the optical properties of individual cells. The scattering of laser light provides gross information about cell size and shape. The highly specific fluorescent properties of fluorochrome chemicals are used to provide more detailed

information about each cell. Fluorescence describes the optical property whereby excitation of an electron with a specific wavelength of light leads to a change in electron energy levels and subsequent emission of a second photon of light at a different, but equally specific wavelength. The flow cytometer contains a series of lasers that are used to excite these electrons, and a series of detectors, which consist of photomultiplier tube detectors covered by monochromatic filters.

Fluorochromes may be conjugated to monoclonal antibodies to measure specific antigens either outside, or within cells. A range of fluorescent probes is also available, whose emission spectra change during highly specific chemical reactions, and may therefore be used to measure the rates of these reactions and make inferences about the quantities of reactants and reaction rates. It is possible to combine fluorochromes and probes, provided emission spectra are different – however, even if peak fluorescence's are different, the spectra may still overlap. To isolate each signal, individual control samples are run to measure relative contributions from each signal, and to also measure background/autofluorescence. The flow cytometry techniques used throughout this thesis follow International Society for Analytical Cytometry MIFlowCyt guidelines (Lee et al., 2008), and recommendations by Tung et al (Tung et al., 2007).

Measurements were performed using the Beckman Coulter CyAn ADP flow cytometer (Beckman Coulter, High Wycombe, UK), using Summit 4.3.1 software to gather flow cytometry files (.fcs). Off-line analysis was performed using Beckman Coulter Cyan Kaluza v1.3 software.

Laser alignment was checked every day using Multicheck beads (Moflo, Beckman Coulter) and every two weeks using SpectrAlign beads (CyAn) to a target fluorescence resolution coefficient of variation <3% of full peak for 488nm excitation and <4% for 633nm excitation by a formally trained support scientist. Lasers were allowed to warm up for 30 minutes before sample acquisition. A routine cleaning protocol was run by each user at the end of each session, and an extended protocol run at the end of each working day. A maintenance contract with Beckman Coulter provided yearly servicing and on-request engineer visits.

CyAn ADP specifications:

Laser source	Emission Filter	Fluorochrome / probe
488nm, 25mW	FL1 (530/40nm)	FITC, rhodamine-123, JC-1
488nm, 25mW	FL2 (575/25nm)	PE, JC-1, TMRM, DHE, MitoSOX Red
488nm, 25mW	FL3 (613/20nm)	PI
642nm, 60mW	FL8 (665/20nm)	APC

Table 2.7 Optical specifications of CyAn ADP flow cytometer.

Taken from (Beckman-Coulter, 2009)

Feature	CyAn ADP
Maximal processing speed	Up to 70,000 events per second
Sample flow rate	Up to 150µL/min
Quartz cuvette	Fused silica, 250µM square sectioned internal channel
Compensation	9x9 matrix, auto compensation
Signal resolution	4096 channels on all parameters
Sensitivity (Molecules of equivalent soluble fluorochrome)	FITC <100; PE <50
Beam geometry	Elliptical / spherical
Sheath management system	Electronic fluidic control of sheath, waste and cleaning fluid with continuous adjustable flow rate
Operating system	Windows XP Professional

Table 2.8 Operating features of CyAn ADP flow cytometer

2.6.1 General conduct

Individual control samples were used for each patient sample. Photomultiplier tube voltages (gain) were set using control samples, and the voltage was adjusted to ensure the control (background) population fell within the first log order of fluorescence. These settings, along with gating strategies, were saved as acquisition protocols to ensure comparability between data collected on different days. All .fcs files were anonymised and saved onto the local hard drive and backed up onto the local network server, onto a home computer, and are available on request.

The choice of control sample depended upon the experimental assay. In the case of monoclonal antibodies isotype controls were used (O'Gorman and Thomas, 1999). Isotype controls are of the same subtype from the same animal (e.g. Mouse IgG1), but are polyclonal and therefore directed to many different epitopes. They account for class or non-specific binding of antibodies, and are particularly important when investigating cells with Fc receptors, such as neutrophils. They were used in the same concentration as test antibodies. For fluorescent cellular probes, such as Rhodamine-123, unstained or untreated control samples were used instead.

2.6.2 Filter compensation and fluorescence-minus-one

The emission spectra of fluorochromes may overlap, leading to detection by an unintended photomultiplier tube. Therefore, when two overlapping probes are used simultaneously a compensation process must be applied to isolate individual fluorochrome responses. Compensation is the first part of any analysis procedure. Compensation uses each of the two overlapping antibody/probes individually as single compensation control samples, allowing the proportion of light detected by the unintended detector to be measured. This proportion is then subtracted from the combined test sample containing both probes. Within modern flow cytometry machines compensation is a post-recording mathematical transformation and does not affect the protocol voltage settings during primary sample acquisition (Roederer et al., 2004). The effects of compensation are demonstrated within the Annexin section below, where Annexin-FITC antibody overlaps with the PI cellular probe.

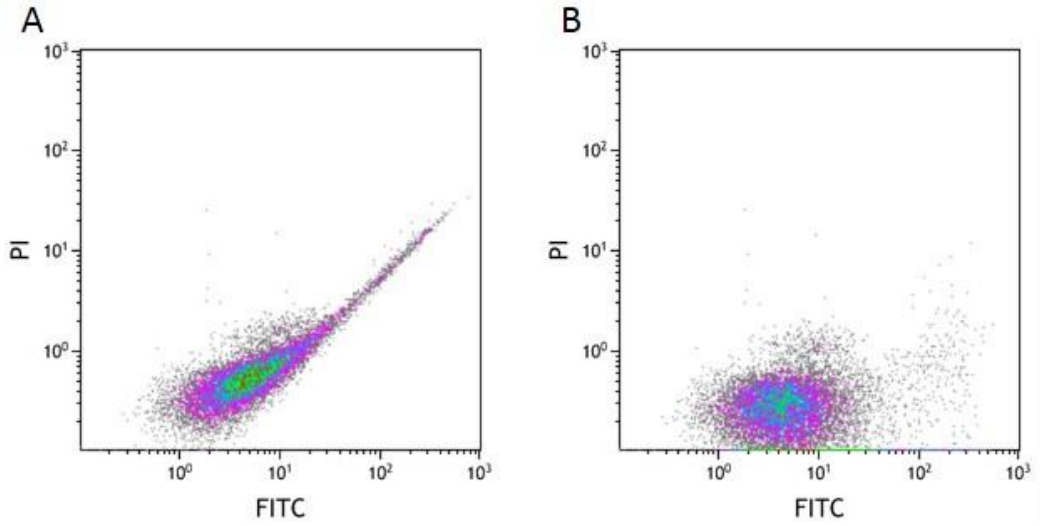

Figure 2.1 Compensation of FITC/PI sample.

(A)In uncompensated sample the FITC emission spectra overlaps with the detection filter (B) With compensation applied PI detection of FITC signal is eliminated.

Fluorescence-minus-one is an alternative method of eliminating spectra overlap. It is particularly useful for samples with low level probe/antibody fluorescence where the relative contribution of autofluorescence to probe/antibody fluorescence will be high, or when several overlapping fluorochromes are being used together. Rather than running each fluorochrome separately, a control sample is created which contains every fluorochrome except the probe of interest. This may then be used to set background fluorescence gates, before the actual test sample is run, containing every probe of interest.

2.6.3 Reporting and presentation of flow cytometry data

FACS data are presented as fluorescence, in dimensionless arbitrary units, typically on a logarithmic scale. However, since logarithmic values are asymptotic to zero, they cannot represent fluorescence values that are close, or approximate to zero. Here, a logicle – or bi-exponential – scale is used, where values around zero have a linear scale. Transformed data from filter compensation often leads to values that fall to zero, necessitating the use of logicle scales.

Neutrophil death and apoptosis were assessed by specific assays, including measurement of mitochondrial membrane potential, early and late apoptosis and cell death. The addition of specific markers of cell death to other assays (e.g. of neutrophil function) were not performed, as has been generally advised by authors commenting on lymphocyte flow cytometry (Tung et al., 2007).

Where possible, co-labelling of conjugated CD16 antibody was used to identify neutrophils during whole blood and isolated neutrophil flow cytometry experiments.

2.6.4 Gating and positive staining

Light scattered by cells passing through the machines laser beam is determined by cellular morphology. Larger cells scatter more light in a forward direction, facing the laser beam (narrow angle, forward scatter, FSC), and denser cells (due to more cytoplasmic granules or organelles) scatter more light sideways (wide angle, side scatter, SSC). Scattering of light may therefore be used to identify granulocytes within isolated samples (Fig 2.2) and lysed whole blood (Fig 2.3) since granulocytes possess distinct morphological features.

Light scattering may also be used to identify cell clumping with the sample stream, and was the first gate set for all analyses. The 'pulse width' contains size information which is independent of many of the factors which contribute to forward and side scattering (Hoffman, 2009), and may be used to exclude clumps and doublets.

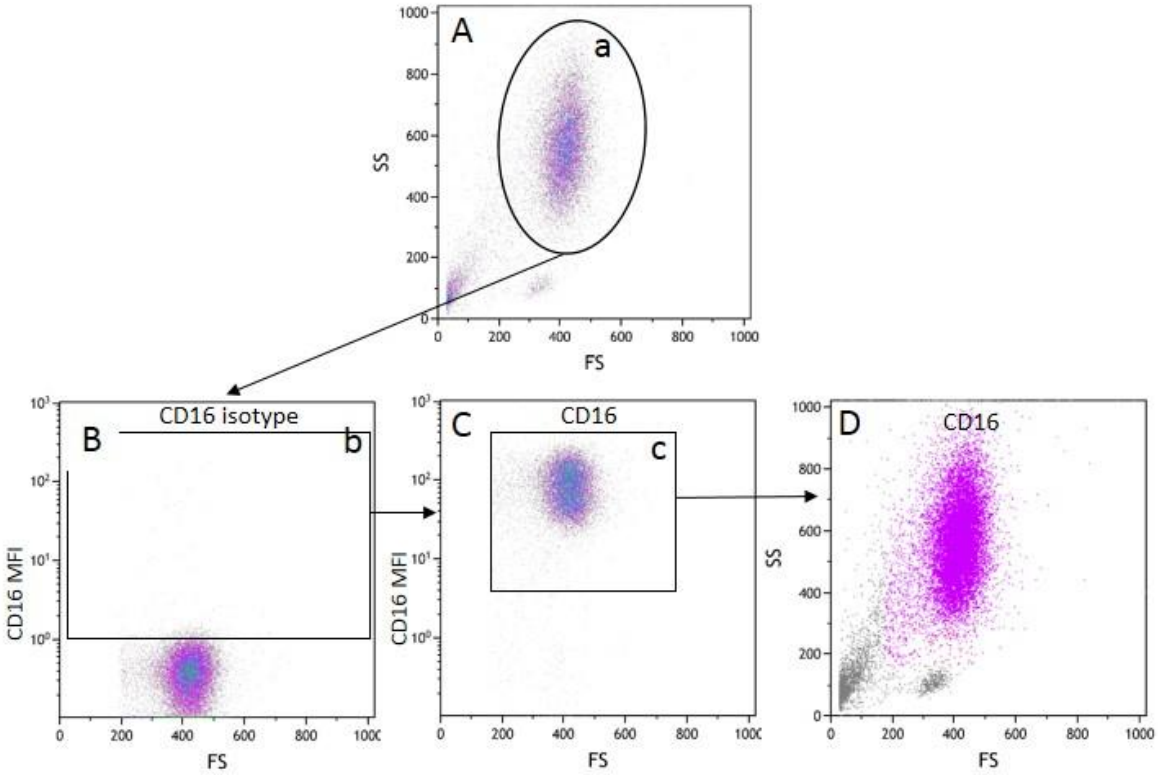

Figure 2.2 Flow cytometric identification of neutrophils within an isolated granulocyte sample.

(A)Isolated granulocyte selected from contaminating lymphocytes/monocytes/debris. (B) CD16^{hi} population selected. (C) CD16^{hi} backgated onto fs/ss plot to confirm purity of isolation procedure.

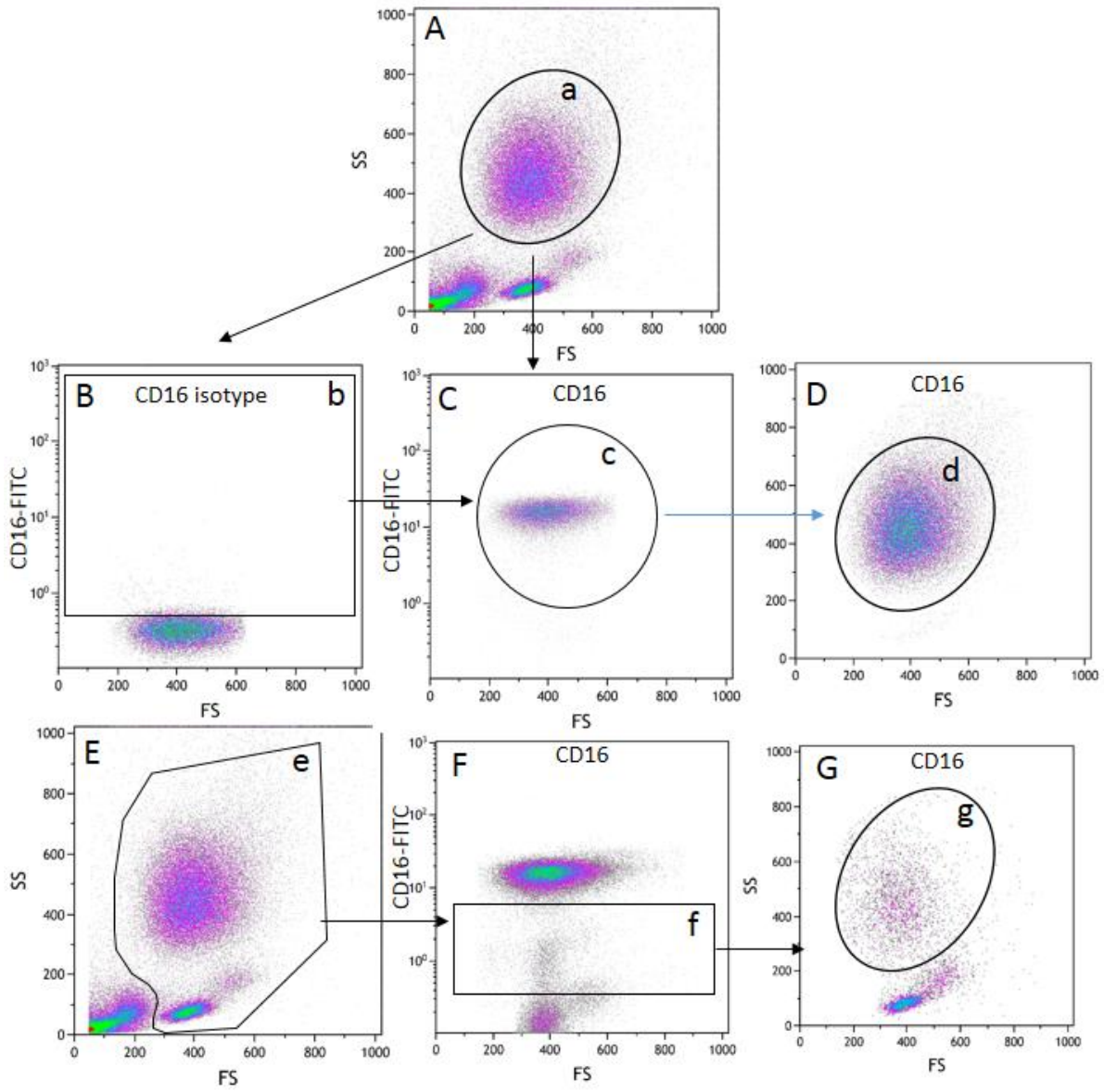

Figure 2.3 Flow cytometric identification of neutrophils within whole blood

(A) Granulocytes identified on fs/ss plot. (B) The CD16 isotype sample was loaded, and a gate set beyond 95% of the isotype population. (C) The test sample was then loaded. (D) Back gating of this population onto the original fs/ss plot confirms these cells are granulocytes. (E) Here, the entire leukocyte population is gated, which shows (F) A population of cells with low expression of CD16 (G) When this population is gated back onto the fs/ss plot, these cells are shown to be lymphocytes/monocytes and a few 'granulocytes'. These CD16^{lo} cells may be apoptosing neutrophils, eosinophils or possibly activated monocytes.

The technique used to describe positive staining was determined by the extent to which the test sample was separated from the relevant control sample. Where there were discrete subpopulations of cells staining positive, the percentage of cells present in the subpopulation and the median fluorescent intensity of the subpopulation were recorded (Fig 2.4 A-E). Samples were considered positive if they fell outside the 95th percentile of the relevant control sample.

Where these samples overlapped, the percentage of cells which fell outside the 95% percentile of the control sample were recorded (Fig 2.4 F-H), unless the population was not normally distributed, in which case median fluorescent intensity (MFI) was reported. Where the test population was completely separated from the control population, MFI was recorded.

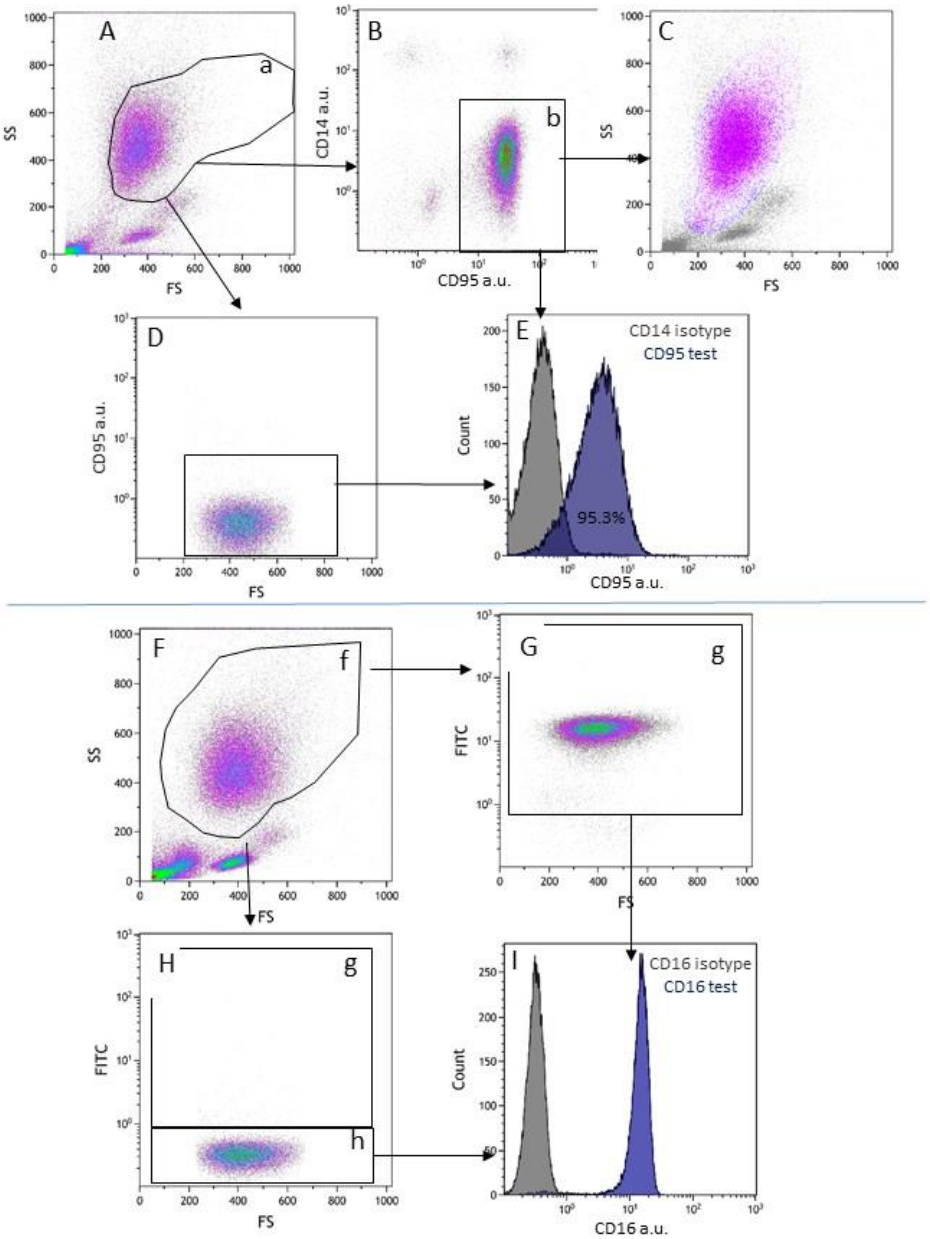

Figure 2.4 Gating strategies for Cluster of Differentiation (CD) antigens.

A-E Measurement of CD14 on neutrophils. Here CD14 expression overlaps the isotype control, and monocytes must be excluded from whole blood sample. (A) Granulocyte population selected. (B) Monocyte subpopulations express variable amounts of CD14, therefore expression of CD16 was used to select the neutrophil population (C) This was backgated onto fs/ss plot to confirm neutrophil population correctly identified. (D) CD14 isotype control run. (E) Overlay plot created, 95% markers created using isotype, then proportion of neutrophils expressing CD14 recorded. F-I Measurement of CD16 on neutrophils. Here the CD16 population is completely separated from the isotype control. (F) Granulocyte population selected. (G) CD16 cells selected (H) Isotype control run (I) MFI of CD16 population recorded.

2.6.5 Monoclonal antibodies

2.6.5.1 Extracellular: Cluster of Differentiation markers (CD)

CD markers used are detailed in table 2.4, and their function and significance described in more detail within chapters 1 and 4. For whole blood samples, 2µL of the relevant conjugated monoclonal antibody (or isotype) was suspended in 100uL 2% BSA then added to 100µL heparinized whole blood and thoroughly mixed. Samples were incubated for 1 hour at 4^{O}C, then fixed with 100µL 2% paraformaldehyde (PFA) for 5 minutes prior to addition of 3mls of Blood Lysis Buffer for a further 10 minutes with regular agitation. Samples were then centrifuged at 300g for three minutes, supernatants discarded, agitated and washed in PBS before repeat centrifuge for 3 minutes at 300g. Supernatants were discarded, then samples agitated plus resuspended in 500µL of PBS and stored in dark at 4°C ready for analysis. All samples were processed within 24 hours of preparation.

Isolated neutrophils were treated similarly. 1x10^{6} cells (1ml) were centrifuged at 300x g for 3 minutes, supernatant was then discarded and the pellet agitated prior to addition of 2µL antibody suspended in 100µL 2% BSA. Samples were incubated for 30 minutes, then fixed and processed as before.

2.6.7 Assays of neutrophil function

2.6.7.1 Phagocytosis

Neutrophil phagocytosis was assessed using PhagoTest (Orpegen Pharma, Heidelberg, Germany). 100µL heparinised whole blood was added to two (test and control) flow cytometry tubes, then cooled to 0°C for 10 minutes. 20µL of precooled, vortexed opsonised FITC-labelled E. coli suspension ($2x10^9$/ml=4000 bacteria) were added to both tubes then vortexed again. The control tube was kept on ice and test tube incubated at 37°C in a water bath for 10 minutes in the dark. At the end of incubation, the test sample was returned to 0°C. 100µL quenching solution (Trypan Blue) was added to each sample, then vortexed. Two wash steps consisting of 3ml cooled wash solution, centrifugation at 300g for 5 minutes at 4°C, and discarding of supernatant were then performed. 2 ml of fix/lysis solution were then added and samples incubated for 20 minutes at room temperature, with regular vortexing. Samples were then washed as before, resuspended in PBS then immediately analysed.

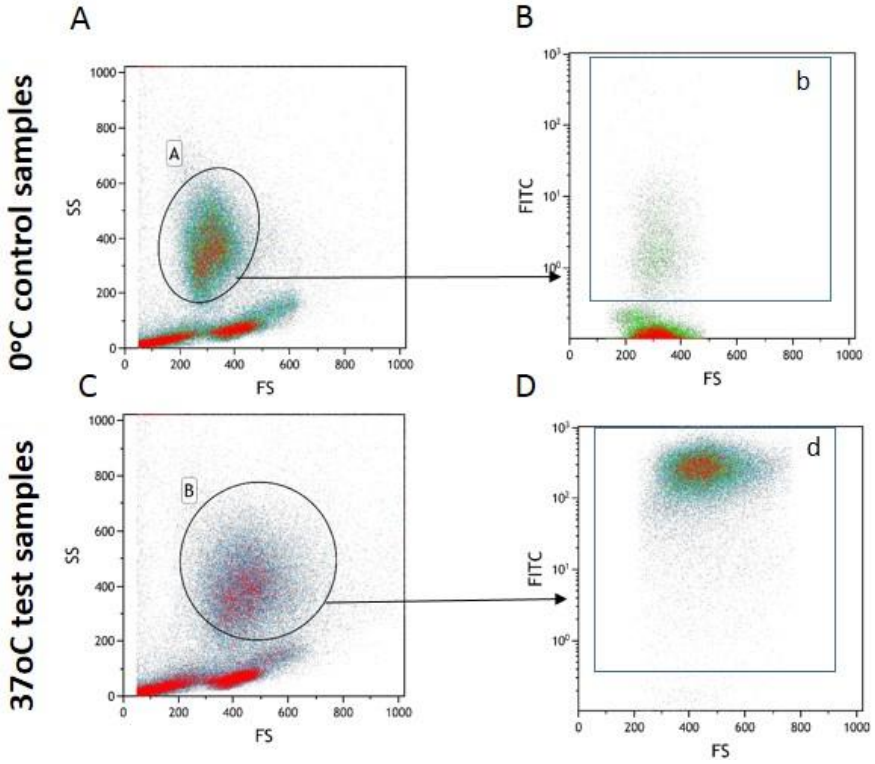

Figure 2.5 Neutrophil phagocytosis assay analysis.

[A] Neutrophils were identified on fs/ss plot. (B) 0°C control sample used to set 95% gate. (C) 37°C test sample run and (D) MFI of test population recorded.

2.6.7.2 Respiratory burst

Neutrophil respiratory burst was assessed using the BurstTest assay (Orpegen Pharma, Heidelberg, Germany). 100µL heparinised whole blood was added to 4 flow cytometry tubes (control, E. coli, fMLP and PMA) and placed on ice. 20µL wash solution were added to the control tube, with 20µL opsonised E. coli bacteria suspension ($1x10^9$/ml), 20µL fMLP working solution (5µM) and 20µL PMA working solution (8.1µM) to respective test tubes. Samples were then vortexed and placed in a 37.0°C water bath for ten minutes. Samples were then returned to ice before addition of 20µL dihydrorhodamine-123 solution, vortexed and incubated for a further 10 minutes at 37°C in a water bath. Samples were then returned to ice and fixed with 100µL PFA for 3 minutes, before addition of 2ml lysing solution and kept at room temperature with regular vortexing. Finally, samples were washed once with 3ml of wash solution, vortexed, centrifuged at 300g for 5 minutes before aspiration of supernatant and resuspension in 100µl wash solution. Samples were then processed immediately on the flow cytometer.

For isolated cells, $1x10^6$/ml (1ml) isolated neutrophils suspended in PBS with 10mg/ml glucose was used in place of whole blood, and the lysis step was omitted. Only PMA test samples were run for isolated cells, since neutrophils do not readily respond to E. coli bacteria in the absence of serum.

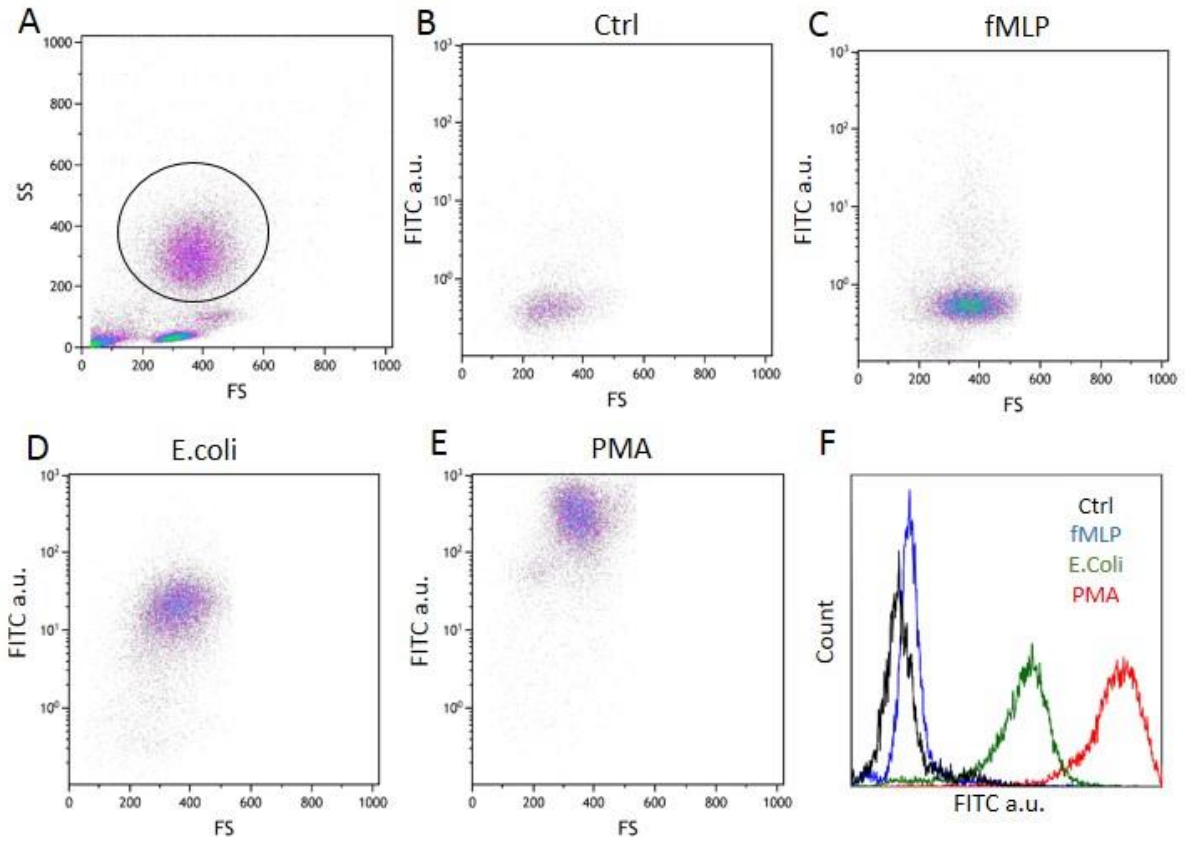

Figure 2.6.Neutrophil respiratory burst assay analysis.

(A) Control sample loaded, and granulocytes identified by fs/ss. (B) Control FITC fluorescence recorded. (C,D,E) Next, fMLP, E. coli and PMA test samples were sequentially loaded, identified on FS/SS (not shown) and FITC fluorescence MFI recorded. (F) Demonstrates overlay plot of all test samples. For fMLP stimulus the percentage of granulocytes positive was also recorded, using a 95% marker previously set on the control sample.

2.6.8 Assays of neutrophil mitochondria

Neutrophils contain functional mitochondria that maintain a mitochondrial membrane potential independent of cytoplasmic ATP production (Fossati et al., 2003; Maianski et al., 2004). The loss of neutrophil mitochondrial membrane potential is believed to be a critical regulator of constitutive apoptosis.

2.6.8.1 Mitochondrial membrane potential

Cell permeable, fluorescent cationic probes may be used with flow cytometry to measure changes within the mitochondrial membrane potential of individual cells. JC-1 is a carbocyanine dye that exists as a green monomer at low concentrations and forms red J-aggregates at high concentrations (Minamikawa et al., 1999; Salvioli et al., 1997). The loss of red fluorescence and gain of green fluorescence may therefore be used to monitor mitochondrial membrane potential. TMRM possesses red-orange fluorescence and accumulates within the positively charged mitochondrial intermembrane space. The magnitude of fluorescence is therefore an indicator of mitochondrial membrane potential. TMRM may be used within whole blood samples, unlike JC-1 which requires an isolated cell system. The uncoupling agent FCCP dissipates the mitochondrial membrane potential independently of ATP production, by providing a route for H^+ to re-enter mitochondrial matrix along a concentration gradient. FCCP may therefore be used as a positive control.

JC-1

I used the MitoPT JC-1 assay kit (Serotec, Kidlington, Oxon, UK, which is only validated within isolated cell preparations (Bassøe et al., 2003). The assay was performed within a darkened room. Working solutions were prepared as per manufacturer's instructions: 1x assay buffer solution and 1x JC-1 solution. Isolated neutrophils were prepared as described above, $1x10^6$/ml in PBS with 10mg/ml glucose, then 1ml neutrophil preparation was added to three flow cytometry tubes (test, control, isotype). These tubes were centrifuged at 300g for 5 minutes, supernatants discarded and re-suspended in 1ml 1x JC-1 buffer. 1µL f FCCP stock (50mM) was added to the control sample (equivalent to 50µM FCCP) and 1µL DMSO to the test sample, all tubes were then incubated at 37°C in water bath for 15 minutes. Then, 1ml 1x JC-1 solution was added to test and control tubes, 1ml JC-1 buffer to the

isotype and 2µL CD16 antibody (or isotype) plus 100µL BSA to test, control and isotype tubes as appropriate. All samples were incubated for a further 15 minutes at 37°C, before being centrifuged at 300g for 5 minutes at room temperature. Supernatants were discarded and all samples washed once with 2ml assay buffer, centrifuged at 300g for 5 minutes, Supernatants were then discarded and re-suspended in 0.5ml of JC-1 buffer and processed immediately on the flow cytometer.

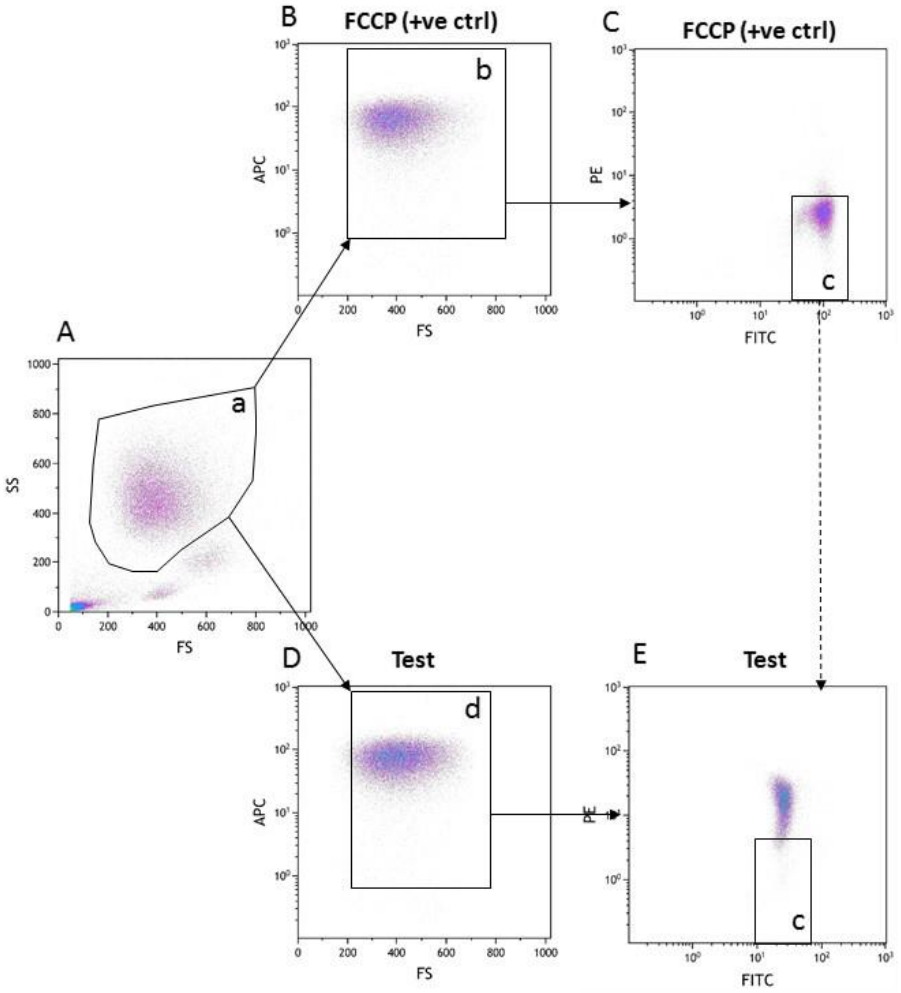

Figure 2.7 JC-1 assay analysis.

(A) FCCP positive control sample loaded, and granulocyte population identified by fs/ss. (B) Positive control neutrophil population identified by CD16hi (C) FITC/PE plot of positive control sample used to identify a gate containing 95% of the neutrophil population. The test sample was loaded and population identified by FS/SS (D) Test neutrophil population identified by CD16^{hi}. (E) The proportion of neutrophils present within the positive control gate, and therefore which had lost their membrane potential, was recorded.

TMRM

TMRM stock solution (10mM) was diluted to form 200nM working solution in JC-1 buffer solution. 100µL heparinised whole blood was added to three tubes (test, control, isotype) and placed on ice. 1µL FCCP stock (50mM) to control sample and 1uL DMSO to the test sample, and incubate at 37°C in water bath for 15 minutes. Next, 1ml of TMRM working solution, and 2µL CD16 antibody (or isotype) suspended in 100uL BSA was added to each tube. Samples were returned to the water bath at 37°C in the dark for a further 15 minutes, before returning to ice. Two wash steps were performed, with 2ml JC-1 buffer, centrifuged at 300g and the supernatant then discarded. Samples were then lysed with 2ml blood lysis buffer before being washed a final time, re-suspended in JC-1 buffer and processed immediately on the flow cytometer.

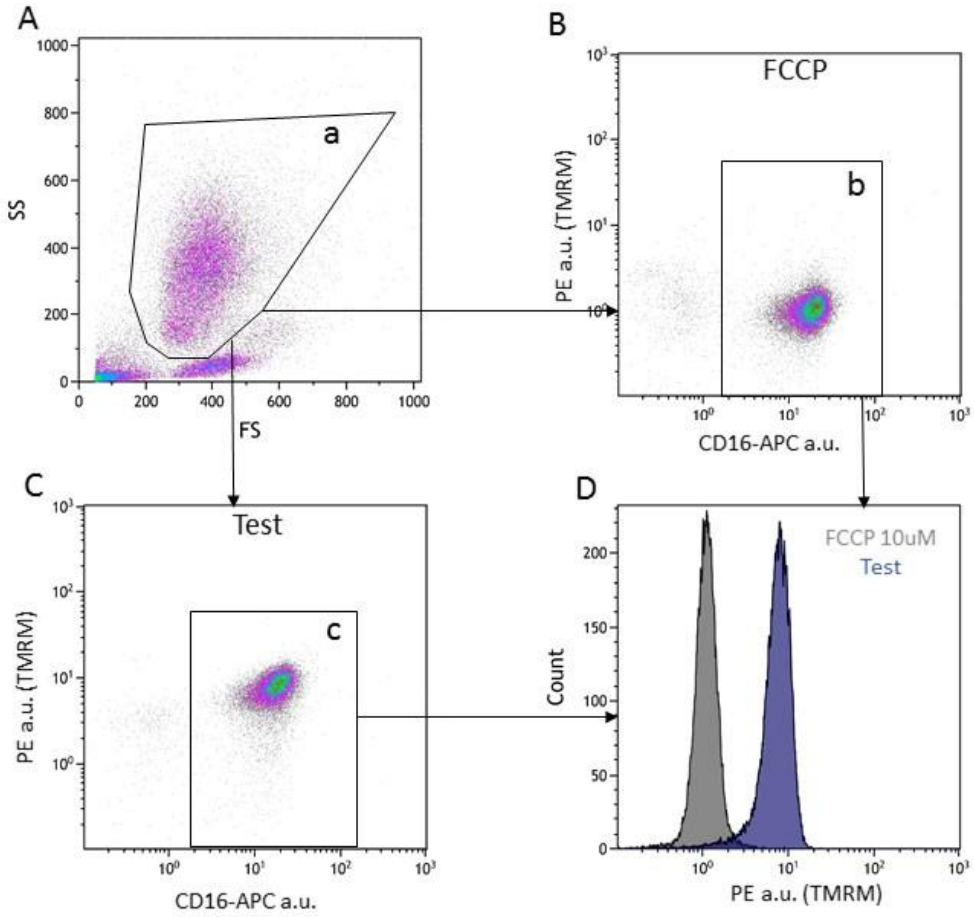

Figure 2.8 TMRM assay analysis.

(A) Positive control sample loaded, and granulocyte population identified by FS/SS (B) Neutrophils then identified by CD16^{hi} (not shown). Test sample loaded and granulocytes identified by FS/SS (C) Test neutrophil identified by CD16^{hi} (D) An overlay plot of positive control and test samples was created. Both the MFI of the test sample, and the % of neutrophils that lost their membrane potential were recorded.

2.6.8.2 Mitochondrial superoxide production

Redox sensitive fluorescent probes may be targeted to mitochondria through conjugation with a negatively charged moiety. MitoSOX Red consists of the hydroethidium probe, which undergoes 2-hydroxylation in the presence of superoxide, attaching to the cationic triphenylphosphonium which promotes electrophoretic uptake by respiring mitochondria (Mukhopadhyay et al., 2007). When oxidised MitoSOX Red binds to cellular DNA it becomes hyper fluorescent (Robinson et al., 2006). This must be considered during flow cytometry analysis. Dihydrorhodamine-123 is less specific, and measures H_2O_2 – including that generated by mitochondrial superoxide dismutase - throughout the cell (Henderson and Chappell, 1993).

MitoSOX Red

The MitoSOX Red assay kit (Invitrogen, Paisley UK) was used. A 50µg vial of MitoSOX was added to 13µl of DMSO, to make 5mM stock solution. 2 µL stock solution and 1µL CD16 antibody were added to100µl MitoSOX assay buffer to create a test solution. 2 µL of DMSO plus 1µL CD16 antibody was added to 100µL of MitoSOX assay buffer to create a control solution and 2 µL DMSO plus1µL CD16 isotype was added to 100µL of MitoSOX assay buffer to make an isotype solution. 100µL of isolated neutrophils (prepared as described above – $1x10^6$ cells/ml in PBS with 10mg/ml glucose) were added to flow cytometry tubes labelled test, control and isotype, with 100µL of the test solution, control solution and isotype solutions added as appropriate. These samples were vortexed, then incubated at 37°C for 15 minutes at 5kPa CO_2. These samples were placed on ice and washed once by adding 2ml of 4°C assay buffer, vortexed, centrifuged at 300g for 5 minutes and supernatants discarded. Samples were resuspended in 100µL of 4°C assay buffer, placed on ice in the dark and processed immediately on the flow cytometer.

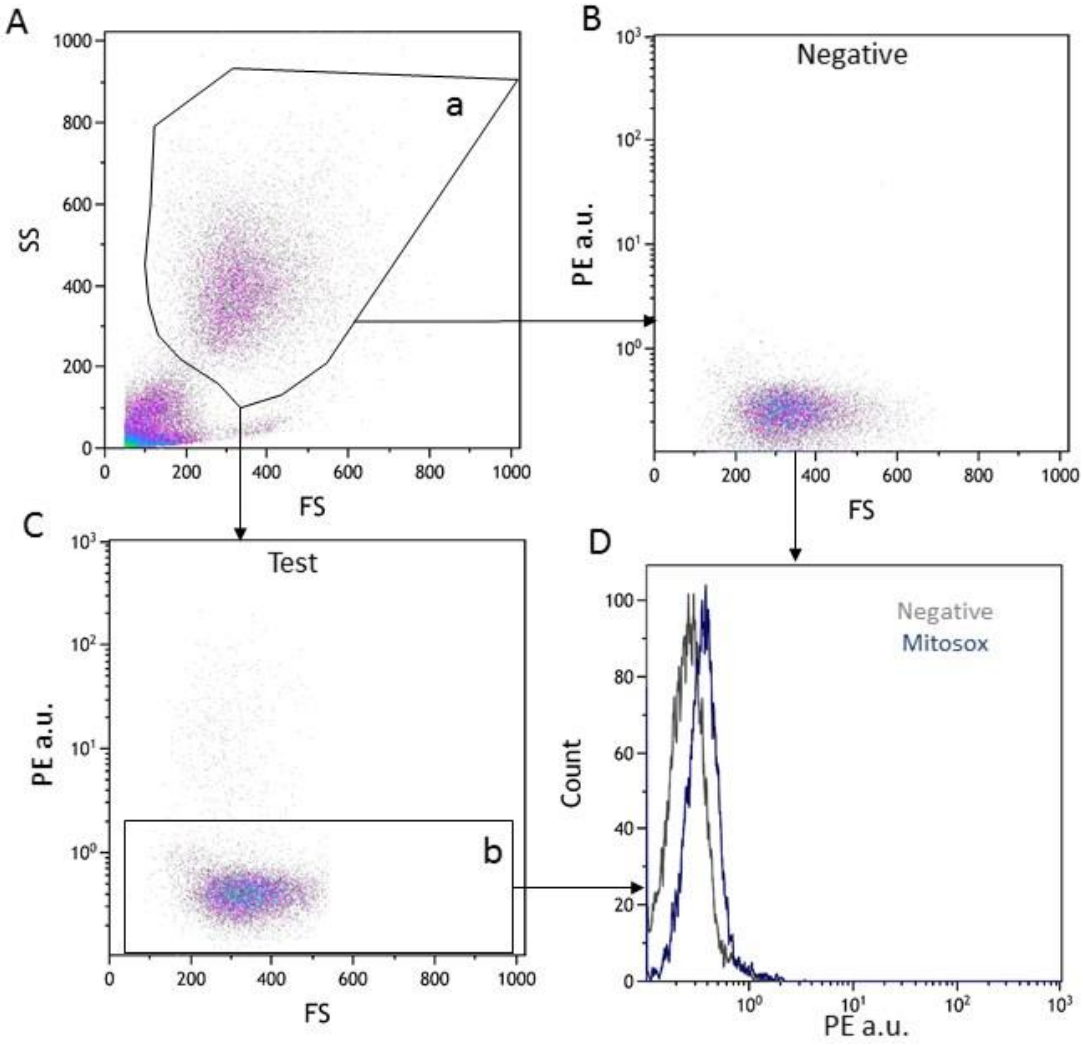

Figure 2.9 MitoSOX Red assay analysis.

(A) Unstained, negative control sample loaded and granulocyte population identified by fs/ss. (B) PE fluorescence of negative sample noted. (C) Test sample loaded, granulocyte population identified and higher staining cells excluded. (D) Overlay plot of negative and test sample. A 95% gate was created from test sample, and the proportion of test neutrophils beyond this recorded.

2.6.9 Apoptosis: Annexin-V-FITC / Propidium Iodide (PI)

Phosphatidylserine is usually present on the cytoplasmic side of the cellular membrane, but becomes externalised early in apoptosis (Vermes et al., 1995). Annexin-V avidly binds to negatively charged phosphatidylserine, and is commercially available in a FITC conjugated form to detect apoptosis. Further progression of apoptosis leads to fragmentation of the cellular membrane, allowing the vital dye PI to enter and bind to DNA. Therefore, combination Annexin/PI staining may be used to classify cellular viability. Cells that have entered early apoptosis will only stain with Annexin-FITC, while those which have progressed to late apoptosis or secondary necrosis will stain for both Annexin-FITC and PI. Meanwhile cells which are primarily necrotic will only stain with PI, while healthy non-apoptotic cells will stain with neither. The Annexin-V assay is widely used, and correlates with other techniques such as TUNEL and the DNA ladder assay (Yasuhara et al., 2003).

$1x10^6$ cells/ml of isolated neutrophils in PBS plus 10mg/ml glucose were prepared as described above, with 1ml added to four flow cytometry tubes labelled Negative, Annexin, PI and Annexin+PI. These samples were centrifuged at 300g for five minutes, supernatants were discarded and re-suspended in 100µL of Annexin buffer that contained either no added agent (negative, PI) or 0.5µL of Annexin-FITC (Annexin, Annexin+PI). These samples were incubated for 20 minutes in the dark at room temperature. A further 500µL of Annexin buffer was added to each tube, and samples were again incubated in the dark for 20 minutes at room temperature. Finally, samples were placed on ice and 5µL PI was added to the PI and Annexin-PI tubes, vortexed and run immediately on the flow cytometer.

Negative control samples were used to identify granulocytes on a FS/SS plot. PMT voltages were adjusted so that this granulocyte population fell within the first log decade on the FITC/PI dot plot and to set the location of quadrant gates. Annexin and PI tubes were then used to set compensation boundaries, prior to processing the Annexin+PI test sample.

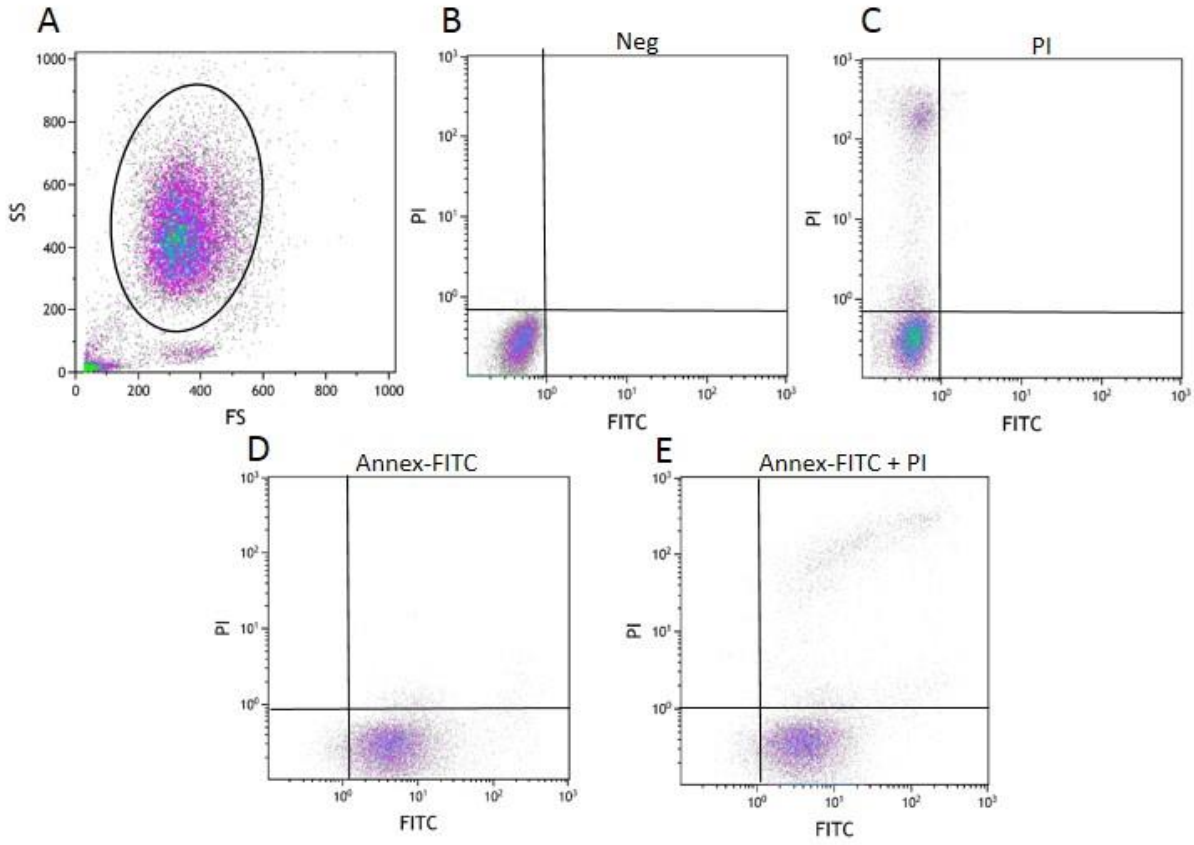

Figure 2.10 Annexin-V/PI apoptosis assay analysis.

(A) Negative control sample loaded and isolated granulocytes identified by fs/ss (B) FITC/PI PMT voltage adjusted so that population falls within 1 log decade for each. Quadrants plotted to encompass 90% of neutrophil population, with 5% FITC positive and 5% PI positive (C) PI control loaded and compensation applied as described above (D) Annexin-FITC control loaded and compensation applied as described above (E) The Annexin-FITC and PI test sample was loaded and proportion of neutrophils within each quadrant recorded.

2.6.10 2-NBDG Glucose uptake assay

The fluorescent glucose analog 2-NBDG [CAS 186689-07-6] may be used to monitor glucose uptake in living cells (Yamada et al., 2007; Yoshioka et al., 1996). Stock 10mg/ml was made by dissolving crystalline solid in DMSO, and stored at -20°C. Isolated neutrophils were prepared as described above ($1x10^6$ cells/ml, PBS both with, and without glucose 10mmol/L), and incubated with 2NBDG at 1, 10 and 100µM for 15 minutes at 37°C in a water bath. Samples were then placed on ice and washed with ice-cold PBS, centrifuged at

300g for 5 minutes, washes again then re-suspended in PBS, and then processed immediately.

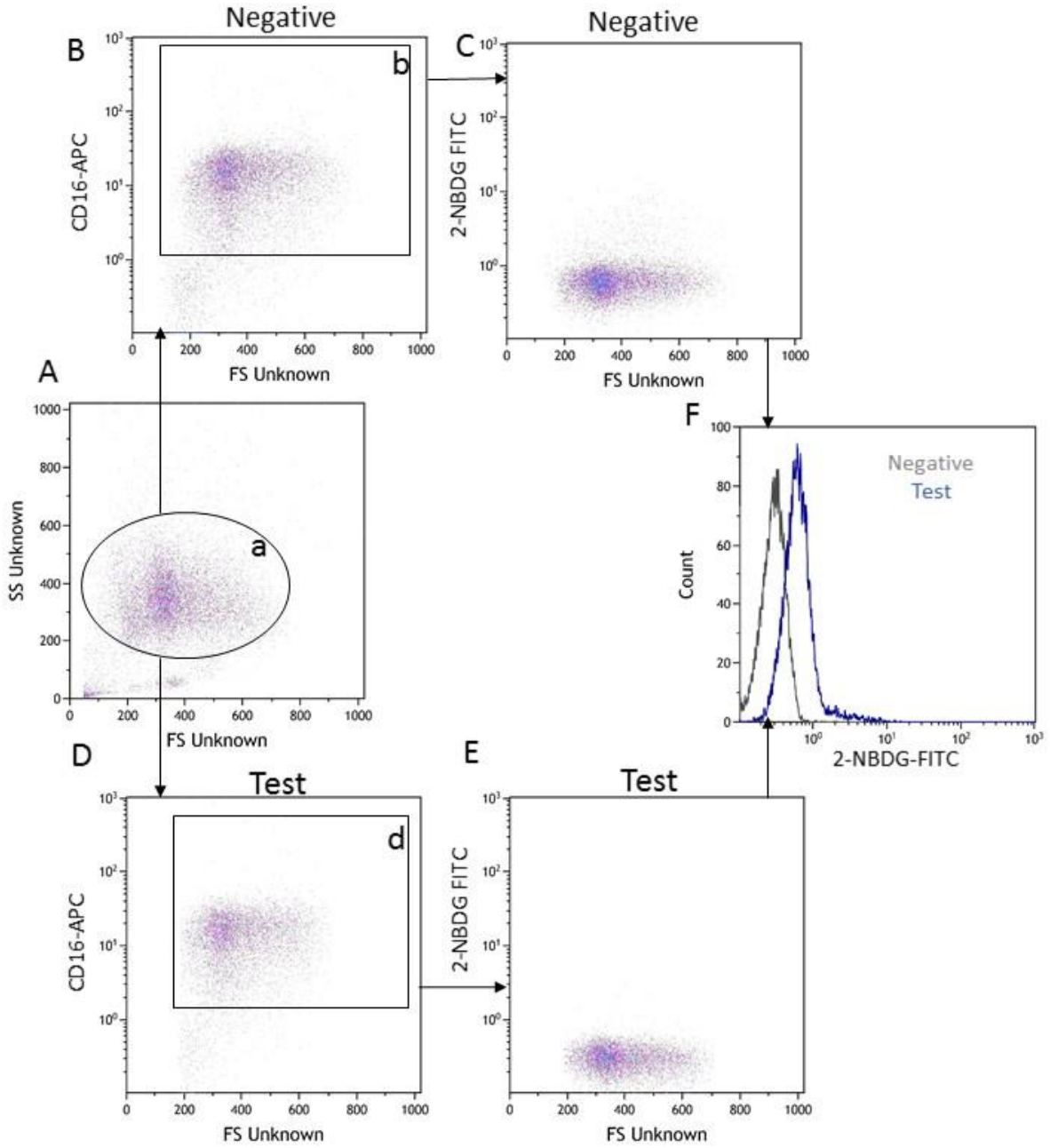

Figure 2.11 2-NBDG glucose uptake assay.

(A) Isolated granulocytes were identified on fs/ss plot (B) Negative control sample loaded and neutrophils identified by CD16^{hi} – isotype control not shown. (C) FITC PMT voltage adjusted so that negative population falls within first log decade. (D) Test sample loaded and neutrophils identified by fs/ss then CD16$^{hi.}$ (E) 2-NBDG FITC fluorescence noted (F) Overlay plot of negative control and 2-NBDG test sample. A gate was created to encompass 95 % of the control population, the proportion of neutrophils falling beyond this gate were recorded as positive.

2.7 Pharmacological manipulation of neutrophil metabolism

2.7.1 Mitochondrial respiratory complex inhibitor assays

To assess whether mitochondrial dysfunction may be responsible for the postoperative neutrophil phenotype, preoperative neutrophils were incubated with respiratory complex inhibitors and the functional assays described above were repeated. Stock solutions of respiratory complex inhibitors rotenone 10mM, antimycin A 10mM and myxothiazol 10mM were prepared in 100% ethanol.

Rotenone inhibits complex I by preventing transfer of electrons from the Fe-S site to ubiquinone. Antimycin A and myoxthiazol both inhibit the Q cycle at complex III, by binding to the Q_i and Q_o sites of cytochrome c reductase, respectively (Thierbach and Reichenbach, 1981).

Isolated neutrophils. $1x10^6$ cells/ml isolated neutrophils in PBS containing 10mg/dL of sterile glucose were incubated for 30 minutes at 37°C in a water bath with relevant respiratory chain inhibiting drugs. Appropriate functional assays were then performed, as described above. Identical volumes of ethanol were added to control samples.

HL60 cells. $500x10^5$ cells washed HL60 cells (6day DMSO differentiated and undifferentiated) were suspended in 1ml plain RPMI 1640 and incubated for 30 minutes at 37°C and 5.0 kPa CO_2 with respiratory chain inhibiting drugs. Functional assays were then performed as described above. Identical volumes of ethanol were added to control samples.

2.7.2 Mitochondrial ROS scavenging: MitoTEMPO

To assess how a reduction of mitochondrial ROS affects neutrophil immune function, neutrophils were preincubated with the mitochondrial ROS scavenger MitoTEMPO prior to performing functional immune assays. MitoTEMPO is a commercially available mitochondrial ROS scavenger, which consists of the antioxidant piperidine nitroxide conjugated to the lipophilic, cationic moiety triphenylphosphonium. This leads to a 1000-fold concentration within mitochondria (Skulachev, 2007). It may be considered a mitochondrial superoxide dismutase mimetic (Dikalov et al., 1997).

MitoTEMPO was dissolved in DMSO to create a stock solution of 10mM and stored at -20°C. MitoTEMPO has been used at concentrations ranging from 1nM (Liang et al., 2010)

to 5μM (Liu et al., 2010), and demonstrated to reduce mitochondria ROS, measured using MitoSOX Red.

Six-day DMSO differentiated and undifferentiated HL60 cells were washed and prepared at a concentration of $3x10^5$/ml in RMPI 1640 + L glutamine without added fetal calf serum. HL60 cells were then incubated with MitoTEMPO 100nM or 500nM for 1 hour at 37°C in CO_2 5.0 kPa, prior to performing functional assays described previously. Control samples contained an equivalent amount of DMSO.

2.7.3 Pyruvate kinase M2 activation: DASA

A cell permeable substituted *N,N'* diarylsulphonamide (NCGC00181061; DASA) was used to selectively activate the M2 isoform of pyruvate kinase (PKM2). PKM2 is the only isoform of pyruvate kinase which demonstrates oxidative inhibition. Briefly, oxidation of cysteine residues at the active site of PKM2 leads to a change in the macrocomplex structure, with active tetramers dissociating to inactive dimers. Pre-treatment of lung cancer cells with 20μM DASA prevents inhibition of PKM2 induced by hydrogen peroxide, diamide and hypoxia (Anastasiou et al., 2012; Boxer et al., 2010).

50 mg/ml DASA stock solution was made by dissolving DMSO and aliquots stored at -20°C. Neutrophil function assays were then performed in the presence of DASA, with equivalent amount of DMSO added to control samples. 10μM and 100μM DASA was added both immediately before functional assays, and incubated with neutrophils for 30minutes at 37°C in water bath. These experiments were repeated in isolated neutrophils and HL60 cells.

2.8 Reactive oxygen species time course experiments

To assess the temporal impact of mitochondrial inhibitors upon neutrophil baseline function and respiratory burst, the experiments described in section 2.8.1 were repeated with the samples loaded onto a 96-well plate and recordings made at 10-minute intervals for 1 hour using a monochromatic plate reader (Synergy MX microplate reader).

2.8.1 Synergy MX Monochromator-based Multi-Mode Microplate Reader

Microplate readers allow experiments to be set up on a multi-well plate, with 96 conditions, and absorbance/fluorescence intensity or luminescence measured for each condition at specified time points.

Features include:

- Four slits on excitation and emission permit upto 16 bandpass combinations for each wavelength pair
- Accurate temperature control (±0.5°C at 37°C)
- Fluorescence (top and bottom), absorbance and luminescence detection systems.

- Absorbance – sample illuminated by a specific wavelength of light, selected by placing a monochromator in front of a light source, and a light detector on the other side of the sample records how much light passes through the sample.
- Fluorescence – a specific wavelength of light, selected by a monochromator, excites the sample, causing light to be emitted at a different that is detected by a photomultiplier tube.
- Luminescence – produced through a chemical or biochemical reaction. Unlike fluorescence no light source is required.

2.8.2 Neutrophil ROS time course protocol: dihydroethidium (DHE)

Human neutrophils were isolated and prepared from a single preoperative patient as described before, at a concentration of $2x10^6$ per ml in PBS with 10mg/dL glucose. The ROS probe dihydroethidium (DHE) was selected to confirm and extend previous findings with the ROS probe DHR. DHE crystalline solid was dissolved in DMSO to create 10mM stock. The same doses of myxothiazol were chosen as for the flow cytometry respiratory burst experiments. A fluorescence protocol was created, with excitation 530/25 nm, fluorescence detection 620/15nm, sensitivity set to 65, temperature 37.0°C and reading every 10 minutes.

A 96-well plate was placed on ice. Experimental conditions were negative, control, myxothiazol 1μM, myxothiazol 10μM, these were repeated with 1μM PMA. 195μL neutrophil suspension were placed in each well. All conditions except negative had DHE added to a final concentration of 5μM. Appropriate volumes of DMSO and ethanol were added to unstimulated and control wells as appropriate. All experimental conditions were repeated in triplicate. The 96-well plate was inserted into the platereader, and the protocol started.

2.9 Bone marrow response to surgery

Having established the effects of major elective surgery upon circulating neutrophils, the effects of anaesthesia and surgery upon the respiratory burst of murine bone marrow neutrophils were assessed. Bone marrow samples were prepared by Gareth Ackland. Cells were processed by density centrifugation prior to seeding, providing >90% purity. The Seahorse XF24 (Seahorse, North Billerica, MA, USA) measures cellular oxygen consumption and acid production, and in combination with specific metabolic inhibitors, may quantify the rate of oxidative phosphorylation and glycolysis. The magnitude of the respiratory burst may also be estimated by recording oxygen consumption in the presence of an activator, such as PMA.

2.9.1 The Seahorse XF24 microplate respirometer

The Seahorse permits the interrogation of whole cell mitochondrial and glycolytic function, through measurement of oxygen consumption and extracellular H^+ through timed/programmed administration of specific metabolic inhibitors.

The Seahorse utilises a 24-well culture plate into which samples are loaded. A separate disposable sensor cartridge mirrors the 24-well plate structure. It contains oxygen and pH sensors and four injection ports for metabolic inhibitors/substrates on a moveable platform. The sensor cartridge is placed on top of the culture plate and loaded into the respirometer. Samples are warmed to 37.0°C, and a series of measurement cycles performed. During each 2-minute measurement cycle the moveable platform lowers down, partially isolating each well below the measurement sensors and injection ports, thereby limiting, but not completely preventing, O_2 and CO_2 exchange. Baseline data are obtained during 3 measurement cycles, then the loaded metabolic inhibitors are hydraulically injected in a sequential manner and changes to the oxygen plus pH profile recorded over further measurement steps.

The O_2 and pH sensors are embedded within a polymeric substrate that selectively permits the diffusion of O_2 and H^+ ions to the fluorescent material. Monochromatic light emitting diode (LED) sources excite the sensor fluorochromes. The oxygen sensor is excited by a 530nm LED source, and maximally emits at 650nm, while pH sensor is excited by a 470nm source and maximally emits at 530nm. Fluorescence is affected by temperature, therefore

temperature control wells, which contain cell free medium, must be included within the plate. A calibration protocol is run before each assay, using calibration solution provided by the manufacturer.

Oxygen acts to quench the sensor fluorescence. A mathematical model that relates fluorescence at 0mmHg O_2 (maximal) to the recorded fluorescence is used to calculate the partial pressure of oxygen in the sample. Oxygen readings are made every 12 seconds during the measurement cycles, permitting calculation of the oxygen consumption rate (OCR). Several replicates are used for each experimental condition to create a mean OCR. OCR accuracy is typically <30pmole/min, therefore optimisation assays were performed with different concentrations of neutrophils and a concentration of $1.5x10^6$ cells/600µL was chosen. Acceptable inter-well variability was considered to be a coefficient of variation of <20%. Measurement of extracellular pH works on a similar principle, only using a fluorescent probe which is quenched by H^+. The Seahorse medium is unbuffered, therefore seeded plates must be stored within an air incubator.

Cells must remain adherent to the plate base so that they are not washed away with medium changes and mixing procedures. Plates are therefore coated with Cell-Tak (BD Biosciences, Bedford, MA, USA), which is a non-immunogenic polyphenolic compound commonly used to immobilise tissues and cells. It has been successfully used for lymphocytes (Edwards et al., 2015). Cell-Tak is reconstituted into a neutral pH solution that leads to precipitation and adsorption onto the polystyrene plastic well wall.

2.9.2 Experimental protocol

Murine bone marrow neutrophils were isolated by Gareth Ackland. Male C57 black mice (age 8-12 week; weight 20-25g) were selected. Animals were housed with food and water available *ad libitum* in the specific pathogen-free central animal facilities at University College London Medical School (London, UK). All experimental protocols were reviewed and approved by the UCL Institutional Animal Care and Use Committee and carried out in accordance with UK Animals (Scientific Procedures) Act, 1986 and ARRIVE guidelines (Animal Research: Reporting of In Vivo Experiments) (Kilkenny et al., 2013)

Six test mice underwent midline laparotomy under isoflurane anaesthesia (oxygen flowmeter 1L/min; isoflurane 3.5% for induction and1.5% for maintenance) for 30 minutes.

Topical bupivacaine was administered for analgesia, and a core temperature of 36.9°C maintained through a closed loop heating mat/rectal temperature probe system. Six control non-operated animals were caged in the same surgical environment. Twelve hours before the assay run the sensor cartridge was hydrated in Seahorse calibration medium at 37°C in the air incubator. An assay plate was prepared with 5µL Cell-Tak /cm^2, which was left for 45 minutes then aspirated and the wells washed twice with sterile distilled water and left to dry in a sterile hood. Mice were systemically heparinised then culled 1 day after surgery and bone marrow was extracted following cervical dislocation. Bone marrow neutrophils were isolated using Ly-6G microbeads, as described above, counted with trypan blue then re-suspended in Seahorse assay medium, with $1.5x10^6$ cells per 100µL added to each Cell-Tak coated well. All experiments were performed in triplicate. Neutrophils were allowed to adhere to Cell-Tak in the air incubator at 37°C, after 30 minutes a further 500µL of assay medium was carefully added to each well and returned to the air incubator for 1 hour. PMA (final concentration 1µM) was added to the first channel of each sensor plate well, then the sensor plate was inserted in the Seahorse XF and the calibration protocol started. Next, assay medium was changed to remove any residual buffer and metabolites from extracellular medium. The protocol was run so that baseline oxygen consumption was measured for 20 minutes prior to addition of PMA.

For these data to be accurate it is vital that the correct number of cells is placed, and remains, within each well. Therefore, at the start of the experiment seeded wells were examined under the microscope to ensure neutrophils were evenly distributed. At the end of the assay the cell plate was examined under a light microscope to check whether neutrophils had become detached. Automated fluorescence microscopy was used to confirm cell counts at the end of the experiment. A selection of plates were stained with Hoeschst 3342, images acquired with the Zeiss Cell Observer imaging system and counted using Velocity 5.0 software.

2.10 Extracellular lactate and glucose measurement.

The effect of mitochondrial respiratory complex inhibitors and stimulation of pyruvate kinase M2 upon extracellular lactate and glucose was measured the Radiometer ABL 800 Flex blood gas analyser. using a blood gas analyser.

2.10.1 The Radiometer ABL 800 Flex

Provides measurement of pH, blood gas tensions, electrolytes, metabolites and oximetry.

- Automated sample processing reduces likelihood of measurement errors.
- A three-layer membrane over the glucose and lactate sensors provides protection against interference.
- Measurement of capillary tube samples up to 35µL volume.
- 128 wavelengths f(478-672nm) for Co-Oximetry

2.10.2 Experimental protocol

Heparinised whole blood was obtained from five preoperative patients as described above. Samples were prepared in 4x4 plates that were placed on ice. The same drug concentrations were used as in previous whole blood experiments. Experimental conditions were control, DASA 100µM and Myxothiazol 10µM, with each repeated in the presence of PMA 1µM. Appropriate volumes of DMSO, ethanol and PBS were added to necessary conditions to ensure volumes and conditions were equal throughout. Incubations were performed for 30 minutes at 37°C, pCO_2 5.0. Samples were then placed on ice, and processed in the blood gas analyser.

Chapter 3 – Establishing a clinical translational model for sepsis

3.1 Introduction

The study of human sepsis presents fundamental challenges to the design of translational immunological investigations. These begin with the very definition of sepsis, which incorporates a number of non-specific bed side parameters and the clinical suspicion of *probable* infection (Dellinger et al., 2013). The results from microbiological investigations may not be available for several days, are often negative and even when positive do not confirm causation nor permit quantitative extrapolation of the infective dose.

The identification of a suitable control population presents the second key challenge. The outcome of case control studies may be affected by a range of confounding factors, which include age (Henriksen et al., 2015, Montecino-Rodriguez et al., 2013; Valente et al., 2009), gender (McGowan et al., 1975; Offner et al., 1999), comorbid disease (Henriksen et al., 2015; Snyder and Greenberg, 2010, Fildes et al., 2009) and therapeutic interventions (Kalghatgi et al., 2013 Mikawa et al., 1998; Kanoh and Rubin, 2010, (Clarke et al., 1993)

Cohort study designs are methodologically superior to case-control studies because study samples maybe compared to individualised pre-illness controls, rather than a separate control population. However, the technologies used are expensive and time consuming, therefore it is not practical for small laboratories to follow healthy patients and await the development of sepsis.

The precise time at which sepsis begins cannot be known, which makes temporal descriptions of the subsequent dynamic inflammatory processes difficult. While healthy volunteer endotoxin/cytokine models do permit cohort design, they cannot reproduce the severity of critical illness present within the ICU (Andreasen et al., 2008).

The use of major elective surgery as a translational model of human sepsis may overcome many of these methodological challenges. Cohort investigations are feasible because surgery happens at a planned time, permitting the acquisition of individualised pre-insult controls samples at specific post-insult time points. Surgical patients are subject to standardised preoperative assessment protocols (Stringer, 2010), which defines the study group with superior accuracy and facilitates the investigation of specific immunologically relevant preoperative disease.

While microbiological investigations permit the identification of possible infections, they provide little information about the quantity or severity of infection – it remains unclear to

what extent sepsis is a product of infective burden and/or an inappropriate host response. By contrast, the 'dose' of surgery is more homogenous, and maybe controlled further by recruiting patients who have similar procedures performed by the same operative teams. This surgical model of sepsis is biologically relevant because – as explored within chapter 1 - human responses to infection and trauma are highly conserved at molecular (Matzinger, 1994; Nathan, 2006), genomic (Haimovich et al., 2014; Xiao et al., 2011) and clinical (Matzinger, 1994; Seong and Matzinger, 2004) levels.

This chapter will establish whether the theoretical methodological advantages of studying surgical patients are realised within the translational immunological literature. A preliminary PubMed search determined that the most frequently investigated functional assay for the principal circulating immune cells was neutrophil respiratory burst, monocyte endotoxin tolerance and lymphocyte apoptosis. The experimental design of these three topics was then compared across sepsis, trauma and elective surgery patients.

3.1.1 Hypothesis

Translational immunological investigations in elective surgery patients are methodologically superior to those in septic and trauma patients, since they facilitate the use of cohort study design and carefully timed sampling from a precisely defined study population.

3.1.2 Aims

To survey the sepsis, trauma and surgical

human immune literature to establish:

i. Use of cohort or case control study design

ii. Differences between study and control group age, ethnicity, sex, comorbid disease and therapeutic interventions

iii. The timing of patient sampling in relation to the inflammatory insult

iv. Variations in the criteria used to define sepsis.

3.2 Methods

PubMed, PubMed Central and Embase databases were searched for the terms "Neutrophil respiratory burst" OR "Monocyte endotoxin tolerance" OR "Lymphocyte apoptosis" AND "Sepsis" OR "Trauma" OR "Surgery", restricted to adult human studies published between 01/03/1998 and 01/03/2013. The abstract of each paper was manually assessed for suitability. Healthy volunteer in-vitro investigations were excluded. Clinical and laboratory data collected are detailed below

Category	Data collected
Demographics	Age, gender, ethnicity, severity, control population, number of patients, a priori power analysis.
Control population	Source (Healthy volunteer/cohort/other patient population), number.
Purpose of study	Experimental context (Clinical outcome, experimental, pathophysiological, biomarker comparison)
Timing of sampling	Sample trigger (hospital admission, ICU admission, onset sepsis, onset of organ failure), time window for sampling, number and timing of subsequent samples.
Clinical severity	Scoring system used, average score and range.
Microbiology	Microbiology results reported, independent adjudication of sepsis diagnosis, definition of sepsis used.
Exclusion criteria	Malignancy, immunosuppressive illness.
Drug therapy	Sedation, antibiotics, steroids.
Primary conclusion of study	Summary of study findings.

3.2.1 Statistics

Data are presented as mean±SD, or median (interquartile range), as appropriate. 95% confidence intervals were constructed from age data, and used to compare control and study population age.

3.3 Results

57 eligible studies were identified and are summarized in Figure 3.1.

3.3.1 Literature search and study selection

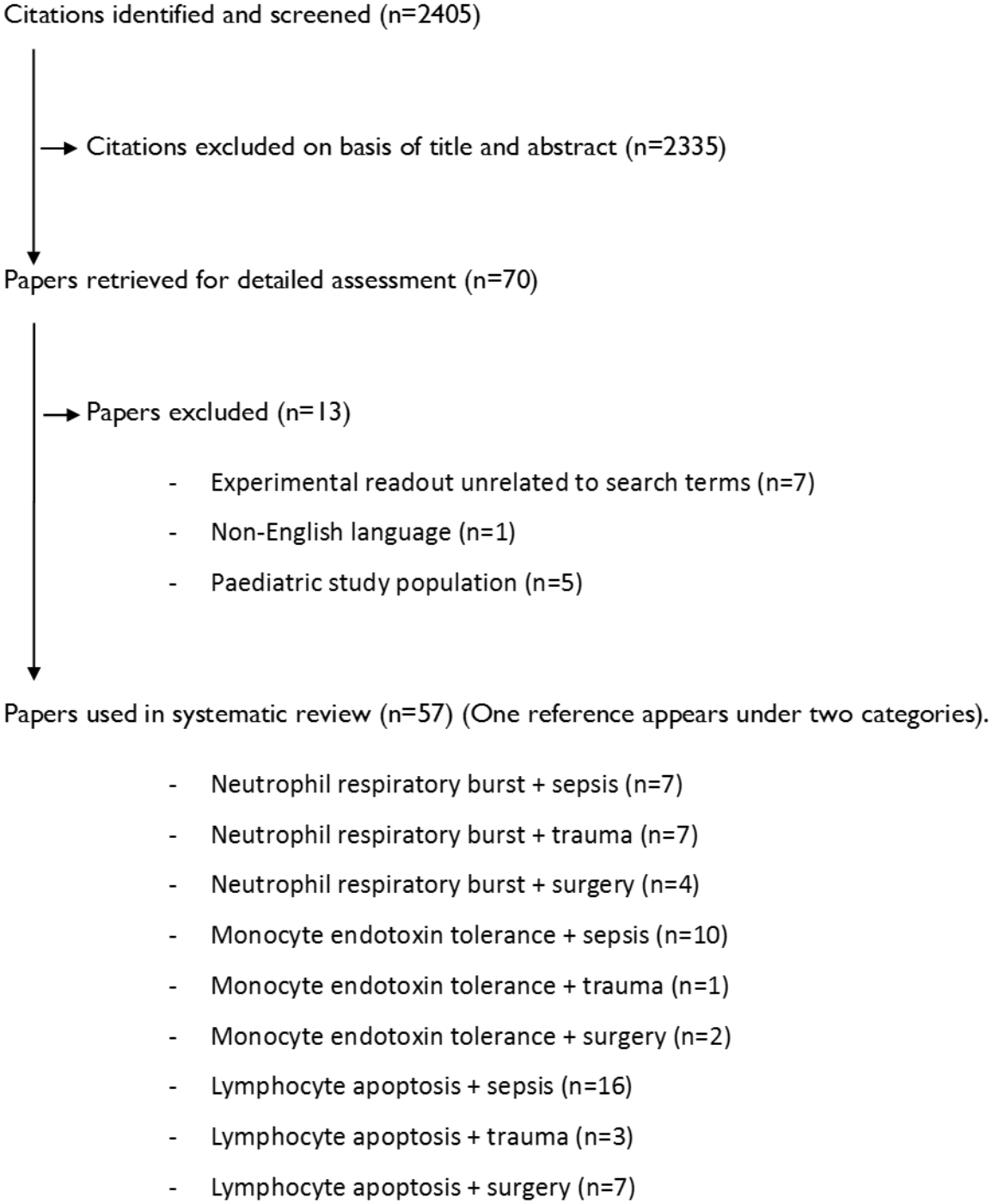

Figure 3.1 Flow diagram of study selection process for literature review.

3.3.2 Data tables

Author (Year)	Study population	Subjects (n)	Control population	Control (n)	Experimental context	Outcome measure correlated with immune readout
(Santos et al., 2012)	Sepsis	49	Healthy volunteer	19	Clinical outcome	Yes
(Paunel-Görgülü et al., 2011)	Trauma	7	Healthy volunteer	6	Experimental	No
(Bruns et al., 2011)	Sepsis (cirrhotics)	45	Healthy volunteer and cohort	9 and 39	Pathophysiological	No
(Shih et al., 2010)	Trauma	32	Healthy volunteer	Not provided	Biomarker comparison	Yes
(Kasten et al., 2010)	Trauma	3	Healthy volunteer	3	Pathophysiological	No
(Valente et al., 2009)	Trauma	24	Healthy volunteer	11	Pathophysiological	No
(Kawasaki et al., 2007)	Elective surgery	20 (10,10)	Cohort	20	Pathophysiological	No
(Fröhlich et al., 2006)	Elective surgery	20	Cohort	20	Experimental	No
(Martins et al., 2003)	Sepsis	16	Healthy volunteer	16	Pathophysiological	Yes
(Barth et al., 2002)	Sepsis	27	Healthy volunteer	11	Biomarker comparison	No
(Mariano et al., 2001)	Sepsis (renal replacement therapy)	7	Haemodialysis patients.	10	Pathophysiological	No

(Quaid et al., 2001)	Trauma	7	Healthy volunteer	Not provided	Pathophysiological	No
(Wiezer et al., 2000)	Elective surgery	22 (6,6,10)	Cohort	22	Pathophysiological / experimental	Yes
(Ahmed et al., 1999)	Sepsis	32	Healthy volunteer	17	Pathophysiological	No
(Shih et al., 1999)	Trauma / Surgery	18	Cohort and healthy volunteer	18	Pathophysiological	No
(Ertel et al., 1999)	Trauma	10 (5,5)	Elective Surgery	10	Pathophysiological	No
(Ogura et al., 1999)	Trauma	24 (7 infected)	Cohort and healthy volunteer	24 and 15	Pathophysiological	Yes
(Pascual et al., 1998)	Sepsis	23	Elective surgery	23	Pathophysiological / experimental	No

Table 3.1 Principal features of neutrophil respiratory burst studies

Subjects: Values within brackets refer to number of sub-populations of study patients (e.g. different anaesthetic technique, presence of infection etc), used within the study

Authors (Year)	Age			Gender (%male)			Subject ethnicity detailed	Severity of subject disease			Subject drug exposure documentation		
	Subjects	Controls	Statistical test result	Subjects	Controls	Statistical test result		Index	Score	No. Groups	Sedatives	Antibiotics	Steroids
(Santos et al., 2012)	60 ±17	55.3 ±18	N	57	53	N	N	APACHE II	17 (4-30)	3	N	N	N
(Paunel-Görgülü et al., 2011)	46 ±4	33± 2	N* (p<0.001)	74	59	N	N	Mortality	9%	1	N	N	N
(Bruns et al., 2011)	58 (40 - 80)	45 (37-82); 58 (?)	0.437	82	73/ 48	0.341	N	-		1	N	N	N
(Shih et al., 2010)	33 ±14	?	N	66	?	N	N	ISS	23	2	N	N	N
(Kasten et al., 2010)	36 ±2	38 ±2	p>0.05	100	100	p>0.05	N	ISS	23	1	N	N	N
(Valente et al., 2009)	75	>65	N	46	?	N	N	ISS	15.00	1	N	N	N

(Kawasaki et al., 2007)	52 ±4 54± 4	N/A	N	70	70	p>0.05	N	ASA	I - II	2	Y	N	N
(Fröhlich et al., 2006)	66 ±10 69 ±6	N/A	N	40	20	N	N	ASA	I	2	Y *t*	Y *t*	Y *t*
(Martins et al., 2003)	50± 21	31 ±6	N* (p=0.0011)	?	?	N	N	Mortality	38%	2	N	N	N
(Barth et al., 2002)	N/S (36 - 82)	24 (22-50)	N	60	36	N	N	Mortality	37%	I	N	N	N
(Mariano et al., 2001)	67 ±4	?	N	?	?	N	N	-		I	N	N	N
(Quaid et al., 2001)	37 (20 - 71)	?	N	?	?	N	N	ISS	24 (17-34)		N	N	N
(Wiezer et al., 2000)	57± 3 62± 2 58± 5	?	N	83, 66, 70		N	N	APACHE III	Graphs (No difference)	3	N	N	N
(Ahmed et al., 1999)	55 ±6	36 ±16	N* (p<0.0001)	46	?	N	N	APACHE II	20 ±1	I	N	N	N

(Shih et al., 1999)	42± 19	N/S	N	55	?	N	N	ISS	26 ±7.2	3	N	N	N
(Ertel et al., 1999)	N/S	?	N	?	?	N	N	AIS	Head 4.5 ±0.2, Chest 4.1 ±0.1	1	N	N	N
(Ogura et al., 1999)	40± 19	35 ±6	N	75	?	N	N	ISS	31 ±10	2	N	N	N
(Pascual et al., 1998)	59 (27 - 81)	45 (27-81)	p>0.05	51	43	N	N	Mortality	21%	1	N	N	Y *t*

Table 3.2 Demographic information of neutrophil respiratory burst studies.

Age – N/S – not summarised (tabulated data for every patient provided), ?-not provided within the manuscript, N/A – not applicable Statistical test result – N=not reported. N=not reported but significant p value identified from the original manuscript data. Severity of subject disease. The average clinical severity score of subjects with an index of listed in brackets. The number of severity groups which subjects were divided into is listed. ISS/AIS= Injury Severity Score/Abbreviated Injury Severity Score (Baker et al. 1974); ASA = American Society of Anesthesiologists Physical Status Classification System (ASA 2014); APACHE II= Acute Physiology and Chronic Health Evaluation II (Knaus et al. 1985), APACHE III = Acute Physiology and Chronic Health Evaluation III (Knaus et al. 1991). Subject drug use detailed – whether patient exposure to known immunomodulating drugs was documented. A 't' signifies that the timing of the drug administration in relation to blood sampling was clear from the study methodology.*

Authors (Year)	Study population	Sample timing		Definition of sepsis	Microbiology results provided.	Independent adjudication of sepsis diagnosis	Exclusion criteria immunosuppressive disease	Exclusion criteria malignancy
		Time of first sample	No. samples (timespan)					
(Santos et al., 2012)	Sepsis	72hrs (Dx sepsis); 48hrs (organ failure); onset of septic shock	2 (7days)	1 A,B,C	N	N	Y	Y
(Paunel-Görgülü et al., 2011)	Trauma	24hrs (Hosp Adm)	1	2 A,B,C	N	N	Y	N
(Bruns et al., 2011)	Sepsis (cirrhotics)	24hrs (Hosp Adm)	1	5	Y	N	Y	N
(Shih et al., 2010)	Trauma	24hrs (Hosp Adm)	2 (3 days)	N	N	N	Y	Y
(Kasten et al., 2010)	Trauma	48-72hrs (Post-trauma)	1	N	N	N	Y	N
(Valente et al., 2009)	Trauma	48hrs (Hosp adm)	3 (5 days)	N	N	N	Y	N

(Kawasaki et al., 2007)	Elective surgery	Pre-insult	5 (4 days)	N	N	N	Y	N
(Fröhlich et al., 2006)	Elective surgery	Pre-insult	2 (end of anaesth)	N	n/a	n/a	Y	Y
(Martins et al., 2003)	Sepsis	48hrs (ICU adm)	1	1 B,C	Y	N	Y	Y
(Barth et al., 2002)	Sepsis	?	6 (5 days)	1 C (>4d)	Y	N	N	N
(Mariano et al., 2001)	Sepsis (renal replacement therapy)	?	4 (1 day)	1 B,D	N	N	N	N
(Quaid et al., 2001)	Trauma	24hrs (Hosp adm)	1	N	N	N	N	N
(Wiezer et al., 2000)	Elective surgery	Pre-insult	5 (7 days)	"clinical criteria"	N	N	Y	N
(Ahmed et al., 1999)	Sepsis	72hrs (Proof of infection)	1	1 A,B	Y	Y	Y	Y
(Shih et al., 1999)	Trauma / Surgery	24hrs (Hosp adm)	3+ (7 days)	1 A,B,C	N	N	Y	Y
(Ertel et al., 1999)	Trauma	24hrs (Hosp adm)	2 (3 days)	N	N	N	Y	N
(Ogura et al., 1999)	Trauma	24hrs (Post trauma)	4+1 (21 days)	2 A B C	Y	N	N	N
(Pascual et al., 1998)	Sepsis	24hrs (ICU adm)	1	1 A C	Y	N	N	N

Table 3.3 Experimental conduct and exclusion criteria of neutrophil respiratory burst studies.

Sample Timing. Were control samples taken at the same time point after the inflammatory stimulus as subject samples? When was the first sample taken from the subject? How many samples were taken for each subject in total and over what time span?

Sepsis criteria. The criteria used to enrol subjects into the study. Where subgroups of these criteria were used (e.g. septic shock) these are detailed. 0=not stated 1= ACCP/SCCM 1992 Consensus Conference(Bone et al., 1992)*, 2=ACCP/SCCM Consensus Conference 2001*(Levy et al., 2003)*, 3=SSC Consensus Conference 2008*(Dellinger et al., 2008)*, 4=CDC NNIC*("CDC definitions for nosocomial infections, 1988.," 1989)*, 5=Microbiology and clinical assessment, 6=Postmortem identification of infection, N=infection not considered, ?=criteria not described. Sepsis severity groups enrolled: A=sepsis, B=severe sepsis, C=septic shock, D=acute renal failure E=SIRS*

Microbiology documentation - Were causative organisms isolated and identified? Were additional steps taken to define whether subject had sepsis beyond the initial clinical diagnosis i.e. retrospective review of case considering subsequent information?

Author/Year	Study population	Subjects (n)	Control population	Controls (n)	Experimental context	Outcome measure correlated with immune readout
(Liu et al., 2011)	Sepsis	2	Healthy volunteer	2	Experimental	No
(Buttenschoen et al., 2009)	Elective surgery	20	Cohort	20	Pathophysiological	No
(Pachot et al., 2008)	Sepsis	47	Healthy volunteer	21	Pathophysiological	Yes
(West et al., 2007)	Sepsis	7	Healthy volunteer, elective surgery and SIRS	16, 5 and 4	Pathophysiological	No
(Härter et al., 2004)	Sepsis	21	Healthy volunteer	12	Pathophysiological	No
(Flohé et al., 2004)	Surgery in trauma patients	16	Healthy volunteer	12	Pathophysiological	No
(Arnalich et al., 2003)	Sepsis	3(5)	Healthy volunteer	3	Pathophysiological	No
(Heagy et al., 2003)	ICU patients (sepsis)	62	Healthy volunteer	15	Clinical outcome	Yes
(Calvano et al., 2003)	Sepsis	18 (10)	Healthy volunteer	15 (6)	Pathophysiological	No
(Sfeir et al., 2001)	Sepsis	10	Healthy volunteer	10	Pathophysiological	No
(Kawasaki et al., 2001)	Elective surgery	20	Cohort	20	Pathophysiological	No
(Heagy et al., 2000)	Sepsis	58	Healthy volunteer	14	Clinical outcome	Yes
(Bergmann et al., 1999)	Sepsis	30 (2)	Healthy volunteer	12	Pathophysiological	No

Table 3.4 Principal features of monocyte tolerance studies

Subjects: Values within brackets refer to number of sub-populations of study patients (e.g. different anaesthetic technique, presence of infection etc), used within the study

Authors (Year)	Age			Gender (%male)			Subject ethnicity	Severity of subject disease			Subject drug exposure documentation		
	Subjects	Controls	Statistical test result	Subjects	Controls	Statistical test result		Index	Score	No. Groups	Sedatives	Antibiotics	Steroids
(Liu et al., 2011)	?	?	N	?	?	N	N	?	?	1	N	N	N
(Buttenschoen et al., 2009)	56 (33-88)	N/A	N	70	N/A	N	N	?	?	n/a	N	N	N
(Pachot et al., 2008)	68 (54-76)	51 (42-65)	N	62	52	N	N	SAPS II	51 (±5)	2	N	N	N
(West et al., 2007)	N/S	N/S	N	42	100; 20; 56	N	N	?	?	2	N	N	N
(Härter et al., 2004)	48 ±20	"comparable"	N	71	12	N	N	APACHE II	13±6	1	N	N	N
(Flohé et al., 2004)	47±18	37±14	N	68	50	N	N	ISS	39±9	1	N	N	N
(Arnalich et al., 2003)	51±12	49±12	N	?	?	N	N	?	?	1	N	N	N

(Heagy et al., 2003)	49±3; 44±8	?	N	?	?	N	N	Mortality	20%, 9.6%	2	N	N	N
(Calvano et al., 2003)	60; 61	58	N	66; 66	66	N	N	?	?	2	N	N	Y *t*
(Sfeir et al., 2001)	63±3	50±7	N*(p<0.0001)	80	50	N	N	APACHE II	27±5	I	N	N	N
(Kawasaki et al., 2001)	?	N/A	N	?	N/A	N	N	ASA	I-II	I	N	N	N
(Heagy et al., 2000)	49±21	?	N	66	?	N	N	?	?	4	N	N	N
(Bergmann et al., 1999)	60; 51	32	N	?	?	N	N	MODS	15±1, 7±1	2	N	N	N

Table 3.5 Demographic information of monocyte tolerance studies

Age – N/S – not summarised (tabulated data for every patient provided), ?-not provided within the manuscript, N/A – not applicable Statistical test result – N=not reported. N=not reported but significant p value identified from the original manuscript data. Severity of subject disease. The average clinical severity score of subjects with an index of listed in brackets. The number of severity groups which subjects were divided into is listed. ISS/AIS= Injury Severity Score/Abbreviated Injury Severity Score(Baker et al., 1974); ASA = American Society of Anesthesiologists Physical Status Classification System (ASA, 2014); APACHE II= Acute Physiology and Chronic Health Evaluation II (Knaus et al., 1985), APACHE III = Acute Physiology and Chronic Health Evaluation III (Knaus et al., 1991)(REF).*

Subject drug use detailed – whether patient exposure to known immunomodulating drugs was documented. A 't' signifies that the timing of the drug administration in relation to blood sampling was clear from the study methodology.

Authors (Year)	Study population	Sample timing		Definition of sepsis	Microbiology results provided.	Independent adjudication of sepsis diagnosis	Exclusion criteria immunosuppressive disease	Exclusion criteria malignancy
		Time of first sample	No. Samples (timespan)					
(Liu et al., 2011)	Sepsis	?	1	? B C	N	N	N	N
(Buttenschoen et al., 2009)	Elective surgery	Pre-insult	4 (2days)	N	N	N	Y	N
(Pachot et al., 2008)	Sepsis	72hrs (onset sep shock)	2	1 C	Y	Y	N	N
(West et al., 2007)	Sepsis	24hrs (ICU adm)	1	1 A, E	Y	N	N	N
(Härter et al., 2004)	Sepsis	?	1	1 A B C	Y	Y	N	N
(Flohé et al., 2004)	Surgery in trauma patients	48hrs (ICU adm)	Mon, Thu.	1 A B C	Y	N	Y	Y
(Arnalich et al., 2003)	Sepsis	48hrs (onset sepsis)	1	1 A	Y	Y	Y	Y

(Heagy et al., 2003)	ICU patients (sepsis)	72hrs (ICU adm)	1	5	N	Y	N	N
(Calvano et al., 2003)	Sepsis	?	1	1 E A	Y	N	N	N
(Sfeir et al., 2001)	Sepsis	24 (Sep Shock)	1	1 C	Y	Y	Y	N
(Kawasaki et al., 2001)	Elective surgery	Pre-ins	7 (7days)	N	N	N	Y	N
(Heagy et al., 2000)	Sepsis	72hrs (ICU adm)	1	5	Y	Y	N	N
(Bergmann et al., 1999)	Sepsis	?		1 B C	N	N	N	N

Table 3.6 Experimental conduct and exclusion criteria of monocyte tolerance studies

Sample Timing. Were control samples taken at the same time point after the inflammatory stimulus as subject samples? When was the first sample taken from the subject? How many samples were taken for each subject in total and over what time span? Sepsis criteria. The criteria used to enrol subjects into the study. Where subgroups of these criteria were used (e.g. septic shock) these are detailed. 0=not stated 1= ACCP/SCCM 1992 Consensus Conference(Bone et al., 1992)*, 2=ACCP/SCCM Consensus Conference 2001*(Levy et al., 2003)*, 3=SSC Consensus Conference 2008*(Dellinger et al., 2008)*, 4=CDC NNIC*("CDC definitions for nosocomial infections, 1988.," 1989)*, 5=Microbiology and clinical assessment, 6=Postmortem identification of infection, N=infection not considered, ?=criteria not described. Sepsis severity groups enrolled: A=sepsis, B=severe sepsis, C=septic shock, D=acute renal failure E=SIRS. Microbiology documentation - Were causative organisms isolated and identified? Were additional steps taken to define whether subject had sepsis beyond the initial clinical diagnosis i.e. retrospective review of case considering subsequent information?*

Author (Year)	Study population	Subjects (n)	Control population	Controls (n)	Experimental context	Outcome measure correlated with immune readout
(Roger et al., 2012)	Sepsis	48	Healthy volunteer	15	Pathophysiological	No
(Bandyopadhyay et al., 2012)	Trauma	113	Healthy volunteer	?	Pathophysiological	No
(White et al., 2011)	Sepsis	60	Gram negative infection and healthy volunteer	15 and 20	Pathophysiological	Yes
(White et al., 2011)	Elective surgery (infective complications)	19	Cohort	41	"	"
(Zhang et al., 2011)	Sepsis	19	Healthy volunteer	22	Pathophysiological	No
(Guignant et al., 2011)	Sepsis	64	Healthy volunteer	49	Pathophysiological	No
(Vaki et al., 2011)	Sepsis	48 (68)	Healthy volunteer	20	Pathophysiological	No
(Słotwiński et al., 2011)	Elective surgery	50 (26, 24)	Cohort	50	Experimental / clinical outcome	No
(Gogos et al., 2010)	Sepsis	PN 183, CAP 97, IA 100, PB 61, HAP 64	N/A		Pathophysiological	Yes
(Hoogerwerf et al., 2010)	Sepsis	16	Healthy volunteer	24	Pathophysiological	No
(Yousef et al., 2010)	Sepsis	32	SIRS and w/o SIRS	35 / 33	Patient outcome	Yes

(Turrel-Davin et al., 2010)	Sepsis	13	Healthy volunteer	15	Biomarker comparison	No
(Pelekanou et al., 2009)	Sepsis	VAP 36	Other infections	32	Pathophysiology	No
(Resident et al., 2009)	Elective surgery	40 (21, 19)	Cohort	40	Pathophysiological	No
(Delogu et al., 2008)	Sepsis	16	? 'individuals'		Pathophysiological	No
(Weber et al., 2008)	Sepsis	16	Non-infected ICU and healthy volunteer	10 and 11	Pathophysiological	No
(Roth et al., 2003)	Sepsis	15	Healthy volunteer	20	Pathophysiological	No
(Le Tulzo et al., 2002)	Sepsis	47 (25, 23)	SIRS and healthy volunteer	7 and 25	Pathophysiological / clinical outcome	Yes
(Hotchkiss et al., 2001)	Sepsis	27 (FC 5) (3 intraop, 24 autopsy)	Critically ill non-septic and trauma	16 and 25 (FC 6) - 3 prospective, 13 retrospective	Pathophysiological	No
(Delogu et al., 2001)	Elective surgery	18	Cohort	18	Pathophysiological	No
(Pellegrini et al., 2000)	Trauma	17 (+13 burns)	Healthy volunteer	17	Clinical outcome / pathophysiological	(Correlate to MODS)
(Delogu et al., 2000)	Surgical	15	Healthy volunteer	10	Pathophysiological / patient outcome	Yes
(Hotchkiss et al., 2000)	Trauma	10	Elective surgery	6 (all prospective)	Pathophysiological	No
(Hotchkiss et al., 1999)	Sepsis	20	Non-septic prospective / non-septic retrospective /	1 / 9 / 6 / 2 / 8	Pathophysiological	No

			prospective trauma splenectomy / prospective colectomy / retrospective colectomy.			
(Sasajima et al., 1999)	Elective surgery	16 (11, 5)	Cohort	16	Pathophysiological	No
(Sugimoto et al., 1998)	Elective surgery	10 (5, 5)	Cohort	10	Pathophysiological	No

Table 3.7 Principal features of lymphocyte apoptosis studies

Subjects: Values within brackets refer to number of sub-populations of study patients (e.g. different anaesthetic technique, presence of infection etc), used within the study

Authors (Year)	Age			Gender (%male)			Ethnicity	Severity of subject disease			Subject drug exposure documentation		
	Subjects	Controls	Statistical test result	Subjects	Controls	Statistical test result		Index	Score	No. Groups	Sedatives	Antibiotics	Steroids
(Roger et al., 2012)	63 (37-82)	55 (37-5)	0.04	50	43	0.76	N	SAPS II	55 (12-92)	2	N	Y *t*	Y *t*
(Bandyopadhyay et al., 2012)	?	"matched"	N	?	"matched"	N	N	APACHE	>21	1	N	N	N
(White et al., 2011)	54 (72-80)	Bacteraemia: 73 (70-82)	>0.05	52	Bacteraemia 40	>0.05	Y	APACHE	25 (21-28)	2	N	N	N
(White et al., 2011)	64± 2	65±1	0.74	68	70	0.86	N			2	N	N	N
(Zhang et al., 2011)	58± 4	59±4	N	52	50	N	N	APACHE II	26±3	1	N	Y *t*	Y *t*
	63 (54-73)	?	N	68	N	N		SAPS II	53(39-64)	1	N	N	Y *t*

(Guignant et al., 2011)													
(Vaki et al., 2011)	71±2	?	N	54	?	N	N	APACHE II	20±9	I (3)	N	N	N
(Słotwiński et al., 2011)	62±9 63±9	-	N	5, 50	-	N	N	TNM	?	I	N	Y *t*	N
(Gogos et al., 2010)	67±17, 68±20, 54±25, 64±16		P<0.0001	52, 62, 57, 67, 64		P=0.011	N	APACHE II	12±7, 16±9, 13±8, 18±8, 20±5	3	N	N	N
(Hoogerwerf et al., 2010)	57±5,	66±5	N* (p<0.0001)	63	50	N	N	APACHE II	19±2	I	N	N	N
(Yousef et al., 2010)	44±9	45±9, 44±10	N	59	60, 57	N	N	SOFA	12 (7-14)	3 (5)	N	N	N
(Turrel-Davin et al., 2010)	60±4	'Age matched'	N	63	'Sex matched'	N	N	SAPS II	51±3	I	N	N	Y
(Pelekanou et al., 2009)	69±16	64±20	0.099	64	43	0.300	N	APACHE II	18±4, 15±5	I	N	N	Y
(Resident et al., 2009)	66±7,		0.8	85, 47		0.54	N	ASA	I-II	I	Y *t*	Y *t*	Y *t*

	67± 10												
(Delogu et al., 2008)	?	?	N	?	?	N	N	?	?	I	N	N	N
(Weber et al., 2008)	56± 4	61±5,?	>0.05	68, 80	?	N	N	SAPS II	26± 2	I	N	N	Y
(Roth et al., 2003)	56± 6	52±14	N	66	"matched"	N	N	APACHE	N/S	I	N	N	N
(Le Tulzo et al., 2002)	55± 4, 64± 4	72 ±4, 55± 4	N* (p<0.00 01)	?	?	N	N	SAPS II	33±3, 58±4	2	N	N	N
(Hotchkiss et al., 2001)	N/S	N/S	N	59	56, ?	N	N	-		I	N	N	Y
(Delogu et al., 2001)	47± 17	"matched"	N	?	"matched"	N	N	ASA	I-II	I	Y	N	Y *t*
(Pellegrini et al., 2000)	44 (20-83)	(18-60)	N	?	?	N	N	ISS	25 (9-59)	I	N	N	N
(Delogu et al., 2000)	?	"matched"	N	?	"matched"	N	N	ASA	I-II	I	N	N	Y *t*
(Hotchkiss et al., 2000)	18-46	?	N	90	?	N	N	ISS	N/S (9-50)	I	N	N	N
(Hotchkiss et al., 1999)	N/S	N/S	N	65	?	N	N	-		I	N	N	Y

(Sasajima et al., 1999)	62 (55-74), 49(37-58)		N		?	N	N	?	?	I	N	N	N
(Sugimoto et al., 1998)	N/S		N	50		N	N	?	?	I	N	N	Y *t*

Table 3.8 Demographic information of lymphocyte apoptosis studies.

Age – N/S – not summarised (tabulated data for every patient provided), ?-not provided within the manuscript, N/A – not applicable

Statistical test result – N=not reported. N=not reported but significant p value identified from the original manuscript data.*

Severity of subject disease. The average clinical severity score of subjects with an index of listed in brackets. The number of severity groups which subjects were divided into is listed. ISS/AIS= Injury Severity Score/Abbreviated Injury Severity Score(Baker et al., 1974)*; ASA = American Society of Anesthesiologists Physical Status Classification System*(ASA, 2014)*; APACHE II= Acute Physiology and Chronic Health Evaluation II* (Knaus et al., 1985)*, APACHE III = Acute Physiology and Chronic Health Evaluation III* (Knaus et al., 1991)*(REF).*

Subject drug use detailed – whether patient exposure to known immunomodulating drugs was documented. A 't' signifies that the timing of the drug administration in relation to blood sampling was clear from the study methodology.

"matched" – paper provided no details but stated the control population was matched to the study population.

Authors (Year)	Study population	Sample timing		Definition of sepsis	Microbiology results provided	Independent adjudication of sepsis diagnosis	Exclusion criteria immunosuppressive disease	Exclusion criteria malignancy
		Time of first sample	No. samples (timespan)					
(Roger et al., 2012)	Sepsis	Before 1st abs	1	3 B C	Y	Y	Y	Y
(Bandyopadhyay et al., 2012)	Trauma	?	Every 4 days (28 days)	N	N	N	Y	N
(White et al., 2011)	Sepsis	24hrs (ICU adm/positive BC)	2 (7 days)	1 B C	N	Y	Y	N
(White et al., 2011)	Elective surgery (infective complications)	Pre-insult	3 (5 days)	4	N	Y	Y	N
(Zhang et al., 2011)	Sepsis	24hrs (sep shock)	1	1 C	N	N	Y	N
(Guignant et al., 2011)	Sepsis	48hrs (sep shock)	3 (10 days)	1 C	Y	Y	N	Y

(Vaki et al., 2011)	Sepsis	12hrs (organ failure)		2 B C	Y	Y	Y	N
(Słotwiński et al., 2011)	Elective surgery	Pre-insult	4 (7 days)	N	N	N	Y	N
(Gogos et al., 2010)	Sepsis	24hrs (signs of sepsis)	1	2 B C	Y	Y	Y	N
(Hoogerwerf et al., 2010)	Sepsis	24hrs (dx sepsis)	1	2 A	Y	Y	Y	N
(Yousef et al., 2010)	Sepsis	?	1	1 A B C	N	N	Y	N
(Turrel-Davin et al., 2010)	Sepsis	48hrs (sep shock)	2 (5 days)	1 C	Y	Y	N	N
(Pelekanou et al., 2009)	Sepsis	24hrs (signs of sepsis)	1	1 2 A B C	Y	Y	Y	N
(Resident et al., 2009)	Elective surgery	Pre-insult	2 (1 day)	N	-		Y	Y
(Delogu et al., 2008)	Sepsis	24hrs (septic shock)	1	? C	Y	N	N	N
(Weber et al., 2008)	Sepsis	4hrs (severe sepsis)	1	1 B	N	N	Y	Y
(Roth et al., 2003)	Sepsis	?	1	1 A B C	N	N	N	N
(Le Tulzo et al., 2002)	Sepsis	+ve microbio-logy ±3days	2 (6 days)	1 B C E	Y	N	N	N
(Hotchkiss et al., 2001)	Sepsis	6hrs (death)	1	6	Y	N	Y	N
(Delogu et al., 2001)	Elective surgery	Pre-insult	3 (4 days)	N	N	N	Y	Y

(Pellegrini et al., 2000)	Trauma	?	2/wk (until death/ discharge)	N	N	N	N	N
(Delogu et al., 2000)	Surgical	Pre-insult	3 (4 days)	N	N	N	Y	Y
(Hotchkiss et al., 2000)	Trauma	10hrs (injury to surgery)	1	N	N	N	N	N
(Hotchkiss et al., 1999)	Sepsis	6hrs (death)	1	6	Y	Y	N	N
(Sasajima et al., 1999)	Elective surgery	Pre-insult	5 (7 days)	N	N	N	N	N
(Sugimoto et al., 1998)	Elective surgery	Pre-insult	4 (4 days)	N	N	N	N	N

Table 3.9 Experimental conduct and exclusion criteria of lymphocyte apoptosis studies.

Sample Timing. Were control samples taken at the same time point after the inflammatory stimulus as subject samples? When was the first sample taken from the subject? How many samples were taken for each subject in total and over what time span?

Sepsis criteria. The criteria used to enrol subjects into the study. Where subgroups of these criteria were used (e.g. septic shock) these are detailed. 0=not stated 1= ACCP/SCCM 1992 Consensus Conference (Bone et al. 1992), 2=ACCP/SCCM Consensus Conference 2001(Levy et al. 2003), 3=SSC Consensus Conference 2008(Dellinger et al. 2008), 4=CDC NNIC(Anon 1989), 5=Microbiology and clinical assessment, 6=Postmortem identification of infection, N=infection not considered, ?=criteria not described. Sepsis severity groups enrolled: A=sepsis, B=severe sepsis, C=septic shock, D=acute renal failure E=SIRS

Microbiology documentation - Were causative organisms isolated and identified? Were additional steps taken to define whether subject had sepsis beyond the initial clinical diagnosis i.e. retrospective review of case considering subsequent information?

Investigator	Source	Control group	Duration follow up (days)	Duration burst increased	Duration burst decreased
(Hughes et al., 2010)	Surgical	Preop	5 days	1 day	0
(Kawasaki et al., 2007)	Surgical	Preop	4 days	0	0
(Fröhlich et al., 2006)	Surgical	Preop	2hrs	0	2 hrs
(Wiezer et al., 2000)	Surgical	Preop	7 days	2 hrs	0
(Paunel-Görgülü et al., 2011)	Trauma	HV	1 day	1 day	-
(Shih et al., 2010)	Trauma	HV	3 days	3 days	-
(Kasten et al., 2010)	Trauma	HV	4 days	3 days	?
(Valente et al., 2009)	Trauma	HV	5 days	3 days.	0
(Quaid et al., 2001)	Trauma	HV	1 day	1 day	-
(Oehler et al. 2000)	Trauma	HV	5 days	5 days	-
(Ertel et al., 1999)	Trauma	Surgery	3 days	3 days	-
(Ogura et al., 1999)	Trauma	HV	21 days	21 days	-
(Shih et al., 1999)	Trauma	HV	7 days	3 days	0
(Botha et al., 1995)	Trauma	HV	3 days	1 day	0
(Tanaka et al., 1991)	Trauma	HV	7 days	3 days	0
(Santos et al., 2012)	Sepsis	HV	7 days	7 days	-
(Bruns et al., 2011)	Sepsis	HV/patients	Single measurement	Reduced	
(Martins et al., 2003)	Sepsis	HV	Single measurement	Increased	
(Barth et al., 2002)	Sepsis	HV	Daily assessment	Increased throughout	
(Mariano et al., 2001)	Sepsis	Patients	Single measurement	Increased	
(Ahmed et al., 1999)	Sepsis	HV	Single measurement	-	-

(Pascual et al., 1998)	Sepsis	HV	Single measurement	Increased
(Kaufmann et al., 2006)	Sepsis	HV	Single measurement	Increased fMLP
(Wenisch and Graninger, 1995)	Sepsis	HV	Single measurement	Reduced
(Bass et al., 1986)	Sepsis	HV	Single measurement	Increased

Table 3.10 Respiratory burst assay results.

3.3.3 Study power and source of experimental control subjects

Sample size calculations utilising existing laboratory or clinical data were not performed by any study. Cohort methodology was only employed by 25% (14/57) studies, of which 86% (12/14) were performed in elective surgery patients, meaning only one elective surgery study did not use this method. Case-control study design was used by 74% (42/57) of studies, of which 83% (35/42) recruited healthy volunteers and the rest utilised variously and loosely described clinical samples (Figure 3.2, Tables 3.1, 3.4, 3.7). The remaining 25% (14/57) studies used cohort methodology

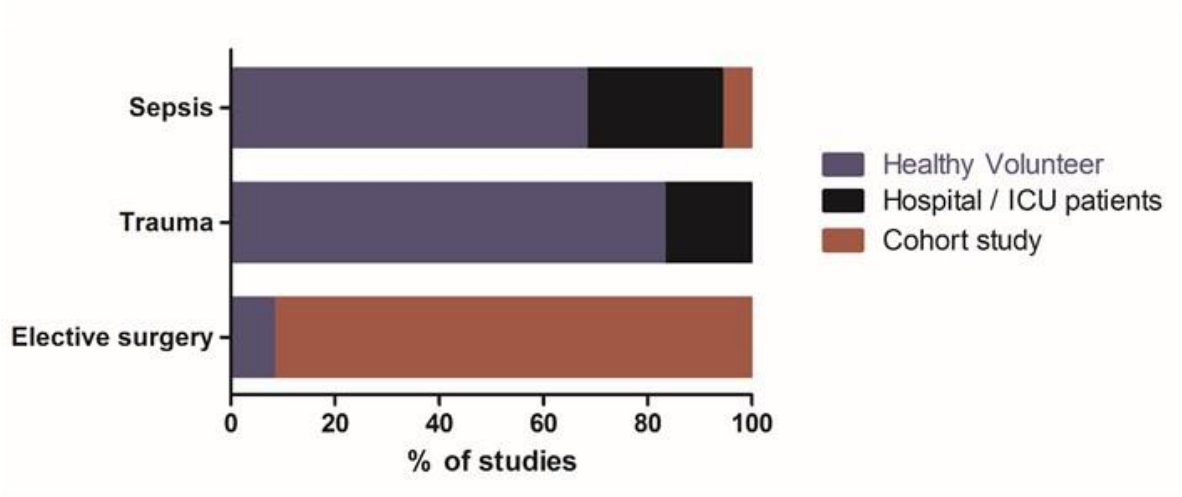

Figure 3.2 Identity of control groups used by for different study populations

3.3.4 Age, gender and ethnicity

Only 48% (20/42) of case control studies included age data for both study and control populations. Of the 35 healthy volunteer studies, 27 reported age data for both study and control populations. Within these 27 studies the average age of study populations was higher than control populations on 17 occasions, but this difference was only significantly different on one occasion (Roger et al., 2012), due to the wide range of ages recruited. Gender distribution was reported by 62% (26/42) of studies and ethnicity by only one. Collectively these data demonstrate frequent underreporting, and within reported data widespread heterogeneity between and within case control study groups, of immunologically relevant parameters.

3.3.5 Comorbid disease

60% (34/57) of studies excluded patients with confirmed immune suppression, and 14% (8/57) excluded those with malignancy (Fig 3.3)

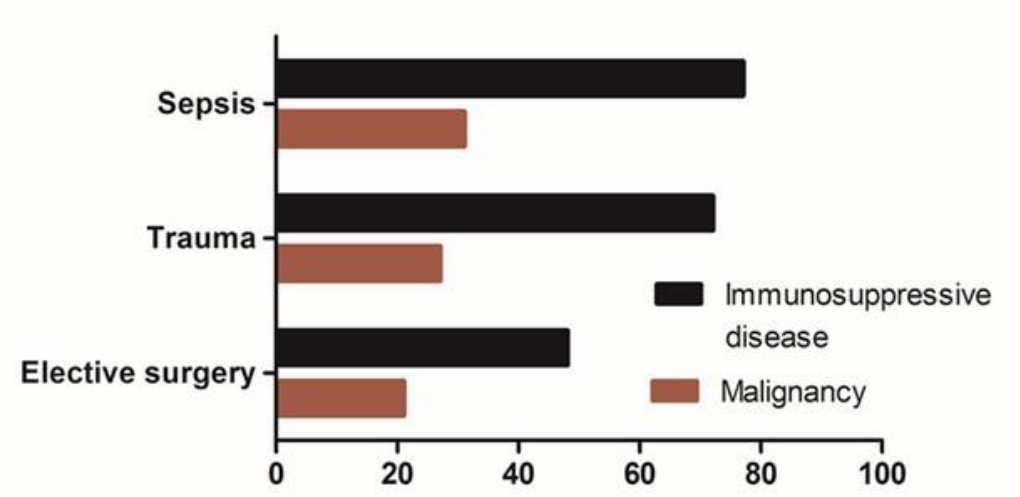

Figure 3.3 Proportion of studies which excluded, or documented the presence of major immunological comorbidities.

3.3.6 Definitions of sepsis

Most studies (79%; 26/33) used established consensus conference criteria to define sepsis syndrome (ACCP/SCCM or Surviving Sepsis Campaign) (Bone et al., 1992; Dellinger et al., 2008; Levy et al., 2003). 58% (15/26) of these studies enrolled patients with 'sepsis', 77% (20/26) those with 'severe sepsis' and 92% (24/26) those with septic shock. These subcategories were combined for analysis by 42% (11/26). One study only included patients with septic shock of 4 days duration (Barth et al., 2002). Of the studies within trauma and surgery patients, only 25% (6/24) reported whether infection developed during the study course, with 5/6 using consensus conference criteria.

3.3.7 Microbiology

Although 44% (25/57) studies provided microbiological data, only 30% (17/57) of studies described independent post hoc evaluation of this data and the sepsis diagnosis. Two studies by Hotchkiss provided autopsy evidence of infection (Hotchkiss et al., 2001, 1999)

3.3.8 Organ dysfunction and illness severity

Indices of organ dysfunction, such as SAPS-II or APACHE-II were reported by 33% (19/57) of studies, which in turn reported a wide range of illness severity. Only 7% (4/57) reported whether patients died and none of these described the severity of illness in survivors.

3.3.9 Sample timing

While the majority (72%; 24/33) of sepsis studies reported the timing of the index blood sample, sampling triggers varied widely (Figure 3.4). These included the onset of septic shock (7/24), diagnosis of sepsis (5/24), ICU admission (5/24), onset of organ failure (3/24) evidence of infection (2/24), death with autopsy sampling (2/24), onset of sepsis (1/24) and hospital admission (1/24) (Fig 3.4). Several studies combined the above triggers to create composite inclusion criteria.

The index patient sample was acquired within 24 hours of hospital admission within 58% (14/24) of sepsis studies. One study restricted inclusion to patients identified within 24hrs of the onset of sepsis symptoms, which included accident and emergency patients, plus followed them up for 28 days (Gogos et al., 2010). Overall, serial sampling was frequently undertaken, but over variable time intervals which were not defined a priori. Similar patterns of sample timing were described for trauma patients. Conversely all 12 elective surgery studies collected preoperative control samples and further samples on predetermined postoperative days. Two studies investigated whether the time delay between patient sampling and laboratory processing affected the results (Gogos et al., 2010; Hotchkiss et al., 1999).

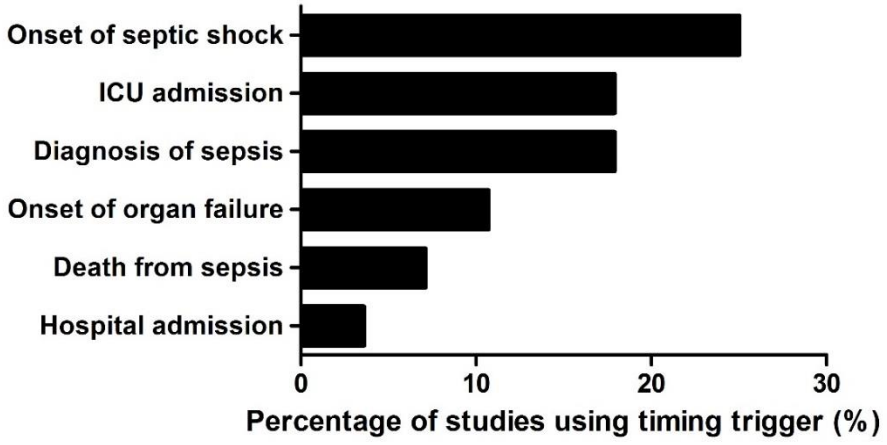

Figure 3.4 Septic patient sample timing

3.3.10 Confounding impact of therapeutic interventions

The concurrent administration of drugs with widely recognised immune modulating properties was reported by 25% (14/57) studies, and of these sedative agents were reported by 7% (4/57), antibiotics by 11% (6/57) and steroids by 26% (15/57) (Fig 3.5). In one study of trauma patients the range for administration of blood products was 0-49 units, with data reported from blood sampled after transfusion (Quaid et al., 2001).

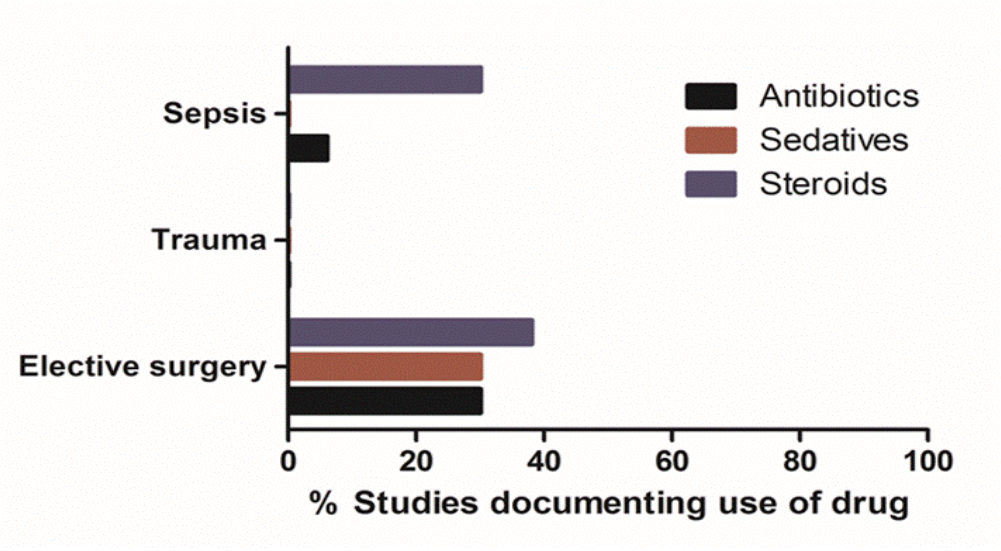

Figure 3.5 Documentation of administered drugs which affect immune function.

3.3.11 Relationship between study population, experimental context outcomes and experimental assay results.

Neutrophil respiratory burst studies conducted in septic patients repeatedly revealed an increased burst response within the septic patient groups (Table 3.10). However, these studies invariably employed a healthy volunteer control group, therefore it is uncertain whether this is attributable to the presence of sepsis, other differences between the groups or perhaps even predisposes to sepsis. None of the three surgical studies investigating the neutrophil burst identified any perioperative change. Monocyte and lymphocyte studies described similar patterns. An index of clinical outcome was related to immune cell function by 23% (13/57) of clinical studies.

Simultaneous sampling of blood and tissue neutrophils from live patients was employed by only one study (Ahmed et al., 1999), which reported that tissue neutrophils had diminished functionality when compared to blood derived neutrophils.

3.3.12 Response of respiratory burst to surgery, trauma and sepsis

Table 3.10 summarises respiratory burst assay results for septic, trauma and surgical patients. All trauma studies identified an increase in respiratory burst when compared to healthy volunteers, or in one case surgical patients, which persisted for between 1 and 21 days. Conversely investigations of surgical and septic patients yielded more variable results, with both increased and decreased respiratory burst commonly described. The experimental assay methodology for these studies is summarised in Appendix A, and demonstrates a wide variation in experimental protocols.

3.4 Discussion

3.4.1 Key findings

1. Translational investigations of surgical patients use individualised pre-insult control samples for each patient, unlike equivalent studies within septic and trauma patients. Every study of surgical patients used cohort study design, with each patient serving as their own control sample. In addition, surgical studies described the study population with more detail and used accurately timed sampling in relation to the primary insult.

2. Some patients enrolled into sepsis studies may not have an infection. Many studies of septic patients failed to report microbiological data and few utilised post-admission clinical/microbiological information to confirm the initial diagnosis.

3. Sepsis studies employ control populations that are not age, race or gender matched. Therefore, studies of septic patients cannot determine whether observed differences within experimental readouts are due to sepsis, predispose to sepsis or are a consequence of the many other differences between the study and control groups.

4. There is a wide variation in enrolment criteria between studies of sepsis patients, with immunologically relevant patient information often not reported. This is partly generated by subtle differences in sepsis diagnosis between various iterations of sepsis guidelines, and may limit inter-study comparisons. Several studies combined different subcategories of sepsis to create composite study groups, e.g. severe sepsis and septic shock. Since these are essentially clinical syndromes that require institution of specific therapeutic interventions with known immunological effects – such as septic shock and noradrenaline - it is not possible to determine whether the observed differences are due to sepsis. It follows that combining these different subpopulations into composite groups may diminish or distort sepsis experimental signals.

3.4.2 Interpretation of results

Experimental conclusions from translational sepsis investigations are limited by intrinsic challenges of study design and a failure to consider or report potentially controllable factors which may impact upon immune function. By contrast, translational investigations within major elective surgery overcame many of these limitations. As described, observations from surgical patients are relevant to those with sepsis because of the established molecular, genomic and clinical similarities between these groups. Therefore, the study of surgical patients may provide a translational model of human critical illness, which overcomes the methodological challenges of human sepsis, and the biological limitations of model organism investigations.

3.4.2.1Intrinsic limitations presented by the unpredictable and indeterminate onset of human sepsis.

This prevents pre-insult control samples from being taken, meaning alternative control populations must be chosen that differ from the study population in many immunologically relevant ways, aside from the presence of infection. Such uncertainty also precludes the accurate timing of patient sampling and consequently the description of putative dynamic immune processes. The importance of accurately timed patient samples is supported by genomic data describing differences in inflammatory markers at 24 hours of intensive care admission which predict survival (Xiao et al., 2011).

3.4.2.2 The diagnostic challenge of sepsis

The most important controllable factor which was ignored by sepsis investigations was confirmation of the sepsis diagnosis i.e. review of latterly returned microbiological results. Some patients enrolled into sepsis studies may not have an infection, since most sepsis studies did not make full use of available information to confirm the sepsis diagnosis, such as post-hoc microbiology results. Critically ill patients are colonised with potentially pathogenic organisms. Failure to demonstrate infection is important, since recent laboratory investigations have revealed that the clinical signs/symptoms of sepsis are frequently mimicked by non-pathogenic molecules (Menu and Vince, 2011). Even then, the binary presence of infection may be insufficiently accurate, since the location and type of micro-organisms may generate specific immune responses (Menu and Vince, 2011; Vandenesch et al., 2012) - only one study of the 57 described above specified infection site

or pathogen type. This criticism is also true of many surgical and trauma studies, which failed to record whether infections developed in the study population.

3.4.2.3 Underexplored, but potentially controllable variables

Other under explored controllable factors included the selection of an appropriate control population, enrolment criteria and reporting of therapeutic interventions. Therefore, although the finding of almost universally increased respiratory burst within septic patients is interesting (table 3.10), deeper consideration of the many limitations within these studies questions their validity.

The variation of assays used to measure specific immune processes is fundamental problem of translational studies. There are no agreed standards, with both major and subtle differences between assay protocols (Table 3.11-3.12) that may impact upon the assay result. For example, heparin has been shown to affect lipopolysaccharide priming of the respiratory burst (Bortolussi et al., 1997). This underscores the importance of using multiple assays of same process within studies, to improve the internal validity.

3.4.2.4 Experimental and clinical implications of findings

Incorporation of the surgical model into the translational investigative process does not mean identical studies must first be performed in surgical patients. What it allows is robust characterisation of standardised immune processes which can be used as readouts for other patient types, for example the use of immune modulating agents may be trialled in septic patients with appropriate immunological readouts. A study of vasoactive medications would never be performed without measuring blood pressure, therefore it is remarkable that so many immunological studies have been performed without the appropriate immune readouts.

Findings from studies of immune function cannot be extrapolated to describe clinical outcome unless they are adequately powered, since the up-regulation of immune function may improve bacterial killing but at expense of increased host cell injury, ultimately causing more harm. Power calculations were not described by any studies for immune function or clinical outcome.

Beyond directly modelling inflammation with the surgical insult, the incidence of sepsis is sufficiently high in surgical patients to permit cohort investigations of postoperative sepsis. Depending upon health care system and database chose, the incidence of clinically defined

sepsis varies between 6.98% and 12.25% (Lawson et al., 2012). Since approximately 240 million surgical procedures are performed worldwide each year, large numbers of well characterised patients may be followed.

3.4.3 Strengths and weaknesses

The studies chosen are representative of the current translational literature, spanning the three most popular areas of translational immunological research over the past fifteen years. Despite this, the number of studies included in this investigation is relatively small. Therefore, it was only possible to perform descriptive rather than quantitative comparisons between the different study types.

These findings support the use of the surgical model as a bridge between model organism and septic patient research, where understanding of immune processes may be refined prior to definitive evaluation in carefully selected and characterised septic patients, which may then be subject to pragmatic real-world testing. They do not imply this model is a replacement for animal model or septic patient research.

This study has only examined the methodological advantages of studying surgical patients over septic patients. While the many similarities between human responses to infection and trauma have already been described, no studies have reported how functional immune assays, such as those described within this study, vary between these two patient groups. For example, there are clinical scenarios where clear differences exist. The fulminant septic responses of patients with meningococcal septicaemia are never encountered following major surgery. Whilst presenting a limitation, studies which directly compare surgical and sepsis patients may identify the key molecular switches which define the differences between the two groups. Although original papers were retrieved for each study, these only contained summary data, therefore the raw data for each investigation was not examined.

3.4.4 Conclusions

The translational investigation of septic patients presents potentially controllable but often ignored methodological limitations. The study of major elective surgery overcomes many of these. The molecular, genomic and clinical similarities that exist between surgical and septic patients provide biological plausibility to this model. The study of major elective surgery may therefore provide an additional translational model between model organism research, healthy volunteer studies and clinical evaluation. Within other areas of translational research, such as cancer cell biology, experimental protocols have been standardised to facilitate comparisons between different studies. Translational sepsis research would benefit from similar standardisation. In addition, the incidence of post-operative sepsis is sufficiently common to permit cohort investigations of septic patients that include individualised pre-sepsis control data.

Chapter 4. The perioperative neutrophil phenotype.

4.1 Introduction

Neutrophils may have an important role in determining the human response to surgery, because they are the predominant cell type of the early inflammatory response (J. C. Marshall, 2005). Although neutrophil function is critical to the timely resolution of infection/inflammation (Almyroudis et al., 2013; Morgenstern et al., 1997) these effector processes may also cause collateral tissue injury (Chollet-Martin et al., 1996; Miller et al., 1992; Steinberg et al., 1994). Therefore, excessive and diminished neutrophil function may both be associated with harm, depending upon the context within which they are operating.

Within laboratory models of neutrophil impairment, using both neutrophil depletion or chemical inhibition, the timing of neutrophil impairment in relation to the index inflammatory insult and further inflammatory stimuli ('second hits') alters experimental outcome (Deitch et al., 1990; Hoesel et al., 2005, Chen et al., 2015, Liu et al., 2006). The coordination of these various immune activities during systemic inflammatory responses remains poorly understood.

Sampling from major elective surgical patients may allow an accurate temporal description of the putative dynamic changes in neutrophil immune function which follow a major inflammatory insult, where individualised human subjects are directly compared to their own pre-insult control samples, thereby accounting for pre-existing comorbidity. Within previous studies the baseline variability in neutrophil respiratory burst assays overlaps with post insult samples, suggesting important experimental signals may be missed unless paired statistical analysis is used (Fröhlich et al., 2006; Kawasaki et al., 2007; Shih et al., 1999; Wiezer et al., 2000).

Key neutrophil immune processes include the respiratory burst and phagocytosis. These effector processes rely upon common cellular mechanisms such as energy generation, cellular remodelling and the fusion of cellular plus vesicular membranes. Two investigations have identified diminished neutrophil phagocytosis following major surgery (Jones et al., 2014; Kawasaki et al., 2007) and three investigations have failed to show any change in perioperative neutrophil respiratory burst function (Fröhlich et al., 2006; Kawasaki et al., 2007; Wiezer et al., 2000). In addition to these effector processes, a range of cell surface proteins are necessary for coordinated activity of neutrophil within experimental

investigations. These include adhesion receptors, cytokine receptors, pattern recognition receptors and immunoglobulin heavy chain receptors, and are described below.

Previous investigations have described how individual cell surface proteins and effector processes change within patients undergoing specific surgical procedures (e.g. liver resection, cataract surgery). None of these studies have documented a wide range of markers/processes within a highly phenotyped general surgical cohort patients undergoing major elective surgery, who are likely to acquire postoperative sepsis.

4.1.1 Hypothesis

Major elective surgery is associated with impaired neutrophil immune function.

4.1.2 Aims

To describe how major elective surgery alters:

1. Circulating numbers of major immune blood cells,
2. Effector processes: the respiratory burst and phagocytosis (including CD16 expression).
3. Cell surface expression of key ligands necessary for the entry of neutrophils into inflamed tissues (CD62L, CD11b, CXCR2) and the detection of tissue injury and infection (TLR-4, TREM-1)

4.2 Methods

Patients were recruited from the Post-Operative Morbidity Oxygen study (POMO, MREC 09/H0805/58) and VISION-UK (MREC 10/WNo03/25) studies. All samples were compared to preoperative controls. POMO was a randomised study of goal directed therapy. I examined patient responses independently of their treatment/control group allocation, and remained blinded to group allocation during the experimental part my investigation - table 4.4 describes the relative contribution of treatment/control patients within each experimental assay. VISION-UK was an observational study. Patient selection, blood sampling, clinical data collection and further details of these studies are described in General Methods.

Blood samples was collected on the morning before surgery, postoperative day two and on postoperative day five. A five-day follow-up period was chosen because this is the time frame in which major elective surgery patients have frequently clinically recovered (mobilising freely, switched to oral medications, urinary catheter out etc). For tests where significant differences were identified on postoperative day two, further patients were recruited for analysis of blood samples after surgery was complete and anaesthesia reversed, within the theatre recovery areas.

Whole blood assays were performed to minimise iatrogenic impact of cell isolation upon results. To validate the isolated neutrophil model used in subsequent chapters, key findings were retested in isolated cells, and described at the end of this chapter. Flow cytometric assays are described with General Methods.

4.3 Results

4.3.1 Patient details

The total study population characteristics are described within tables 4.1 and 4.2, perioperative care details within table 4.3. Due to the large number of experimental assays used, each assay was not performed within all patients. The characteristics of patients used for each assay are compared within chapter 4.4.

Clinical and demographic details	
Demographics	
Number of patients studied	59
Age (median, [IQR]) (years)	64 [55-72]
Male (n, [%])	36 [61.0]
Surgery Type	
Hepatobiliary (n, [%])	42 [71.2]
Upper gastrointenstinal (n, [%])	9 [15.3]
Colorectal (n, [%])	2 [3.3]
Urology (n, [%])	2 [3.3]
Gynaecological oncology (n, [%])	2 [3.3]
Perioperative risk evaluation	
Body Mass Index (median, [IQR]) kg/m^2	27.4 [24.5-30.3]
Current smoking (n, [%])	16 [27.1]
POSSUM predicted morbidity (%, [IQR])	53.9 [41.1-72.5]
POSSUM predicted mortality (%, [IQR])	5.24 [1.8-6.3]
Duke Activity Status Index (median, [IQR])	35.0 [21.1-50.7]
VSAQ METS (median, [IQR])	8 [5-8]

Table 4.1 Demographic and clinical features of study patients.

Co-morbidities and preoperative medications	
Co-morbidities	
Malignancy	38 [64.4]
Estimated glomerular filtration rate (GFR) mls/min/1.73m^2	72.8 [16.2]
Abnormal electrocardiogram/ECG	26 [44.1]
Hypertension	25 [42.3]
Diabetes Mellitus	14 [23.7]
Asthma / Chronic obstructive pulmonary disease	10 [17.5]
Ischaemic heart disease	9 [15.3]
Arrhythmia	6 [10.2]
Rheumatoid arthritis	4 [6.8]
Cerebrovascular disease	2 [3.4]
Thyroid disease	2 [3.4]
Chronic congestive heart failure	2 [3.3]
Renal impairment (GFR <50 mls/min/1.73m^2)	1 [1.7]
Crohns	0 [0.0]
Ulcerative colitis	0 [0.0]
Cirrhosis	0 [0.0]
Medications	
ACE inhibitor / angiotensin receptor blocker	24 [40.7]
Statin	23 [39.0]
Aspirin	14 [23.7]
Beta-blocker	12 [20.3]
Calcium channel blocker	11 [18.6]
Diuretic	11 [18.6]
Clopidogrel	3 [5.1]
Warfarin	2 [3.4]
Proton pump inhibitor / H_2 antagonist	19[32.2]
Chemotherapy (past 3 months)	7 [11.9]
Paracetamol	5 [8.5]
Oral opiates	4 [6.8]

Non-steroidal anti-inflammatory agents	2 [3.4]
Sulfasalazine	1 [1.7]
Antibiotics	1 [1.7]
Steroids	0 [0.0]
Azathioprine	0 [0.0]
Anti-Tumour necrosis factor drugs	0 [0.0]
Intravenous opiates	0 [0]

Table 4.2 Comorbidities and preoperative medication use of study patients.

*All values refer to (number of patients, [%]) unless otherwise stated. *Abnormal ECG defined by any of left ventricular hypertrophy, left bundle branch block, ST/T-wave abnormalities.*

Perioperative care			
	Perioperative day		
	Intraoperative	**POD 2**	**POD 5**
Duration of operation hrs: mins (mean, [sd])	4:25 [1:57]		
Fluid mls/kg (median, [IQR])	22.3 [13.1-27.2]		
Patients received blood products	6 [10.2]		
Vasopressor / inotropic infusion	9 [15.3]	-	-
Sedation	10 [17.0]	-	-
Epidural	37 [62.7]	29 [49.2]	24 [40.7]
Intravenous opiates	18 [30.5]	26 [44.1]	25 [42.4]
Low molecular weight heparin	-	39 [66.1]	46 [78.0]
Positive microbiology*	1 [1.7]	0 [0]	2 [3.3]
Location within postoperative care / intensive care unit	59 [100]	9 [15.3]	7 [11.9]

Table 4.3 Perioperative care of study patients.

*All values refer to (number of patients, [%]). * Microbiology cultures from blood/sputum/urine/wound/bile of potentially pathogenic organisms. Management of patients with suspected sepsis was left to discretion of bedside clinicians.*

Individual assay patient demographics, co-morbidity and medication data								
Assay	**Total number#**	**Age** (median, [IQR]) (years)	**Male** (n, [%])	**Malignancy** (n, [%])	**Immune medications** (n, [%])	**POMS morbidity** (median, [IQR]) (%)	**Hepatobiliary surgery** (n, [%])	**POMO Goal Directed Therapy*** (n, [%])
Respiratory burst (preop-pod2)	18	63 (58-73)	12 [67]	6 [33]	2 [11]	49.2 [23.1-67.1]	12 [67]	10 [55]
Respiratory burst (preop-postop)	4	73.5 (68.5-77.25)	1 [25]	4 [100]	0 [0]	58.54 [53.9-62.3]	2 [50]	1 [25]
Respiratory burst (preop-POD2-POD5)	6	64 (63-73)	3 [50]	1 [17]	0 [0]	54.3 [47.6-63.1]	4 [67]	4 [67]
Phagocytosis (preop-POD2)	9	59 (48-66)	4 [44]	-	-	-	2 [22]	VISION
CD16 (preop-POD2)	19	69 (61-74)	10 [52]	11 [58]	1 [5]	51.2 [45.5-62.3]	11 [58]	7 [37]
CD16	4	73.5	1 [25]	4 [100]	0 [0]	58.54 [53.9-62.3]	2 [50]	1 [25]

(preop-postop)		(68.5-77.25)						
CD16 (preop-POD2-POD5)	11	66.5 (60-73.75)	5 [45]	7 [64]	1 [9]	56.3 [50.6-62.3]	5 [45]	5 [45]
CXCR2 (preop-POD2)	4	61 (58.5-66.25)	3 [75]	-	-	-	1 [25]	VISION
TREM1 (preop-POD2)	6	58.5 (54.75-61.5)	3 [50]	-	-	-	1 [17]	VISION
TLR4 (preop-POD2)	6	61 (59.25-71)	4 [67]	-	-	-	2 [33]	VISION
CD62 (preop-POD2)	4	67 (61-76.5)	3 [75]	2 [50]	0 [0]	61.8 [47.2-71.1]	1 [25]	2 [50]
CD11b (preop-POD2)	15	73 (67-74)	6 [40]	7 [47]	0 [0]	49.8 [44.0-69.8]	8 [53]	8 [53]

Table 4.4 Key demographic, co-morbidity and medication data for individual experimental assays.

*Each assay was not performed on every patient, therefore data from tables 4.1-4.3 is summarised here so that patients within each assay group maybe compared. #Only complete data sets for each time point are included within study analysis, therefore there are more preop-POD2 than preop-POD2-POD5, owing to missing patient samples. *Within POMO studied patients were randomised to receive standardised goal directed therapy or standard, anaesthetist directed, care* (Ackland et al., 2015), *"VISION" denotes patients were recruited through the VISION study. The inclusion criteria for these two studies are detailed within Table 2.1*

4.3.2 Perioperative change in blood leukocyte numbers

Figure 4.1 describes perioperative changes in leukocyte subsets. The absolute neutrophil count was elevated, and the absolute lymphocyte reduced throughout the recorded perioperative period. Monocyte counts increased from postoperative day two. The biological significance of these changes in cell number is uncertain.

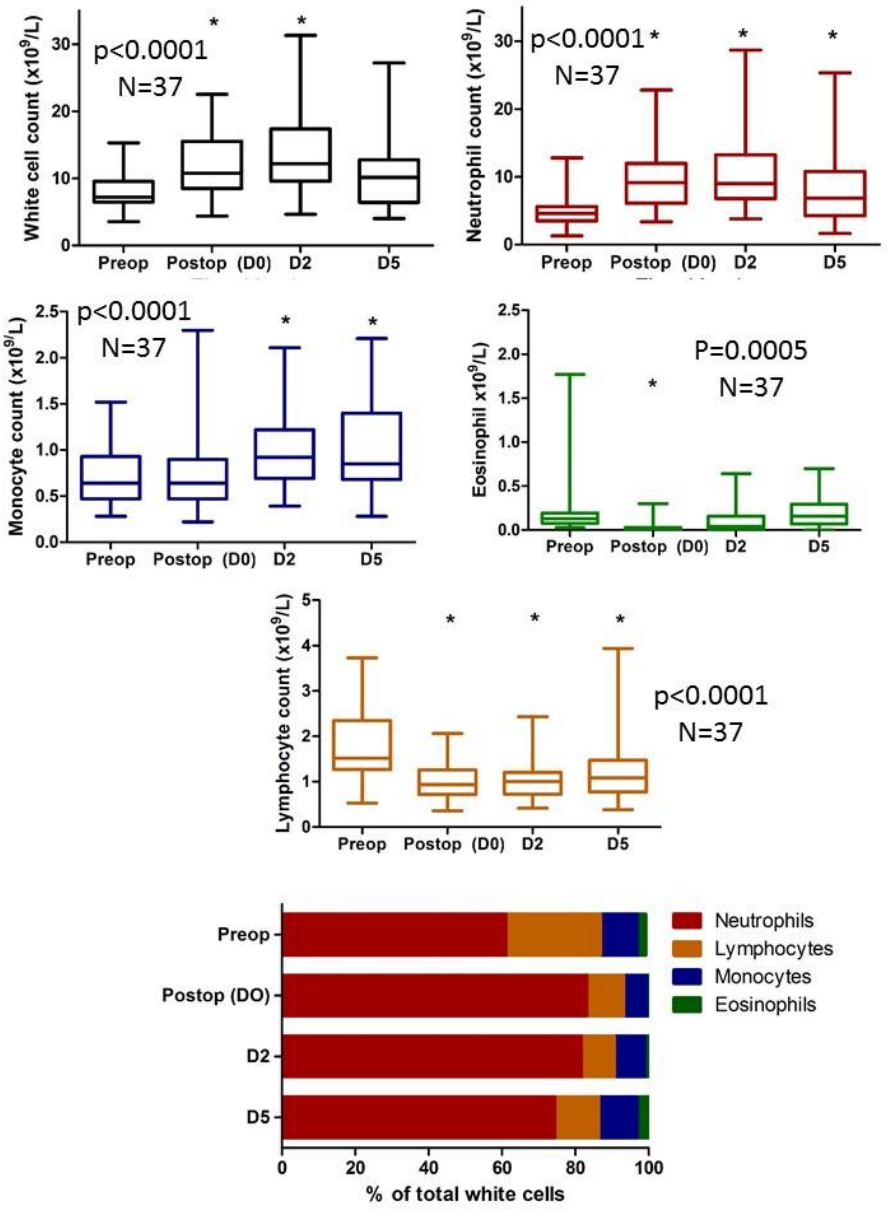

Figure 4.1 Perioperative change in blood leukocyte counts.

*Box and whisker plots represent median and IQR. Postoperative (D0) samples were taken on arrival of patient within intensive care / postoperative care unit, D2=postoperative day 2, D5=postoperative day 5. Friedman's ANOVA with Dunn's post-test comparing preoperative with each postoperative value. * p<0.05. Only complete data sets represented, therefore total patient numbers described are less than total studied.*

4.3.3 Perioperative neutrophil respiratory burst

The E. coli and PMA stimulated respiratory burst capacity were reduced on postoperative day 2 (Fig 4.2, 4.3), and began to return to baseline by postoperative day 5. No differences were identified with fMLP (Fig 4.4). fMLP is a gentler stimulant than E.coli and PMA, and has been used by other investigators to identify small differences in neutrophil priming. The distribution of burst responses within individuals was normally distributed - FACS plots did not identify any evidence for distinct subpopulations / multi-modal distributions. (Fig 4.2, 4.3, 4.4).

Next, using a murine model, I sought to establish whether the postoperative decrease in respiratory burst was also evident in bone marrow derived neutrophils which have not egressed into the systemic circulation. Mice were randomised to either 30 minutes of isoflurane anaesthesia plus midline laparotomy, or sham groups and bone marrow harvested 24hr later. The isoflurane/laparotomy group demonstrated reduced PMA stimulated oxygen consumption when compared to the sham group (Fig 4.5). The apparent increase in control sample (resting cell) ROS production on postoperative day two when compared to preoperative samples (Fig 4.2C, 4.3C) is explored further in Chapter 5.

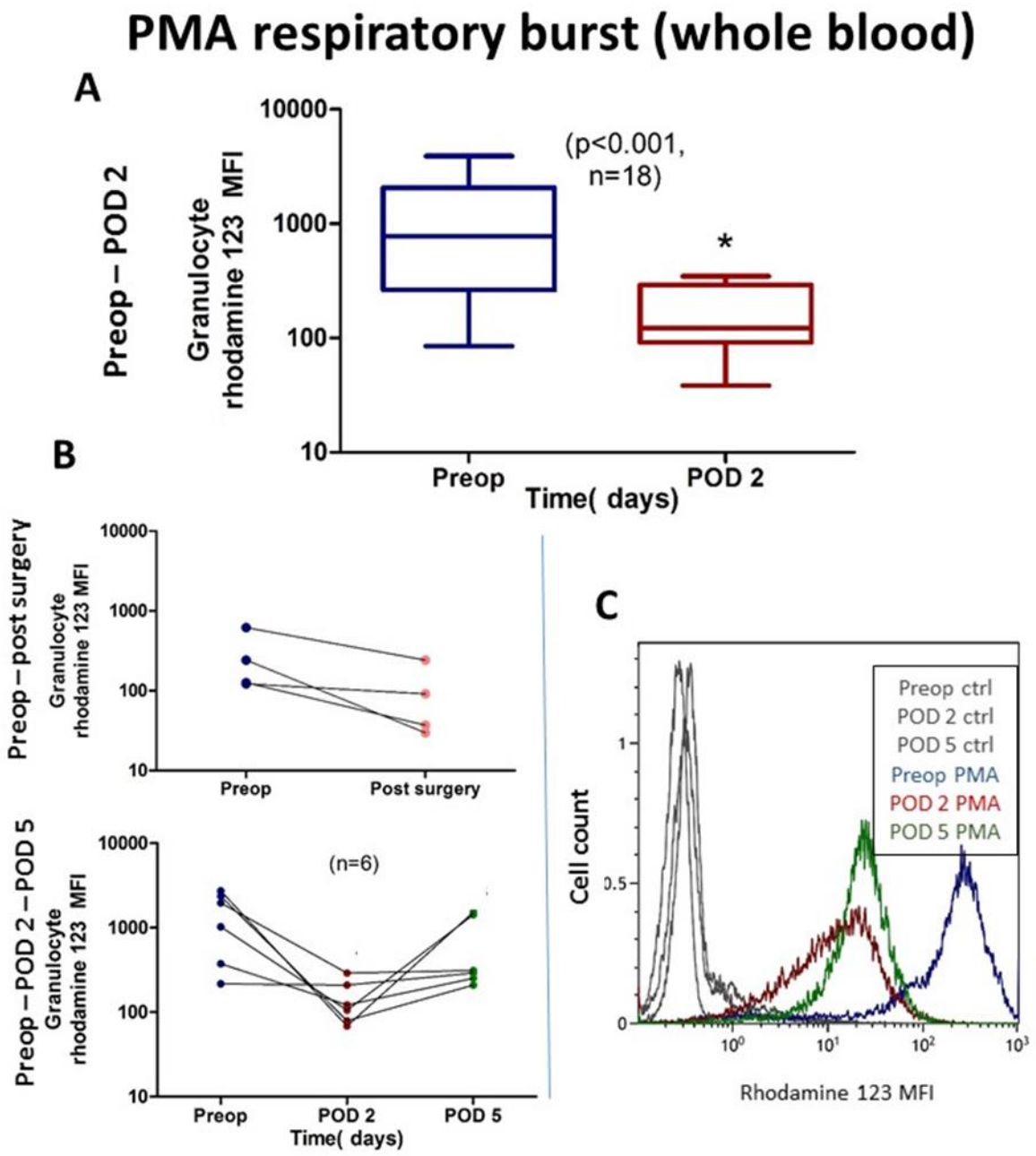

Figure 4.2 Perioperative change in whole blood granulocyte PMA stimulated respiratory burst

All data are paired preoperative and postoperative samples. Only complete data sets for each time point are presented, therefore total numbers within each graph vary. A Preop-POD 2 population data. Box-whisker plot is median-IQR-max/min; Wilcoxon matched pairs test used. B Population data for other time points. C Overlay plot showing typical paired preop-POD 2-POD 5 data.

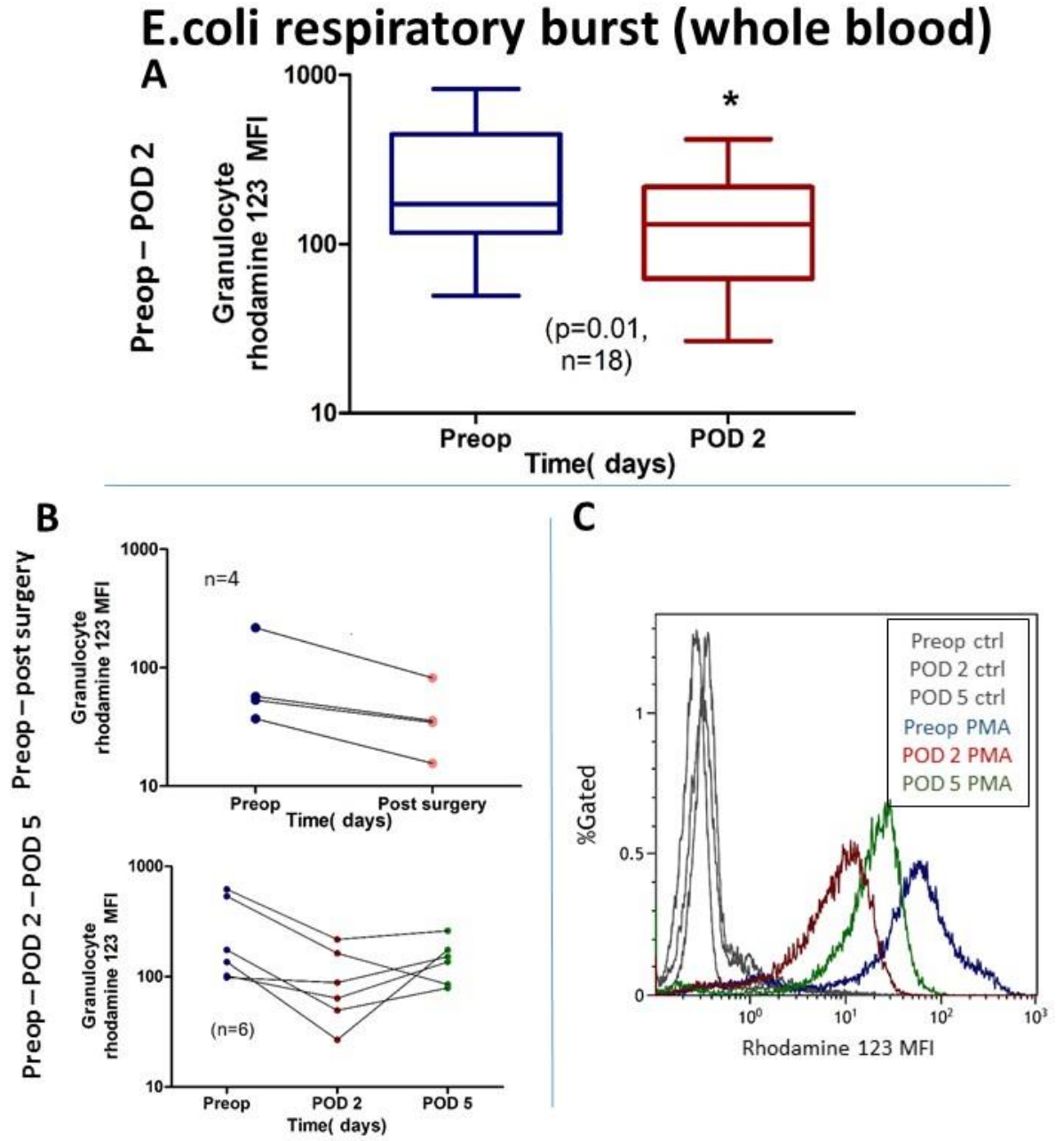

Figure 4.3 Perioperative change in whole blood granulocyte E. coli stimulated respiratory burst

All data are paired preoperative and postoperative samples. Only complete data sets for each graph are presented, therefore total numbers within each graph vary. A Population data for preop-POD2 time points. Box-whisker plot is median-IQR-max/min with Wilcoxon matched pairs test used. B Preop-post surgery and preop-POD2-POD5 time points. C Overlay plot showing typical paired preop-POD 2-POD 5 data.

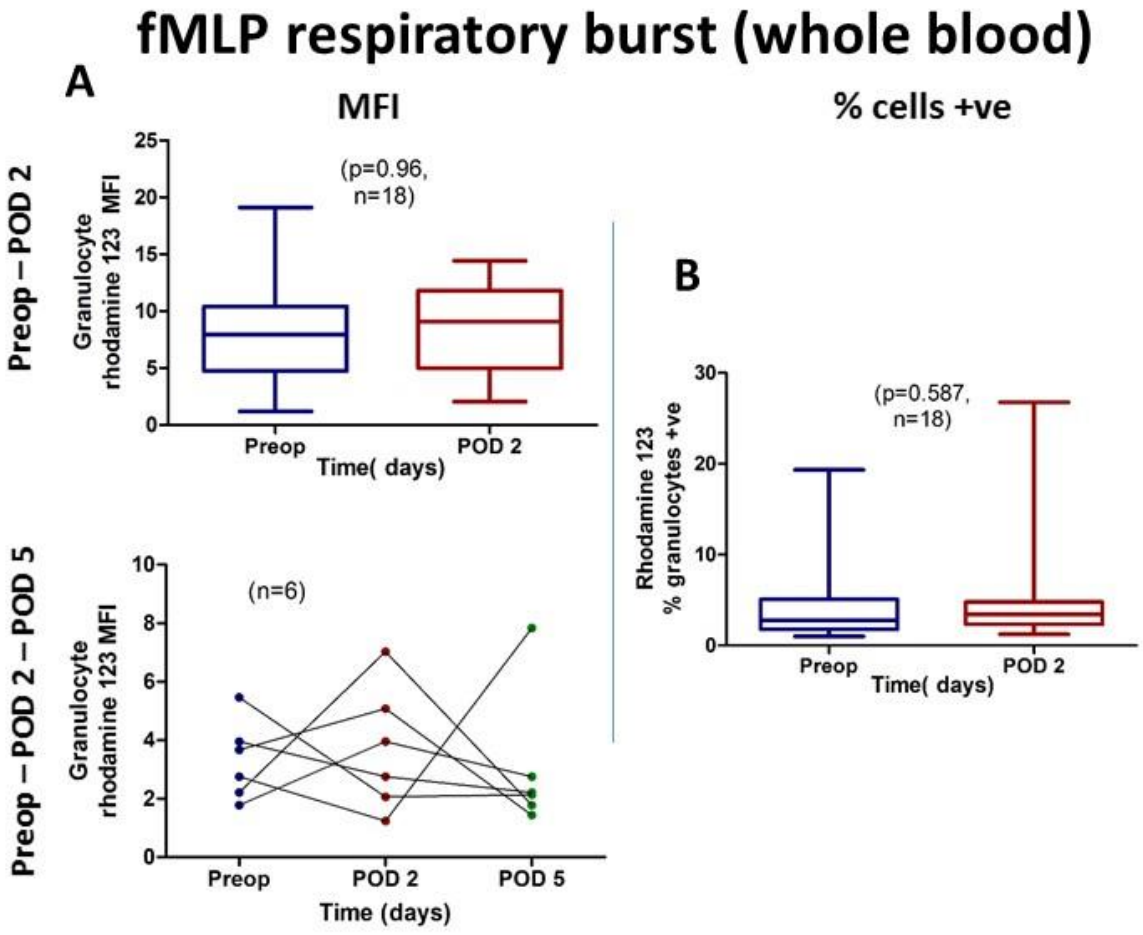

Figure 4.4 Perioperative change in whole blood granulocyte fMLP stimulated respiratory burst

All data are paired preoperative and postoperative samples. Only complete data sets for each graph are presented, therefore total numbers within each graph vary. A. Population data for rhodamine 123 MFI at specified time points, box-whisker plots are mean-IQR-max/min with Wilcoxon matched pairs for preop-POD2. B Population data for % granulocytes with rhodamine123 fluorescence, median-IQR-max/min.

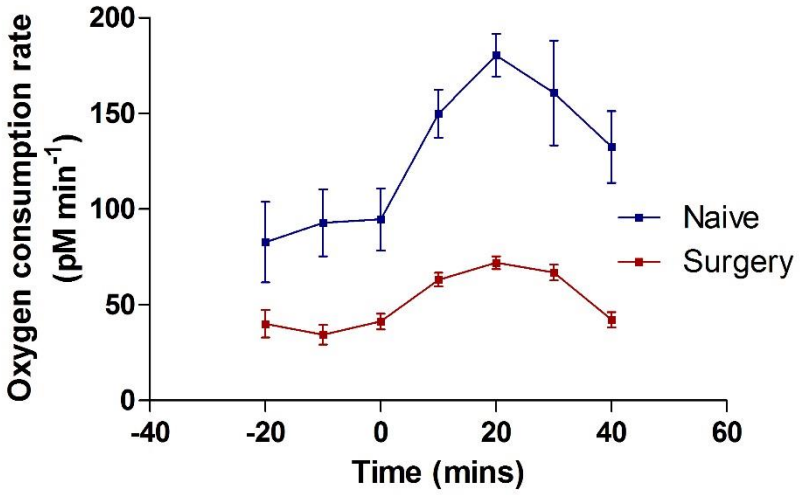

Figure 4.5 PMA stimulated oxygen consumption of murine bone marrow neutrophils 24hrs after surgery.

Two groups were compared: 30 minutes of isoflurane anaesthesia plus midline laparotomy or sham (anaesthesia without surgery) groups. N=3 per group and each experiment performed in triplicate. Bone marrow neutrophils were plated 24hrs later. Animals were randomised through number identification and blinded selection of numbers. Further experimental details are provided within section 2.4. Oxygen consumption rates following PMA stimulation were measured with the Seahorse Bioscience XF respirometer. Error bars represent mean +/- sd.

4.3.4 Perioperative neutrophil phagocytosis.

Neutrophil phagocytosis of E. coli bacteria on postoperative day 2 were unchanged when compared to preoperative values (p=0.084) (Fig 4.6). However, cell surface expression of the Fc receptor CD16 was significantly reduced on postoperative day two, with no apparent relationship between postoperative day two and five levels (Fig 4.7).

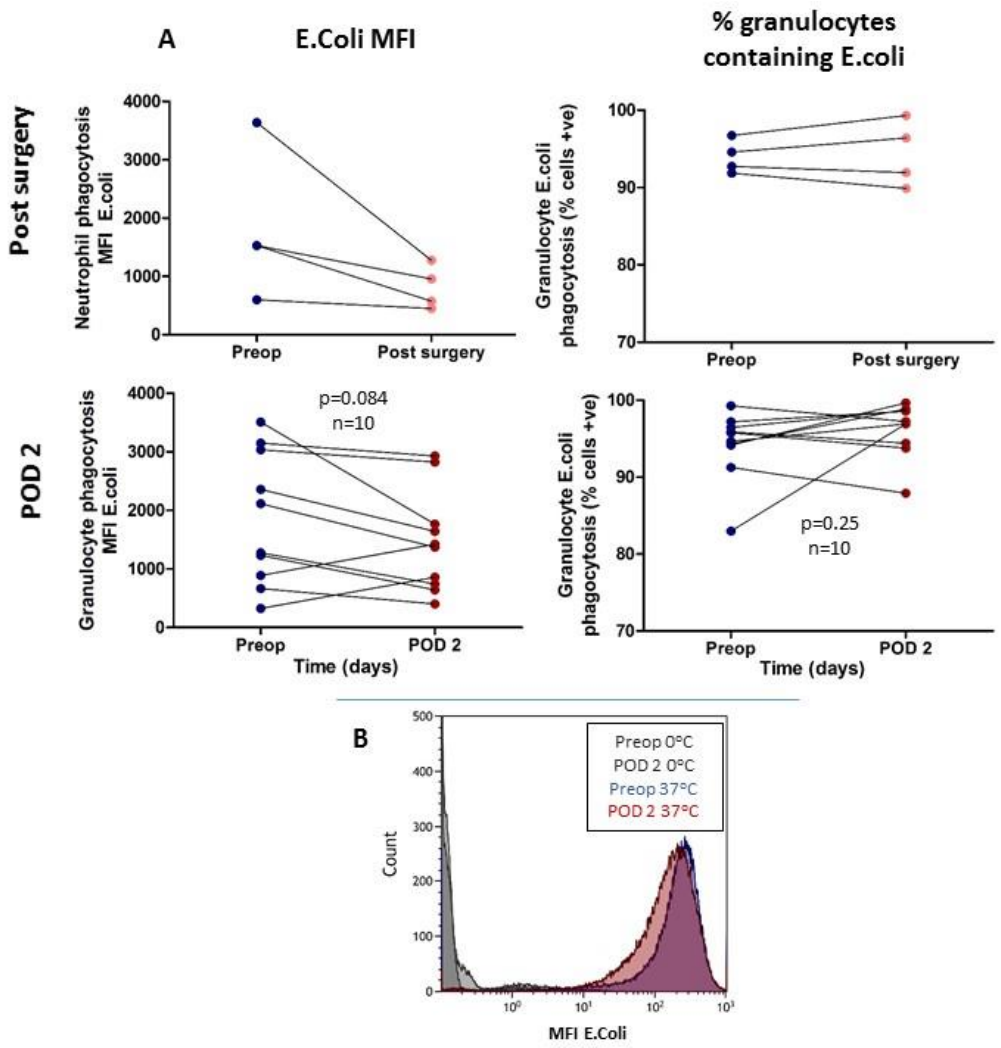

Figure 4.6 Perioperative change in whole blood granulocyte phagocytosis of heat-inactivated FITC-labelled E. coli bacteria.

All data are paired preoperative and postoperative samples. Only complete data sets for each graph are presented, therefore total numbers within each graph vary. ***A*** *Population data at defined time points. Post-surgery samples taken within two hours of the end of surgery. Paired line charts are presented and Wilcoxon matched pairs test used for Preop-POD2 data.* ***B*** *Overlay plot of typical preoperative – postoperative day 2 sample, showing 0°C control and 37°C test samples.*

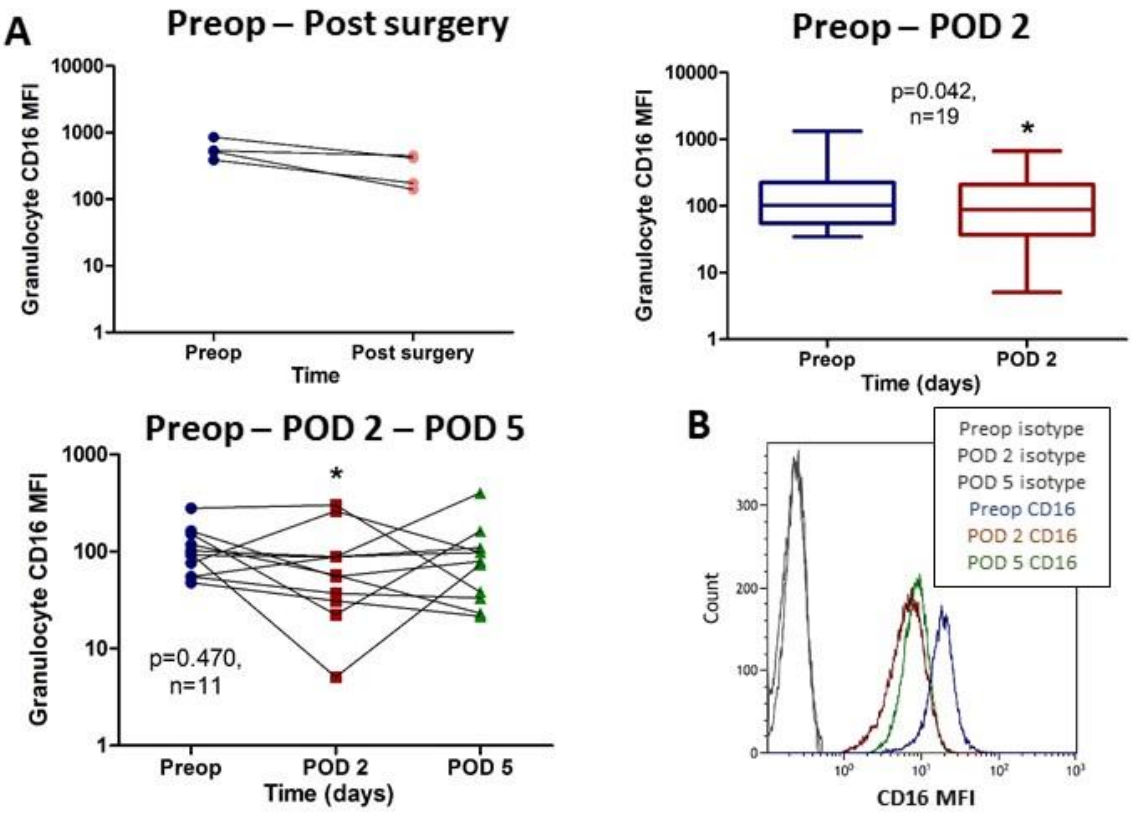

Figure 4.7 Perioperative change in granulocyte cell surface expression of CD16.

All data are paired preoperative and postoperative samples. ***A*** *Population data at specified time points. Post-surgery samples taken within two hours of the end of surgery. Paired t-test used for Preop-POD2 data, and Friedman matched pairs analysis used for Preop-POD2-POD5 data.* ***B*** *Overlay plot demonstrating typical paired patient samples.*

4.3.5 Perioperative change in neutrophil adhesion and chemotaxis receptors

No significant differences in cell surface expression of CD62L, CD11b or CXCR2 were identified on postoperative day two (Fig 4.8).

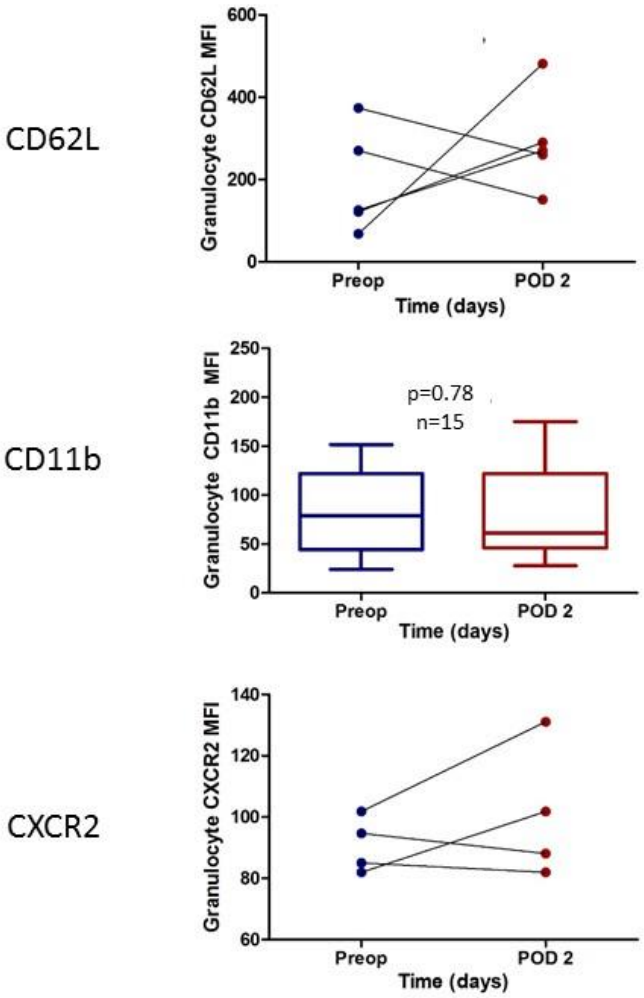

Figure 4.8 Perioperative expression of neutrophil cell surface receptors facilitating transmigration and chemotaxis.

All data are paired preoperative and postoperative samples. Where n>10 box-whisker plots are provided (median-IQR-max/min) and Wilcoxon matched pairs test used, if n<10 paired line plots are provided alone.

4.3.6 Perioperative expression of neutrophil pattern recognition receptors.

Neutrophil expression of TREM-I was significantly increased on postoperative day 2, although no difference in TLR4 expression was identified (Fig 4.9).

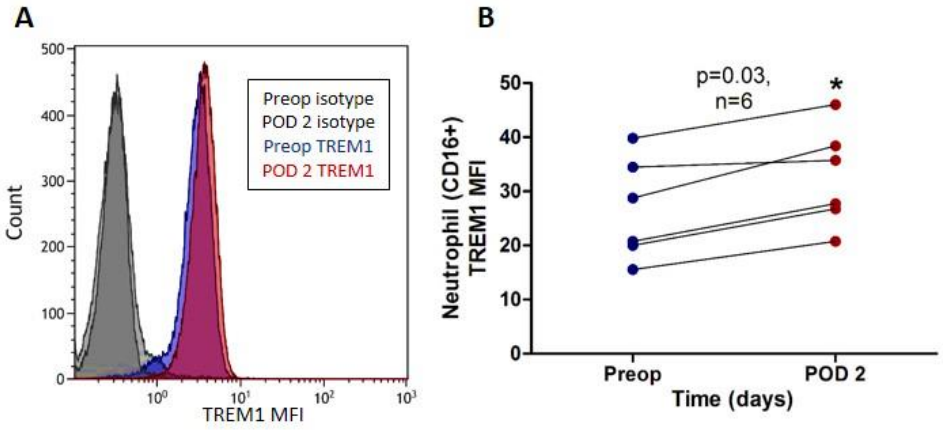

Figure 4.9 Perioperative change in neutrophil cell surface expression of TREM1.

All data are paired preoperative and postoperative samples. A Typical overlay plot B Population data. Paired line plot and Wilcoxon matched pairs test used.

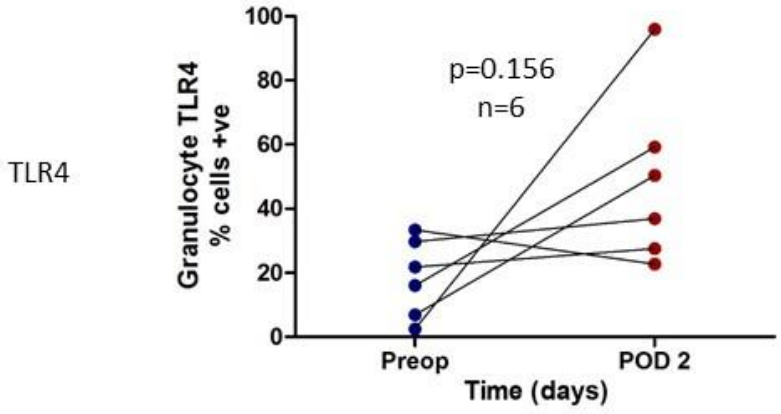

Figure 4.10 Perioperative change in neutrophil cell surface expression of TLR4.

All data are paired preoperative and postoperative samples.

Paired line plot and Wilcoxon matched pairs test.

4.3.7 Isolated granulocyte experiments.

There are many assays of neutrophil functions which have only been validated within isolated, rather than whole blood cells. Therefore, I repeated positive experimental findings from whole blood cells within isolated granulocytes. Isolated postoperative neutrophil displayed diminished CD16 and PMA simulated respiratory burst (Fig 4.11) – the same pattern as previous whole blood data. E.coli respiratory burst does not work within an isolated cell system.

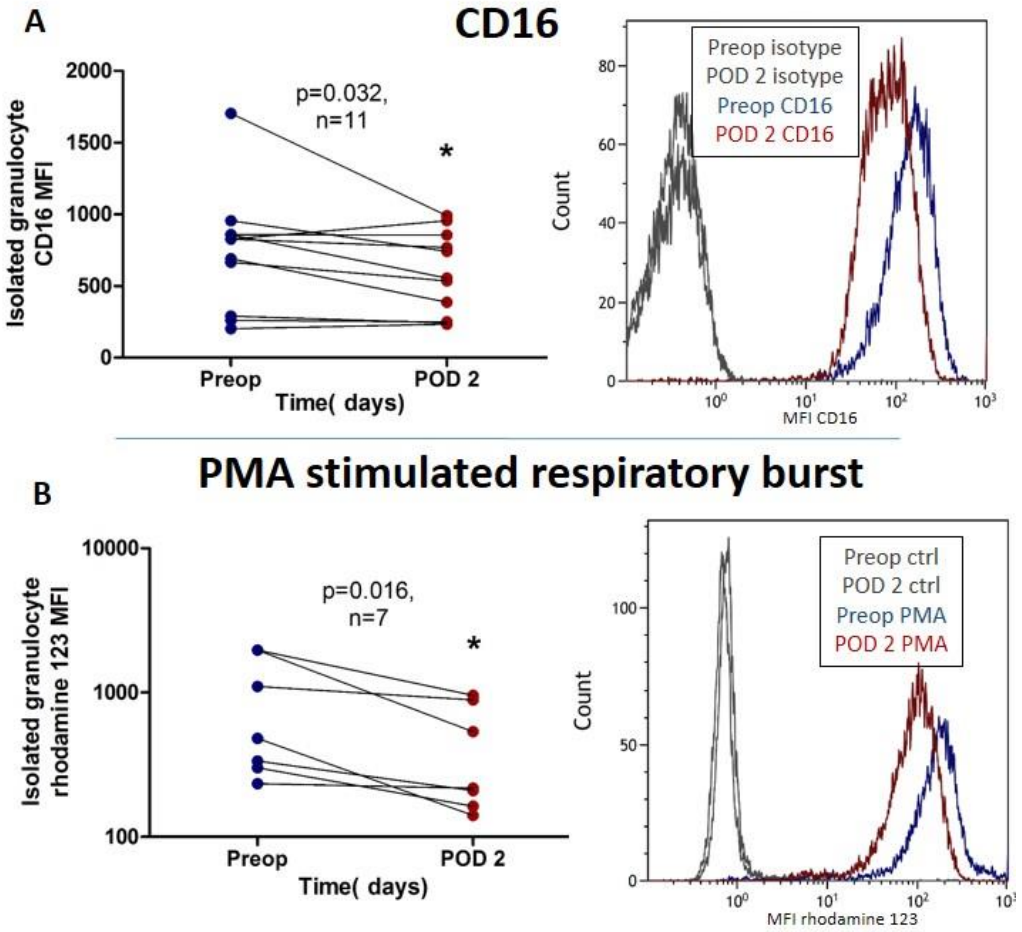

Figure 4.11 Perioperative change in isolated neutrophil respiratory burst and CD16 expression.

Granulocytes were isolated and prepared as described in general methods. All data are paired preoperative and postoperative samples. Overlay plots detail typical paired patient samples. A Perioperative change in isolated granulocyte CD16 expression. Wilcoxon matched pairs tests used. B Perioperative change in isolated granulocyte PMA stimulated respiratory burst. Wilcoxon matched paired test used.

4.4 Discussion

4.4.1 Key findings

1. **Patients on postoperative day two have a reduced respiratory burst and cell surface CD16 expression.** The PMA ($p<0.001$; n=18) and E. coli ($p=0.01$; n=18) respiratory burst and cell surface CD16 ($p=0.042$; n=19) were both reduced on postoperative day two when compared to individualised preoperative control samples. The fMLP respiratory burst, a marker of more subtle changes in neutrophil function (priming), was unchanged. The PMA and E.coli respiratory burst samples were typically increased on postoperative day five. The baseline / resting variation in these data which is evident in every assay used suggests that subtle experimental signals may be missed if case control study design were used, with unpaired statistical tests. These findings are compatible with impaired Fc receptor mediated phagocytosis and bacterial killing/activation of neutrophil granular contents on postoperative day two. The postoperative expression of neutrophil surface TREM-1 was increased ($p=0.03$; n=6), which may reflect TREM-1 amplifying inflammatory responses following the detection of endogenous PAMPs.

2. **The neutrophil count was increased on postoperative day two.** The absolute and relative neutrophil counts were increased on postoperative day two and five ($p<0.001$; n=37). Therefore, my data reveal a difference between a widely used index of inflammation (neutrophil count) and laboratory assays of circulating neutrophil immune function (PMA/E.coli respiratory burst). The activity of egressed tissue neutrophils was not recorded.

3. **Isolated neutrophils demonstrate diminished cell surface CD16 and PMA stimulated respiratory burst, similar to whole blood assays**
   The respiratory burst ($p=0.016$; n=7) and cell surface CD16 ($p=0.032$; n=11) were similarly reduced within an isolated system. These observations are mechanistically relevant, since they suggest that the postoperative functional impairment is not due to the tonic effects of circulating neurohumoral mediators or drugs. Additionally, they broaden the range of available assays which may be used in subsequent

chapters, since many experimental assays are not validated for use with whole blood samples.

4.4.2 Interpretation of key findings

The data provide experimental evidence of reduced postoperative neutrophil immune function, and demonstrate how the inter-individual variation in baseline function may lead to missed experimental signals unless an individual's postoperative responses are compared to their own preoperative control samples. Whether this functional extends between postoperative days one to four is unknown, because samples were not recorded at these time points. This baseline variation is evident within other perioperative investigations of neutrophil immune function (Fröhlich et al., 2006; Takashi Kawasaki et al., 2001; Wiezer et al., 2000). It is possible that this variation is due to differences in patient's baseline health, such as comorbid disease, presence of malignancy or administration of immune altering medications. Such accurate timing and access to baseline data are not possible during the study of established critical illness, such as trauma and sepsis. It is therefore possible that studies within these patient groups may therefore miss subtle experimental signals.

This period of impaired neutrophil functionality – with reduced PMA and E. coli stimulated respiratory burst – may be plausibly related to the development of postoperative complications. The respiratory burst is not only responsible for direct bacterial killing but also optimises activity of phagocytic granules (Segal, 2005), while observations from patients with chronic granulomatous disease suggest a diminished respiratory burst may generate states of chronic non-resolving sterile inflammation (Morgenstern et al., 1997). The magnitude of postoperative respiratory burst depression is comparable with that found in alcoholic hepatitis superimposed upon cirrhosis (Mookerjee et al., 2007) and in established sepsis (Pascual et al., 1998).

Greater depression of postoperative PMA burst responses than E. coli burst responses may have mechanistic implications. PMA mediates a persistent, maximal activation of the respiratory burst through direct binding to protein kinase c. Therefore, the postoperative functional impairment is not simply due to reduction in DAMP receptor signalling pathways, for example through reduced CD14 expression. Confirmation of the PMA data within the isolated cell system further excludes the possibility of tonic effects of circulating neuro-

humoral mediators. At a molecular level, the diminished respiratory burst may therefore be mediated through impaired PHOX assembly, subunit activity or substrate availability.

At a cellular level, the decreased quantity of neutrophil surface CD16 is compatible with previous descriptions of immature neutrophil release (Brown et al., 1989; Orr et al., 2005), implying postoperative day two neutrophils will be functionally immature also. CD16 expression is acquired at the metamyelocyte stage, and increases progressively with neutrophil maturation in bone marrow (Terstappen et al., 1990). Alternatively, exposure of bone marrow cells to neuro-humoral mediators, or drug exposure, may result in mature but functionally impaired cells.

Measurement of circulating neutrophil lifespan in humans is difficult to perform. Studies using ex vivo transplant of modified neutrophils suggest circulating lifespan is less than 1 day in health (Dancey et al., 1976). Since the changes I recorded were evident at postoperative day two, the presence of functionally impaired mature cells may be more likely than circulating immature cells. The murine bone marrow data provided is compatible with both explanations. On average postoperative CD16 MFI was reduced by 20% of preoperative levels, far less than the 90% downregulation that is observed during early apoptosis (Dransfield et al., 1994). Therefore, the postoperative reduction in CD16 is unlikely to signify the presence of neutrophil apoptosis.

Together, these data suggest that established tests of systemic inflammation - white cell count and CRP – do not correlate with a functional assay of circulating neutrophil immune function. The clinical significance of these changes warrants further investigation. If immunomodulatory therapies are to be delivered to clinical benefit, then they must be administered according to reliable assays of immunological function. Therefore, the surgical model provides an opportunity to investigate and define the role of immunomodulatory therapy.

None of the three previous investigations into perioperative neutrophil respiratory burst identified any significant postoperative changes (Fröhlich et al., 2006; Kawasaki et al., 2007; Wiezer et al., 2000). There are several key differences between these studies and mine. One study was conducted in patients undergoing cataract surgery (Fröhlich et al., 2006) and only sampled neutrophils on the operative day. Here, the minor surgical insult coupled with short follow up time may be insufficient to promote a change in global immune function.

The other two studies were conducted in comparable patient populations, partial gastrectomy and major liver surgery, and used identical reagents E. coli/PMA stimulated Dihydrorhodamine-123 assays (Kawasaki et al., 2007; Wiezer et al., 2000). However, Kawasaki et al analysed patient responses on postoperative days 1 and 4, and will have missed the day 2 effect this investigation has revealed. Wiezer's data sampled blood on postoperative day two, however the assay methodology was different, using only isolated neutrophils, a different isolation technique, lower doses of PMA (10ng/ml) and the addition of calcium which will alter calcium dependent respiratory burst response. The number of patients studied with this investigation is comparable to the other respiratory burst investigations (table 3.1).

Respiratory burst studies in trauma and septic patients have typically demonstrated an increased burst response when compared to healthy volunteer control group populations (Table 3.10). Given the fundamental similarities of human innate immune responses to disparate stimuli revealed by genomic investigations (Xiao et al., 2011), the data presented in this chapter initially appears counterintuitive. However, chapter 3 identified several intrinsic limitations within studies of trauma and sepsis patients which may explain this apparent difference. Although these methodological limitations provide sufficient explanation for differences between these populations, it is possible that specific differences mediated by PAMP c.f. DAMP signalling, or perhaps the prior/simultaneous administration of anaesthetic/analgesic agents at the time of inflammatory insult significantly modifies functional responses.

In contrast, diminished CD16 expression has been described following surgery and traumatic injury (Fung et al. 2008; Holzer et al. 2010, Wakefield et al. 1995). Reduced postoperative expression of CD16 is compatible with impaired opsonin mediated phagocytosis, but may only be confirmed with further investigation. This investigation did not reproduce the commonly described neutrophil cell surface CD11b increase and CD62L decrease. Several of the studies reporting the CD11b/CD62L relationship recorded data from the immediate perioperative period, and reported a return to pre-surgery values by postoperative day two. Therefore, these changes may have occurred too early to be identified within this study.

4.4.3 Strengths and limitations

This study describes a reduction in PMA and E.coli neutrophil respiratory burst, and reduction in neutrophil surface CD16, on postoperative day two. These changes may be mechanistically relevant to the development of both infectious and non-infectious complications, however this would require further clinical correlation. The breadth of assays performed is larger, and the number of patients studied broadly comparable with other investigations of innate immune cell function (Table 3.1).

Patients were part of a controlled, protocolised clinical trial and therefore received highly standardised care and underwent extensive preoperative assessment. Whole blood assays were used with significant findings subsequently confirmed within an isolated model. Confirmation within the isolated system provides mechanistic insight since it removes the tonic effects of circulating neurohumoral mediators and validates the use of isolated cellular assays for the mechanistic experiments within chapters 5 and 6. The ROS probe dihydrorhodamine is generally agreed to be the most reliable cellular probe with which to measure the respiratory burst (van Pelt et al., 1996).

However, the in vitro measurement of a specific aspect of cellular function does not necessarily equate to in vivo changes. True assays of human bacterial killing are difficult to perform. Even then, such killing assays will not identify the impact of dysregulated chronic inflammation. Neutrophils are highly susceptible to injury and activation during isolation procedures, and it is not ethical to invoke direct infection. Diminished neutrophil immune functionality may lead to both protection and harm, however this was a pilot study, and not powered to explore relationships with clinical outcome.

This study examined circulating neutrophil immune function. The activity of tissue neutrophil function is unknown. Crucially, differences in endothelial activation at different time points may lead to more activated neutrophils being pulled from circulation. It is therefore possible that tissue neutrophils would behave differently to circulating cells.

The results I have provided do not describe a universal postoperative phenotype, because all assays were not performed within the same patients. Specifically, patients were recruited from two other clinical studies (POMO and VISION), with the POMO group being split into treatment (goal directed therapy) and control (standard care) populations. The overall patient features are described within tables 4.1-4.3, and the patient features for each assay

described within table 4.4. For example, less patients from the respiratory burst preop-POD2 group had malignancy than within the respiratory burst preop-POD2-POD5. Patients were not sampled upon postoperative days one and three, therefore it is uncertain how long the respiratory burst is depressed for.

4.4.4 Conclusions and further work

This study has identified an impairment of the neutrophil respiratory burst at postoperative day two, as well as a reduction in the Fc receptor CD16. This impairment is compatible with diminished bacterial killing and possible prolongation of inflammation which may both predispose to postoperative complications. The data support a functional cellular defect within the burst machinery itself, which is present within bone marrow as well as circulating cells following elective surgery. Microscopy examination of blood samples maybe performed to identify the proportion of neutrophil precursors at different time points.

Chapter 5 – Postoperative neutrophil mitochondrial dysfunction and regulation of the respiratory burst

5.1 Introduction

Oxidative stress is a feature of the human response to surgical injury (Biglioli et al., 2003; Lefer and Lefer, 1996; Stipancic et al., 2005). This chapter will investigate the role of reactive oxygen species (ROS) within postoperative neutrophils, focussing upon the mechanistic relationship between resting cellular ROS levels and activated functional activity.

Mitochondria are a common source of ROS within cells which synthesise bioenergetic ATP through oxidative phosphorylation (Fig 1.4). While neutrophils are considered to be solely glycolytic (Borregaard and Herlin, 1982; Karnovsky, 1968; Segal and Abo, 1993) they do contain mitochondria which maintain a membrane potential independent of cytoplasmic ATP production (Fossati et al., 2003; Maianski et al., 2003; Pryde et al., 2000), and regulate neutrophil life span through constitutive apoptosis.

In patients with established sepsis, monocytes and platelet mitochondrial function is impaired (Japiassú et al., 2011; Protti et al., 2014; Weiss et al., 2015). Similar impairment of neutrophil mitochondria is supported by genomic investigations of healthy volunteer endotoxin exposure, which reveal a dramatic reduction in the expression of genes associated with mitochondrial function (Calvano et al., 2005). Within a monocyte cell line, mitochondrial ROS production at complex III has been shown to transduce hypoxic and outside inflammatory stimuli (Brunelle et al., 2005) through stabilisation of HIF and the integration of inflammatory signals that promote inflammasome assembly (Menu and Vince, 2011).

Since the majority of priming agents simultaneously augment neutrophil effector function and prolong lifespan (El Kebir et al., 2009; Lee et al., 1993; a C. Lee et al., 1999), it is plausible that neutrophil mitochondria may be involved in inflammatory/immune effector signalling in addition to apoptotic signalling. Specifically, the generation of mitochondrial ROS within inflamed neutrophils may be responsible for the diminished postoperative respiratory burst response.

The regulation of neutrophil PHOX by mitochondrial ROS has not been directly addressed within the literature before. However, several investigations have addressed roles for (mitochondrial) ROS during activation of PHOX, and other immune processes within a range of immune cells. The direct addition of H_2O_2 to human neutrophils increases PHOX derived superoxide (El Jamali et al., 2010). Within human lymphoblasts, which contain

functional PHOX, the addition of the complex I inhibitor rotenone 10μM and complex III inhibitor antimycin A 10μM increases NADPH oxidase derived extracellular ROS (Dikalov et al., 2012). Within human neutrophils the inhibition of complex I and III increases resting neutrophil cellular ROS, impairs cytoskeletal rearrangement (Fossati et al., 2003) and inhibits LPS induced NF-kB activation and proinflammatory cytokine production (Zmijewski et al., 2008).

5.1.1 Hypothesis

Postoperative neutrophils exhibit mitochondrial dysfunction, which may inhibit the respiratory burst.

5.1.2 Aims

1. To describe postoperative changes in resting neutrophil mitochondrial function.

2. To determine whether postoperative neutrophil mitochondrial oxidative stress is mechanistically responsible for the impaired respiratory burst response.

5.2 Methods

Patient samples were collected from POMO (ISRCTN76894700) and VISION-UK (10/WNo03/25) studies, and the patient characteristics for each experimental assay are detailed in table 5.1. Laboratory methods, including HL-60 cell line and differentiation, are detailed in General Methods. Neutrophil isolation and assays in sections 5.2.1 and 5.2.2 are more completely described in General Methods.

5.2.1 Perioperative human mitochondrial function

The measurement of cellular and mitochondrial ROS and neutrophil apoptosis are described in General Methods.

5.2.2 Model 1: Respiratory complex inhibition within human neutrophils

Respiratory complex inhibitor experiments were performed within a standardised, isolated cell system consisting of $1x10^6$/ml neutrophils suspended within PBS plus glucose 10mM. The complex I inhibitor rotenone and complex III inhibitors Myxothiazol and Antimycin A were added to preoperative and postoperative samples initially at 10μM doses, as described previously (Zmijewski 2008, Dikalov 2012). 30 minute incubation times were chosen to minimise the impact of ex-vivo incubation upon neutrophil function (Wenisch et al., 2001). Time course assays were performed with a plate reader and the ROS probe dihydroethidium.

5.2.3 Model 2: ROS manipulation within cell line model

Undifferentiated and DMSO-differentiated HL-60 cells were prepared as described within general methods. The resting and PMA stimulated ROS profiles of undifferentiated and DMSO differentiated HL-60 cells were characterised using probes MitoSOX-Red, dihydroethidium, rhodamine-123, and TMRM.

The redox status of resting HL60 cells was manipulated using ROS generating myxothiazol, and the ROS scavenger MitoTEMPO. Dose response experiments with MitoTEMPO, within an isolated system using cytochrome c, identified MitoTEMPO 500μM to provide the greatest antioxidant protection within an isolated system (Trnka et al., 2009). Similarly, my own dose response / time course experiments with undifferentiated ('postoperative') HL-60 cells supported the 500μM dose, with an incubation time of 240 minutes.

5.3 Results

5.3.1 Patient characteristics

Assay	Total number	Age (median, [IQR]) (years)	Male (n, [%])	Surgical speciality (n, [%])							Patient study source
				Hepato-biliary	Gynae-cology	Urology	Maxillo-facial	Gastro-intestinal	Ortho-paediacs	Breast	
Whole blood granulocytes DHR (preop-pod2) Fig 5.1AB	18	63 (58-73)	12 [67]	12 [67]	1	2	-	1	-		POMO
Isolated granulocytes Mitosox. (preop-POD2) Fig 5.1CD	8	61 (51-69)	4 [50]	1 [13]	1 [13]	3 [13]	-	2 [25]	-		VISION
Whole blood TMRM (preop-POD2) Fig 5.2 AB	15	61 [54-65]	8 [53]	3 [20]	1 [7]	7 [47]	2 [13]	1 [7]	1 [7]		VISION
Isolated granulocytes TMRM (preop-POD2) Fig 5.2C	4	66.5 [61-72]	2 [50]	1 [25]	1 [25]	2 [50]	-	-	-		VISION
Isolated granulocytes JC-1	8	69.5 (60-75)	3 [38]	6 [80]	1	-	-	1	-		POMO

(preop-POD2) Fig 5.3AB											
Isolated granulocytes Annexin (preop-POD2) Fig 5.4AB	9	68 (64-79)	3 [33]	1 [11]	3 [33]	-	-	1 [11]	4 [44]		VISION
Fas (CD95) (preop-POD2) Fig 5.5 AB	17	71 (65-75)	7 [41]	12 [71]	2	2	-	1	-		VISION
Rotenone 10µM + DHR Fig 5.6 A	16	60 (53-72)	6 [38]	2 [13]	4 [25]	6 [38]	-		2 [13]	2 [13]	VISION
Rotenone 10µM + Mitosox Fig 5.6 BC	7	55 (51-69)	4 [57]	1 [14]	0	3 [43]	-	2 [29]	-	1 [14]	VISION
Rotenone 10µM + PMA + DHR Fig 5.6 DE	18	61.5 (53-74)	8 [44]	3 [17]	5 [28]	6 [33]	-	-	2 [11]	2 [11]	VISION
Rotenone 10µM + PMA + DHR (preop-POD2) Fig 5.7	6	57 (54– 59)	3 [50]	1 [17]	1 [17]	2 [33]	-	-	1 [17]	1 [17]	VISION
Myxothiazol 10µM + Rhodamine Fig 5.8 AB	7	65 (61-77)	4 [57]	-	1 [14]	4 [57]	-	2 [29]	-	-	VISION
Myxothiazol 10µM + Mitosox	7	55 (51-69)	4 [57]	1 [14]	-	3 [43]	-	2 [29]	-	1 [14]	VISION

Fig 5.8 CD											
Myxothiazol 10µM + PMA + DHR Fig 5.8 EF	5	63 (59-90)	2 [40]	-	1 [20]	2 [40]	-	2 [40]	-	-	VISION
Myxothiazol 10µM + CD16 Fig 5.9 AB	6	60 (55-69)	3 [50]	1 [17]	-	3 [50]	-	-	2 [33]	-	VISION
Myxothiazol 10µM + Annexin Fig 5.9 CDE	3	54 (54-68)	2 [67]	-	-	3 [100]	-	-	-	-	VISION
Antimycin A 10µM + DHR Fig 5.10 AB	11	62 (58-74)	6 [55]	2 [18]	1 [9]	2 [18]	1 [9]	3 [27]	2 [18]	-	VISION
Antimycin A 10µM + PMA + DHR Fig 5.10 CD	11	62 (58-74)	6 [55]	2 [18]	1 [9]	2 [18]	1 [9]	3 [27]	2 [18]	-	VISION
Antimycin A 10µM + CD16 Fig 5.11 AB	7	61 (58-65)	3 [43]	2 [29]	-	-	1 [14]	2 [29]	2 [29]	-	VISION
Antimycin A 10µM + Annexin Fig 5.11 CDE	6	62 (60-67)	3 [50]	2 [33]	-	-	1 [17]	1 [17]	2 [33]	-	VISION

Myxothiazol 1µM + DHR Fig 5.12 AB	14	65 (59-72)	8 [57]	1 [7]	1 [7]	4 [29]	-	1 [7]	7 [50]	-	VISION
Myxothiazol 1µM + PMA + DHR Fig 5.12 CD	12	66 (61-75)	6 [50]	-	1 [8]	3 [25]	-	1 [8]	7 [58]	-	VISION
Myxothiazol 1µM + CD16 Fig 5.12 E	10	65 (59-67)	6 [60]	1 [10]	1 [10]	3 [30]	-	-	5 [50]	-	VISION
Myxothiazol 1µM + Annexin Fig 5.12 F	6	69 (61-76)	3 [50]	-	1 [17]	-	-	1 [17]	4 [67]	-	VISION
Myxothiazol ± PMA + DHE	2	55, 65	1 [50]	-	1 [50]	-	-	1 [50]	-	-	VISION

Table 5.1 Characteristics of the patients for Chapter 5 experimental assays.

5.3.2 Perioperative neutrophil mitochondrial phenotype

Rhodamine-123 (Fig 5.1 A,B) and MitoSOX Red fluorescence (Fig 5.1 C,D) were increased in postoperative resting whole blood and isolated neutrophils respectively, when compared to matched preoperative control samples, which supports the presence of increased cellular and mitochondrial ROS within postoperative neutrophils.

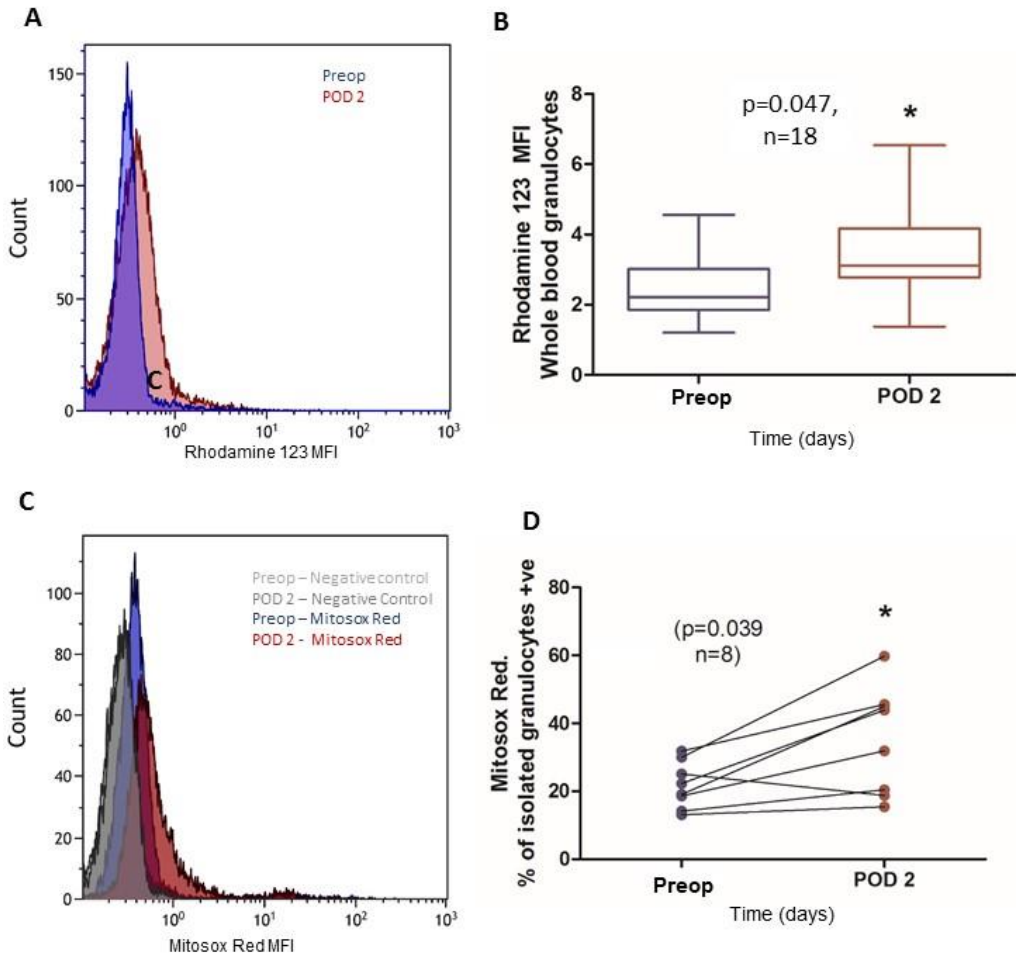

Figure 5.1 Perioperative resting neutrophil reactive oxygen species production.

All data are paired preoperative and postoperative samples. A Overlay plot demonstrating typical change in perioperative Rhodamine-123 fluorescence. B Box whisker plots (median-IQR) demonstrating population change in perioperative Rhodamine-123 production. Paired t-test. C Overlay plot demonstrating typical change in perioperative Mitosox-Red fluorescence, D Paired line plot demonstrating population change in perioperative Mitosox-Red fluorescence, Wilcoxon matched pairs test

Within whole blood neutrophils TMRM staining was increased postoperatively (Fig 5.2 A,B), suggesting hyperpolarisation of the mitochondrial membrane potential within postoperative neutrophils.

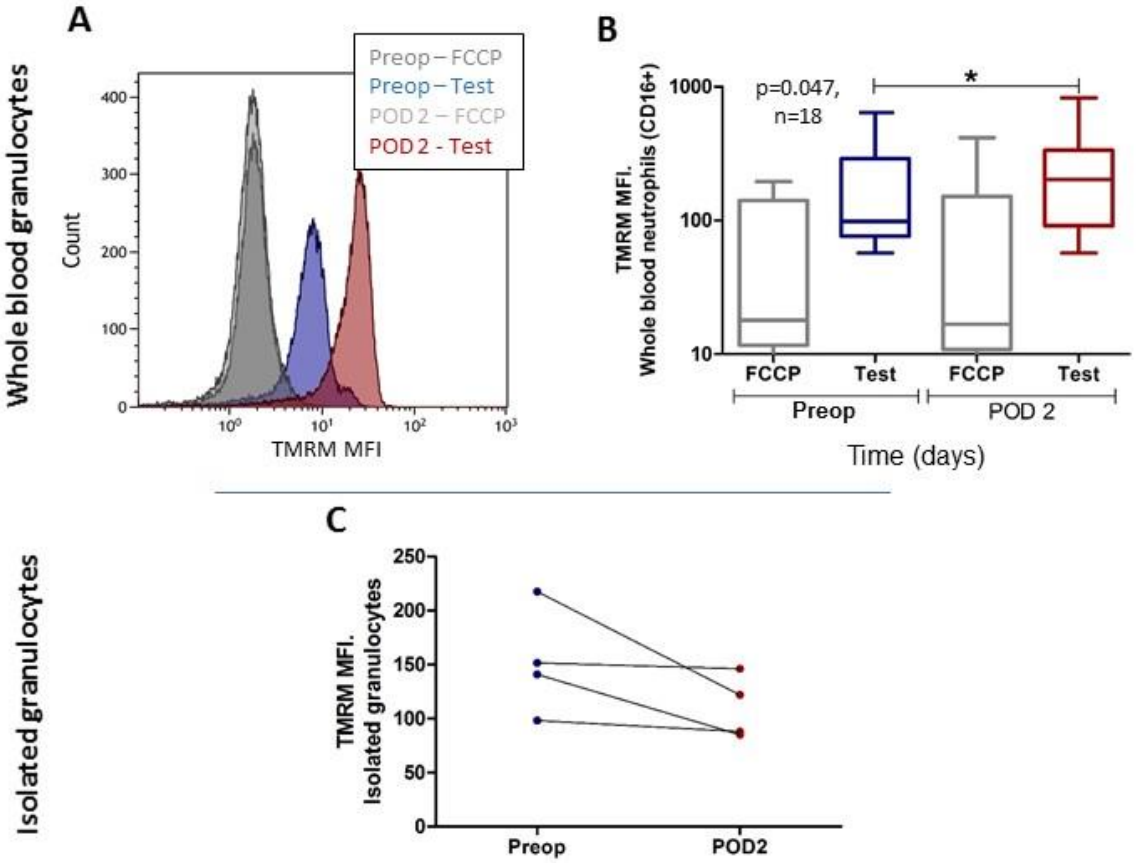

Figure 5.2 Perioperative resting neutrophil mitochondrial membrane potential: TMRM assay (whole blood and isolated granulocytes)

All data are paired preoperative and postoperative samples. A Overlay plot demonstrating typical patients perioperative change in whole blood neutrophil TMRM fluorescence. 10μM FCCP used as positive control. B Population box-whisker plots for perioperative change in whole blood neutrophils TMRM fluorescence. Wilcoxon matched pairs test used. C Perioperative change in isolated neutrophil TMRM fluorescence.

By contrast, isolated neutrophil TMRM fluorescence (Fig 5.2C) and JC-1 fluorescence (Fig 5.3 A,B) was diminished within postoperative neutrophils.

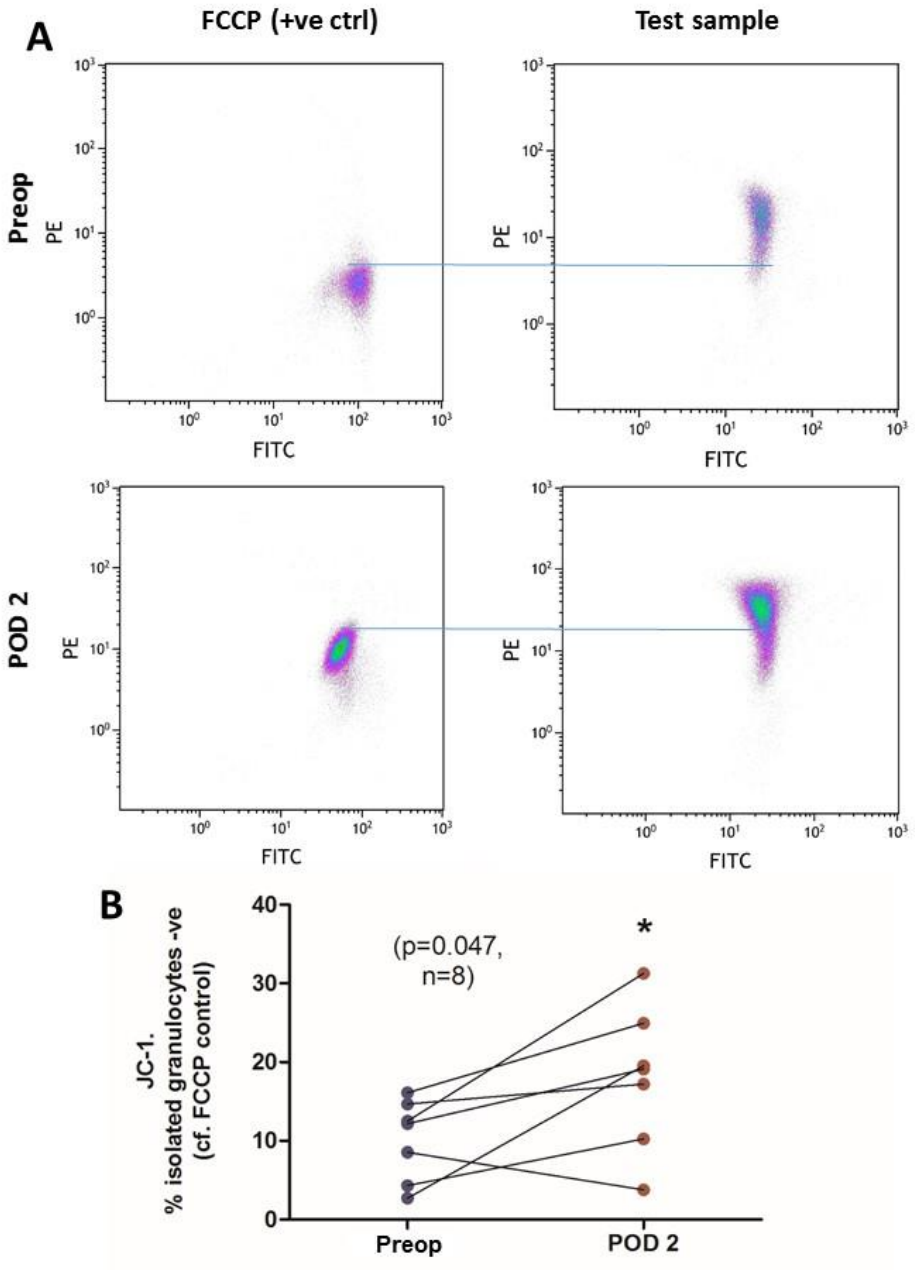

Figure 5.3 Perioperative resting isolated neutrophil mitochondrial membrane potential: JC-1 assay.

A Density plots demonstrating perioperative change in isolated neutrophil JC-1 fluorescence. FCCP 10µm used as positive control to generate 95% threshold for loss of mitochondrial membrane potential B Paired line diagram demonstrating population change in proportion of isolated neutrophils which have lost mitochondrial membrane potential, as determined by JC-1 fluorescence and comparison to individualised FCCP positive controls, Wilcoxon matched pairs test used.

There was no difference between matched preoperative and postoperative neutrophil Annexin-V and PI expression/uptake (Fig 5.4 A,B). These assays were performed immediately following neutrophil isolation. Neutrophil apoptosis is accompanied by a specific, and dramatic (80%+) downregulation of cell surface CD16 (Dransfield et al., 1994). Therefore, although chapter 4 identified an approximate 20% reduction in cell surface CD16, this is not a sufficient decrease to support the presence of apoptosis. Together these data suggest that there are no differences in the rate of neutrophil apoptosis on postoperative day two as preoperatively,

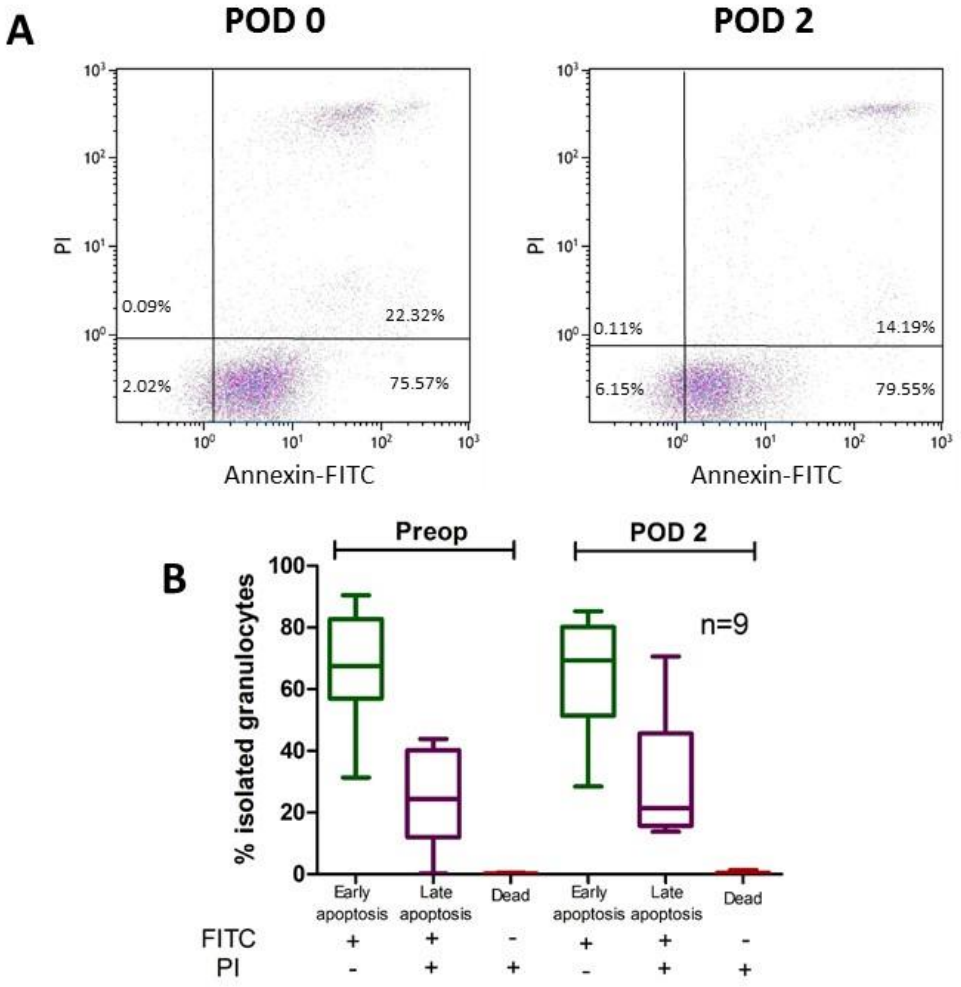

Figure 5.4 Perioperative isolated neutrophil apoptosis: Annexin-V assay.

All data are paired preoperative and postoperative samples. A Density plots demonstrating isolated neutrophil perioperative combined annexinV-FITC/PI staining. B Box whisker plots (median-IQR-max/min) demonstrating perioperative change in isolated neutrophil annexin V/PI expression, overall p-value for one way ANOVA reported.

However, expression of the extrinsic apoptosis regulator protein Fas (CD95) was higher upon postoperative neutrophils (Fig 5.5 A,B). Engagement of Fas activates the extrinsic apoptotic pathway, and may also have synergistic effects in promotion of intrinsic apoptosis (Brown and Savill, 1999; Liles et al., 1996; Renshaw et al., 2000). Postoperative neutrophils may be more susceptible to extrinsic apoptotic signalling.

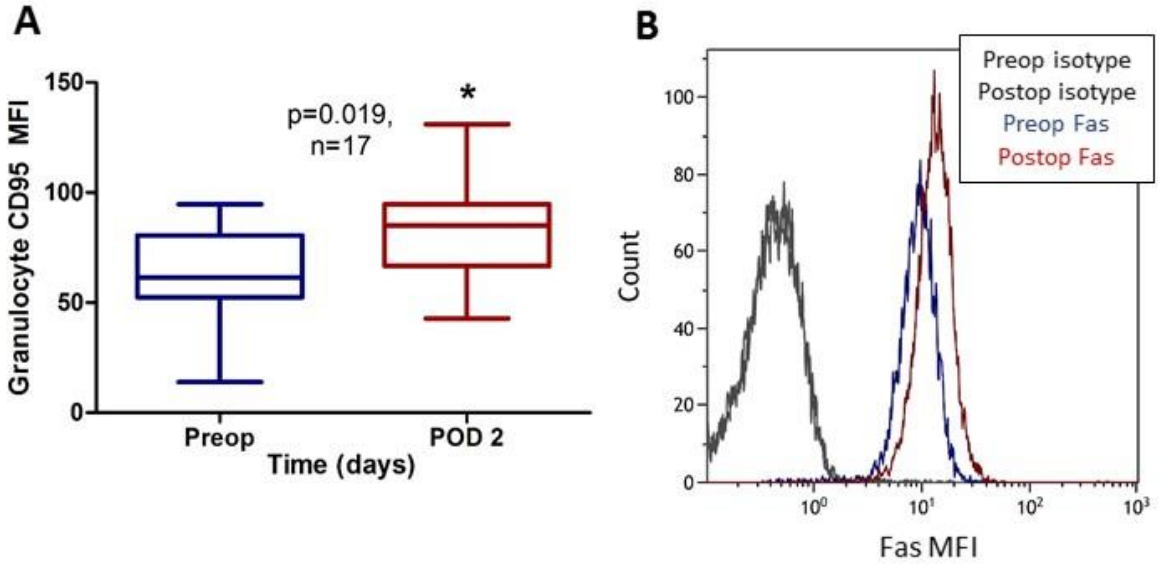

Figure 5.5 Perioperative neutrophil apoptosis: cell surface Fas (CD95).

A Population data demonstrating whole blood granulocyte cell surface CD95 expression, paired t-test used. B overlay plot demonstrating typical patient sample.

Collectively these data support the presence of postoperative neutrophil mitochondrial dysfunction, characterized by increased mitochondrial ROS and relatively hyperpolarised mitochondrial membrane potential within whole blood samples. By contrast isolated postoperative neutrophils display a loss of mitochondrial membrane potential, possibly because of an increased sensitivity to the isolation process. This study found no evidence to support the presence of increased neutrophil apoptosis on postoperative day two, however increased Fas receptor expression suggests these cells may be more sensitive to in-vivo FasL death receptor pathway signalling.

5.3.3 Model 1: Respiratory complex inhibition within human neutrophils

The data presented above supports the presence of mitochondrial oxidative stress within resting postoperative neutrophils. Within chapter 4, I demonstrated that activated postoperative neutrophils possess a diminished respiratory burst. Here, I test the hypothesis that resting oxidative stress is mechanistically responsible for the diminished respiratory burst within postoperative cells, using two different models of oxidative stress.

The principal sites of mitochondrial ROS production are believed to be respiratory complexes I and III (Chen et al., 2003; Lenaz et al., 2006). Previous investigators have demonstrated that the addition of low doses of inhibitors to respiratory complex I and III to neurones promotes mitochondrial hyperpolarisation and ROS generation (Forkink et al., 2014) - the same phenotype I identified within postoperative cells. Therefore, for the first model I incubated granulocytes with respiratory complex inhibitors and measured respiratory burst responses. An isolated neutrophil system was chosen for these experiments to ensure the ratio of cells to inhibitor was constant.

5.3.2.1 Respiratory complex I inhibition: Rotenone.

Incubation of isolated preoperative neutrophils with rotenone 10μM for thirty minutes did not increase resting cell rhodamine-123 fluorescence (Fig 5.6 A) but there was a trend for increased resting MitoSOX-Red fluorescence (Fig 5.6 B,C). Rotenone increased the PMA activated respiratory burst (Fig 5.6 D,E). Rotenone 10μM did not alter expression of Annexin-V or CD16 in isolated preoperative neutrophils (data not shown).

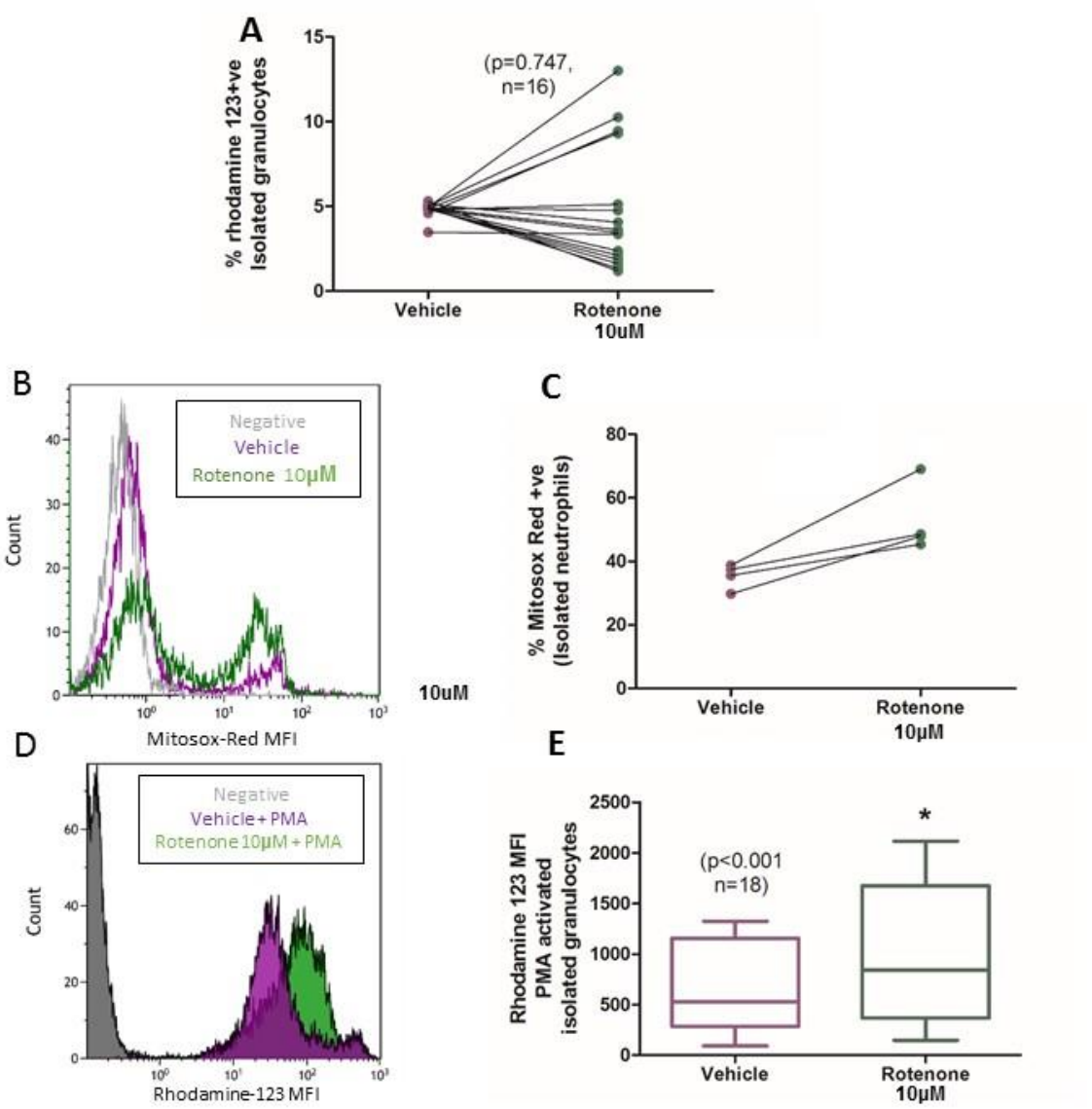

Figure 5.6 The effect of respiratory complex I inhibition with Rotenone 10µM upon isolated neutrophil PMA respiratory burst.

All rotenone incubations performed with rotenone 10µM, at 37°C for 30minutes. Negative samples are unstained, without MitoSOX Red. Vehicle and rotenone samples are with MitoSOX Red. Wilcoxon matched pairs test used throughout. A Paired line diagram demonstrating change in proportion of resting neutrophils generating Rhodamine-123 following addition of Rotenone. B C Overlay plot / paired line diagram demonstrating typical sample / population response of MitoSOX-Red fluorescence following addition of Rotenone D E Overlay plot / Box-whisker (median-IQR-max/min) demonstrating typical response / population response in PMA-activated respiratory burst (Rhodamine-123 fluorescence) after preincubation with Rotenone.

Pre-incubation with rotenone increased the preoperative PMA respiratory burst in every patient, however it was reduced for one patient postoperatively (Fig 5.7)

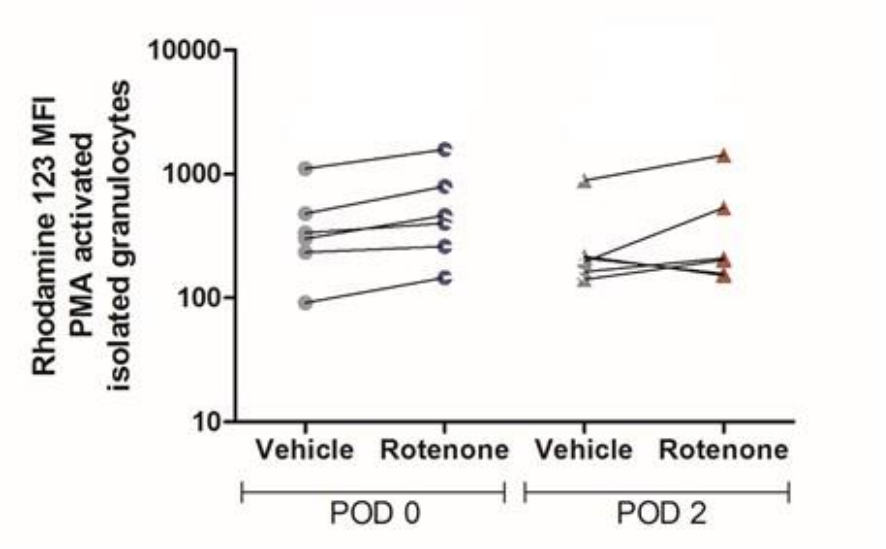

Figure 5.7 Perioperative change in isolated neutrophil PMA stimulated respiratory burst following incubation with rotenone 10µM.

Isolated neutrophils were incubated with rotenone 10µM for 30 minutes prior to respiratory burst assay.

5.3.2.2 Respiratory complex III inhibition: Myxothiazol and Antimycin A

Incubation of isolated resting neutrophils with Myxothiazol 10μM for 30mins increased Rhodamine (Fig 5.8 A,B) and MitoSOX-Red (Fig 5.8 C,D) derived fluorescence and was accompanied by a trend for reduced PMA stimulated Rhodamine fluorescence (Fig 5.8 E,F).

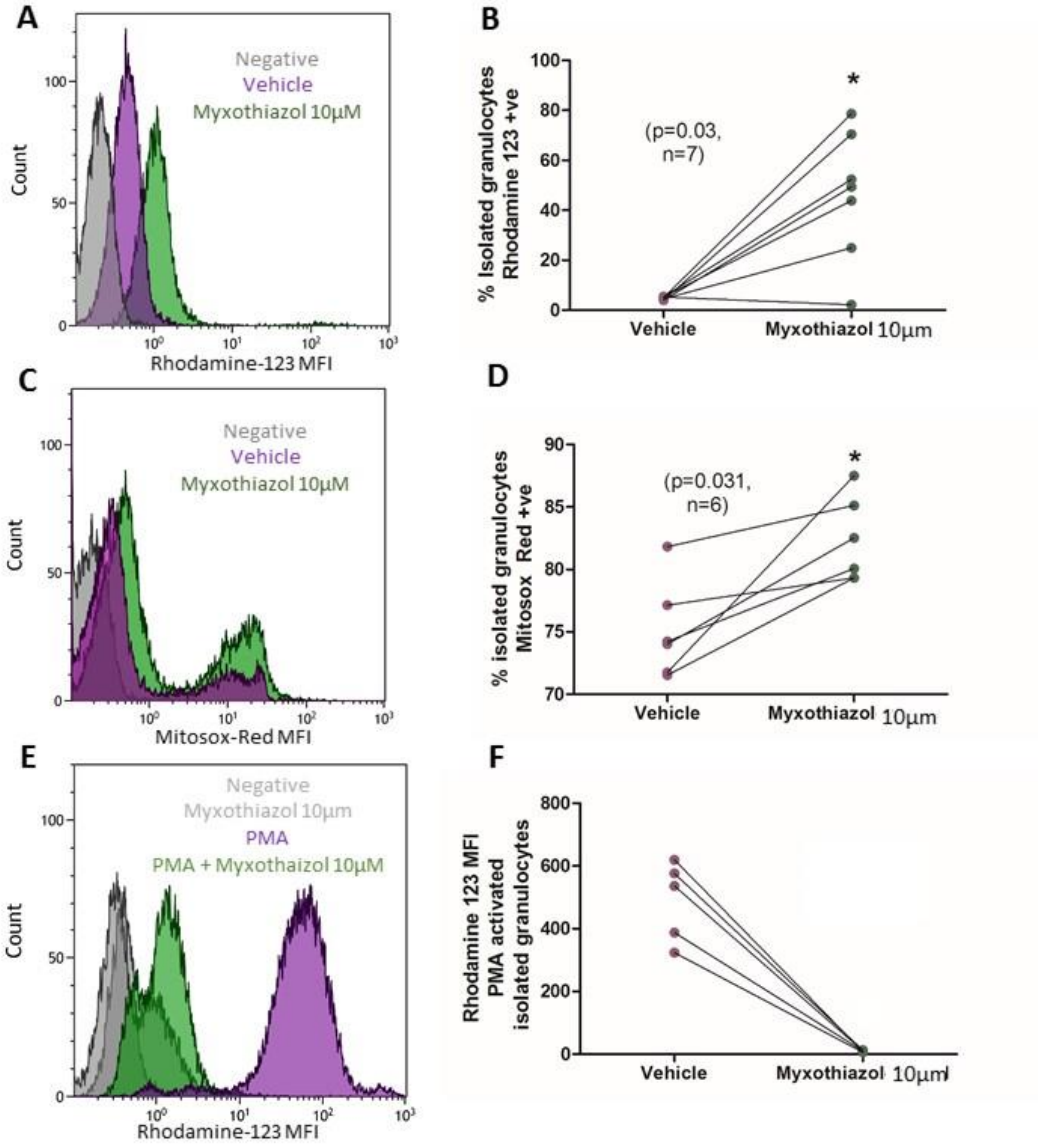

Figure 5.8 The effect of myxothiazol 10μM upon isolated neutrophil ROS.

All Myxothiazol incubations performed at 37°C for 30minutes. Wilcoxon matched pairs test used throughout. A B Overlay plot / paired line diagram demonstrating typical sample / population response of resting neutrophils Rhodamine-123 fluorescence following incubation with Myxothiazol. C D Overlay plot / paired line diagram demonstrating typical sample population response of resting neutrophils Mitosox-Red fluorescence following incubation with Myxothiazol. E F Overlay plot / paired line diagram demonstrating typical sample / population response of PMA stimulated neutrophil burst.

However, the addition of Myxothiazol 10μM to isolated neutrophils also promoted an approximately 80% down-regulation of cell surface CD16 (Fig 5.9 A,B) and a trend for increased Annexin binding (Fig 5.9 C-E). These data suggest that incubation of isolated neutrophils with Myxothiazol 10μM for 30 minutes induces apoptosis.

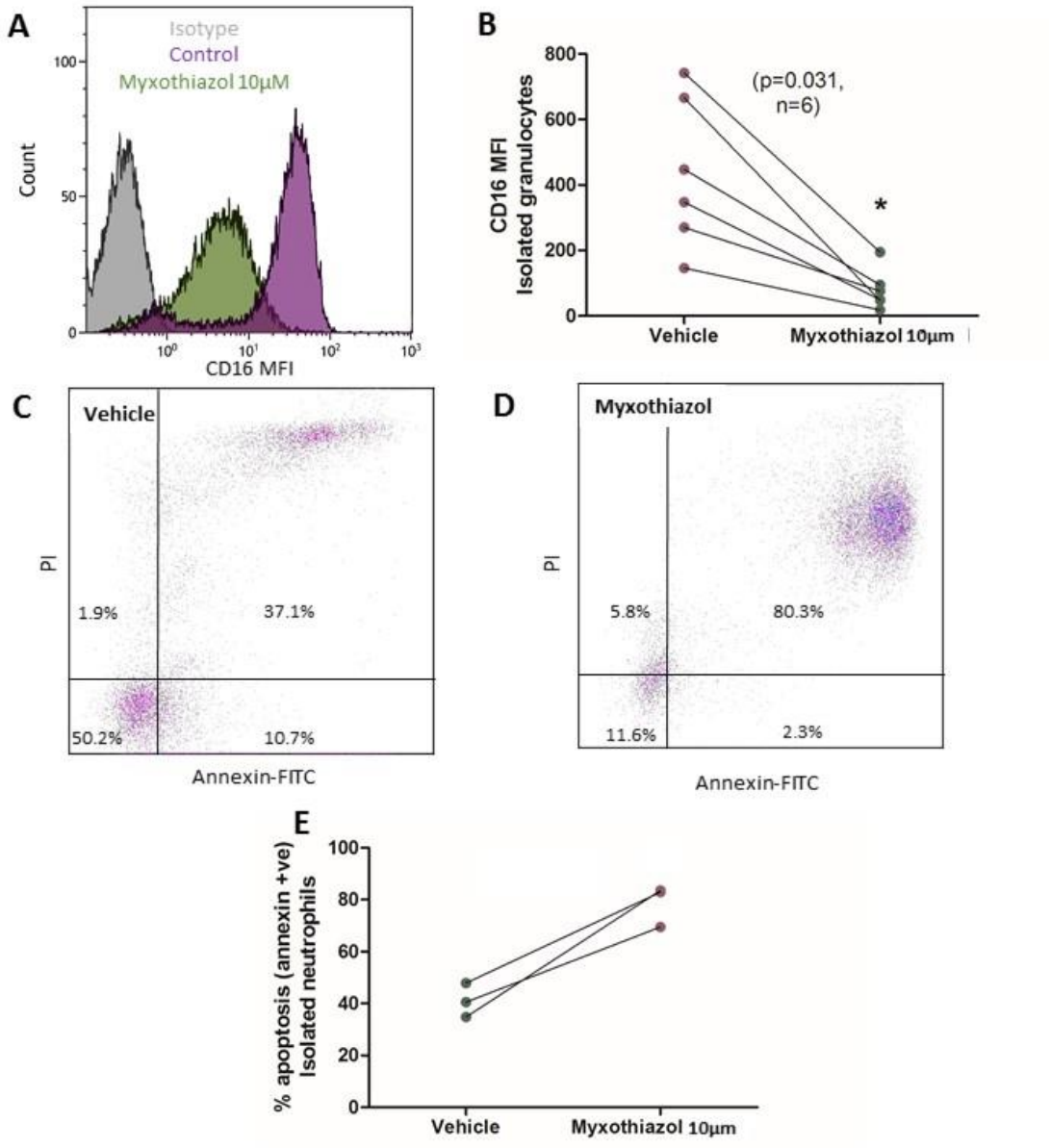

Figure 5.9 The effect of Myxothiazol 10μM upon isolated neutrophil apoptosis.

All Myxothiazol incubations performed at 37°C for 30 minutes. A B Overlay plot / Paired line diagram demonstrating typical sample / population change in neutrophil surface CD16 expression following incubation with Myxothiazol, Wilcoxon matched pairs test used C D E Density plots / paired line diagram demonstrating effect of Myxothiazol upon neutrophil apoptosis (Annexin-V positivity). Quadrant numbers represent percentage of gated population within each quadrant. F Paired line diagram demonstrating change in isolated neutrophil TMRM fluorescence following incubation with Myxothiazol.

Incubation of isolated neutrophils with a second respiratory complex III inhibitor - Antimycin-A 10μM - produced similar effects upon resting and PMA activated neutrophil ROS levels to Myxothiazol 10μM, with an increase in resting Rhodamine fluorescence (Fig 5.10 A,B), reduction in PMA stimulated Rhodamine fluorescence (Fig 5.10 C,D).

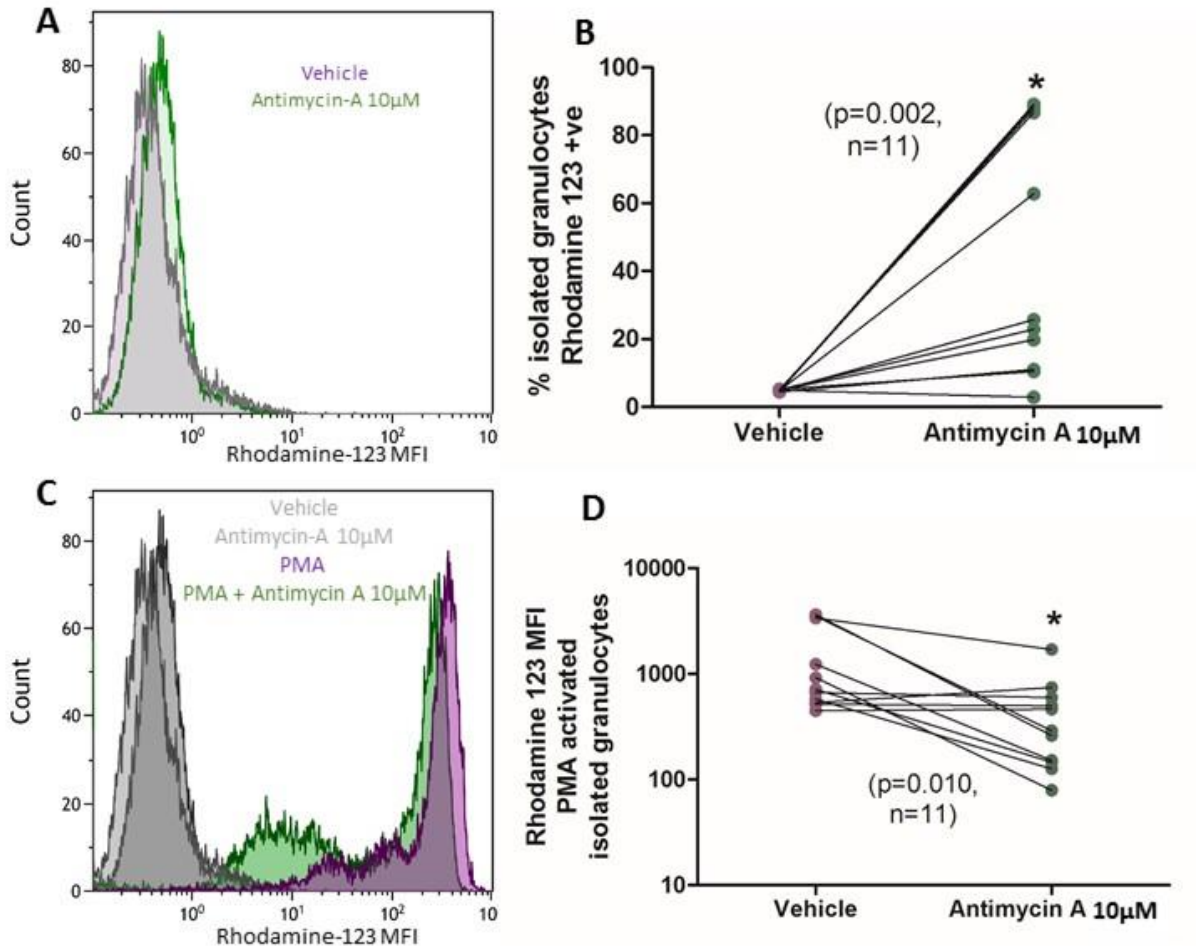

Figure 5.10 The effect of Antimycin A 10μM upon isolated neutrophil ROS.

All Antimycin A incubations performed at 37°C for 30minutes. Wilcoxon matched pairs test used throughout. A B Overlay plot / paired line diagram demonstrating typical sample / population response of resting neutrophils Rhodamine-123 fluorescence following incubation with Antimycin A. C D Overlay plot / paired line diagram demonstrating typical sample / population response of PMA stimulated neutrophil following preincubation with Antimycin A

Incubation of isolated neutrophils with Antimycin A consistently downregulated neutrophil cell surface CD16 (Fig 5.11 A,B), and tended to increase Annexin V binding (Fig5.11 C-E), suggesting than incubation with Antimycin A promotes resting isolated neutrophil apoptosis. However, the magnitude of CD16 downregulation and of annexin binding was less than previously with Myxothiazol (Fig 5.9).

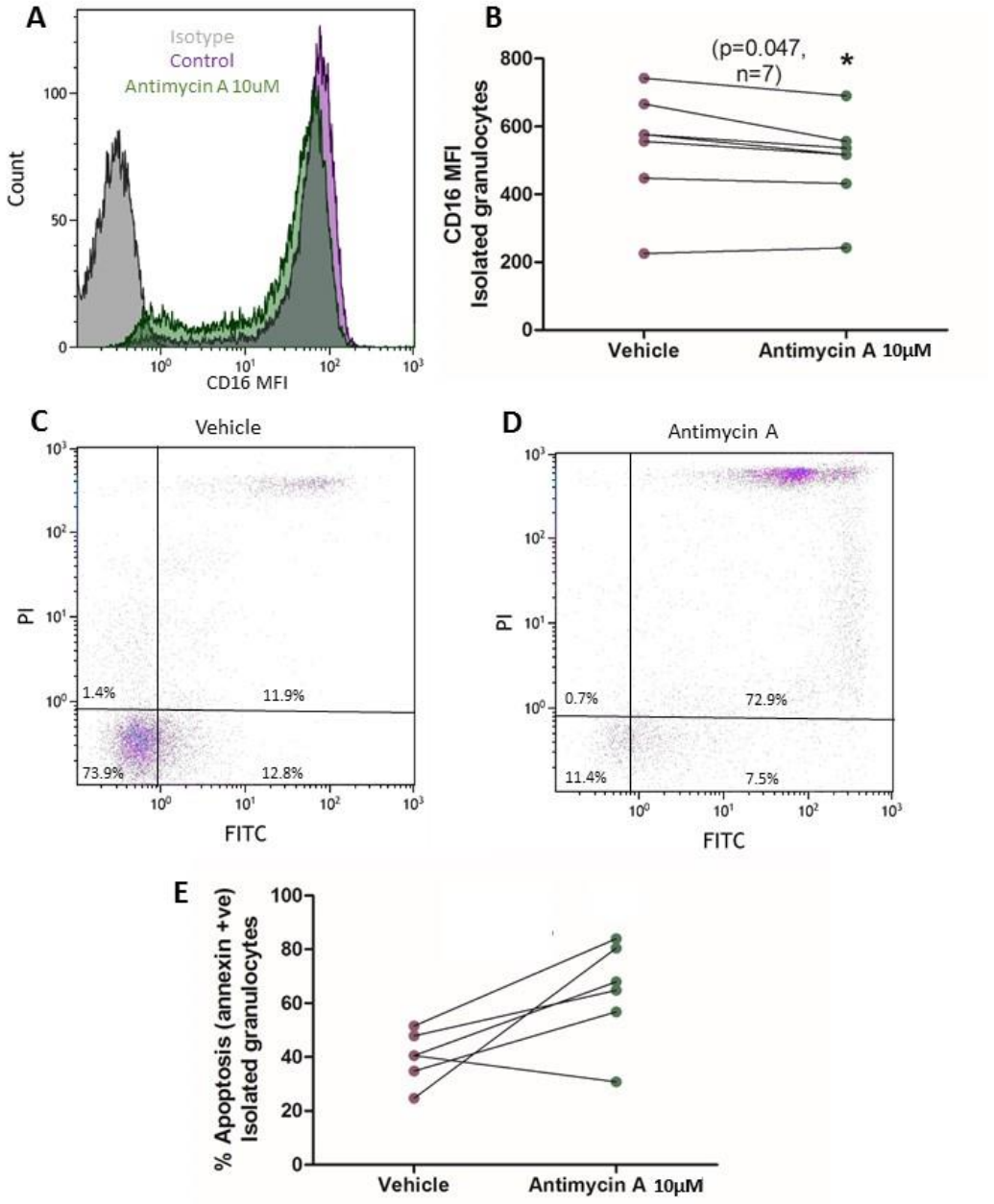

Figure 5.11 The effect of Antimycin A 10µM upon isolated neutrophil apoptosis.

All Antimycin A incubations performed at 37°C for 30minutes. A B Overlay plot / paired line diagram demonstrating typical sample / population change in neutrophil surface CD16 expression following incubation with antimycin A, Wilcoxon matched pairs test used. C D E Density plots / paired line diagram demonstrating effect of antimycin A upon neutrophil apoptosis (Annexin-V positivity).

Within other cell types, respiratory complex III inhibitors demonstrate a dose dependent effect upon mitochondria, with high doses causing lipid peroxidation with complex disaggregation and lower doses increasing the mitochondrial membrane potential while maintaining mitochondrial viability (Lenaz et al., 2006). Therefore, I repeated these experiments using Myxothiazol 1µM. Myxothiazol 1µM did not cause a measurable change in resting neutrophil Rhodamine fluorescence (Fig 5.12 A,B), however PMA stimulated Rhodamine fluorescence was increased (Fig 5.12 C,D), without any change in cell surface CD16 (Fig 5.12 E) or Annexin (Fig 5.12 F).

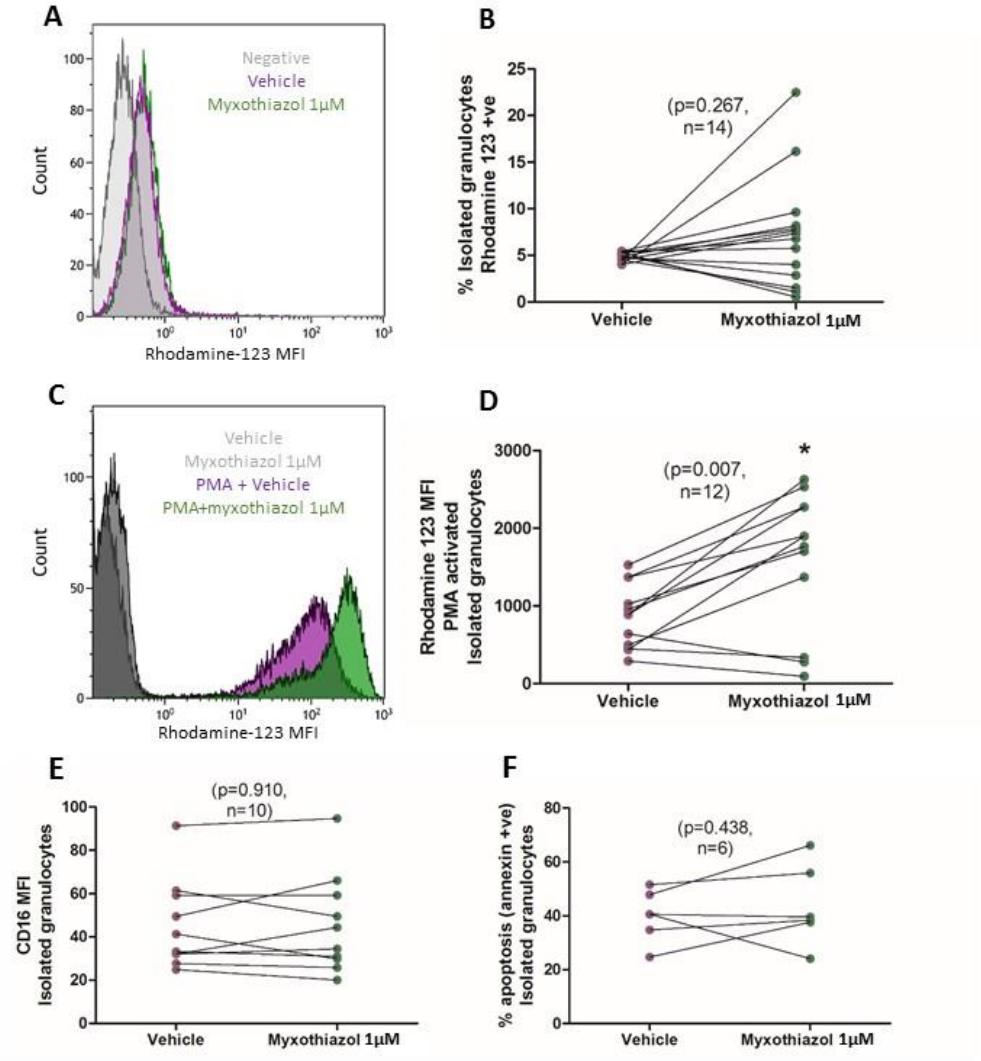

Figure 5.12 The effect of Myxothiazol 1µM upon isolated neutrophil ROS and apoptosis

All myxothiazol incubations performed at 37°C for 30 minutes. Wilcoxon matched pairs test used throughout. A B Overlay plot / paired line diagram demonstrating typical sample / population response of

resting neutrophils Rhodamine-123 fluorescence following incubation with Myxothiazol 1μM. C D Overlay plot / paired line diagram demonstrating typical sample / population response of PMA stimulated neutrophils following preincubation with myxothiazol 1μM. E Paired line diagram demonstrating population change in neutrophil surface CD16 expression following incubation with Myxothiazol. F Paired line diagram demonstrating effect of myxothiazol upon neutrophil apoptosis (Annexin-V positivity).

Having demonstrated differences between Myoxthiazol 1μM and 10μM upon resting and activated isolated neutrophil ROS levels following 30-minute incubation, I next tested the effect of incubation time for these Myxothiazol concentrations using a platereader (Fig 5.13) to permit sequential fluorescence measurements. At 1μM Myxothiazol did not increase resting cell DHE fluorescence, and did not affect PMA stimulated burst. At 10μM Myxothiazol tended to increase resting neutrophil dihydroethidium fluorescence, but reduced PMA stimulated respiratory burst fluorescence (Fig 5.8).

Therefore, myxothiazol may have different effects upon resting DHR and DHE fluorescence. 10μM myxothiazol may increase resting DHR and DHE fluorescence, however 1μM only increases resting DHE fluorescence.

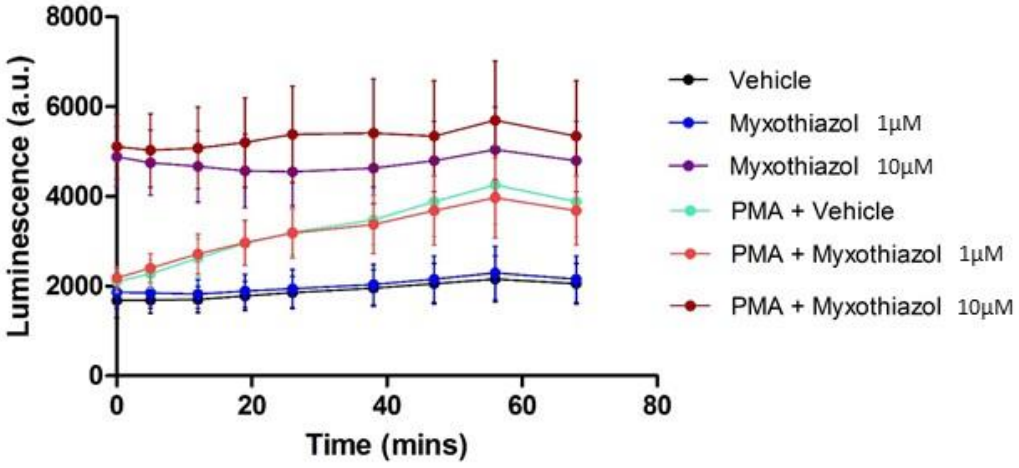

Figure 5.13 The effect of Myxothiazol 1uM and 10uM upon isolated resting and PMA stimulated neutrophil DHE fluorescence.

Two patient samples were used. Each condition was performed in triplicate at each time point. Graph represents mean +/- standard deviation.

5.3.4 Model 2: HL-60 cell line model

The data from model 1 describes how respiratory complex III inhibitors may increase resting neutrophil ROS and impair the PMA stimulated respiratory burst within healthy volunteer/preoperative cells – mimicking the postoperative neutrophil phenotype. Now, I again tested the hypothesis that mitochondrial ROS may impair the neutrophil respiratory burst within a new model, using the HL-60 promyelocyte cell line. DMSO-differentiated HL-60 cells have been widely employed as a model to explore neutrophil biology (Birnie, 1988). The addition of DMSO to undifferentiated promyelocyte precursors promotes their differentiation into neutrophil like cells over a 5-7 day period (Collins et al., 1979; Newburger et al., 1979). DMSO differentiated cells display diminished ATP turnover and an increased respiratory burst when compared to undifferentiated cells (Muranaka et al., 2005; Newburger et al., 1979). Therefore, correlation of HL-60 oxidative stress/respiratory burst profile permits further interrogation of the primary hypothesis that mitochondrial dysfunction (ROS) may regulate the neutrophil respiratory burst.

5.3.3.1 DMSO differentiated and undifferentiated HL-60 cells mimic the oxidative characteristics of preoperative and postoperative neutrophils respectively.

Resting undifferentiated HL60 cells displayed increased MitoSOX Red (Fig 5.14 A,B), Rhodamine-123 (Fig 5.14 C,D) and TMRM (Fig5.15 E,F) fluorescence. These data suggest the resting undifferentiated HL-60 cells contain more mitochondrial and cellular ROS than DMSO differentiated cells.

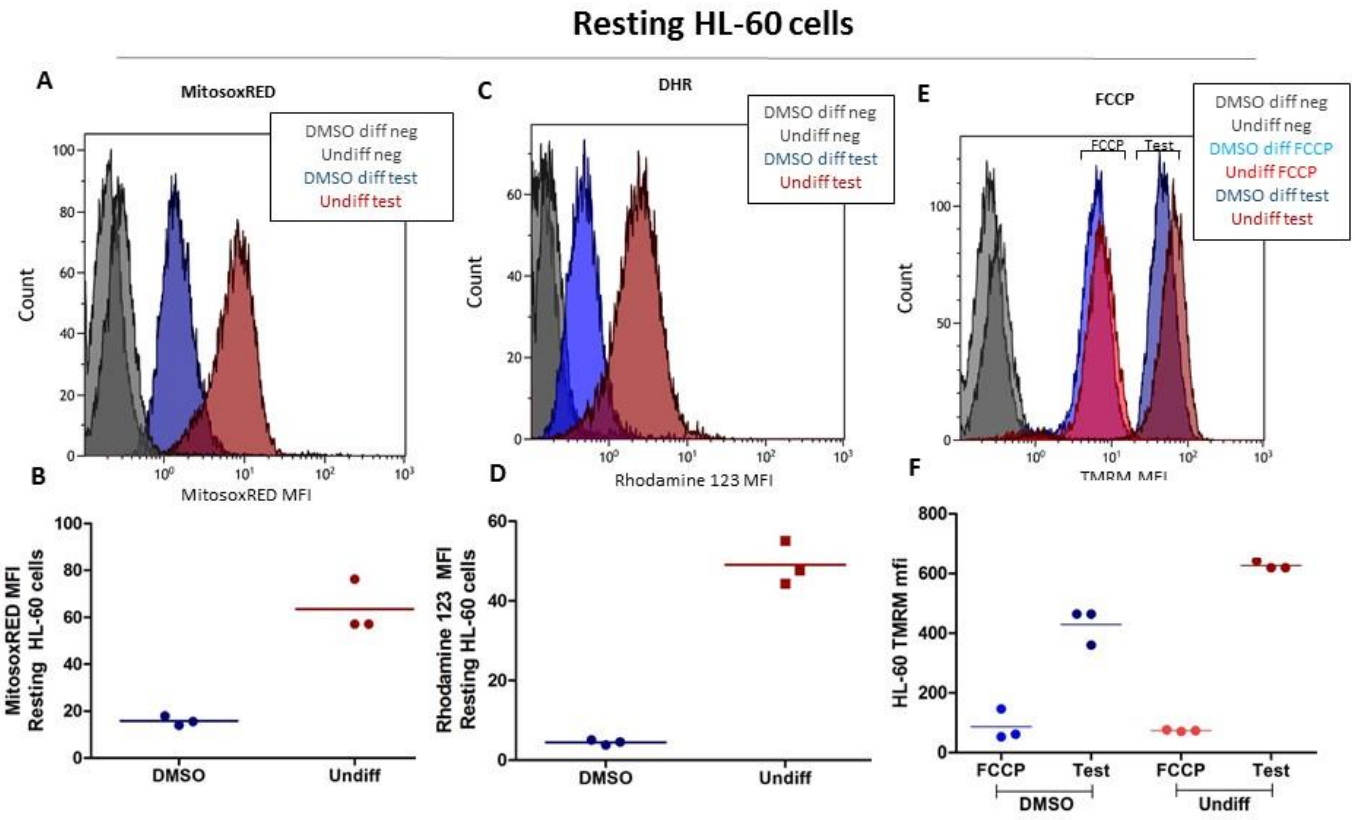

Figure 5.14 Resting HL-60 cell ROS characteristics.

Overlay plots demonstrate typical samples, dot plots population responses. A B MitoSOX Red fluorescence of resting HL-60 cells. C D Rhodamine fluorescence of resting HL-60 cells. E F TMRM fluorescence in resting HL-60 cells, with FCCP 10uM positive control samples.

PMA activation caused a reduction in DMSO differentiated MitoSOX Red fluorescence (Fig5.15 A,B), and an increase in cellular ROS production (Fig 5.15 C,D), that were not evident within undifferentiated cells. DMSO differentiated HL-60 cells and undifferentiated HL-60 cells mimic the resting ROS characteristics I identified within preoperative and postoperative neutrophils.

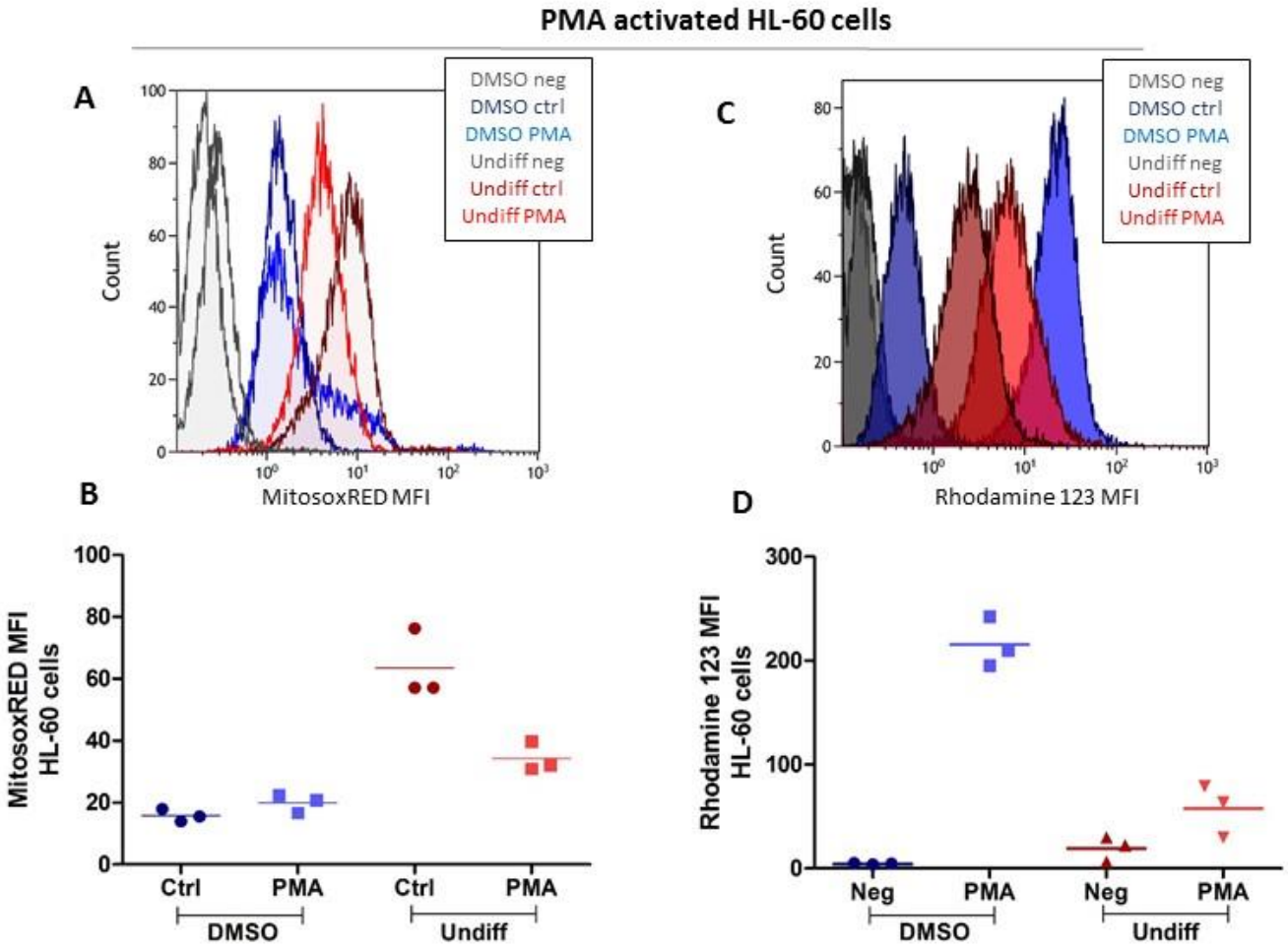

Figure 5.15 PMA activated HL-60 cell ROS characteristics

Overlay plots demonstrate typical samples, dot plots population responses. A B MitoSOX Red fluorescence of PMA stimulated HL-60 cells C D Rhodamine-123 fluorescence of PMA stimulated HL-60 cells

5.3.3.2 The addition of Myxothiazol 10μM to DMSO differentiated HL-60 cells increases resting cell ROS, but not the PMA stimulated respiratory burst.

Next, I sought to determine whether I could recreate the postoperative ROS phenotype within 'preoperative' DMSO differentiated cells by increasing resting ROS levels through the addition of Myxothiazol 10μM and promoting a decrease in activated DMSO differentiated respiratory burst.

Myxothiazol 10μM did not increase resting cellular ROS, but did increase PMA stimulated ROS (Fig 5.13 A,B) - similar to the addition of Myxothiazol 1uM to isolated human neutrophils (Fig 5.12). Therefore, I tested a higher dose of Myxothiazol, and found that the addition of 100μM Myxothiazol to DMSO differentiated HL-60 cells increased baseline ROS, but had no significant effect upon PMA activated cell ROS production (Fig 5.16 A,B). The addition of Myxothiazol to DMSO differentiated HL60 cells did not reproduce the postoperative neutrophil phenotype.

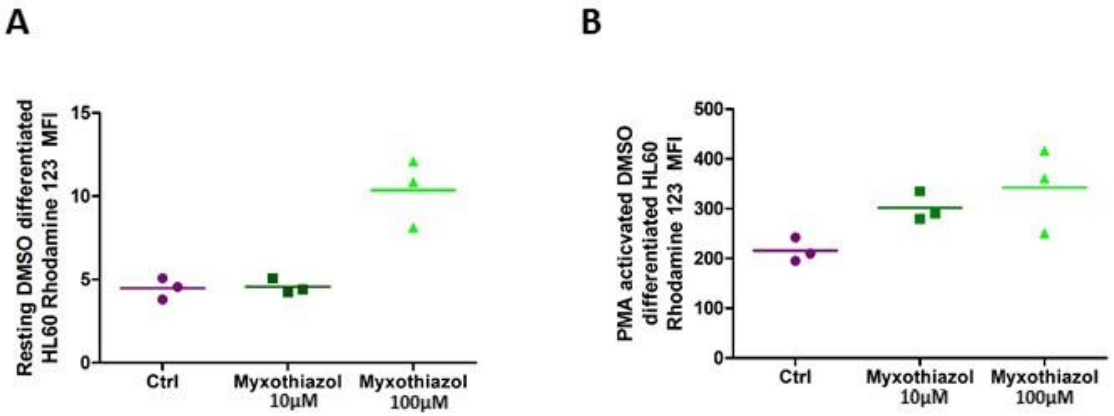

Figure 5.16 The effect of Myxothiazol upon resting and PMA stimulated DMSO differentiated ('preoperative') HL-60 cell ROS production.

Myxothiazol incubations were performed for 20mins, prior dihydrorhodamine assay as described above. A Resting DMSO differentiated HL60 cells. B PMA simulated DMSO differentiated HL60 cells.

5.3.3.3 Addition of the mitochondrial antioxidant MitoTEMPO to undifferentiated HL-60 cells does not increase the PMA stimulated respiratory burst

Next, I attempted to induce the preoperative ROS phenotype (low resting ROS, high activated ROS) within undifferentiated HL-60 cells (analogous to postoperative neutrophils), through the addition of the mitochondrial antioxidant MitoTEMPO.

Previous investigators have published MitoTEMPO incubation data at doses ranging from 25μM to 500μM and times ranging from 30mins to 240mins (Dikalov et al., 2014; Trnka et al., 2009). At lower dose range and incubation times I observed no effects upon undifferentiated HL-60 cell ROS production. Incubation of undifferentiated HL-60 cells with MitoTEMPO 500μM for 240 minutes tended to increase resting cell MitoSOX Red fluorescence (Fig 5.17 A,B), but there was no clear effect upon either resting cell Rhodamine fluorescence (Fig 5.17 C,D) or PMA activated Rhodamine fluorescence (Fig 5.17 E,F).

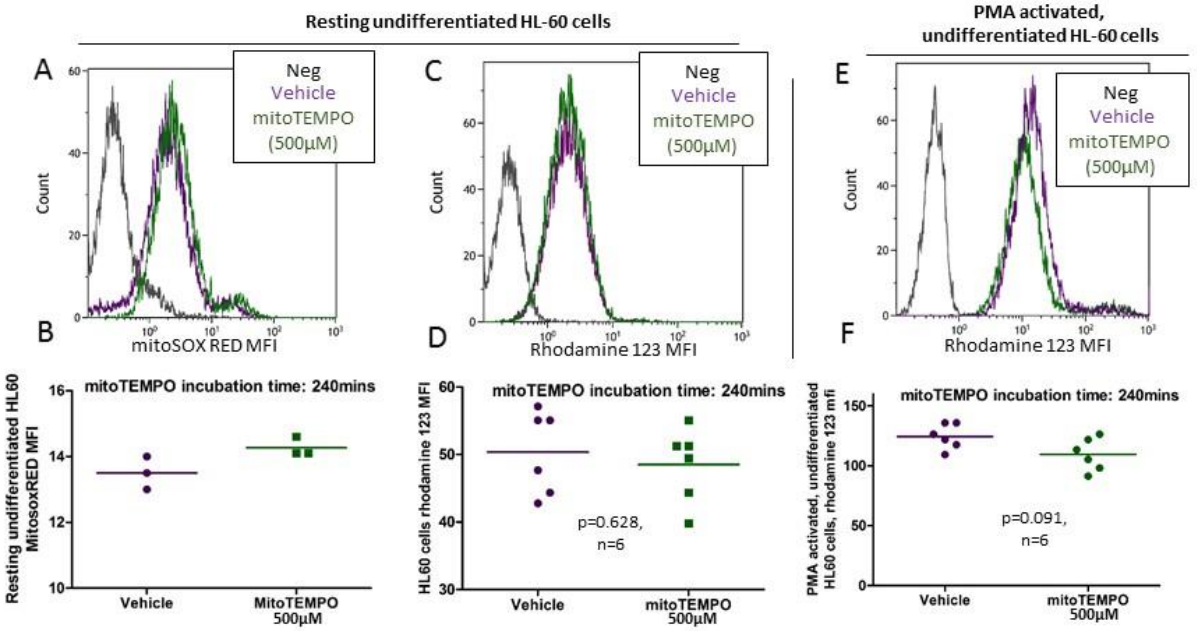

Figure 5.17 The effect of mitochondrial antioxidant mitoTEMPO upon resting and PMA stimulated undifferentiated ('postoperative') HL-60 cells.

Undifferentiated HL-60 cells were incubated with MitoTEMPO 500μM for 240minutes prior to addition of ROS probes or PMA activation for 10mins then subsequent addition of ROS probes. Overlay plots demonstrate typical samples, dot plots population responses. Mann-Whitney test where statistics used. A B MitoSOX Red fluorescence, resting cells. C D Rhodamine fluorescence, resting cells. E F Rhodamine fluorescence, PMA activated cells.

5.4 Discussion

5.4.1 Key findings

This study has made several findings which support the presence of dysfunctional mitochondria within postoperative neutrophils. Mitochondria within postoperative neutrophils display a raised mitochondrial membrane potential and produce increased amount of mitochondrial ROS. Two models were presented which each support a mechanistic association between elevated postoperative mitochondrial ROS and the impaired activated cell respiratory burst.

1. **Mitochondrial dysfunction is acquired within postoperative neutrophils.** Postoperative neutrophil mitochondria produce more ROS and have a hyperpolarised membrane potential when compared to preoperative cells. Together, these observations suggest that damaged mitochondria persist within postoperative cells, and that a distal impairment of oxidative phosphorylation may be preventing the return of hydrogen ions to the matrix. These findings are consistent with dysfunctional mitochondria that release ROS, impair cellular function and may ultimately promote apoptosis and/or inflammation.

   An association between raised membrane potential and increased ROS production has been previously identified within neurones. Although an increase in mitochondrial membrane potential maybe plausibly associated with an increase in ROS production, there are other possible sources of ROS production within postoperative neutrophils, such as endoplasmic reticulum or PHOX activity within partially activated cells (Balaban et al., 2005; Landmesser et al., 2003; Sevier and Kaiser, 2008).

2. **The proportion of neutrophils undergoing (Annexin-V positive) apoptosis is similar in isolated preoperative and postoperative preparations.** Total apoptosis and overt necrosis were unchanged within postoperative samples when compared to paired preoperative controls. Reported rates of in vitro neutrophil

apoptosis widely between different laboratories (Luo and Loison, 2008), e.g. 10 to 75% at 6hours (Blomgran et al., 2007; Cowburn et al., 2005). A previous perioperative study identified an increased rate of neutrophil apoptosis at 12hrs (Delogu et al., 2001a) and decreased at 24hrs (Fanning et al., 1999b), but neither reported on postoperative day two. FasL was upregulated on postoperative cells. As well as mediating extrinsic apoptosis, there is evidence for cross talk between FasL and neutrophil constitutive apoptosis (Brown, 1999; Liles et al., 1996; Renshaw et al., 2000) and may directly promote mitochondrial dysfunction (Watson et al., 1999).

In contrast to whole blood samples, isolated neutrophils demonstrated a postoperative loss of mitochondrial membrane potential - an established measure of apoptosis - suggesting that postoperative cells may be more likely to enter apoptosis under physiologically stressful conditions.

3. **Two different models of neutrophil mitochondrial dysfunction support a mechanistic relationship between resting ROS levels and respiratory burst function:**

   a. **Pharmacological inhibition of respiratory complex III.** Myxothiazol is a competitive inhibitor of ubiquinol, and blocks electron transfer to the Rieske protein of complex III (Thierbach and Reichenbach, 1981). Antimycin binds to the Qi site of cytochrome reductase within complex III (Bolgunas et al., 2006). Both Myxothiazol and Antimycin increased resting neutrophil ROS and decreased the PMA activated respiratory burst. The magnitude of respiratory burst depression and cell surface CD16 expression which followed incubation of preoperative neutrophils with Antimycin 10μM was similar to that found within freshly isolated postoperative neutrophils. Myxothiazol 10μM produced greater depressions in PMA stimulated respiratory burst and CD16 expression than was seen in postoperative neutrophils. Both Antimycin A 10μM and Myxothiazol 10μM caused an increased rate of neutrophil apoptosis when compared to resting postoperative cells.

Conversely, lower doses of respiratory complex III inhibitors and complex I inhibition augmented/primed the neutrophil respiratory burst. These findings are consistent with previously reported effects of rotenone 10μM and Myxothiazol 10μM upon lymphoblast respiratory burst (Dikalov et al., 2014) and Oligomycin 1ug/ml upon neutrophil burst (Fossati et al., 2003). This observation supports the presence of decreased complex I responsiveness within postoperative cells, and may be mechanistically relevant to the decreased postoperative burst. The priming effect of Rotenone was not apparent within postoperative neutrophils, which is compatible with inhibition of the respiratory burst within preoperative neutrophils by higher dose complex III inhibition.

b. **DMSO differentiated and undifferentiated HL-60 cells parallel the resting and PMA stimulated oxidative profiles of preoperative and postoperative neutrophils respectively.** DMSO differentiated HL60 cells demonstrate lower resting cellular ROS production with higher PMA stimulated ROS production. Conversely, undifferentiated HL60 cells demonstrate higher resting cellular ROS production, but lower PMA stimulated ROS production. The addition of Myxothiazol to DMSO differentiated HL60 cells and MitoTEMPO to undifferentiated cells did not mimic the postoperative and preoperative neutrophil phenotypes respectively.

5.4.2 Interpretation of key findings

These data describe several related differences between preoperative and postoperative neutrophil mitochondrial function. Together, these observations support the presence of dysfunctional mitochondria within postoperative neutrophils and furthermore, are consistent with a mechanistic relationship between resting neutrophil ROS and the respiratory burst.

The methodological advantages presented by the study of surgical patients allowed carefully timed postoperative samples to be taken and compared to individualised control results. The only previous investigation of postoperative neutrophil mitochondrial function used a single probe ($DiOC_6$) within isolated cells, and reported an increased rate of apoptosis at 12

hours post insult (Delogu et al., 2001) which had fallen at 24 hours (Fanning et al., 1999), neither sampled at 48 hours post insult.

Previously, the mechanistic impact of mitochondrial ROS upon neutrophil inflammatory function has only been described in terms of NF-kB stabilisation and cytokine expression, not actual immune effector mechanisms (Zmijewski et al., 2009, 2008). The interrogation of effector mechanisms does not necessarily reflect changes observed in alternative markers of inflammation do not necessarily correlate with those of neutrophil effector function [Chapter 4].

The production of mitochondrial ROS following hyperpolarisation of mitochondrial membrane potential has been described before within neurones, and was associated with diminished ATP production and H^+ utilisation (Abramov et al., 2005, 2004). Alternative explanations for the increase in mitochondrial ROS would be an increase in flux through oxidative phosphorylation or impaired ROS detoxification capacity, including reduced mitochondrial superoxide dismutase, cytoplasmic catalase or glutathione peroxidase (Hellmich et al., 2005)(Fosslien, 2003; Hellmich et al., 2005; Lebovitz et al., 1996). However, none of these alternative explanations provides a unifying explanation for all the experimental observations I have made, which the presence of postoperative mitochondrial dysfunction does. Glutathione peroxidase requires NADPH, therefore it is possible that impaired hexose monophosphate shunt may promote both an increase in resting ROS levels and an impaired respiratory burst within activated cells.

The differences between whole blood and isolated data – where isolated data showed loss of postoperative mitochondrial membrane potential – may be explained by an increased sensitivity of postoperative cells to the isolation process. This sensitivity may be a manifestation of limited postoperative bioenergetic reserve, thereby limiting effector function (explored further within chapter 6).

The increase in PMA stimulated respiratory burst following addition of Rotenone 10μM or Myxothiazol 1μM is supported by previous work which revealed similar increases in PMA burst responses following the direct addition of H_2O_2 (El Jamali et al., 2010) or Oligomycin (Fossati et al., 2003) to neutrophils, and Rotenone or Myxothiazol to lymphoblasts (Dikalov et al., 2012). However, other investigators have demonstrated that the incubation of neutrophils with complex I and III inhibitors causes diminished NF-kB signalling and cytokine

production (Zmijewski et al., 2009, 2008). Hypoxia has been shown to inhibit the respiratory burst and killing of S. aureus in human neutrophils (McGovern et al., 2011). The addition of rotenone 10μM delivered measurable increases in resting neutrophil mitochondrial superoxide production (MitoSOX Red), but not overall cellular ROS (Rhodamine-123), supporting a mechanistic role for mitochondrial ROS.

The reduction in postoperative respiratory burst following the addition of the complex I inhibitor Rotenone suggests that complex I may act as a signal transducer during the respiratory burst, and toward a loss of complex I responsiveness postoperatively. Diminished mitochondrial complex I function has been previously described within septic peripheral blood mononuclear cells (Garrabou et al., 2012).

Conversely, the reduction in preoperative respiratory burst following the addition of complex III inhibitors Antimycin A 10μM and Myxothiazol 10μM suggests that loss of complex III function may mediate the observed postoperative mitochondrial dysfunction. Since complex III is the principal source of mitochondrial ROS, distal inhibition of oxidative phosphorylation (e.g. absence of terminal electron recipient oxygen) will lead to liberation of electrons at complex III, and therefore ROS formation. Beyond its role in oxidative phosphorylation, complex III is critical for wider range of cellular functions that effectively function independently of oxidative phosphorylation, such as oxygen tension sensing at complex III and initiation of HIF signalling (Brunelle et al., 2005; Klimova and Chandel, 2008).

Although HIF was first identified as a mediator of hypoxic inflammatory signalling, it is now understood to be activated during many other inflammatory insults (Klimova and Chandel, 2008), which may or may not be dependent upon mitochondrial ROS. Within other cell types HIF signalling and AMPK regulate the interaction between mitophagy and glycolysis. Cellular AMP accumulates with bioenergetic stress and is detected by AMPK, which in synergy with growth signal integrating mTOR (Papandreou et al., 2006; Semenza et al., 1994; Zhang et al., 2007) regulates mitochondrial biogenesis (Jäger et al., 2007). HIF-1 regulates cellular oxygen consumption, in part through the promotion of glycolysis and inhibition of mitochondrial biogenesis and function. Healthy volunteer endotoxin experiments reveal a reduction in leukocyte ATP that is associated with simultaneous decline in AMPK and increase in autophagy (Haimovich et al., 2014; Z. Zhang et al., 2010)) at very low doses of endotoxin that are insufficient to cause a SIRS response.

These two models support a mechanistic role for mitochondrial ROS in the regulation of the respiratory burst. ROS are not only injurious, but at lower concentrations mediate signal transduction for a diverse range of cellular functions, where specific redox reactions within target proteins causes a change in shape and function (Dröge, 2002). There are many possible targets which could impact upon the respiratory burst function beyond HIF signalling e.g. NADPH oxidase assembly and function, inflammatory cytokine binding (Davidson et al., 2013; Segal et al., 2010), or regulation of associated cellular processes such as NADPH production. Alternatively, the increase in mitochondrial ROS may not be directly related and could be on feature of a wider mitochondrial impairment. For example, maintenance of mitochondrial membrane potential is necessary for calcium/potassium transport and calcium homeostasis (Graier et al., 2007), and respiratory burst is a calcium dependent process. Calcium flux through the VDAC family of mitochondrial membrane proteins is critical to ROS production, and inhibition of VDAC causes a reduction in mitochondrial ROS (Colombini, 2004).

While human neutrophils are traditionally considered to be solely glycolytic, the experiments which determined this were all performed within healthy, non-inflamed, human subjects. Perhaps the bioenergetic profiles of neutrophils during established inflammatory responses differ to healthy circulating counterparts. Indeed, some of these studies have reported a change in neutrophil ATP balance following the addition of respiratory complex inhibitors (Minakami, 1968; Sbarra and Karnovsky, 1959). Therefore, it is possible that mitochondrial dysfunction may impair postoperative neutrophil immune function through by limiting the supply of ATP.

5.4.3 Strengths and weaknesses

Comorbid disease, age and therapeutic interventions are associated with altered mitochondrial function, and may confound experimental observations without the selection of an appropriately matched control population. This study used patients matched preoperative and postoperative samples, so that each test sample could be compared to its own, individualised preoperative control sample. Theoretically this allows the effect of the experimental insult – surgery and anaesthesia – to be individually isolated from baseline

variations in immune function. However, it remains possible that specific comorbidities or medications may interact with perioperative insult differently, therefore it is not possible to expand the results of individual assays to the wider surgical population. Since different assays were performed in different patients, it is not possible to generalise observations into a universal postoperative phenotype. Samples were taken within the same postoperative window that was associated with impairment of neutrophil function described in Chapter 4.

Assays of mitochondrial function were performed in whole blood whenever possible to minimise processing artefacts. The fluorescent probes used have been extensively characterised by other investigators, and are generally agreed to be the most specific available. Visualisation with confocal microscopy would identify the specific subcellular location of fluorescent signals, and may help determine whether ROS signals were coming from mitochondria or other cytoplasmic sources, such as resting NOX/PHOX activity.

The two in vitro models of oxidative stress both support the hypothesis for mitochondrial ROS regulating the postoperative neutrophil phenotype, with each demonstrating an increase in baseline ROS with a decrease in the respiratory burst/activated cell ROS production, through different mitochondrial insults. Pharmacological inhibition of respiratory complex III within preoperative cells profoundly inhibits the respiratory burst. The redox characteristics of DMSO differentiated and undifferentiated HL-60 cells mirror those of postoperative and preoperative human neutrophils respectively.

Taken together, these models reproduce the redox relationship between resting and activated/respiratory burst neutrophils to that found in perioperative samples. Specific limitations of each model should be considered. Mitochondrial inhibitor experiments could only be incubated for short periods of time (30 minutes), due to the fragility of isolated incubated neutrophils – the rate of apoptosis within freshly isolated neutrophils was 70% at this time point. The transition of priming to depression of the respiratory burst as Myxothiazol dose increased from 1uM to 10uM suggests a critical dose exists, beyond which ROS scavenging mechanisms become overwhelmed and lipid peroxidation ensues with ROS amplification. While undifferentiated HL60 cells have been shown to contain functional PHOX machinery (Muranaka et al., 2005), it is possible that the quantity of PHOX present is very much less in undifferentiated as compared to DMSO differentiated cells, and that this is responsible for the reduced burst responses, rather than tonic activity of resting cellular ROS levels.

Further corroborative evidence could not be provided through manipulation of HL-60 ROS levels – attempts to augment and scavenge HL-60 ROS and alter respiratory burst function produced opposite results to those expected. The addition of MitoTEMPO increased baseline mitochondrial ROS in undifferentiated cells, which may possibly be explained by complex interactions between oxidative and reductive complexes with MitoTEMPO, or by non-specific cellular location or molecular targets of the probes and antioxidants used.

5.4.4 Conclusions

1. Whole blood postoperative neutrophil mitochondria possess a hyperpolarised mitochondrial membrane potential and have greater levels of mitochondrial ROS than matched preoperative samples.

2. Increased resting mitochondrial ROS may impair the neutrophil respiratory burst.

Chapter 6 – Neutrophil pyruvate kinase and perioperative respiratory burst

6.1 Introduction

Investigations into the regulation of the neutrophil respiratory burst have largely focussed upon the phosphorylation and assembly of PHOX subunits (Segal, 2005). However, substrate level control is also plausible because of the dramatic changes in neutrophil metabolism which accompany the respiratory burst. Pyruvate kinase, a rate limiting step of neutrophil glycolysis (Fauth et al., 1993), is upregulated within neutrophils during inflammatory responses (Oehler et al., 2000) and the isoform expressed within human neutrophils is subject to redox regulation (Kechemir et al., 1989). Having demonstrated a plausible relationship between resting neutrophil ROS and activated neutrophil respiratory burst within Chapter 5, Chapter 6 will explore whether oxidative inhibition of the glycolytic enzyme pyruvate kinase regulates the human postoperative respiratory burst by limiting the supply of NADPH substrate

Granulocytes may utilise both exogenous glucose and intracellular glycogen (Weisdorf et al., 1982). Neutrophil activation promotes glucose uptake by MAP Kinase/HIF-α driven translocation Glucose Transport 1 (GLUT1) – responsible for passive/constitutive uptake of glucose (Carruthers, 1990) to the cell surface (Schuster et al., 2007), while exogenous glucose, up to a plateau of 10mmol/L, increases both the PMA respiratory burst and lactate production (Oehler et al., 2000; Schuster et al., 2007; Tan et al., 1998).

The principal substrate of respiratory burst is NADPH, synthesised through the hexose monophosphate shunt (Figure 1.4). G6PDH regulates the entry of glucose substrate into the hexose monophosphate shunt, and within murine stem cells G6PDH activity is regulated by NADPH:NAD (Filosa et al., 2003). The G6PDH inhibitor dehydroepiandrosterone abolishes the PMA stimulated respiratory burst plus associated glucose consumption (Bender and Van Epps, 1985; Oehler et al., 2000), but leaves other processes such as chemotaxis unaffected (Bender and Van Epps, 1985). Neutrophils have a six-fold higher expression of glucose-6-phosphate dehydrogenase than lymphocytes (Beck, 1958).

The hexose monophosphate shunt competes directly with glycolysis for glucose-6-phosphate (Fig 1.3). Fluorometric analysis of glycolytic mass action ratios within human and guinea pig neutrophils has identified that the enzymes hexokinase, phosphofructokinase and pyruvate kinase are the rate limiting steps (Beck, 1958; Minakami, 1968). These guinea pig neutrophils were harvested from a chemical peritonitis model, meaning they are primed/activated cells and may therefore be of greater relevance to human postoperative

neutrophil investigations. By contrast, many studies into neutrophil metabolic control have been performed within animal and cell line models which do not address the changes that occur during established inflammation.

Several human genomic investigations of human leukocytes have identified a rapid change in expression of genes related to metabolic processes, with upregulation of glycolytic and downregulation of mitochondrial genes (Fessler et al., 2002; Malcolm et al., 2003; Zhang et al., 2004). Furthermore, the addition of low doses of endotoxin to human volunteers promotes cellular metabolic changes without an increase in circulating cytokine levels, underlining the importance of metabolic reprogramming during inflammation (Haimovich et al., 2014). Within a series of trauma patients an increase in respiratory burst from day 1 which peaked at day 5 was accompanied by similar increases in pyruvate kinase protein expression and activity (Oehler et al., 2000). Collectively these genomic, proteomic and metabolic investigations suggest that pyruvate kinase is upregulated during pro-inflammatory responses, and is a rate limiting step in the formation of NADPH (Fig 1.4).

Pyruvate kinase is composed of up to four subunits which combine to form three distinct isoforms and whose expression is tissue specific. The different isoforms share several regulatory mechanisms, including the phosphorylation and allosteric modulation of subunit shape that determines the transition from inactive dimers to active heterodimers. For example, binding of fructose 1,6 bisphosphate, to a series of tyrosine (tyr) residues favours heterodimer formation ('feed forward regulation') (Mazurek et al., 2005), while phosphorylation of tyr105 prevents fructose 1,6 bisphosphate from binding (Hitosugi et al., 2009). Conversely, ATP acts as a negative allosteric regulator. The tetramer must disassemble for subunits to alter their function.

In addition to these shared regulatory mechanisms of all pyruvate kinase isoforms the PKM2 isoform of pyruvate kinase is also sensitive to oxidative regulation. Diverse types of cancer cells share a common metabolic phenotype termed the Warburg effect, where glycolytic activity is increased and mitochondrial oxidative phosphorylation depressed. This metabolic phenotype maintains ATP production while simultaneously increasing flow to the hexose monophosphate shunt, generating NADPH for glutathione antioxidant pathway, and ribose sugars for cell cycle progression / anabolism. PKM2 has also been identified in neutrophils (Kechemir et al., 1989).

Physiological concentrations of specific oxidative products – including O_2^- and H_2O_2 – may interact with a host of cellular signalling cascades and metabolic processes (Dröge, 2002; Janssen-Heininger et al., 2008; Jones, 2008). Cysteine residues are particularly susceptible to oxidation, and dimerise via a disulphide bridge to form cystine (McDonagh et al., 2009) (Fig1.2). PKM2 is inhibited by oxidation at cys358 within the catalytic site, which diverts glucose-6-phosphate to hexose monophosphate shunt (Mazurek et al., 2005, 2002). Transgenic substitution of PKM2 Cys358 for Ser358 renders the enzyme insensitive to ROS inhibition and impairs tumour growth (Anastasiou et al., 2012). Oxidative control of pyruvate kinase orthologs is widely preserved between species (Cumming et al., 2004; Maeba and Sanwal, 1968; McDonagh et al., 2009) .

DASA10 is a small molecular activator of PKM2 Cys358 that may be used to investigate redox regulation of PKM2. DASA10 is a substituted N, N diarylsulfonamide which prevents oxidative H_2O_2 inhibition of PKM2 Cys358, preserving heterodimerisation and increasing baseline function 2.8-fold (Anastasiou et al., 2012). The DASA10 compound must be applied before the ROS insult in order to provide protection (Anastasiou et al., 2012), because subunit reassembly prevents access the regulatory site.

Since neutrophils also express the PKM2 (Kechemir et al., 1989) isoform of pyruvate kinase, the neutrophil hexose monophosphate shunt may be subject to redox regulation in a manner similar to cancer cells. Redox regulation of PKM2 may therefore be a key regulator of postoperative neutrophil metabolism and innate immunity.

6.1.1 Hypothesis

Oxidative inhibition of the glycolytic enzyme pyruvate kinase determines postoperative impairment of respiratory burst by limiting supply of NADPH substrate.

6.1.2 Aims

1. To describe perioperative resting and activated neutrophil glucose uptake and glycolytic activity.

2. To determine how preservation of pyruvate kinase activity with DASA affects the perioperative respiratory burst.

3. To determine whether DASA may prevent oxidative stress induced inhibition of the respiratory burst.

6.2 Methods

Patient sample collection, laboratory techniques and protocols are described in General Methods.

6.2.1 Perioperative neutrophil glucose uptake

The effect of extracellular glucose concentration upon the respiratory burst was determined by comparing the PMA stimulated respiratory burst of isolated neutrophils in the absence, or presence, of glucose 10mM in preoperative samples.

Next, whole blood neutrophil glycolytic activity was determined at rest, following PMA activation and under oxidative stress (Myxothiazol 10μM). Briefly, PMA and Myxothiazol were added for 10 minutes each, and all samples incubated for an equal amount of time overall. Whole blood glucose, lactate, pH and ionised calcium were measured using Radiometer ABL800 Flex blood gas analyser.

Perioperative glucose uptake was similarly determined at rest, following PMA activation and after addition of Myxothiazol 10μM using the fluorescent glucose analog 2-NBDG.

6.2.2 Redox regulation of pyruvate kinase

The effect of PMK2 small molecule activator DASA upon neutrophil redox status and glycolysis was determined in whole blood. DASA was prepared in DMSO vehicle. DASA (100μM) was added immediately before each assay was performed. Resting oxidative stress and PMA stimulated respiratory burst were quantified with Rhodamine-123, as previously described, and extracellular glucose and lactate concentrations measured with Radiometer ABL800 Flex blood gas analyser. DASA respiratory burst experiments were then repeated within paired preoperative and postoperative samples.

The capacity for DASA to maintain neutrophil respiratory burst in the presence of a subsequent oxidative challenge was then assessed. Since application of Myxothiazol (10μM) to isolated neutrophils for 10 minutes was shown to reduce the respiratory burst (Chapter 5), this dose was chosen. Dose response experiments with DASA were performed in the isolated neutrophil system, and a 10μM dose was shown to increase respiratory burst by a similar magnitude as 100μM dose in whole blood system. Isolated neutrophils were incubated with DASA 10μM for 10 minutes, prior to addition of myxothiazol for 10 minutes and resting plus PMA activated responses recorded.

6.3 Results

6.3.1 Patient characteristics

Assay	Total number	Age (median, [IQR]) (years)	Male (n, [%])	Surgical speciality (n, [%])							Patient study source
				Hepato-biliary	Gynae-cology	Urology	Maxillo-facial	Gastro-intestinal	Ortho-paediacs	Breast	
Glucose + PMA + DHR Fig 6.1 AB	7	60 (54-64.5)	5 [71]	2 [29]	-	1 [14]	-	3 [43]	1 [14]	-	VISION
Myxothiazol: blood glucose, lactate and pH Fig 6.1 CDE	5	71 (69-73)	2 [40]	-	-	2 [40]	-	1 [20]	2 [40]	-	VISION
2-NBDG dose response Fig 6.2 A	2	61, 71	1 [50]	-	-	1 [50]	-	-	1 [50]	-	VISION
2-NBDG + PMA (Preop-POD1) Fig 6.2 BC	5	63 (56-69)	5 [20]	-	-	1 [20]	-	1 [20]	3 [60]	-	VISION
2-NBDG + Rotenone/Myxothiazol Fig 6.2 DE	4	61 (53-68)	3 [75]	-	-	2 [50]	-	2 [50]	-	-	VISION
DASA + DHR Fig 6.3 A	8	59 (53-62)	3 [38]	1 [13]	-	-	-	5 [63]	2 [25]	-	VISION

DASA + PMA + DHR Fig 6.3 BC	8	59 (53-62)	3 [38]	1 [13]	-	-	-	5 [63]	2 [25]	-	VISION
DASA + PMA: Blood glucose & lactate Fig 6.3 DE	5	71 (69-73)	2 [40]	-	-	2 [40]	-	1 [20]	2 [40]	-	VISION
DASA + PMA/E.coli + DHR (preop-POD1) Fig 6.4 ABCD	5	71 (69-73)	2 [40]	-	-	2 [40]	-	1 [20]	2 [40]	-	VISION
DASA 100µM ± PMA + DHR Fig 6.5 AB	4	66 (62-74)	1 [25]	-	1 [25]	2 [50]	-	1 [25]	-	-	VISION
DASA 10µM ± PMA + DHR Fig 6.6 CDF	5	65 (58-65)	3 [60]	1 [20]	1 [20]	2 [40]	-	-	1 [20]	-	VISION
DASA 10µM + CD16	10	63 (57-67)	6 [60]	1 [10]	1 [10]	2 [20]	-	1 [10]	5 [50]	-	VISION
DASA 10µM ± Myxothiazol + DHR	5	71 (69-73)	2 [40]	-	-	2 [40]	-	1 [20]	2 [40]	-	VISION

Table 6.1 Characteristics of the patients for Chapter 6 experimental assays.

6.3.2 Perioperative neutrophil glucose uptake

Figures 6.1 and 6.2 suggest that the neutrophil respiratory burst uses extracellular glucose as a metabolic substrate, supporting similar observations from other authors (Oehler et al., 2000; Tan et al., 1998). Specifically, the presence of extracellular glucose increases the respiratory burst (Fig6.1A,B) and PMA neutrophil activation promotes a fall in extracellular glucose concentration (Fig 6.1C) plus increased transportation of the fluorescent glucose analog 2-NBDG (Fig6.2B). Extracellular lactate concentrations were unchanged. The induction of oxidative stress with Myxothiazol 10μM did not alter resting or activated neutrophil glucose uptake, but did increase extracellular lactate concentration and reduce extracellular pH (Fig6.1 D,E).

Having established that the respiratory burst utilises extracellular glucose, next I investigated whether glucose uptake differed between preoperative and postoperative neutrophil preparations. The fluorescent glucose analog 2-NBDG has been widely used to investigate glucose uptake in a diverse range of cell types (Gandhi et al., 2009; Yamada et al., 2007). Dose response experiments demonstrated that 1μM 2-NBDG provided optimal fluorescence conditions for FACS analysis (Fig 6.2 A); 2-NBDG is intensely fluorescent and saturated the detector at higher concentrations. Postoperative resting and PMA activated 2-NBDG were increased when compared to preoperative control samples (Fig 6.2 B,C).

In chapter 5 I demonstrated that Rotenone 10μM (respiratory complex I inhibitor) primed the respiratory burst and Myxothiazol 10μM (respiratory complex III inhibitor) inhibited it. Here, the addition of rotenone and myxothiazol to neutrophil preparations did not affect resting neutrophil glucose uptake (Fig 6.2 D,E).

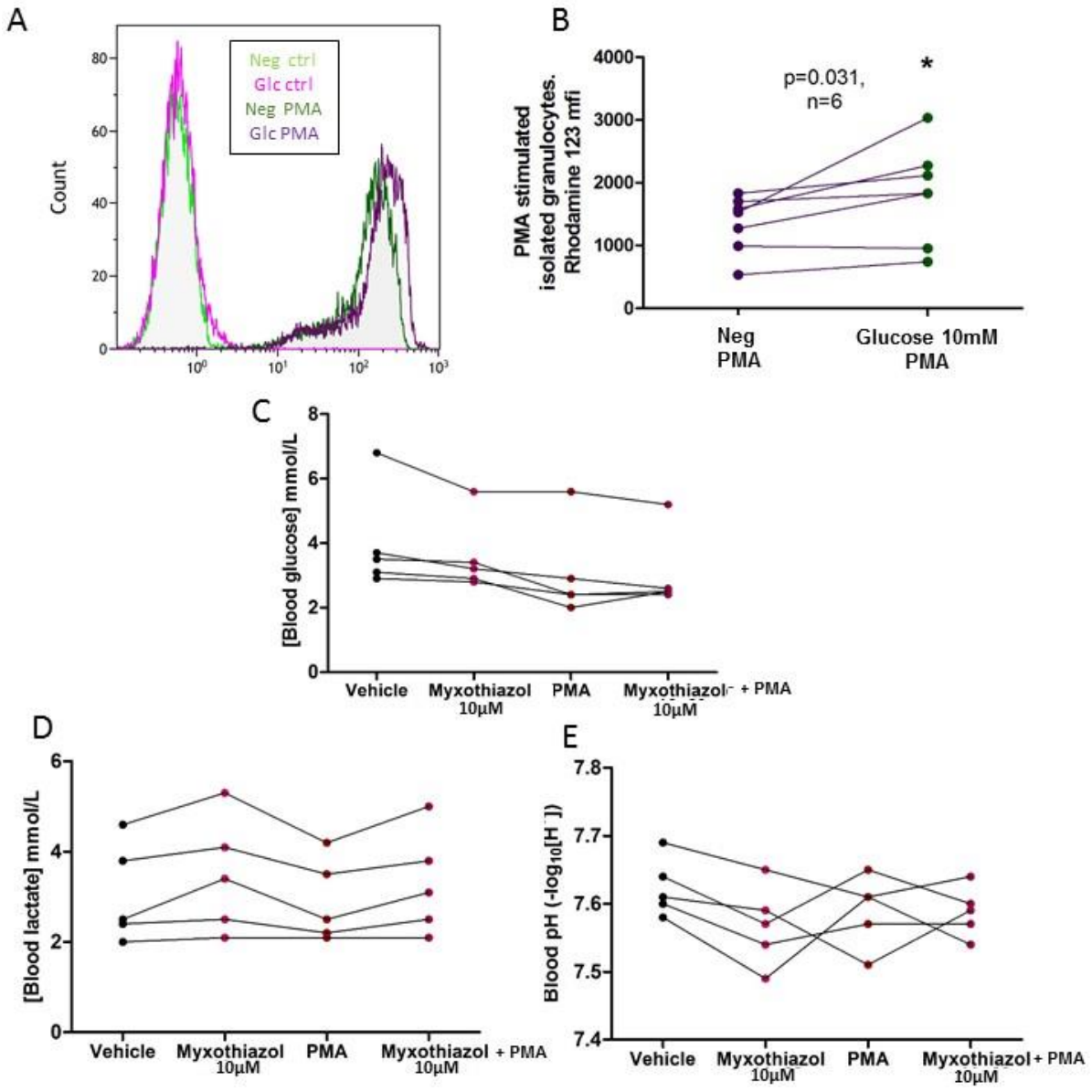

Figure 6.1 Glycolytic profile of neutrophils at rest, following PMA activation and under myxothiazol 10µM redox stress.

AB Typical plot and population data for PMA stimulated respiratory burst following the addition of extracellular glucose to isolated granulocytes, suspended in phosphate-buffered saline. Glucose added immediately before burst assay. Wilcoxon matched pairs test used. C D E Change in whole blood extracellular glucose/lactate/pH following the addition of Myxothiazol and PMA activation. Assays performed simultaneously on each test sample using Radiometer ABL 800 blood gas analysed. Myxothiazol incubations for 30 minutes, PMA incubations for 10minutes. The total incubation times were all identical.

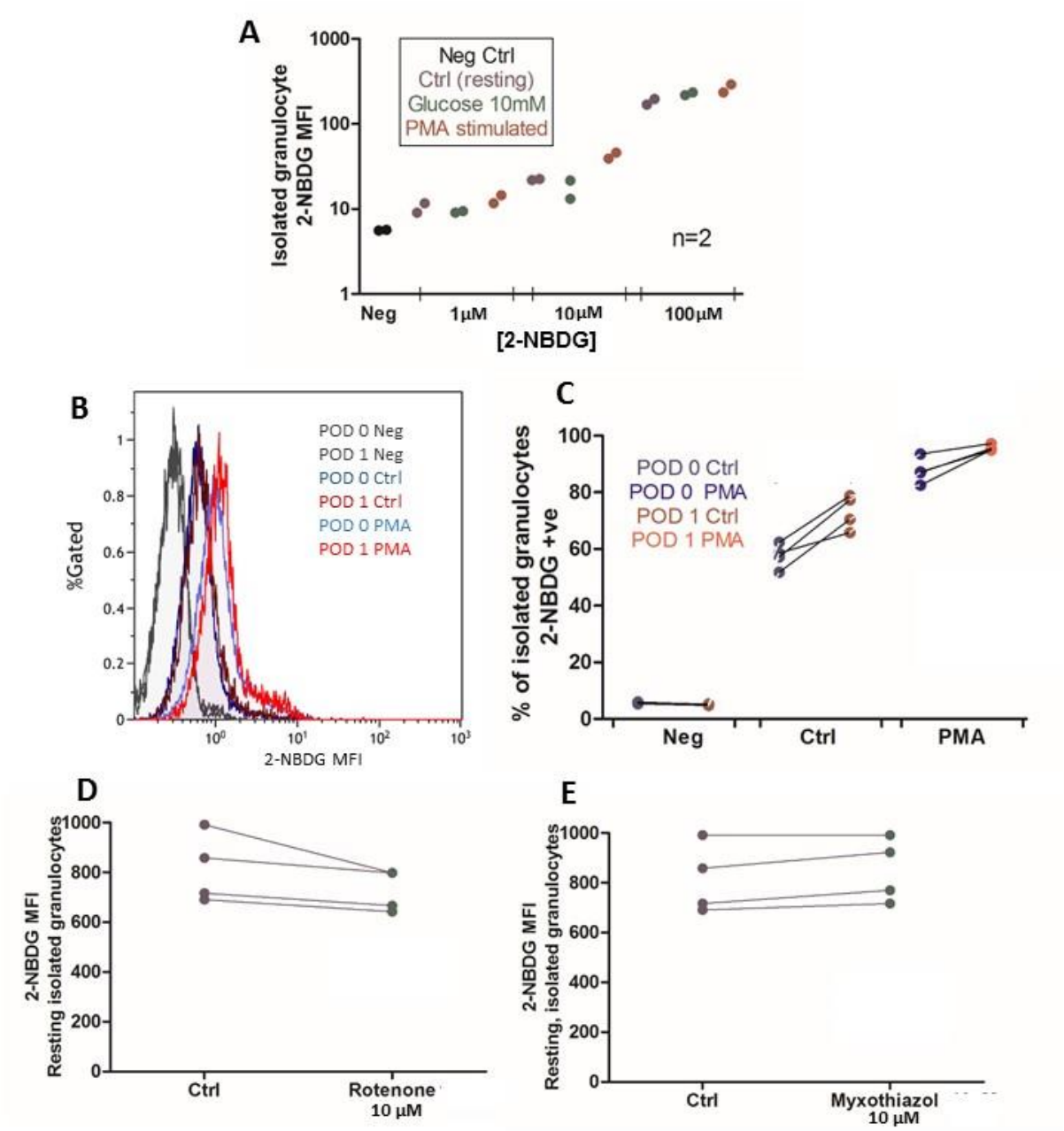

Figure 6.2 Neutrophil uptake of glucose analog 2-NBDG following surgery and addition of respiratory complex inhibitors.

Isolated neutrophils used throughout. 2-NBDG was added for 10 minutes and incubated at 37°C, then washed twice and resuspended in PBS. A. Dose response experiment to find optimal concentration of 2-NBDG. B C Perioperative change in 2-NBDG in resting and PMA activated granulocytes. D E The effect of Rotenone and Myxothiazol upon resting granulocyte uptake of 2-NBDG. Cells were incubated with Rotenone/Myxothiazol for 30 minutes prior to addition of 2-NBDG.

6.3.3 DASA stimulation of pyruvate kinase.

DASA is a highly specific molecule which both prevents the oxidative inhibition of, and activates, PKM2. The addition of small molecule activator DASA increased whole blood PMA respiratory burst without affecting baseline ROS production (Fig 6.3 A,B,C) or extracellular glucose concentration (Fig 6.3 D).

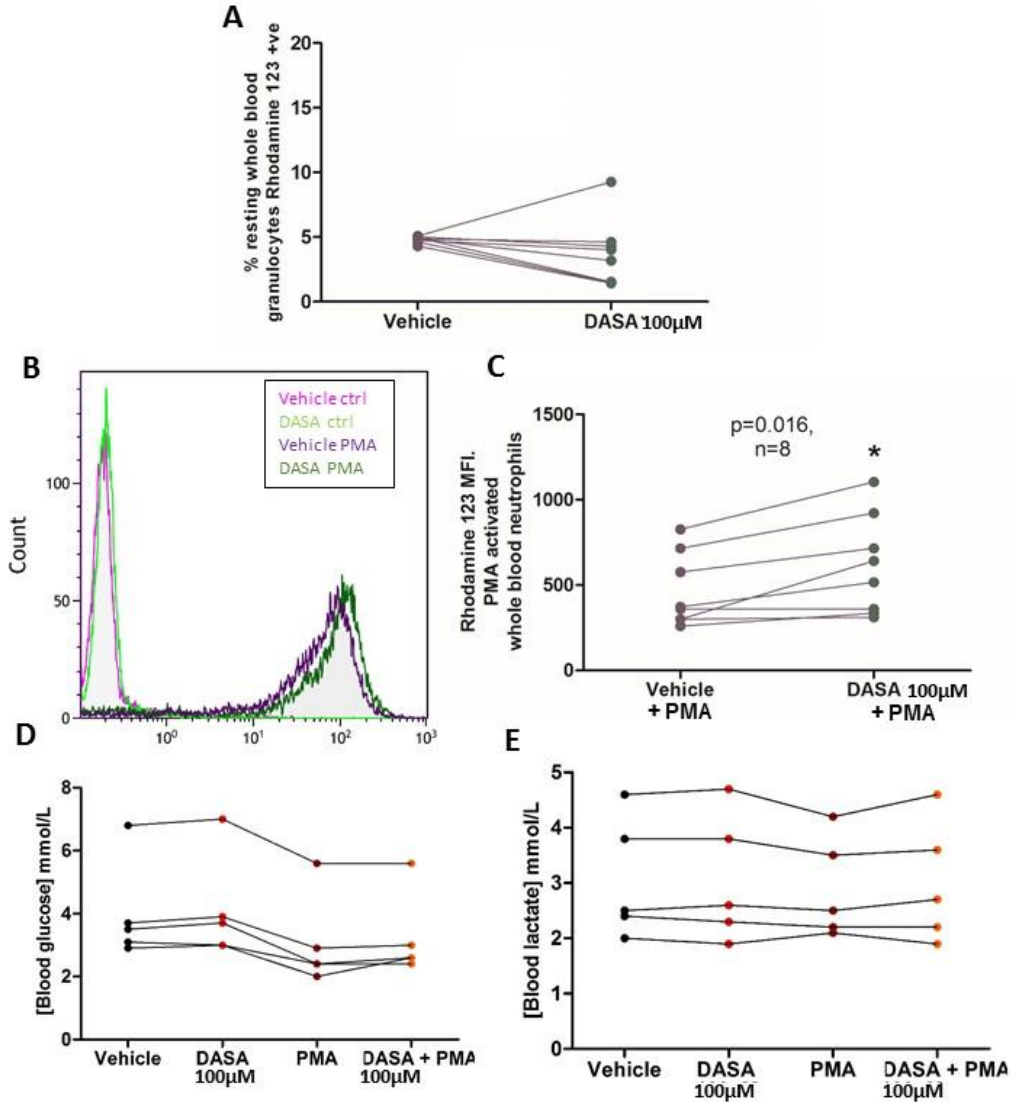

Figure 6.3 The effect of PKM2 activator DASA upon whole blood respiratory burst and glycolytic profile.

DASA was added immediately before each assay – no additional incubation time. A Baseline ROS production following addition of DASA 100uM. B PMA stimulated respiratory burst following addition of DASA 100µM. C D Extracellular glucose and lactate concentration following addition of DASA and PMA, Wilcoxon matched pairs test used. Here, whole blood samples were incubated with DASA for 10 minutes and PMA for 10 minutes. Total incubation times the same.

Next, paired preoperative and postoperative whole blood respiratory burst responses were compared in the presence of DASA. DASA consistently increased preoperative but not postoperative PMA (Fig 6.4 A,B) and E. coli burst responses (Fig 6.4 C,D).

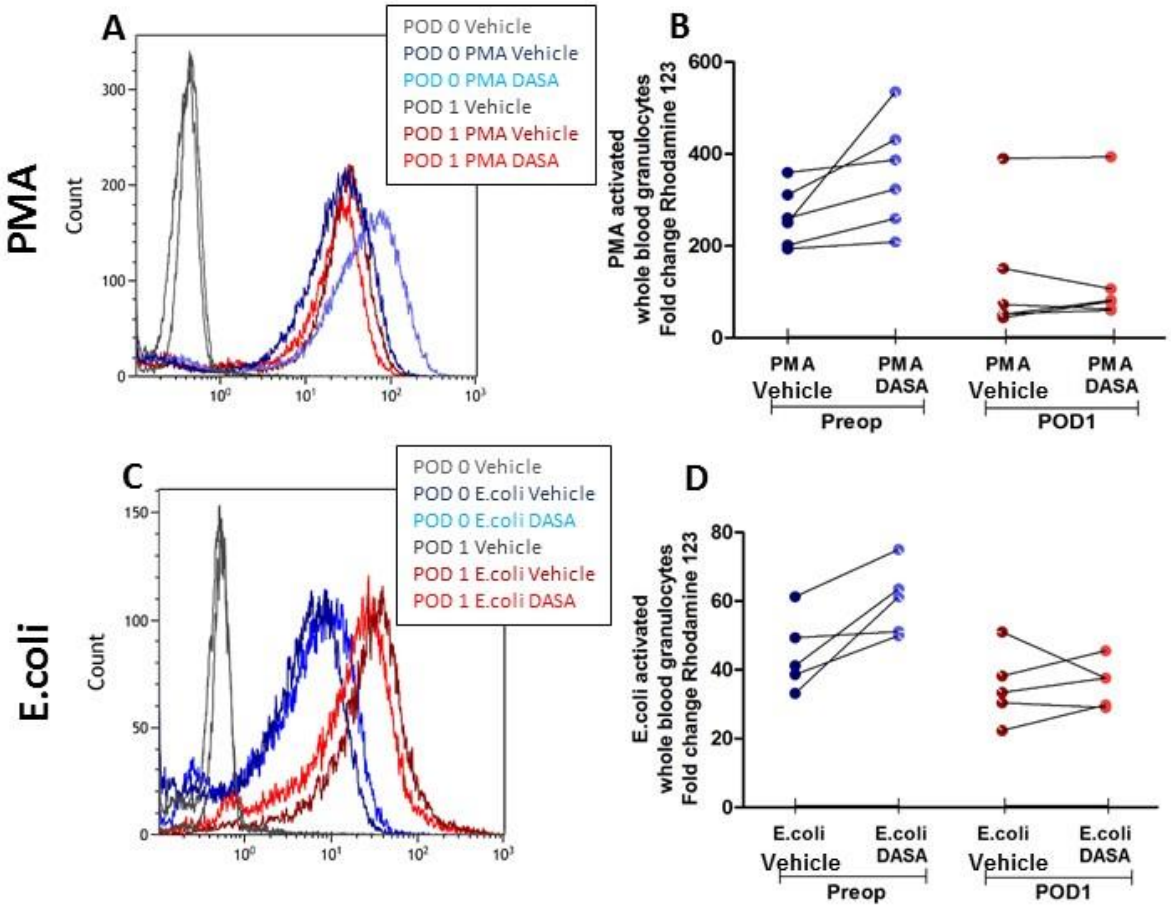

Figure 6.4. Perioperative effect of DASA 100μM upon whole blood PMA and E. coli stimulated respiratory burst.

DASA 100μM was added immediately before each burst assay – no additional incubation time. A B C The effect of DASA upon perioperative PMA stimulated respiratory burst; (A) typical plot, (B) fold-change in respiratory burst, = $MFI_{PMA}/MFI_{resting}$ C D The effect of DASA upon perioperative E. coli stimulated respiratory burst, = $MFI_{E.\ coli}/MFI_{resting}$.

6.3.4 DASA reversal of Myxothiazol mediated respiratory burst inhibition

DASA must be added to PKM2 prior to oxidative challenge for oxidative protection (Anastasiou et al., 2012), which has been explained through conformational change and access to relevant allosteric site. Therefore, the ability of DASA to augment preoperative, but not postoperative respiratory burst suggests that postoperative PKM2 may be subject to oxidative inhibition. In chapter 5, I demonstrated that Myxothiazol causes an increase in baseline neutrophil ROS and impairs the respiratory burst. Therefore, I tested the hypothesis that preincubation of resting preoperative neutrophils with DASA could prevent the Myxothiazol driven inhibition of the respiratory burst.

My investigations have shown that myxothiazol works reliably within isolated neutrophil preparations, but not within whole blood (Chapter 5). Therefore, I began by testing the effects of DASA within an isolated system. DASA 100μM – as used in whole blood experiments above – reduced the PMA burst (Fig 6.5 A,B). However, subsequent resting with a lower dose, DASA 10μM, increased the PMA burst (Fig 6.5 C,D,E), similar to whole blood experiments above.

Isolated neutrophils were then incubated with DASA 10μM for 10 minutes prior to addition of Myxothiazol 10μM and characterisation of respiratory burst responses. Neither preincubation with DASA 10μM or 100μM preserved the PMA respiratory burst in the presence of Myxothiazol (Fig 6.6).

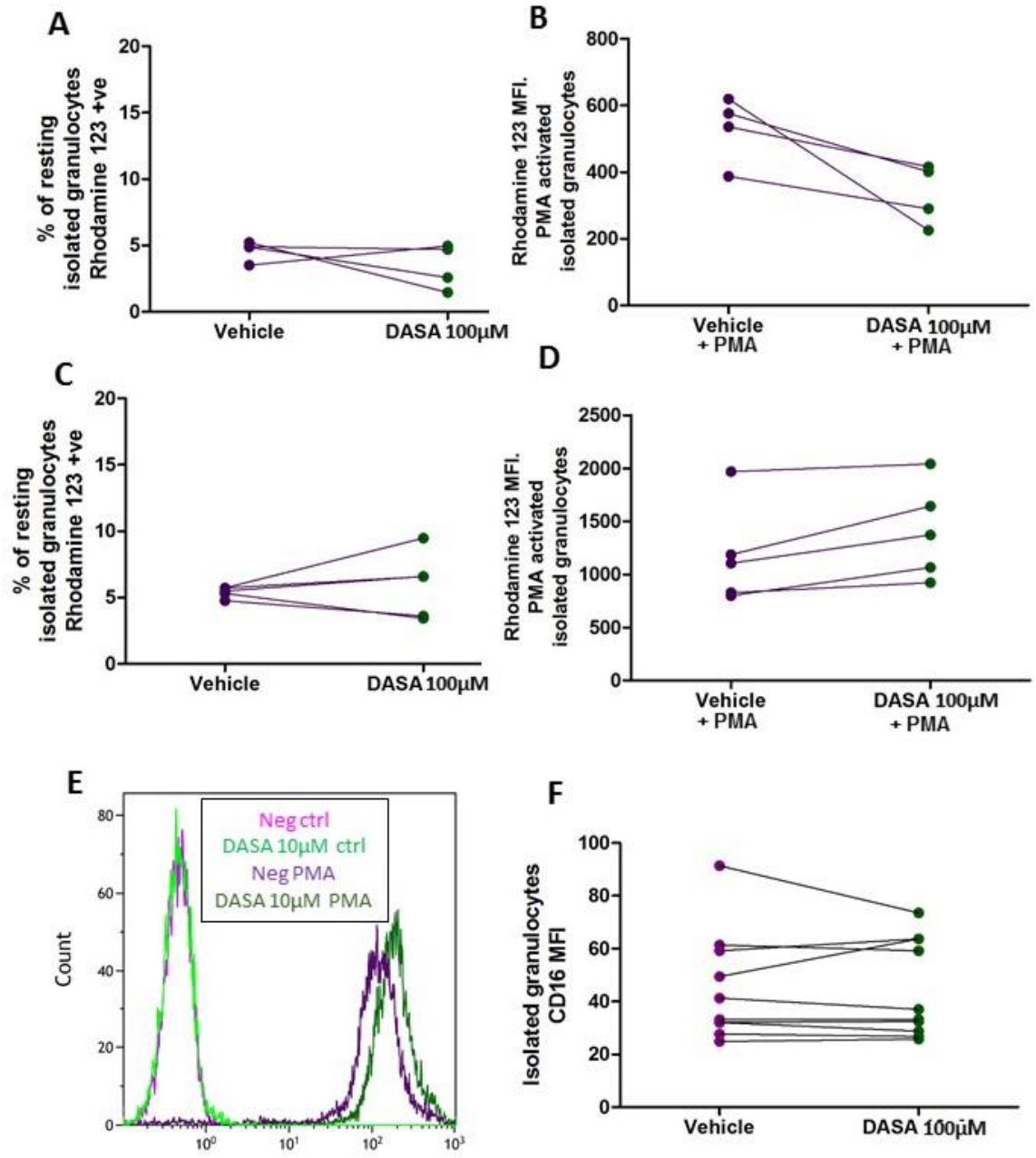

Figure 6.5 The effect of DASA upon isolated granulocyte PMA stimulated respiratory burst.

Paired t-test used throughout. DASA was added immediately before each assay – no additional incubation time. A B The effect of DASA 100µM upon resting (A) ROS production and (B) PMA stimulated respiratory burst. C D E F The effect of DASA 10µM upon (A) resting ROS production, (B) cell surface CD16 expression (C, D) typical plot and population data for PMA stimulated respiratory burst.

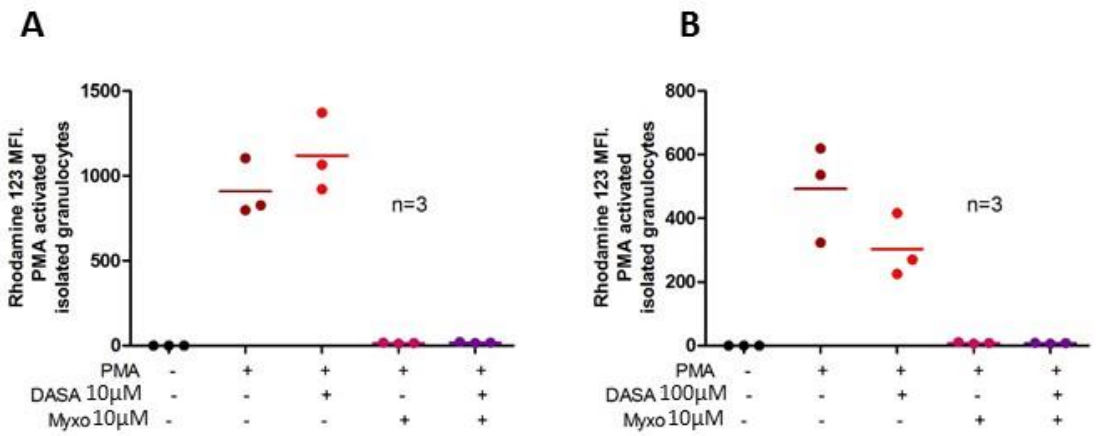

Figure 6.6 The effect of preincubation with DASA upon myxothiazol mediated inhibition of the PMA stimulated respiratory burst in isolated granulocytes.

DASA added for 10 minutes, Myxothiazol for 30minutes and PMA for 10 minutes. Total incubation times were all identical. A DASA 10µM. B DASA 100µM.

6.4 Discussion

6.4.1 Key Findings

1. **The magnitude of the neutrophil respiratory burst is partly determined by the availability of metabolic substrate.** PMA activation promoted a fall in the concentration of extracellular glucose and an increase in the intracellular transport of the fluorescent glucose analog 2-NBDG within isolated neutrophil preparations, supporting findings from other authors that suggest activated neutrophils utilise extracellular glucose (Oehler et al., 2000; Tan et al., 1998). Isolated neutrophil preparations demonstrated a greater PMA respiratory burst in the presence of glucose 10mM, although the recording of measurable burst responses in the absence of extracellular glucose suggests that both intracellular and extracellular glucose reserves are used during neutrophil activation.

   Together, these data suggest that the availability of extracellular metabolic substrate, and consequently competition between glycolysis and hexose-monophosphate shunt for glucose-6-phosphate, may affect the magnitude of the respiratory burst.

2. **Resting and PMA activated postoperative neutrophils demonstrate increased 2-NBDG uptake when compared to paired preoperative controls.** These data suggest either an increase in baseline metabolic rate, or a reduction in intracellular glucose reserves, within postoperative neutrophils. The divergence between this increase in glucose uptake and the previously identified reduction in postoperative respiratory burst would support an increase in resting neutrophil substrate use.

3. **The PKM2 activator DASA consistently increased the preoperative neutrophil respiratory burst.** DASA is a highly specific molecule which activates the PKM2 isoform of pyruvate kinase but not the more common PKM1. These data suggest that PKM2 activity may regulate the respiratory burst, supporting findings from trauma patient neutrophils (Oehler et al., 2000).

4. **DASA does not consistently increase the postoperative neutrophil respiratory burst.** These data suggest that the postoperative metabolic profile and regulation of respiratory burst is different to preoperative cells. Since DASA can only prevent, but not reverse, oxidative inhibition of PKM2, this finding supports the hypothesis that raised resting oxidative stress within postoperative cells is responsible for inhibition of the respiratory burst. Alternatively, postoperative neutrophils may contain an additional rate limiting step which leaves them unaffected by DASA stimulation.

5. **DASA cannot prevent Myxothiazol 10μM / ROS induced impairment of the respiratory burst within isolated neutrophils.** These data do not support the hypothesis that postoperative oxidative inhibition of PKM2 is responsible for the impaired respiratory burst. However, as described in chapter 5, this short-term Myxothiazol ROS model is not completely consistent with the postoperative phenotype, and that these differences may be responsible for the observed DASA resistance.

6.4.2 Interpretation of key findings

These experiments have identified several differences within the perioperative metabolic responses of neutrophils which are associated with functional changes in neutrophil immune function. The preoperative neutrophil respiratory burst is consistently increased in the presence of DASA, a highly specific small molecule activator of PKM2. By contrast, postoperative neutrophils are often resistant to DASA and demonstrate increased glucose uptake at rest and when activated.

DASA activates and prevents oxidative inhibition of the PKM2 isoform. Therefore, the ability of DASA to increase the respiratory burst in preoperative neutrophils suggests that the neutrophil respiratory burst may be subject to metabolic (glycolytic) regulation.

DASA was specifically designed to investigate the role of PKM2 regulation within cancer cells. Within my experiments, the effect of DASA upon metabolic flux in neutrophils appears to be opposite to that in lung cancer cells. Within cancer cells DASA activation of PKM2 reduces NADPH synthesis by increasing flow through glycolysis. Here, within neutrophils DASA appears to increase flux through the hexose monophosphate shunt because the respiratory burst is increased, which implies an increase in NADPH formation.

These divergent results within neutrophil and cancer cells may be explained by differences in the outflow of hexose monophosphate shunt between terminally differentiated neutrophils and highly anabolic cancer cells. In cancer cells the carbon skeletons from hexose monophosphate shunt may form ribose sugars thereby supporting nucleotide synthesis and ultimately promote cell cycle progression. However, within neutrophils these carbon skeleton return to glycolysis proximal to pyruvate kinase (Figure 1.4), supported by the reported increase in neutrophil lactate production which follows PMA activation (Oehler et al., 2000).

The relative resistance of postoperative neutrophils to DASA supports the presence of oxidised PKM2 within postoperative cells, since it is established that DASA prevents, but cannot reverse, oxidative inhibition within the PKM2 isoform (Anastasiou et al., 2012). Therefore, beyond the specific established allosteric regulators of pyruvate kinase function by both immediate and distant substrates and products, these data support an additional regulatory mechanism, whereby resting neutrophil oxidative stress may act as a transducer to regulate activated cell immune effector mechanisms.

Since DASA may prevent but not reverse oxidative inhibition of PKM2, I attempted to prevent Myxothiazol mediated inhibition of the respiratory burst through the prior addition of DASA. However, DASA did not prevent Myxothiazol induce inhibition of the respiratory burst within this model. Although this observation does not support the hypothesis for oxidative regulation of PKM2 within neutrophils, limitations within the experimental model may also be responsible. In earlier experiments, I found that Myxothiazol 10μM increased apoptosis as well as impairing the respiratory burst. Therefore, apoptotic demands for ATP may limit flux through pentose phosphate pathway. Alternatively, the ROS generation within this Myxothiazol model may overwhelm physiological buffering, consume NADPH through glutathione and cause non-specific lipid peroxidation and cellular injury.

Cellular metabolism is subject to complex regulatory processes, with multiple steps that involve substrate availability, feedback inhibition and compartmentalisation (Srere, 1994), that are difficult to fully replicate in vitro. There are alternative explanations for these postoperative experimental observations. These include the development of an alternative rate determining step within the formation of NADPH, competition for NADPH and an inability to utilise NADPH.

The rate determining step maybe competition for glucose-6-phosphate between glycolysis and the hexose monophosphate shunt. Together, observations of DASA resistance and increased glucose uptake suggest an increased rate of cellular ATP consumption. Alternatively, postoperative mitochondrial dysfunction may place an increased demand on less efficient glycolytic ATP production – if mitochondria do indeed make a meaningful contribution to (postoperative) neutrophil ATP production.

Increased glucose uptake has been reported in neutrophils from trauma patients (Oehler et al., 2000). These observations would also be compatible with a population of immature circulating neutrophils. Data from a HL60 model found increased Glucose-6-Phosphate Dehydrogenase and hexose monophosphate shunt activity within DMSO differentiated cells when compared to undifferentiated cells (Ahmed et al., 1993). Alternatively, partially activated postoperative cells may have depleted glycogen reserves. In these settings glycolysis is already running at maximal capacity to meet the raised metabolic demands of these cells, providing the additional rate limiting step described above.

NADPH is required for other cellular processes beyond the neutrophil respiratory burst, such as the maintenance of glutathione – an important antioxidant pathway. The presence of increased postoperative resting oxidative stress is compatible with increased glutathione turnover. Therefore, the failure of DASA to increase the respiratory burst DASA may be explained by competition for NADPH. Finally, the PHOX function may be impaired such that it is insensitive to increases in NADPH. Again, immature precursors may have insufficient amounts of PHOX – a hypothesis supported by HL-60 data comparing PHOX content of undifferentiated promyelocytes and DMSO differentiated cells (Ahmed et al., 1993) - although other regulatory processes that alter enzyme function may also be responsible.

This study supports the more extensive observational literature concerning the genomic responses that occur within the circulating leukocyte transcriptome from humans with systemic inflammation, which collectively report an upregulation of glycolytic, and down regulation of mitochondrial genes (Calvano et al., 2005; Xiao et al., 2011). Healthy volunteer experiments have revealed how low dose endotoxin challenge – at doses insufficient to promote release of systemic cytokines – are associated with altered expression of metabolic genes (Haimovich et al., 2014). Taken with the mechanistic data from this study, these observations support the concept of metabolic regulation of neutrophil immune function.

6.4.3 Strengths and weaknesses

This study identified metabolic differences in postoperative neutrophils when compared to matched preoperative control samples. Paired sampling is important because comorbid disease, age and therapeutic interventions may affect neutrophil metabolism. By using individually matched control samples, the direct effect of these premorbid factors upon the experimental signal can be minimised. However, how these premorbid factors interact with surgery/anaesthesia remains unknown. Since all assays were not performed in the same patients, it is not possible to extrapolate the study findings to a universal postoperative phenotype.

These postoperative data extend the literature concerning metabolic function of human neutrophils during established inflammation, since most human data has been obtained from healthy volunteers – including the widely accepted fluorometric data describing the rate limiting steps of neutrophil glycolysis. My findings suggest a shared regulatory mechanism with cancer cells, which adds to the wider literature describing similarities between cancer cell biology and inflammatory processes (Hotchkiss et al., 2014).

Whole blood samples were used where possible, including for preoperative and postoperative DASA experiments. DASA is a highly specific activator molecule, widely investigated and published within cancer literature and considered to act solely upon PKM2. Therefore, its ability to manipulate neutrophil function at the same doses used within the cancer literature directly supports the presence of PKM2 within neutrophils. Nonetheless, as with any drug therapy, alternative off-target effects may be responsible. Some authors have proposed that 2NBDG may become metabolised to a non-fluorescent colour (Yamada et al., 2007), however the experiments in this study compared different treatments over identical time periods, therefore these differences should be controlled for.

The complex nature of metabolic networks makes the interpretation of relatively isolated experimental results challenging, and are compounded by the modest evidence base describing metabolic profiles of human neutrophils from humans with active inflammation. Assuming DASA is acting upon PKM2, the data presented within this chapter may be explained by either the presence of oxidised PKM2 within postoperative samples, or alternatively by the presence of an additional rate determining step. These two explanations may be differentiated by measuring the rates of glycolysis and hexose monophosphate shunt reactions within preoperative and postoperative samples, as originally performed in resting cells in the 1960s and 1970s using fluorometric measurement techniques (Beck, 1958; Minakami, 1968).

6.4.4 Conclusions

1. The metabolic profile of postoperative neutrophils is different to their matched preoperative counterparts, and may include increased resting glucose uptake.

2. The glycolytic enzyme PKM2 may be involved in the regulation of the neutrophil respiratory burst and may be subject to postoperative oxidative inhibition by mitochondrial ROS.

Chapter 7 – Discussion

7.1 Introduction

This thesis has demonstrated how the study of patients undergoing major elective surgery may provide a translational model of human sepsis. This surgical model provides the methodological advantages of laboratory research within human subjects. Ex-vivo assays may therefore be performed in a controlled manner using human tissues and cells. Surgical patients receive a relatively homogenous inflammatory insult at a known time, permitting the collection of individualised pre-insult control samples and precisely timed post-insult samples.

Using the surgical model, this thesis has identified impairments of neutrophil immune and metabolic function on postoperative day two. These impairments had improved by postoperative day five, and may predispose to the development of postoperative infections, non-infective complications and chronic, non-resolving inflammation.

Two different laboratory models of mitochondrial oxidative stress reproduced key features of the postoperative functional immune impairment. The most notable immune defect was a reduction in the neutrophil respiratory burst, a process which consumes NADPH that is supplied by the hexose monophosphate shunt. Substrate flow through hexose monophosphate shunt and glycolysis are intrinsically linked, and a key glycolytic regulatory enzyme was identified as a possible target for this mitochondrial redox signal. Chemical manipulation of PKM2 function reproduced many features of the perioperative immune phenotype.

7.2 Summary of key findings

1. **Major elective surgery may provide a translational model of human sepsis which overcomes many of the methodological challenges of sepsis studies.** Most translational sepsis studies in humans do not use all the information available to determine whether infection is present, and employ control groups whose baseline immunological profiles are dramatically different to the study population. By contrast, patients undergoing major elective surgery are extensively phenotyped and receive a planned and relatively homogenous inflammatory insult - provided specific enrolment criteria are used. Consequently, each patient may act as their own preoperative control and measurements/blood sampling may occur at specified post-insult time points, facilitating accurate interrogation of dynamic inflammatory processes. Highly conserved genomic and metabolic responses to DAMP and PAMP signals provide biological plausibility to this model.

2. **The neutrophil respiratory burst is impaired on postoperative day two, when the circulating white cell counting is increased.** Whole blood and isolated neutrophil investigations revealed a significant reduction in respiratory burst following activation with clinically relevant E. coli bacteria and PMA, a potent activator of Protein Kinase C. Neutrophil cell surface CD16 was significantly reduced on postoperative day two, compatible with diminished phagocytosis. These defects had begun to improve by postoperative day five.

3. **Postoperative neutrophil mitochondria may have impaired functionality.** Postoperative neutrophil mitochondria are hyperpolarised, and liberate increased amounts of superoxide.

4. **Mitochondrial dysfunction may be mechanistically responsible for the impairment of postoperative neutrophil effector function.** Two distinct models of mitochondrial dysfunction that increased ROS production both down

regulated the respiratory burst. Mitochondria may therefore act as inflammatory signal transducers, with mitochondrial ROS acting upon cytoplasmic targets to regulate the respiratory burst.

5. **Mitochondrial ROS may regulate the respiratory burst by limiting the supply of NADPH substrate, through oxidative inhibition of pyruvate kinase M2.** Pyruvate kinase is a rate limiting step in neutrophils isolated from human healthy volunteers and from peritoneal fluid of guinea pigs with peritonitis. Within cancer cell biology PKM2 function is an established regulatory mechanism that determines flux through glycolysis and the hexose monophosphate shunt. The PKM2 small molecule activator DASA - which can activate non-oxidised PKM2 - increased the preoperative but not postoperative respiratory burst.

7.3 Translational relevance of results

7.3.1 The surgical model of inflammation

Decades of translational critical care research has failed to deliver a single immunomodulatory agent that has been reliably demonstrated to improve clinical outcome (Angus et al., 2000; Lehmann et al., 2014; Ranieri et al., 2012; Rimmer et al., 2012; Sprung et al., 2008). This thesis has identified several challenges to the rational design and of sepsis studies, including uncertainties surrounding diagnosis, sample timing to the index insult and the identification of appropriate control populations. Therefore, translational investigations of sepsis are maybe being performed within patients who have not been reliably demonstrated to have an infection, and comparisons made to control groups whose baseline immunological status may be very different to the study population.

The importance of comparing paired preoperative control samples with carefully timed postoperative samples is underlined by the data presented within chapters 4 to 6, which describes the perioperative neutrophil immune and metabolic phenotype. The variation in baseline immune function and the specific time window during which functional impairment occurs mean these experimental signals would have been missed if all the postoperative samples were grouped together and compared to a separate and poorly defined control population, as can happen during sepsis studies.

Biological plausibility of the surgical model is provided by studies that have shown how similar human inflammatory responses to traumatic and infective injury are at genomic (Haimovich et al., 2014; Xiao et al., 2011), molecular (Matzinger, 1994), local and systemic (Matzinger, 1994; Seong and Matzinger, 2004) levels. This surgical model would not replace the use of animal models during early investigations, nor the need for pragmatic effectiveness studies of novel therapies within critically unwell patients. What the surgical model offers is a translational bridge between these two extremes, providing the methodological advantages of laboratory studies within a human immune environment.

7.3.2 Postoperative neutrophil immune impairment (tolerance)

This thesis has revealed that patients undergoing major elective surgery have an impaired neutrophil respiratory burst on postoperative day two, which has begun to improve by postoperative day five, as well as a down regulation of CD16 receptors which mediate the recognition and phagocytosis of pathogenic microorganisms. The magnitude of respiratory burst depression is similar to that found in patients with alcoholic hepatitis superimposed upon cirrhosis (Mookerjee et al., 2007). Therefore, the nature, magnitude and timing of this functional impairment make it a plausible candidate for development of infectious and non-infectious complications, through the following mechanisms:

7.3.2.1 Predisposition to infectious complications

Gross reductions in human neutrophil numbers, and absolute absence of the respiratory burst are associated with an increased incidence of sepsis and soft tissue infection (Anderson et al., 1985; Kuderer et al., 2006; Morgenstern et al., 1997). Similarly, within critically ill patients who are not being treated for an infection, those with diminished neutrophil burst and phagocytosis are more likely to develop a subsequent nosocomial infection (Stephan et al., 2002). Within animal models of haemorrhagic shock, neutrophil depletion increases systemic bacterial translocation (Deitch et al., 1990). Therefore, it is possible that the 90% reduction in postoperative neutrophil burst responses, and other deficits, described in chapter 4 may lead to an increased risk of postoperative infection.

Impairment of the respiratory burst will likely lead to a wider impairment of other neutrophil immune functions. The respiratory burst is not just responsible for direct killing of bacteria, but also provides the electromotive potential for optimisation and activation of granule contents and is absolutely required for the formation of neutrophil extracellular traps (NETs) (Segal, 2005).

7.3.2.2 Protection from tissue damage and non-infectious complications

Postoperative neutrophil impairment may protect against non-infectious complications by limiting autotoxic neutrophil activities. Bacterial killing and removal may cause collateral tissue injury (Chollet-Martin et al., 1996; Nathens et al., 1997; Steinberg et al., 1994), and

activated neutrophils may also enter non-inflamed tissues to cause remote damage (Lowell and Berton, 1998).

Experimental blockade of neutrophil CD11b/CD18 receptor prevents neutrophil transmigration across endothelium and limits tissue oedema and injury. In contrast to endotoxin translocation data, lung injury models which include blunt chest trauma (Raghavendran et al., 2005) and 'double-hit' chest trauma plus caecal ligation and puncture (Perl et al., 2012) and lung stretch injury all report a protective effect from neutrophil depletion (Kotani et al., 2004). Similarly, neutrophil depletion protects against hepatic ischaemic reperfusion injury (Poggetti et al., 1992) and may hasten sterile wound closure (Dovi et al., 2003). Knockout of tyrosine kinase signalling – necessary for adhesion dependent activation of the respiratory burst – confers renal and hepatic protection within a murine LPS model of sepsis (Lowell and Berton, 1998). These model data are supported by observational clinical data from critically ill patients (Chollet-Martin et al., 1996; Steinberg et al., 1994).

The increased susceptibility of postoperative neutrophils to lose mitochondrial membrane potential, and therefore enter apoptosis, under conditions of stress (described in chapter 5) may confer additional protective properties. Apoptotic neutrophils downregulate expression of many proinflammatory genes, and their ingestion by macrophages favours an anti-inflammatory macrophage phenotype (Ariel et al., 2006; Fadok et al., 1998; Kobayashi et al., 2003). Injection of apoptotic neutrophils provides protection within an LPS model of inflammation (Ren et al., 2008).

7.3.2.3 Predisposition to chronic inflammation

Failure to switch off inflammation in a timely manner may lead to a non-resolving state of chronic inflammation (Fullerton et al., 2013; Lawrence and Gilroy, 2007). Data from patients with chronic granulomatous disease – who possess an absent respiratory burst – suggest that capacity to mount an adequate initial inflammatory response is necessary for the timely resolution of that response. As well as a predisposition to infection, patients with chronic granulomatous disease also develop foci of sterile inflammation, (Hampton et al., 2002; Hatanaka et al., 2004; Kasahara et al., 1997; Morgenstern et al., 1997). Neutrophils isolated from these patients demonstrate increased secretion of IL-8 (Hatanaka et al., 2004), prolonged survival (Kasahara et al., 1997) and reduced uptake by macrophages (Hampton et al., 2002). Murine models of chronic granulomatous disease demonstrate sustained and

exaggerated lung inflammation (Davidson et al., 2013; Deng et al., 2012; Schauer et al., 2014; Segal et al., 2010, 2007), as well as a predisposition to autoimmune arthritis that may be prevented through reactivation of PHOX (Hultqvist et al., 2004). Impaired NET formation may partly explain this phenomenon. The respiratory burst is necessary for formation of NETs, which act to retain inflammatory mediators, thereby preventing systemic propagation of local inflammatory responses.

7.3.3 Postoperative neutrophil metabolic dysfunction

The genomic revolution that has swept through cancer cell biology has led to an expanded understanding of gene product function, and how these products interact in complex networks to regulate cellular processes. The classification and nomenclature of these gene products has changed accordingly. It is now appreciated that many oncogenes and tumour suppressor genes actually have a primary metabolic role within non-cancerous cells (Boroughs and DeBerardinis, 2015). It follows that these metabolic changes are now considered prime movers of phenotypic change, rather than simply suppliers of metabolic products.

The concept of metabolic regulation of cellular functions may similarly revolutionise how we conceptualise inflammation, and is supported by genomic descriptions of leukocytes from trauma patients and healthy volunteers who have received endotoxin, which include a decline in mitochondrial gene expression (Calvano et al., 2005). In particular, the administration of low doses of endotoxin which are insufficient to induce measurable changes in systemic cytokine levels may promote metabolic reprogramming of circulating leukocytes, while higher doses of endotoxin promote metabolic reprogramming before classical immunological changes are evident (Haimovich et al., 2014). Activated neutrophils are highly metabolically active, therefore even modest changes in metabolic profile may have dramatic effects in function.

My experiments that postoperative neutrophil mitochondria may have impaired functionality, which would support the genomic investigations above. Specifically, postoperative mitochondria were hyperpolarised and displayed an increased production of mitochondrial ROS, the same phenotype which has been identified within neuronal mitochondria (Abramov et al., 2005, 2004).

7.3.3.1 Parallels with cancer cell metabolism: The Warburg effect

The Warburg effect describes a metabolic signature common to many cancers. The principal features are an increased rate of glycolysis, depression in the rate of oxidative phosphorylation with increased flux through the hexose monophosphate shunt (Hsu and Sabatini, 2008; Vander Heiden, M. G., Cantley, L.C., Thompson, 2009). Therefore, perioperative neutrophils demonstrating mitochondrial dysfunction in parallel with an increased rate of glucose uptake, mirrors this phenotype. It is therefore possible that other core features of Warburg metabolism may be relevant to postoperative neutrophil biology:

- **PI3/Akt/mTOR pathway.** Within cancer cells this promotes cellular proliferation and anabolism. In neutrophils PI3K/Akt activates a range of inflammatory transcription factors such as NF-kB, c-jun and FosB (Ratajczak-Wrona et al., 2013), and in context of Fas engagement promotes apoptosis (Alvarado-Kristensson et al., 2002).
- **AMP-kinase (AMPK) activation**. AMPK is activated by an increase in AMP:ATP ratio, which signals a bioenergetic debt and promotes utilisation of alternative energy sources to glucose (Winder and Hardie, 1999). Within neutrophils AMPK activation reduces TLR4 induced neutrophil activation and is protective in an LPS model of lung injury, consistent with the postoperative phenotype.
- **JAK/STAT stimulation of PIM1-PIM2**. JAK/STAT is a common signalling pathway in many malignancies, activated by a range of cell surface receptors and promotes gene transcription. In neutrophils JAK/STAT pathway is engaged by a range of cytokine receptors, including G-CSF , GM-CSF and IL-6 (Futosi et al., 2013), promoting differentiation and inhibiting apoptosis. PIM1-PIM2 genes and products are not well characterised in neutrophils.

7.3.3.2 The functional significance of mitochondrial dysfunction

Regulation of constitutive apoptosis by neutrophil mitochondria is widely described, and established within the literature (Liu et al., 2003; Maianski et al., 2004; Murphy et al., 2003; Pryde et al., 2000. The initiation of neutrophil apoptosis promotes the down-regulation of many neutrophil immune processes at level of gene expression. This thesis provides

evidence that neutrophil mitochondria may have an extended signalling role, and act as signal transducers during inflammatory responses which activate and inhibit the respiratory burst responses through interaction with cytoplasmic metabolic responses. Two different models of mitochondrial dysfunction were presented. Both induced resting mitochondrial oxidative stress and reduced activated respiratory burst responses. Within macrophages, depletion of autophagy proteins has been shown to promote mitochondrial ROS synthesis (Nakahira et al., 2011).

7.4 Unifying explanations for immunological and metabolic impairments within postoperative neutrophils

This thesis has presented and explored the relationship between functional impairment of neutrophil immune and mitochondrial function through transmission of oxidative stress signals. Regulation via PKM2 was proposed as the target for this oxidative signal. In addition to this proposal, there are several alternative, but overlapping explanations for the experimental observations I have made.

Many of these explanations focus upon the supply, demand and competition for metabolic substrates ATP and NADPH. Although the respiratory burst directly utilises NADPH, the supply of ATP may be directly relevant to the respiratory burst, since it is necessary for the many cellular processes which occur in parallel during neutrophil activation, such as cytoskeletal rearrangement for trafficking of organelles and phagolysosomes. ATP demand may also directly impact upon the supply of NADPH to the respiratory burst, since glycolysis and hexose monophosphate shunt both compete for glucose-6-phosphate. Although outflow from hexose monophosphate shunt does return to distal glycolytic pathway, this is at the expense of the first synthetic step for ATP (Figure 1.4).

Since the hexose monophosphate shunt feeds off and returns into glycolysis, these two processes will be subject to reciprocal regulation, which may manifest directly through open competition for shared substrate, or indirectly through extrinsic regulation of enzyme activity.

Competition for substrate may be interpreted as a limitation of bioenergetic ATP supply. My data suggest increased resting and activated neutrophil glucose uptake, in the presence of a diminished burst, which is compatible with increased metabolic utilisation for glucose-6-

phosphate, limiting respiratory burst substrate within postoperative neutrophils. The loss of mitochondrial membrane potential may be a manifestation of limited bioenergetic reserve. Failure of DASA, a PKM2 activator, to stimulate the postoperative respiratory burst may also be interpreted as substrate limitation, i.e. postoperative neutrophils are functioning at metabolic capacity therefore cannot increase output any further.

Increases in postoperative neutrophil ATP demand may be explained by the presence of immature neutrophils or by partially activated neutrophils, both of which will be subject to intensive gene expression and protein synthesis.

7.4.1 The relevance of mitochondrial dysfunction

The dysfunctional postoperative mitochondrial phenotype suggested within chapter 5 may limit neutrophil immune function through limited synthesis of ATP or other synthetic/transport processes dependent upon mitochondrial membrane potential. The presence of impaired mitophagy would reduce efficiency of ATP synthesis.

Although neutrophils are traditionally considered to be solely dependent upon glycolytic ATP, the addition of respiratory complex inhibitors does diminish neutrophil ATP concentration (Minakami, 1968; Sbarra and Karnovsky, 1959). Most of these experiments were performed within neutrophils collected from healthy volunteers (i.e. non-inflamed) humans, or model animals. The bioenergetic behaviour of neutrophils in established human inflammation has not been adequately addressed before. Therefore, it is possible that postoperative neutrophils do rely upon mitochondria for generation of ATP, and that dysfunctional mitochondria may be a limiting factor in neutrophil function through bioenergetic mechanisms.

Alternatively, mitochondrial ATP may serve a non-bioenergetic role. There is a precedent for this through Pannexin I/P_2Y_2 mediated chemotaxis, which relies upon the autocrine release of mitochondrial ATP (Bao et al., 2014). The release of nucleotides by necrotic cells is an important clearance signal (Elliott et al., 2009), therefore this mechanism may also facilitate neutrophil clearance of apoptotic cells.

Mitochondria may act as inflammatory signal transducers. Neutrophil mitochondria are known to coordinate apoptosis, integrating a range of intrinsic/extrinsic signals to determine output of Bcl-2 homologs (Brenner and Mak, 2009; Reed, 2006). In a similar manner,

mitochondrial may act as inflammatory signal transducers through the release of mitochondrial ROS. Several processes relevant to inflammation have been described which may lead to the release of mitochondrial ROS species. In this manner, ROS are not simply a toxic by-product, but an important signal that regulates expression of the inflammatory phenotype. For example, mitochondrial potassium channels may provide a mechanism for ROS induced mitochondrial dysfunction since O_2^- and H_2O_2 directly promote their opening (Gao and Mann, 2009), alternatively mitochondrial ROS could cause generalised mitochondrial impairment through peroxidation.

The production of mitochondrial ROS may be explained through several mechanisms related to oxidative phosphorylation, including hyperpolarisation due to reduced distal electron acceptors leading to inefficient electron transport (Vincent et al., 2004), specific dysfunction of individual respiratory complexes, loss of supracomplex organisation limiting efficiency of electron transfer or impairment of scavenging processes such as mitochondrial superoxide dismutase. Respiratory complexes are also involved in biochemical pathways that proceed independently of oxidative phosphorylation, for example oxygen sensing through complex III-ROS–HIF signalling (Brunelle et al., 2005). My data describe differential perioperative effects of complex I inhibition. It is also possible that impaired mitophagy, for example through diminished PINK1/Parkin expression, may lead to persistence of damaged mitochondria and unchecked mitochondrial ROS release.

Although ATP production is considered the primary role of mitochondria, there are other essential biochemical and metabolic processes which rely upon the electromotive potential generated by respiratory complexes. The presence of increased ROS production may therefore be considered a barometer for mitochondrial health, with a proportional impairment of these other processes. Relevant processes to the postoperative phenotype include calcium homeostasis, anion flux through VDAC channels and integrin signalling (Colombini, 2004). In reality these processes are closely related, for example VDAC activity favours a reduction in ROS formation (Zhou et al., 2011). Alternatively mitochondrial dysfunction may be associated with release of mtDNA, an established DAMP providing a universal inflammatory stimulus (Nakahira et al., 2011) in a manner analogous to gut derived endotoxin.

7.4.2 Cellular targets for mitochondrial ROS signalling

This thesis has focussed upon redox regulation of PKM2 as a regulator of postoperative neutrophil immune function. However, in addition to PKM2 there are several other possible targets. The molecular specificity of ROS signalling makes it an ideal mechanism to propagate cell-wide signals, coordinating the activity of structurally distant targets which share common amino acid residues around functional or allosteric sites.

7.4.2.1 Pyruvate kinase M2 (PKM2)

Oxidative regulation of substrate flow through glycolysis and hexose monophosphate shunt is an established feature of the cancer cell biology (Warburg effect). This thesis has presented data strongly supporting presence of redox sensitive PKM2 within neutrophils. Specifically, postoperative neutrophils are subject to redox stress and are less responsive to the highly specific PKM2 activator DASA. Therefore, it is plausible that PKM2 may act to regulate flow through glycolysis and the hexose monophosphate shunt. Central to this argument is regulation of PKM2 through heterodimerisation, where oxidation of cys358 leads to a change in quaternary structure which prevents further access to the site. Consequently, DASA can protect from, but not reverse oxidative inhibition.

The PKM2 isoform has a second unique property, it may translocate to the nucleus and act as a transcription factor. Within cancer cells EGFR-activated ERK2 phosphorylates PKM2, but not PKM1, to promote nuclear translocation (Yang et al., 2012). Nuclear PKM2 induces c-myc expression, leading to GLUT1 and positive feedback PKM2 expression. Transgenic translocation-deficient PKM2 blocks the Warburg effect within a mouse glioblastoma model. It follows that neutrophil PKM2 may direct gene transcription in a similar manner.

7.4.2.1 Hypoxia inducible factor (HIF)

HIF was initially identified as a mediator of hypoxic adaptation and inflammatory signalling, but has since been shown to be activated following many other inflammatory stimuli (Klimova and Chandel, 2008). The key signal for stabilisation of HIF is the production of mitochondrial ROS at respiratory complex III (Brunelle et al., 2005), or possibly respiratory complex II (Dikalov, 2011). Pharmacological inhibition of respiratory complex III is an established experimental tool for investigation of HIF signalling (Dikalov, 2011).

HIF activation has pleiotropic molecular effects, many of which combine to promote glycolysis and inhibit oxidative phosphorylation and mitochondrial biogenesis. HIF is

intimately associated with the control of autophagy, which may liberate endogenous metabolic reserves during times of energy starvation, as well as being responsible for physiological cellular turnover. Here a decline in leukocyte ATP and rise in AMP signals metabolite stress, promoting a decline in AMP-kinase and increase in HIF1-α, which signals autophagy (Zhang et al., 2010). Sub-inflammatory doses of endotoxin have been shown to promote the same changes in AMP-kinase and HIF-1(Haimovich et al., 2014). AMP-kinase regulated mitochondrial biogenesis (Jäger et al., 2007) acts in synergy with growth signal integrating mTOR (Papandreou et al., 2006; Semenza et al., 1994; Zhang et al., 2007).

7.4.2.2 Redox regulation of inflammatory gene expression

Neutrophils are subject to intense gene regulation and protein synthesis (Calvano et al., 2005; Teles et al., 2012; Xiao et al., 2011). Several transcription factors display altered DNA binding following ROS driven oxidation (Kolls, 2006), including NF-kB (Han et al., 2013) and Nrf2 (Davidson et al., 2013; Segal et al., 2010). The addition of respiratory complex I and III inhibitors to neutrophils has been shown to stabilise NF-kB and promote cytokine expression (Zmijewski et al., 2009, 2008). NRF2 is involved in antioxidant and cytoprotective responses, revealing how ROS may promote their own detoxification (Davidson et al., 2013; Segal et al., 2010). These processes may be intrinsically linked to regulation of mitochondrial function, since transcription of key apoptotic signals, which target mitochondria, are altered by NF-kB and PI3K (Edwards et al., 2004). As discussed above, PKM2 may enter the nucleus and serve as a transcription factor. The time scale of transcriptional changes is compatible with the functional changes on postoperative day two, and may account for shorter term classical priming or tolerance through interaction of transcription factors with other signalling pathways, such as MAPK pathway. Gene silencing has been previously reported as a possible mechanism of immune tolerance (Liu et al., 2011; McCall and Yoza, 2007).

7.4.2.3 The NLRP3 inflammasome

Within macrophages the NLRP3 inflammasome has emerged as a key regulator of innate immunity, responsible for the release of proinflammatory mediators IL-1B and IL-18 through cleavage of caspase-1 (Stutz et al., 2009). The depletion of autophagy mediators has been shown to promote NLRP3 mediated inflammation, through preservation of dysfunctional mitochondria (Nakahira et al., 2011). Here, the depletion of mitophagy signalling proteins

leads to the persistence of ROS generating mitochondria, and activation of the NLRP3 inflammasome. The NLRP3 inflammasome has been identified within neutrophils and is necessary for the synthesis of neutrophil IL-1β, however beyond this it remains less well characterised than within monocytes (Karmakar et al., 2015).

7.4.2.4 Direct regulation of NADPH oxidase

There is evidence for direct signalling between mitochondrial NADPH oxidase and NADPH oxidase (Dikalov, 2011), although most of these studies have investigated the Angiotensin-II-NOX pathway (rather than PHOX) and the exact site of ROS/NADPH interaction remains speculative. Within HAEC cells, SOD2 knockdown prevents angiotensin-II stimulated NADPH oxidase activity (Dikalova et al., 2010) and accumulated mitochondrial damage within rabbit aortic smooth muscle cells is associated with diminished NOX1 mRNA expression (Wosniak et al., 2009). The mitochondrial-NOX communication may be reciprocal, promoting positive feedback and further ROS production. Depletion of $p22^{phox}$ causes a significant reduction in Angiotensin-II stimulated mitochondrial ROS production (Mehta and Griendling, 2007). There is no evidence from CGD patients than diminished respiratory burst function alters metabolic function, since glycolysis/oxidative phosphorylation appear unchanged (Borregaard and Herlin, 1982).

7.4.3 A neutrophil specific manifestation of a global metabolic phenotype.

Systemic inflammatory responses are associated with body wide metabolic reprogramming. Since the bioenergetics and synthetic requirements of different cells types vary, the various changes of cellular function which accompany inflammatory responses may be the product of a common metabolic phenotype interacting with each cell's specific metabolic requirements. For example, within highly anabolic cells the hexose monophosphate shunt is a source of ribose sugars, while platelets possess few mitochondria and have no nucleus to support mRNA synthesis. In this setting acquired mitochondrial dysfunction could represent a common pathological mechanism of clinically different states (Neustadt and Pieczenik, 2008).

7.4.4 A consequence of circulating, immature neutrophil precursors.

The short life span of circulating neutrophils, typically estimated to be one day (Luo and Loison, 2008; Maianski et al., 2004; Pillay et al., 2010), combined with the release of immature neutrophils during systemic inflammatory responses suggests that the postoperative phenotype may be partly explained by an increase in the proportion of circulating immature cells. Within septic patients the presence of immature neutrophil precursors (myelocytes and metamyelocytes) may have prognostic significance (Mare et al, 2015)

Immaturity is unlikely to be the sole explanation, because postoperative bone marrow derived neutrophils demonstrate a diminished burst response when compared to preoperative control bone marrow (Chapter 4), which supports a global change in the entire neutrophil pool. Nonetheless there are similarities between immature neutrophils and the postoperative cells I have described.

7.5 Clinical relevance

7.5.1 Clinical study design

This thesis supports the use of major elective surgery as a translational model for sepsis, serving as a bridge between model organism studies and clinical sepsis research. The addition of this extra layer of investigations would help refine experimental hypotheses and questions so clinical studies within critically ill may be better designed to isolate/identify the experimental signals in question. This also implies that the many studies already performed within surgical patients may serve as a valuable resource for sepsis research. Looking to the future, perioperative medicine is a rapidly expanding field. Firstly, this will hopefully provide information and observations which may push sepsis research onwards. Secondly, collusion between perioperative and critical care research will be mutually beneficial.

Moving beyond surgery, similarities between other patient groups and septic patients may mean that observations within these other groups are relevant to septic patients. For example, mechanisms underlying anergic immune responses within cancer patients may be relevant to similar responses within long stay ICU patients. The effect of surgery upon cancer patients may therefore provide a tool to investigate effects of acute-on-chronic inflammation.

7.5.2 Assays of inflammation

The postoperative functional impairments of neutrophil function described within this thesis occur at a time when routine laboratory markers of inflammation, such as CRP and white cell count, are increased.

The development of clinical assays of more specific markers of immune function may improve patient care in many ways. At present, clinicians may only guess at how effective the immune response a patient is mounting. This is because the two critical variables are unknown: infective burden/potency and the magnitude of the immune response. Therefore, a septic patient may deteriorate because they mount an insufficient or excessive immune response. The development of validated immune assays would also be directly relevant to future studies of immune modulating agents.

7.5.3 Novel targets for immunomodulation

Metabolic changes are a feature of the inflammatory response. Therefore, metabolic targets may potentially be used to regulate inflammation within patients. Many established therapeutic agents have direct metabolic actions, such as metformin which inhibits mitochondrial respiratory complex I. Within the cancer literature many chemotherapy agents have been shown to have metabolic activities, for example cyclosporin-A inhibits lymphocyte glycolysis (Röpke et al., 2000).

7.6 Further work

7.6.1 Extension of the postoperative immune function and metabolic postoperative phenotype

Functional analysis may be extended through specific assays of bacterial killing, chemotaxis and NET formation. The metabolic phenotype may be explored in several ways. Firstly, in vitro assays of isolated neutrophil mitochondria may provide insight into how function of specific respiratory complexes changes over perioperative period. These assays may be performed within whole neutrophils, using the Seahorse respirometer (Chapter 2), or within isolated mitochondria using a Clarke electrode. Similarly, for glycolysis, the function of individual enzymes may be quantified to determine precisely how these activities change during human inflammatory responses – these assays have only been performed within 'healthy' human cells before.

7.6.2 Correlation with clinical outcome

It is possible that increased and decreased neutrophil functions may be beneficial or harmful. Therefore, this study may be extended to follow the relationship between functional changes and clinical outcome. This is a necessary step before studies of immune modulation could be attempted.

Beyond this, the surgical model may also provide a tool to investigate the immunosuppressive phase of sepsis, using tools such as latent virus reactivation (Limaye et al., 2008; Luyt et al., 2007) and colonisation by opportunistic and gut derived pathogens (Guan et al., 2013). Cancer patients share many of the immunological defects of sepsis, therefore elective surgery within cancer patients offers a further opportunity to simulate the critically unwell patient.

7.7 Conclusions

Major elective surgery may provide a valuable translational model of human sepsis, which overcomes many of methodological challenges presented by patients with an established illness. Postoperative neutrophils demonstrate a reversible impairment of neutrophil respiratory burst, as well as features that support mitochondrial dysfunction, that may be relevant to development of postoperative complications, and therefore outcomes in sepsis. These metabolic changes may regulate immune function through novel signalling molecules such as mitochondrial ROS, as well as the delivery of metabolic substrate to these processes. Investigations into metabolic regulation of cancer cells have revolutionised cancer research, and further characterisation of metabolic responses within human sepsis may offer similar benefits and identify new therapeutic targets.

APPENDIX A – Experimental assay details for studies included in Chapter 3.

Author (Year)	Clinical Setting*	Experimental protocol[#]	ROS substrate [$]	Burst stimulus
(Santos et al., 2012)	Septic patients		DCFH-DA	P.aeruginosa, S.aureus
(Paunel-Görgülü et al., 2011)	Trauma patients	WB, ?AC [FC:morph/-vecd14]	DHR	PMA
(Bruns et al., 2011)	Septic patients (cirrhosis)	Gran, Hep, DG: Percoll,? (Abs) [FC]	DHR	E. coli, fmlp, PMA
(Shih et al., 2010)	Trauma patients	WB, Hep [FC:morph]	SOD inhibition of Cyt c	fMLP
Kasten et al. 2010)	Trauma patients	Gran, Hep, DG:Ficoll-Hypaque+Dextran, HL (abs) [trypan blue]	Scopoletin, HPO	FN attached
(Valente et al., 2009)	Trauma patients	PBL, EDTA, HL, (FN attached) [confocal]	DHR	PMA
(Kawasaki et al., 2007)	Surgical patients	PBL, Hep [FC:morph]	DHR	E. coli

Fröhlich et al. 2006)	Surgical patients	WB, Hep [FC:morph]	DHR	fMLP, PMA
(Martins et al., 2003)	Septic patients	PBL, Hep, DG:Ficoll lymph sep 1.077 [FC:morph]	DCF	fMLP, LPS, PMA, S.aureus
(Barth et al., 2002)	Septic patients	WB, Hep [FC:morph]	DHR	E. coli
(Mariano et al., 2001)	Septic patients (RRT)	WB, Hep [FC:morph]	Luminol (HV neu)	Patient sera
(Quaid et al., 2001)	Trauma patients	Gran, EDTA, Lo/Hi centrifugation, HL [Giesma stain]	Scopoletin, HPO	FN attached, PMA, TNF-α
(Wiezer et al., 2000)	Surgical patients	Gran,EDTA, DG: Histopaque1077, HL, (FN) [">95% pure"]	DHR	PMA (stop with BSA)
Ahmed et al. 1999)	Septic patients	PBL, hep, DG: Lymphoprep, IL [FS:morph]	DHR (tissue+blood neu)	fMLP
(Shih et al., 1999)	Surgical patients	Gran, hep, DG: dextran-70, Ficoll-Hypaque, HL [FS:morph]	SOD inhibition of Cyt c	fMLP

(Ertel et al., 1999)	Trauma patients	Gran, EDTA, DG: Ficcol-Paque, HL [Wright stain]	DHR (HV neu)	BALF,CSF,fMLP
(Ogura et al., 1999)	Trauma patients	Gran, hep, DG: Histopaque 1077, IL [FC:cd-15]	DCFH	fmlp
(Pascual et al., 1998)	Septic patients	WB, ?, "lysis soln" [FC:morph]	Lucigenin (Patient+HV neu),	Patient+HV serum

Table A1 Experimental assay details for respiratory burst studies

#Experimental protocol: format: cell used, anticoagulation, separation technique:colloid used, lysis step. (Additional substances added) [Identification of neutrophils]. WB=Whole blood, Gran=granulocytes, PBL=peripheral blood leukocytes. ?AC=anticoagulant not detailed, Hep=heparin, EDTA=Ethylenediaminetetraacetic acid. DG:density gradient. HL=hypotonic lysis, IL=isotonic lysis. Abs=cultured with antibiotics, FN=cultured in fibronectin coated plates. FC=flow cytometer, morph=forward scatter/side scatter on flow cytometer used to identify neutrophils

$DCFH-DA=2',7'-dichlorodihydrofluorescein diacetate; DHR=Dihydrorhodamine 123; PMA=Phorbol 12-myristate 13-acetate; fMLP=Formyl-Methionyl-Leucyl-Phenylalanine; SOD=Superoxide Dismutase; Cyt c=Cytochrome C, HPO=Horseradish peroxidase; FN=Fibronectin; HV=Healthy volunteer; TNF-α=Tumour necrosis factor α; BSA=Bovine serum albumin; BALF=Bronchoalveolar lavage fluid; CSF=cerebrospinal fluid.

Author/Year	Clinical Setting	Experiment protocol[#]	Tolerance induction*	Tolerance assay[$]	Experimental Readout[&]
(Liu et al., 2011)	Septic patients	Plasma: EDTA. WB: [FC:CD 14	LPS, ?dose, ex-vivo, 1hr, WB	PBL, qRT-PCR, TNF-α.	PBL, ChIP, SIRT1 and RelB @ TNF-α promoter.
(Buttenschoen et al., 2009)	Surgical patients	PBMC: ?, DG: Ficoll/Isopaque.	LPS 0.2ng/ml,ex-vivo, 6hrs, WB.	WB SN, ELISA, TNF-α Il-1ß IL-6.	MNF, qRT-PCR, TLR-2 TLR-4
(Pachot et al., 2008)	Septic patients	PBMC: ?, DG:Ficoll-plaque, BD Pharm Lyse Buffer. Monocyte: cd14 MACS isolation.	Unstimulated	monocyte, mAb, HLA-DR	WB, qRT-PCR, CX3CR1. Monocyte, mAb, CX3CR1.
(West et al., 2007)	Septic Patients	WB: Hep. [FC:morph]	LPS, 10ng/ml, ex-vivo, 15min WB	Plasma, ELISA, TNF-α.	WB, mAb, MAPK ERK1/2
(Härter et al., 2004)	Septic patients	PBMC: Hep, DG:histopaque, IL, [FC:cd14]	LPS 1ug/ml / MALP2 2nM, ex-vivo, 4/16hrs , neu+monocyte	Neu+monocyte, ELISA, IL-8 TNF-α	Neu+mono, mAb, TLR2 TLR4
(Flohé et al., 2004)	Surgery in trauma patients	WB: Hep. [FC:CD-14]	LPS, 10ng/ml, ex-vivo, 14hrs, WB. (abs)	Plasma, ELISA, TNF-α IL-10. WB mAb HLA-DR CD14	

(Arnalich et al., 2003)	Septic patients	Monocytes: ?, DG:Ficoll-Hypaque Plus, adh-sep (abs).	LPS, 10/ng/ml / 100ng/ml, ex-vivo, 1hr, monocyte.	Monocyte, qRT-PCR, TNF-α IL-6	IRAK-M TLR-4
(Heagy et al., 2003)	ICU patients	WB (discarded):, Hep.	LPS, 10ng/ml,ex-vivo, 3hrs, WB.	Plasma, ELISA, TNF-α, IL-6	LOS, VFD, WCC, Infection
(Calvano et al., 2003)	Septic patients	WB: ?AC, TNF-α. Monocytes: ?, Rosette Sep enrichment, cell sort with CD15, confirm CD14	LPS, 10ng/ml, ex-vivo, 24hrs	Plasma, ELISA, TNF-α	Monocyte, mAb, TLR2 TLR4 CD14. Monocyte, qRT-PCR cd14 TLR4 TLR2, MD-2.
(Sfeir et al., 2001)	Septic patients	Monocytes: Citrate, DG:Nycoprep, adh-sep [FC:CD14]	LPS, 1ug/ml, ex-vivo, 16hrs, monocytes.	Monocyte, ELISA,TNF-α IL10 TGF-ß	Repeat with anti-IL-10+anti TGF-ß
(Kawasaki et al., 2001)	Surgical patients	WB: Hep/EDTA.	LPS, 10ng/ml, ex-vivo, 4hrs, WB.	Plasma, L929 cell cytotoxicity, TNF-α. Plasma, ELISA, IL-10.	WB, mAb, HLA-DR CD14
(Heagy et al., 2000)	Septic patients	WB (discarded): Hep. [FC:CD14]	LPS, 10ng/m, ex-vivo, 3hrs, WB (abs)	Plasma, ELISA, TNF-α	WB, mAb, CD14 45 TNF-α_{IC}
(Bergmann et al., 1999)	Septic patients	PBMC: Citrate, DG:Ficoll.	LPS, 1ug/ml, ex-vivo, 1-3hrs, WB.	Plasma, ELISA, TNF-α, IL-1, IL-6	MNF, Nblot, TNF-α IL-1 IL-6

Table A2 Experimental assay details for monocyte endotoxin tolerance studies

#Experimental protocol: format: cell used, anticoagulation, separation technique: colloid used, lysis step. (Additional substances added) [Identification of neutrophils]. WB=Whole blood, Discarded=blood used from aspiration of dead-space in venous cannulas, PBMC=Peripheral blood mononuclear cells. ?AC=anticoagulant not detailed, Hep=heparin, EDTA=Ethylenediaminetetraacetic acid. DG: density gradient. HL=hypotonic lysis, IL=isotonic lysis. Abs=cultured with antibiotics, FC=flow cytometer, morph=forward scatter/side scatter on flow cytometer used to identify monocytes.

**Tolerance induction: Agent, dose, location, exposure time, blood product (additional agents). LPS=Lipopolysaccharide, WB=Whole blood, neu=neutrophil, abs=antibiotics*

$Tolerance assay: Blood product, assay, target. PBL=Peripheral blood leukocyte, ELISA= Enzyme-linked immunosorbent assay, qRT-PCR = quantitative real time polymerase chain reaction, mAb = monoclonal antibody,

&Experimental Readout: Blood product, assay, target. ChIP=Chromatin Immunoprecipitation, Wblot = Western blot, Nblot = Northern blot, LOS=Length of ITU stay, VFD=Ventilator free days, WCC=White cell count.

Author (Year)	Clinical Setting	Experimental# protocol	Apoptosis readout*	Cell types$
(Roger et al., 2012)	Septic patients	WB: ?AC	Hoeschst dye	CD4, CD8($CD3^+$ $CD4^-$)
(Bandyopadhyay et al., 2012)	Trauma patients	PBMC: ?AC, DG:Ficoll-Hypaque, NTSR.	Annexin-V, 7AAD. Caspase-10_{IC}	T cell
(White et al., 2011)	Septic patients	PBMC: EDTA, Lymphoprep.	mRNA: BAX, BCL-2, BIM.	PBMC
(White et al., 2011)	Surgical patients (infective complications)	PBMC: EDTA, Lymphoprep.	mRNA: BAX, BCL-2, BIM.	PBMC
(Zhang et al., 2011)	Septic patients	PBL: ?AC, VersaLyse). Lympho. (?,DG: Ficoll + cd14 neg selection.	Annexin-V. Caspase-3, PD-1	CD4, CD8, CD19
(Guignant et al., 2011)	Septic patients	WB: ?AC.	PD-1	CD4
(Vaki et al., 2011)	Septic patients	HV Lympho + subject serum: Hep, DG: Ficoll-Hypaque. Serum incubation for 24hrs, adh-sep (non adherant analysed).	Annexin-V. Caspase-3 activity. CD95.	CD4
(Słotwiński et al., 2011)	Surgical patients	WB. Lympho,: Hep, DG:Lymphoprep, ?, ?.	Bax, Bcl-2, Caspase-3 9, Fas, PARP-1, TNFR1.	Lymphocytes

(Gogos et al., 2010)	Septic patients	PBL: EDTA, IL.	Annexin-V, 7-AAD	CD4, CD8, 19, CD56
(Hoogerwerf et al., 2010)	Septic patients	Lympho: Hep, IL, cell sort CD4.	Microarray: 30 genes	CD4
(Yousef et al., 2010)	Septic patients	PBL: EDTA, IL.	Annexin-V, 7-AAD	CD3, CD19
(Turrel-Davin et al., 2010)	Septic patients	WB: EDTA. PBMC: EDTA, DG: Ficoll-Paque, 'lysed'.	Annexin-V. Bcl-2, Caspase-3. mRNA: *BAK, BAX, BCL-XL,BID, BIM, FAS*	CD4, CD8, CD19
(Pelekanou et al., 2009)	Septic patients	PBL: Hep, IL.	Annexin-V, PI	CD4, CD8, CD19, CD56
(Resident et al., 2009)	Surgical patients	PBL: Citrate, VersaLyse.	Annexin-V,PI	Lymphocytes (FC morphology)
(Delogu et al., 2008)	Septic patients	"Isolated PBMC"	Annexin-V, 7AAD, $DiOC_{6.}$ Bcl-2, Caspase-3 8 9, Fas, FasL.	PBMC
(Weber et al., 2008)	Septic patients	PBL. (EDTA, HL). PCR=Paxgene	Annexin-V, 7AAD. Bcl-2, Caspase-3. mRNA: *BCL-2, BCL-XL, BID, BAK.*	CD4, CD8, CD19. mRNA=WB
(Roth et al., 2003)	Septic patients	PBMC: EDTA, DG:Ficoll	Annexin-V, 7AAD. ACE, IL-1ß, IL-10. sCD95, sTNFR1.	CD3. Serum cytokines.

(Le Tulzo et al., 2002)	Septic patients	PBL: EDTA, Othro-lysis.	Annexin-V, 7AAD. CD95, CD95L, TRAIL-R1, TRAIL-R2.	CD4, CD8 ($CD4^-$ $CD3^+$), CD19
(Hotchkiss et al., 2001)	Volunteer endotoxin	PBMC: EDTA, DG:Ficoll.	Annexin-V, 7AAD	PBMC
(Delogu et al., 2001)	Septic patients	Fixed cadaveric/(intraoperative) spleens	Lightmicroscopy. Immunohistochemistry (inc caspase 9). Annexin-V, PI.	Splenic tissue. B cells, CD20, CD4, CD8, CD3, NK.
(Pellegrini et al., 2000)	Surgical patients	Lymphocytes: Hep, DG:Lymphoprep	7AAD	CD4, CD8
(Delogu et al., 2000)	Trauma patients	PBMC +T-cells: (Hep, DG:Ficoll-Hypaque, adh-sep NTSR)	PI (loss of)	T cell, PBMC
(Hotchkiss et al., 2000)	Surgical	Lympho: Hep, DG: Lymphoprep, ?.	7AAD. Bcl-2, Fas FasL, p35.	CD4, CD8
(Hotchkiss et al., 1999)	Trauma patients	Fixed intraoperative bowel tissue.	Light microscopy. Immunohistochemistry (Cytokeratin 18). Hoechst dye.	Lymphocyte aggregates within bowel biopsy.
(Sasajima et al., 1999)	Septic patients	Fixed cadaveric/(intraoperative) spleen tissue.	Light microscopy. Immunohistochemistry (inc Caspase-3, Bcl-2). DAGE, TUNEL.	Lymphocyte aggregates within bowel and spleen biopsies.

(Sugimoto et al., 1998)	Surgical patients	Lympho: ?,Lymphoprep, IL.	DAGE	Lymphocytes
(Roger et al., 2012)	Surgical patients	PBMC: Hep, DG:Lymphoprep.	Fas. mRNA: *FASL*	CD4

Table A3 Experimental assay details of lymphocyte apoptosis studies

#Experimental protocol. Format: "cell used: (anticoagulation, separation technique: colloid used, lysis step). <Additional substances added>". WB=Whole blood, PBMC=Peripheral blood mononuclear cells, PBL= Peripheral blood leukocytes, Lympho=lymphocytes. ?AC=anticoagulant not detailed, Hep=heparin, EDTA=Ethylenediaminetetraacetic acid. DG=density gradient:colloid used. HL=hypotonic lysis, IL=isotonic lysis. Abs=cultured with antibiotics, FC=flow cytometer, morph=forward scatter/side scatter on flow cytometer used to identify monocytes.

**Apoptotic readout. Italics indicate genes/ribonucleic acid gene products. PI = Propidium iodide, 7AAD= 7-Aminoactinomycin D, FLICA = fluorochrome inhibitors of caspases, TUNEL = Terminal deoxynucleotidyl transferase dUTP nick end labelling, DNA agarose gel electrophoresis*

$Cell type studied. PBMC - peripheral blood mononuclear cell, FC=flow cytometry (i.e. forward scatter/side scatter), WB=Whole Blood.

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