The role of antibiotics, catecholamines and sedatives on immune cell functionality and mitochondrial activity.

Dr Muska Miller

A dissertation submitted in partial fulfilment of the requirements for the degree of

Doctor of Philosophy

of

University College London.

Bloomsbury Institute of Intensive Care Medicine Division of Medicine

University College London

Gower Street, London WC1E 6BT

17th August 2022

Declaration

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

Abstract

Introduction and aim:

Sepsis is defined as life-threatening organ dysfunction caused by a dysregulated host response to infection. In the absence of host-modulating therapies, specific management is focussed upon eradication of the pathogen by antibiotics ± source control, organ support, and prevention of iatrogenic harm. The use of antibiotics, catecholamines and sedatives is associated with off-target effects including immunomodulation. These drugs are routinely used together in critically ill patients with sepsis so there may be cumulative effects. Mechanisms underlying immunomodulation are still to be fully elaborated, however there is increasing interest in the role of mitochondria in organ dysfunction and drug-induced immunomodulation. My aim was to investigate the role of ciprofloxacin, propofol and norepinephrine (NE), alone and in combination, on immune cell functionality and mitochondrial activity in septic and healthy peripheral blood mononuclear cells (PBMCs).

Methods:

PBMCs isolated from healthy volunteers were incubated with either ciprofloxacin (100 µg/mL), propofol (50 µg/mL), NE (10 µg/mL) or all three drugs combined, in the presence and absence of endotoxin (100 ng/mL) for 6 or 24 hours. Comparison was made against untreated cells. Measurements were made of IL-6, IL-10 and TNF-α production (ELISA), cell viability, phagocytosis, HLA-DR, mitochondrial membrane potential and reactive oxygen species production (flow cytometry), and mitochondrial O_2 consumption (Seahorse respirometry). This was repeated in PBMCs from healthy volunteers co-incubated with serum from ED patients with suspected sepsis or septic ICU patients. Immune and metabolic pathways were investigated in PBMCs from healthy volunteers co-incubated in septic or healthy volunteer serum using Nanostring technology to identify potential pathways that may explain underlying mechanisms.

Results and conclusion:

While the exemplar antibiotic, sedative and catecholamine reduced TNF-α, IL-6 and phagocytosis in PBMCs isolated from septic ICU patients and from healthy volunteers co-incubated with septic serum, no cumulative effect was seen with the combination. No consistent changes were seen in PBMCs' mitochondrial functionality. Similarly, gene expression analysis did not highlight any involvement of specific immune or metabolic pathways as underlying mechanisms. Further studies are required to fully characterise immune dysfunction caused by these drugs and whether these *in-vitro* findings translate to the *in-vivo* situation.

Impact statement

Sepsis is a major global health concern with high morbidity and mortality. The underlying pathophysiology is unclear and management of sepsis still remains largely supportive, with the exception of antibiotics and source control to treat the infection. Numerous studies have demonstrated that antibiotics, catecholamines and sedatives have immunomodulatory effects (1-18). However, these studies have largely been performed *in-vitro* or *ex-vivo* using immune cells taken from healthy volunteers or animals, and not on cells taken from septic patients or animals. In addition, these have generally been examined in isolation, while treatment in patients usually consists of a combination of such drugs.

Mechanisms are poorly elaborated however there is increasing interest in the role of mitochondrial dysfunction, both in terms of the development of organ dysfunction in sepsis and in the immunomodulatory effects of antibiotics, catecholamines and sedatives. These two aspects have previously been looked at separately rather than in conjunction.

In this thesis, I have investigated the impact of these drugs, individually and in combination, on immune cell functionality and mitochondrial function in the context of sepsis. I have also compared findings in circulating immune cells taken from healthy volunteers co-incubated (or not) with septic serum, from ED patients with suspected sepsis, and from ICU patients with confirmed sepsis. To my knowledge, no such comparison has been previously undertaken.

My research demonstrates that ciprofloxacin, propofol and norepinephrine individually suppress immune function *in vitro*, however I could not demonstrate a cumulative effect. These findings were most profound in peripheral blood mononuclear cells isolated from septic ICU patients and from healthy volunteers co-incubated with septic serum. There were no obvious mitochondrial changes associated with these drug-induced immune changes.

These research findings have been highlighted in local meetings to colleagues. Further dissemination will include publication and presentation in international journals and at relevant conferences. These findings serve as useful groundwork for further exploration of current *in-vitro* and *ex-vivo* models of sepsis and how these can be modified to better describe the pathophysiology and natural history of sepsis. My results require further comprehensive investigation of immune and mitochondrial function in response to such drugs. In the long term, this may influence the choice and duration of drugs used in septic patients and may lead to development of novel therapeutics to mitigate unwanted effects.

Acknowledgements

I am thankful to a huge number of people for their generous support and encouragement during the course of my PhD. Firstly, thanks to my esteemed supervisors Professor Mervyn Singer and Professor Manu Shankar-Hari for their invaluable supervision, enthusiastic support and tutelage. Your immense knowledge, plentiful experience and open-door policy have been enormously helpful during my research. Thank you for taking a punt on me and for giving me the opportunity to see how a clinician can learn from and contribute to a basic science lab. I feel infinitely richer for having had this experience.

My gratitude extends to all members of the Singer lab (Anna, Miranda, Giacomo, Vera, Alex, Nish, Tim and Naveed), thank you for your patience, technical support and important life lessons. A special thank you to Dr Anna Kleyman for her incredible mentorship and friendship; her expertise, critique and encouragement have been invaluable. Thank you also to Miranda Melis for being a stellar peer and friend, for sharing ideas, resources and workspace, and for keeping me going in times of darkness. I would also like to thank the Gentian study team especially Dr Brealey in collecting patient samples and the brilliant research nurses for all their help. None of this work would have been possible without the patients and healthy volunteers who kindly donated their blood; I am indebted to you all. Thanks also to Jamie Evans for support with flow cytometry experiments, to Anita Chacko and Claire Duff for helping with Seahorse experiments, and to Dr Gareth Ambler for providing insight into data analysis and interpretation.

My sincere thanks to The London Clinic, in particular Dr John Goldstone, for their generous support and treasured clinical opportunity. Thanks to the wonderful nursing and housekeeping staff for your kindness, generosity and understanding over the past few years.

I would like to thank my friends and family (especially Staci, Daniel, Eric, Sonia, Kathleen, Simon, Andrew, Justin, Carla, Peter, Lesley, Natasha, Yinka and

Pamela) for the relentless support, encouragement and advice. Thank you to my parents for instilling the desire to enrich myself with knowledge from a young age, it paid off in pointing me in this direction. Thank you to Willow and Joshua for coming to see me and cheering me up with cuddles. I am deeply grateful to Staci for the decades of patience and love, no statement would be adequate to recognise all the contribution you have made in my life.

Finally, the biggest thanks to my wonderful husband Jamie and son Alex. Jamie thank you for your unconditional love, relentless encouragement and tremendous support in all aspects of life. Thanks for looking after Alex when I've been home late or on my clinical shifts. Your dedication and enthusiasm towards our little boy have put my heart at ease at times when I haven't been able to be there myself. Mr Tingles thank you for being the sweetest little boy in the universe and for making our time together joyous. Thank you for the heart-warming cuddles, sloppy kisses and 'Laaa luuuus' that transform any day to one of my happiest. This thesis is dedicated to you both.

Contents Page

List of Tables

List of Figures

Chapter 1 Introduction

Intensive care units are increasingly admitting complex patients with advanced age, multiple comorbidities and declining functional status. The overarching aim is to improve survival rates and survivorship while balancing the risks and benefits of intervention to individual patients. Sepsis comprises a significant proportion of intensive care admissions and remains a global challenge due to its high mortality rate and poor early identification. Despite decades of translational research and clinical trials of novel therapeutic interventions, management remains limited to the conventional tenets of timely antibiotics and fluid resuscitation, source isolation, recognition and prevention of iatrogenic harm (e.g. excessive ventilation, excess fluid therapy, overuse of drugs including catecholamines and sedatives).

The use of antibiotics, catecholamines and sedatives is associated with numerous off-target effects, of which immunomodulation is of great importance given the increased susceptibility of critically ill patients to secondary infections and a consequently increased risk of mortality and morbidity (1-18). Mechanisms of immunomodulation are still to be fully elaborated however there is increasing interest in the role of mitochondria in organ dysfunction and drug-induced immunomodulation. To date, these two aspects – the disease process and iatrogenic interventions - have been looked at separately rather than in conjunction.

This chapter begins with an overview of sepsis, the immune system and mitochondria, before focusing on the effects of individual drug groups on the immune system and mitochondria.

1.1 Sepsis

1.1.1 Definition

The word sepsis is derived from the Greek *σήψη* meaning '*decay or decomposition of organic matter*'. The first modern definition, coined by Bone *et al*. in 1991 (19), focused on the hyperinflammatory phase of sepsis pathophysiology, which they termed the systemic inflammatory response syndrome (SIRS). SIRS was characterised clinically by abnormalities measured in ≥2 of four clinical variables (heart rate, respiratory rate (or $PaCO_2$), temperature and white cell counts) (Table 1.1) (19). As understanding of sepsis pathophysiology has improved, there has been increasing appreciation of the importance of anti-inflammatory pathways alongside pro-inflammatory. Modern definitions of sepsis have thus evolved as a result.

Table 1.1. SIRS criteria.

At least two of the following parameters are required for a diagnosis of SIRS (19):

Parameters	**Values**
Temperature	>38 or <36 °C
White cell count	>12,000 or <4000/mm^3 or >10% bands
Heart rate	>90 beats/min
Respiratory rate (or $PaCO_2$)	>20 breaths/min or $PaCO_2$ <32 mmHg (4.3 kPa)

The current definition (Sepsis-3) defines sepsis as life-threatening organ dysfunction caused by a dysregulated host response to infection (20). This places the main emphasis on the host response to the pathogen rather than the pathogen itself. Organ dysfunction is characterized as a ≥2 point increase from the patient's normal baseline in the Sequential [Sepsis-related] Organ Failure Assessment (SOFA) score (Table 1.2) (20). Septic shock is now defined as a subset of sepsis comprising marked circulatory, cellular and metabolic abnormalities. This is clinically characterised by persisting hyperlactatemia (>2 mmol/l) and a vasopressor requirement despite correction of hypovolaemia; this carries a much greater mortality risk compared to sepsis

alone (20). This definition has contributed to improved consistency for coding, epidemiology and clinical research.

Table 1.2. Sequential [Sepsis-related] Organ Failure Assessment (SOFA) scoring system.

This is used to determine the functional status of critically ill patients. The score is based on six organ systems (respiratory, cardiovascular, hepatic, coagulation, renal and neurological systems) and the score ranges from 0 at best to 24 at worst (21).

Systems	**Scores**				
	0	1	2	3	4
Respiratory system PaO_2/FiO_2 mmHg (kPa)	≥400 (53.3)	<400 (53.3)	<300 (40)	<200 (26.7) with respiratory support	<100 (13.3) with respiratory support
Coagulation Platelets ($\times 10^3/\mu L$)	≥150	<150	<100	<50	<20
Hepatic system Bilirubin mg/dL (µmol/L)	20	20-32	22-10	102-204	204
Cardiovascular system	MAP ≥70 mmHg	MAP <70 mmHg	Dopamine <5 or dobutamine (any dose)[b]	Dopamine 5.1-15, epinephrine ≤0.1, or norepinephrine ≤0.1[b]	Dopamine >15, epinephrine >0.1, or norepinephrine >0.1[b]
Central nervous system GCS score	15	13-14	10-12	6-9	<6
Renal system Creatinine (µmol/L) or urine output (ml/day)	110	110-170	171-299	300-440 <500 ml	440 <200 ml

PaO_2: partial pressure of arterial oxygen; FiO_2: fraction of inspired oxygen, MAP: mean arterial pressure, GCS: Glasgow Coma Scale score. b- Doses are presented as µg/kg/min.

1.1.2 Epidemiology

Sepsis is associated with significant mortality, morbidity, and socio-economic burdens. Current estimates of sepsis-related incidence and outcomes vary markedly due to the complex natural history, differences in definitions and data capture, and distinctions in geographical locations and healthcare provision (21). An estimated ~48.9 million cases occurred in 2017, leading to 11 million deaths; this represents 19.7% of all global deaths (22). Recent estimates in North America, Europe and Australia report average 30- and 90-day mortalities as 24.4% and 32.2%, respectively (23).

Mortality is multifactorial and influenced by increasing age, co-morbidities, pathogen characteristics and the number and severity of organ dysfunctions (24, 25). In England 77.5% of sepsis-related deaths are considered to occur in patients aged ≥75 years, compared to 0.075% in children up to 18 years (26). No significant sex differences in mortality are seen, however sepsis generally occurs later in life in females (27). There has been a general decline in mortality related to Gram-negative bacteria, however Gram-positive related mortality has remained static (27). Organ dysfunction is strongly associated with patient mortality, with death rates as high as 70% when ≥3 organs are affected (27). Septic shock carries mortality rates of at least 40-50% (28).

The clinical course is also influenced by pathogen characteristics, e.g. type of microorganism, virulence and site of infection. A point prevalence study of adult patients from 75 countries found 64% of cases were respiratory, 20% abdominal, 15% primary bacteraemia and 14% genitourinary (29). Seventy percent had positive microbiological cultures: 62% for Gram-negative organisms, 47% for Gram-positive organisms (some were mixed infections) and 19% fungal. *Staphylococcus aureus* (20%) was the predominant Gram positive pathogen and *Pseudomonas* species (20%) and *Escherichia coli* (16%) were the most prevalent Gram negative pathogens (29).

Sepsis-related mortality may be reducing due to improvements in medical care (e.g., earlier intervention and reduction of iatrogenic harm such as

polypharmacy), however the economic burden remains significant (30-34). The York Health Economics Consortium in 2017 estimated the annual cost of sepsis to the UK as £7.76 billion (35). A systematic review of global studies between 2005-15 suggested that average hospital-wide and ICU costs per patient were $32,421 and $27,461, respectively (36).

1.1.3 Pathophysiology of sepsis

The pathogenesis of sepsis is complex and heterogenous. It begins with exposure of the innate immune system to pathogens leading to activation of pro- and anti-inflammatory pathways. The innate system is the first line of defence as it is rapid, antigen-independent and mediates a non-specific immune response. It has several physical, chemical and cellular barriers that prevent pathogen invasion and propagation (e.g. skin, mucous membranes, complement, lysozymes and various myeloid progenitor derived cells). Innate immune cells include neutrophils, monocytes, macrophages, and dendritic cells (DCs). These express specialised, germ line-encoded pattern recognition receptors (PRRs). Four main receptor groups are identified: toll-like (TLRs), C-type lectin-like (CLRs), nucleotide binding oligomerization domain (NOD)-like (NLRs), and retinoic acid inducible gene 1-like (RIG-1) (RLRs). PRRs recognise evolutionarily conserved components of pathogens termed pathogen-associated molecular patterns (PAMPs). Examples include endotoxin, peptidoglycan, lipoteichoic acid and β-glucan (37). PAMPs vary between organisms and enable the host to mount an appropriate response. PAMPs may be expressed on the cell surface or intracellularly, the latter requiring phagocytosis or cell lysis by complement to liberate and release them. PRRs also recognise endogenous stress signals produced by tissue injury or necrotic cells known as danger-associated molecular patterns (DAMPs) (e.g. histones, mitochondrial DNA (mtDNA), heat shock protein and haem) (38-40). Sensing of PAMPs or DAMPs triggers multiple signalling pathways that result in transcription of genes involved in the inflammatory response.

TLRs are the most common and best characterised PRRs. Ten and 13 TLRs have been identified in humans and mice, respectively. TLRs 1-9 are conserved in both species, TLR-10 is non-functional in mice and TLRs 11-13 are lost in the human genome (41). TLRs are broadly divided into two groups depending on their location: TLRs-1, -2, -4, -5, -6, and -11 are found on the plasma membrane and TLRs-3, -7, -8, and -9 are found intracellularly in endosomes, lysosomes or endolysosomes (41). The former TLRs recognise cell surface-bound PAMPs while the latter recognise viral and bacterial nucleic acids as well as self-nucleic acids degraded by DNAses. TLRs have structural homology to the interleukin-1 receptor (IL-1R) in their cytoplasmic signalling domains, which means both activate the same signalling molecules (42).

After ligand binding, TLRs dimerize and undergo conformational change that allows recruitment of TIR-domain-containing adaptor molecules such as MyD88, TIRAP TRIF and TRAM which activate downstream pathways. For example, MyD88 activates transcription factors NF-κB, MAPK and PI3K which, in turn, regulate distinct genetic responses to infection and injury (37, 43, 44). Examples include pro-inflammatory cytokines (e.g. TNF-α, IL-1β, IL-6), anti-inflammatory cytokines (e.g. IL-4, IL-10, TGF-β), chemokines (e.g. IL-8, MIP-1 and and MCP-2, -3 and -4) and cell adhesion molecules (e.g. ICAM-1 and VCAM-1) (43). The TRIF-dependent pathway is used by TLR -3 and -4, and activates NF-κB and interferon regulatory factor 3 (IRF3); this induces both inflammatory cytokines and type I interferons (figure 1.1).

Collectively, the pro-inflammatory mediators produced in response to the activation of PRRs by PAMPs recruit other innate immune cells. These phagocytose and kill the pathogen and propagate the inflammatory response by activating the adaptive immune system.

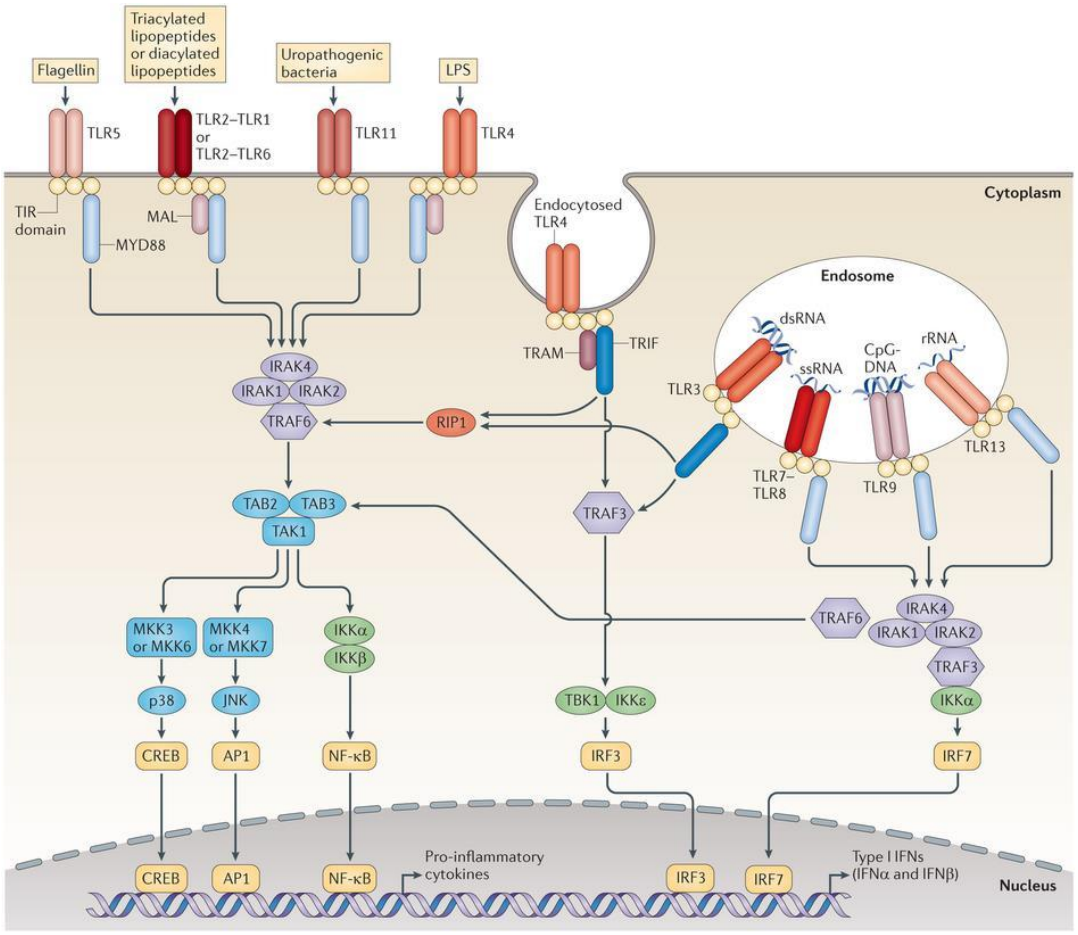


Figure 1.1. Summary of TLR signalling cascades.

Following activation by LPS, TLR-4 activates multiple intracellular signalling cascades that ultimately regulate transcription of inflammatory genes. Reprinted from O'Neill L et al, Nature Rev Immunol 2013; 13:453-60, with permission from Nature Publishing Group (46).

The adaptive immune system is delayed in onset but is highly specific and provides longer lasting protection. It consists of B- and T-lymphocytes which carry out antibody- and cell-mediated-responses, respectively. Antigen-presenting cells (e.g. monocytes, macrophages, dendritic cells and B-lymphocytes) present antigenic peptides via major histocompatibility complex (MHC)-II molecules to naïve T cells to initiate adaptive immune responses. These include rapid proliferation and differentiation of lymphocytes into diverse effector cells or memory cells, with the latter providing long term protection against the same antigen. Both arms of the immune system combine to produce an immunological tour de force of pathogen neutralisation.

1.1.4 Sepsis-induced organ dysfunction

The presence of organ dysfunction is central to the diagnosis of sepsis. Other than injury and modulation by circulating inflammatory mediators, there is often a mismatch between tissue perfusion and metabolic demands. This is exacerbated by impaired tissue oxygen utilisation which may be related to mitochondrial dysfunction (45).

Vascular changes in sepsis are characterised by endothelial and microvascular dysfunction, and reduced vascular tone. Endothelial cells are activated by inflammatory mediators, increasing vascular permeability and capillary leak resulting in tissue oedema (46). Vascular smooth muscle becomes hyporesponsive to catecholamines with often significant vasodilatation, hypoperfusion and circulatory failure (46-48). Mechanisms suggested for endothelial relaxation include excess production of nitric oxide (NO) by inducible nitric oxide synthase (iNOS) (49), hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD), and prostacyclin (PGI_2) by cyclooxygenase (50). NO causes smooth muscle relaxation by increasing cyclic guanosine monophosphate (cGMP) via guanylate cyclase activation.

These vascular changes are often compounded by a reduction in cardiac output secondary to reduced cardiomyocyte contractility. Multiple factors are implicated including pro-inflammatory cytokines, mitochondrial dysfunction, and down-regulation of β-adrenoceptors (51-53).

Microcirculatory changes are also multifactorial and involve activation of the coagulation cascade. There may be macro- and/or microvascular occlusions but these are generally not seen in either *in-vivo* microcirculation studies or at post-mortem (Figure 1.2). Intermittent and stop-go microcirculatory flow are much more frequent in sepsis compared to the healthy normal state.

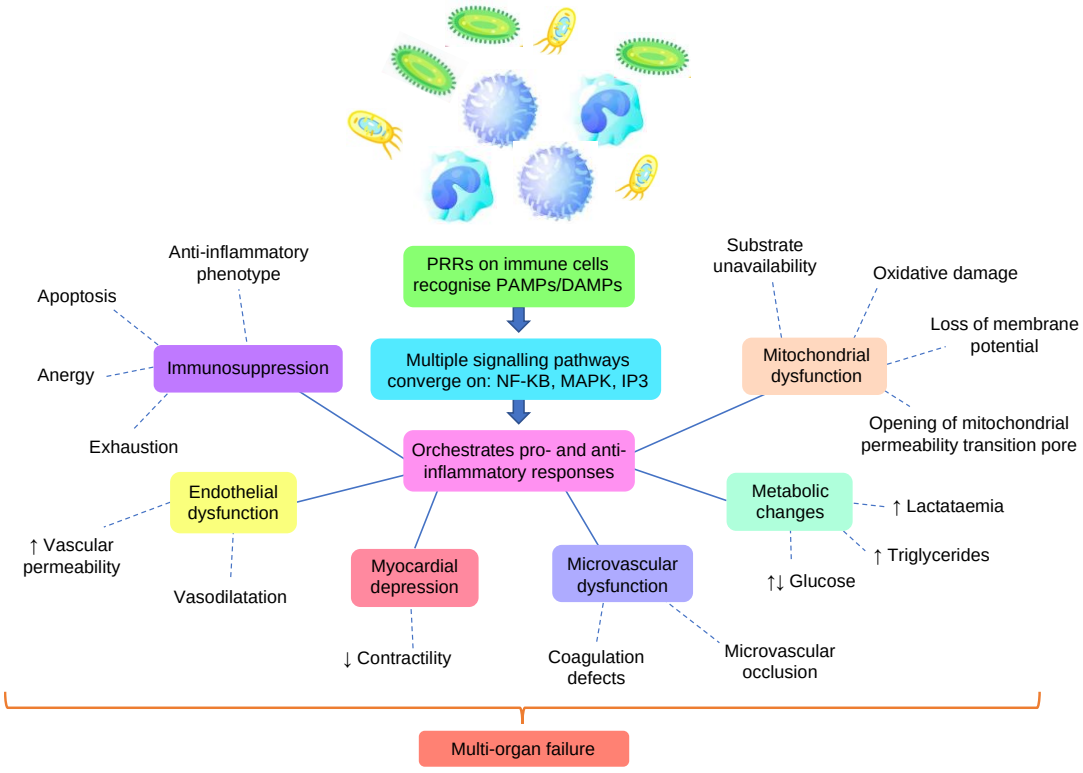


Figure 1.2. Schema of the pathways involved in organ dysfunction in sepsis.

1.2 Sepsis-related immunosuppression

During sepsis, both pro- and anti-inflammatory signalling pathways are exaggerated, leading to excessive systemic inflammation and immunosuppression. Immune cells are reprogrammed, rendering them less effective at eradicating pathogens and increasing the risk of secondary infection and mortality (54-57). This risk may persist for months after the initial infection (58). Underlying mechanisms of immunosuppression and the contribution of iatrogenic factors to poor patient outcomes remain uncertain. Common features of immunosuppression include the presence of functionally defective phenotypes with immune tolerance, anergy and apoptosis.

1.2.1 Neutrophils

Neutrophils constitute up to 70% of myeloid blood cells (59). Under homeostatic conditions, neutrophils differentiate in the bone marrow and are

released into the blood where they circulate for 6-24 hours. With infection, neutrophils migrate to tissues where they exert their anti-microbial effects. Neutrophils marginate and roll along the luminal surface of blood vessels towards the site of infection, facilitated by low-affinity interactions between their cell surface L-selectins and endothelial P- and E-selectins (60). They then adhere via β-integrins to endothelial ICAMs and VCAMs before transmigrating through tight junctions into the required tissues. Once there, neutrophils phagocytose and kill microbial pathogens by fusion of intracellular vesicles containing elastase, collagenase, defensins, lysozymes and histamine. NETs are large extracellular structures composed of vesicles with proteins that are scaffolded on decondensed chromatin (e.g. neutrophil elastase, myeloperoxidase (MPO), and defensins) (61). These trap and kill microorganisms, and can regulate inflammatory cytokines directly or indirectly by modulating other immune cells (62). Neutrophils subsequently undergo cell death known as NETosis.

In sepsis, apoptosis is delayed. There is upregulation of anti-apoptotic molecules (e.g. myeloid cell leukaemia (Mcl)-1 and B-cell lymphoma-extra-large (Bcl-xL) (63)) and downregulation of pro-apoptotic molecules such as bam, a member of the Bcl2 protein family (64). Pro-inflammatory cytokines including TNF-α, IL-1β, and IL-6 increase GM-CSF production; this in turn drives production and release of immature neutrophils from the bone marrow. Release is mediated by increased CXC motif chemokine ligand 1 (CXCL1) and decreased CXCL12 (65-68). Neutrophils have impaired migration due to increased β-integrin expression, reduced L-selectin expression and reduced chemotactic cell surface markers (e.g. CXCR2) (60, 69, 70). Furthermore, phagocytosis and the respiratory burst (ROS production) are impaired (65). These changes reduce the neutrophil's ability to clear pathogens, thereby increasing susceptibility to secondary infection (71). There may also be exaggerated NETosis; NET formation can promote coagulation, vascular occlusion and thrombosis.

While neutrophils contribute a vital protective role in sepsis, they present several significant technical challenges making laboratory investigation and

data interpretation difficult. These include their short lifespan in culture and the presence of heterogenous circulating neutrophil populations at a given time (each with differing maturity and functional states). Neutrophils are easily activated with minimal experimental handling. They also release immunogenic molecules such as NETs which, in turn, trigger other immune cells in the absence of a septic stimuli. For these reasons, I have focused my studies primarily on isolated peripheral blood mononuclear cells (PBMCs).

1.2.2 Monocytes, macrophages and dendritic cells

Monocytes, macrophages and dendritic cells (DCs) are antigen-presenting cells (APCs) that bridge the innate and adaptive immune responses. On encountering a pathogen, these cells secrete cytokines and chemokines to attract other immune cells. They also phagocytose and kill pathogens to present digested peptides on their MHC II molecules. They upregulate co-stimulatory molecules that activate T-helper (T_H) cells. Each cell subtype has additional distinct roles during an immune response e.g. macrophages are efficient phagocytes, DCs primarily present to and activate naïve T-cells while monocytes regulate the inflammatory response to limit tissue damage while assisting in fighting the pathogen.

Monocytes comprise ~5-10% of circulating leukocytes. They are a heterogenous group of cells differentiated by the cell surface expression of cluster of differentiation 14 (CD14; a component of the lipopolysaccharide (LPS) receptor complex) and CD16 (the FcγRIII immunoglobulin receptor). Classical cells express $CD14^{++}CD16^{-}$ which constitute 80-90% of monocytes. Non-classical monocytes express $CD14^{+}CD16^{+}$ and represent up to 10% of the monocyte population, while intermediate cells express $CD14^{++}CD16^{+}$ and also represent up to 10%. The biological roles of these subsets overlap and vary with the classification used. Broadly, classical cells are pro-inflammatory but also reported to be scavengers that phagocytose unwanted organisms; non-classical monocytes are involved in anti-inflammatory markers in response to infections while intermediate cells highly express MHC II and are efficient at antigen presentation. Collectively, in the presence of infection,

monocytes can migrate into tissues and differentiate into macrophages and DCs, although these can also arise directly from the bone marrow.

Macrophages are a diverse group of cells with tissue-specific functions as well as circulatory roles (e.g., brain microglia, liver Kupffer cells and skin Langerhans cells). At these sites macrophages help with clearance of senescent cells, repair and remodel tissues, and carry out phagocytosis. Circulatory macrophages are heterogenous and can be polarised into distinct subpopulations depending on the stimulus involved. M1 or classical macrophages are pro-inflammatory cells which are induced by IFN-γ and LPS, while anti-inflammatory M2 (alternative) macrophages are induced by IL-4 and IL-13.

DCs are also heterogeneous populations that arise from both myeloid and lymphoid haematopoietic lineages (myeloid or plasmacytoid subtypes). These subtypes have distinct markers that influence their location, migratory pathways and specific functions. Immature DCs patrol peripheral tissues to capture pathogens by phagocytosis or pinocytosis. Upon contact with pathogens, DCs form mature subtypes specialised for antigen processing and presentation to naïve T cells. The maturation of DCs depends on the presence of infectious or inflammatory stimuli. DCs undergo significant apoptosis in sepsis and have reduced antigen-presenting capacity (72-74). They also play a role in inducing T-cell anergy and selectively promote proliferation of T-regulatory cells via increased TGF-β (73, 75). These collectively reduce the immune response in sepsis and increase the risk of mortality.

During sepsis, there is a significant expansion of non-classical monocytes and M2 macrophages. The exact proportions and phenotypes vary with infection type, timing and severity. Monocytes and macrophages undergo endotoxin tolerance, which blunts their ability to release pro-inflammatory cytokines (76-78). These cells have reduced antigen-presenting capacity and reduced human leukocyte antigen-DR isotype (HLA-DR) expression (79, 80). HLA-DR is a cell surface glycoprotein that forms part of the MHC class II system;

reduced expression in sepsis is associated with an increased risk of secondary infection and mortality (81, 82).

1.2.3 Lymphocytes

Lymphocytes represent 20-40% of circulating leukocytes and 99% of cells in the lymph. There are three main types: B-lymphocytes, T-lymphocytes and innate lymphoid cells that include natural killer (NK) cells. Lymphocytes originate from bone-marrow derived progenitors; T-cells develop in the thymus and B-cells in the bone marrow (in adults) or liver (in the foetus). Lymphocytes can also be differentiated by cell surface expression of CD markers; these vary depending on the developmental and activation state of the lymphocyte (e.g., CD3 is expressed on T-helper (T_H) and T-cytotoxic (T_C) cells, CD19 is expressed by B-cells and CD56 and CD161 by NK cells). B- and T-lymphocytes are also identified by expression of antigen-specific receptors known as B-cell and T-cell receptors, respectively.

The B-cell receptor is a membrane-bound immunoglobulin that binds soluble and particulate antigens. Each B-cell also expresses up to 300 000 surface antibodies with unique specificity for particular antigens. B-cells can undergo somatic hypermutation to increase antigen binding efficiency and produce antibodies of different functional classes by class switching. When activated these cells internalise, process and present specific antigens along with co-stimulatory molecules to T-cells. T-cells produce cytokines that differentiate B-cells into antibody-producing plasma cells and memory cells. The former secrete antibodies to opsonise pathogens and increase phagocytosis by innate cells.

T-lymphocytes consist of two main groups of cells, T_H and T_C, distinguished by the presence of surface CD4 or CD8 molecules, respectively. T_H cells recognise antigens in complex with MHC II while T_C cells recognise antigens in complex with MHC I. In both cases T-cell receptors recognise only processed antigens (e.g. peptides). When activated, T_H cells differentiate into lineages including T_H1, T_H 2, T_H17 and T-follicular helper cells. These cells

produce cytokines that activate macrophages, B and T_C cells. T_C cells directly destroy infected cells by recognising proteins they either secrete or display on the cell surface. T-regulatory cells ($T\text{-}_{Reg}$ cells) are a subset of CD4 T-cells; these inhibit immune responses and are distinguished by expression of the transcription factor FoxP3.

During sepsis, lymphocytes undergo profound apoptosis, cellular anergy and exhaustion. T-cells demonstrate reduced proliferation and cytokine production and increased programmed death-1 (PD-1) expression; the latter is associated with nosocomial infections and mortality (66, 83-85). Transcription factors driving production of T_H related cytokines are reduced (e.g., T-bet for T_H1 cells and GATA-binding protein 3 for T_H2 cells) (86-88). T_H17 cytokine response and differentiation is reduced , the latter due to diminished expression of retinoic acid receptor related orphan receptor-γt (RORγt) (87) and is associated with increased secondary fungal infection (89). Immunosuppressive T-regulator cells are upregulated in sepsis, their Foxp3 expression is reduced (90).

My thesis focuses on PBMCs which consist of monocytes, DCs, lymphocytes and NK cells. The exact cellular populations vary between individuals and activation states but generally consist of mostly lymphocytes (70-90%) with a smaller number of monocytes (10-20%) with minimal DCs (1-2%). This milieu is ideal as it makes it possible to reliably study the interactions between the innate and adaptive immune system *ex vivo*. The cellular yield and viability is also consistent which means study of phenotype and cell functionality is more reliable.

1.3 **Mitochondria**

1.3.1 Mitochondrial physiology

Mitochondria are small organelles (~0.5-3 μm in diameter) that according to the endosymbiotic theory, are thought to have evolved from α-proteobacteria (91-93). They consist of two functionally different membranes that create distinct compartments; an outer semi-permeable membrane and an inner

impermeable membrane separated by an intermembrane space. This double membrane encloses the mitochondrial matrix where multiple vital metabolic reactions including the tricarboxylic acid (TCA) (also known as the Krebs' or citric acid) cycle take place (Figure 1.3).

Mitochondrial structure is uniquely suited for production of ATP. In the cytosol substrates including carbohydrates, fatty acids and proteins are converted to acetyl coenzyme A (CoA) and/or α-ketoglutarate. These enter the TCA cycle to produce reduced nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$) which transfer their electrons to the inner mitochondrial membrane respiratory complexes I and II, respectively. There are five respiratory complexes along the inner mitochondrial membrane that constitute the electron transport chain (ETC). Electrons are transferred via several redox centres along the ETC to complex IV, where they react with oxygen to form water. Simultaneously, at complexes I, III and IV, the electron motive force generated is converted to a proton motive force that drives protons into the intermembrane space against their concentration gradient. This generates a transmembrane potential which is harnessed by complex V (ATP synthase) to produce ATP from ADP and inorganic phosphate. This process is known as oxidative phosphorylation (OxPHOS).

In most cell types, mitochondria produce >90% of cellular ATP with the remainder coming from glycolysis within the cytosol. Under physiological conditions, small numbers of protons cross back into the mitochondrial matrix, bypassing complex V and releasing their stored energy as heat; this is known as proton leak or mitochondrial uncoupling. A significant increase in proton leak can open the mitochondrial permeability transition pore (mPTP) and this is associated with cell death. Similarly, electrons can leak at complexes I and III, reacting with oxygen to produce reactive oxygen species (ROS). In low concentrations, ROS plays a role in cellular signalling. However, at high concentrations these overwhelm endogenous antioxidant defences (e.g. SOD and catalase) and can cause significant oxidative damage. In addition to ATP production and cellular signalling, mitochondria also play a pivotal role in other

processes including intracellular calcium homeostasis, steroidogenesis, thermoregulation, haem biosynthesis and apoptosis.

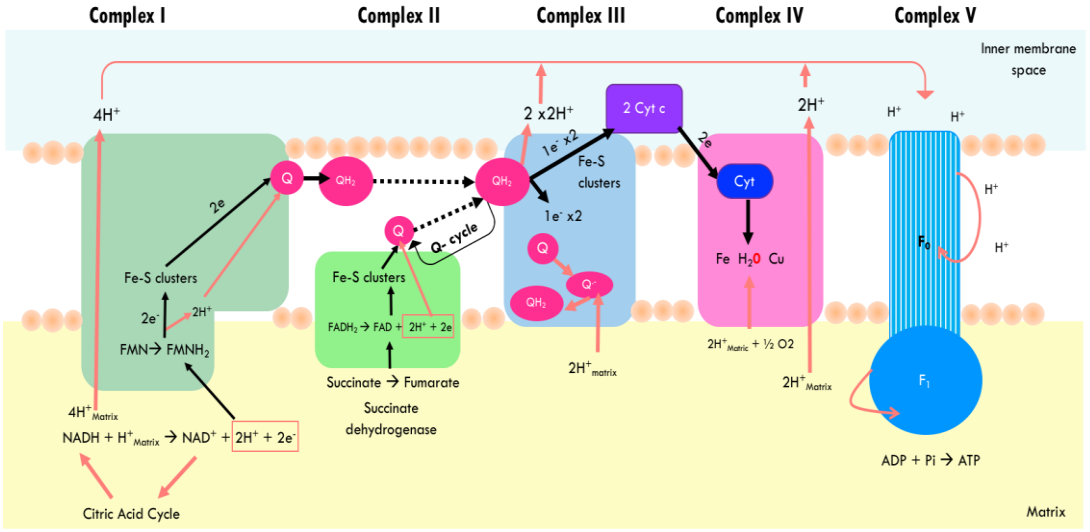


Figure 1.3. Illustration of mitochondrial electron transport chain structure and function.

There are five enzyme complexes on the inner mitochondrial membrane. Glucose, fats and amino acids are broken down into electron donors NADH and FADH2. These transfer electrons via several redox reactions to complex IV which reduces molecular oxygen to form water. Simultaneously, complexes I, III and IV actively pump protons from the mitochondrial matrix into the intermembrane space. The resultant proton gradient across the inner membrane drives protons back into the matrix at Complex V. This releases energy which phosphorylates ADP to ATP.

1.3.2 Mitochondrial genetics and dynamics

Mitochondrial function relies on a complex interaction between nuclear and mitochondrial genomes. Mitochondria consists of circular double stranded DNA that is tightly packed with proteins to form nucleoids. This process is mediated by transcription factors such as mitochondrial transcription factor A (TFAM). There are approximately 100- 10000 nucleoids per cell, each containing a copy of mitochondrial DNA (mtDNA) that are inherited maternally (94). In humans, mitochondrial genome consists of 16 569 base pairs which encodes 37 genes -13 for components of the OXPHOS machinery, 22 tRNAs and 2 rRNAs (95). Nuclear DNA encode the majority of mitochondrial proteins

(~1500 proteins in total); these play a critical role in OxPHOS, mtDNA replication and mitochondrial distribution within the cell (96).

Mitochondria form an interconnected reticular network that undergoes continual fission and fusion in response to changes in cellular energy demands and stress levels. Mitochondrial fusion is the merging of two mitochondria; this improves bioenergetic efficiency as resources between mitochondria are shared. Fission is the separation of one mitochondrion into two; this is associated with increased oxidative stress and mitochondrial depolarisation, and reduced ATP production. These processes are tightly regulated by proteins on the inner- and outer- mitochondrial membranes (IMM and OMM respectively). Fusion is coordinated by mitofusins (MFN-1 and MFN-2) on the OMM and by optic atrophy-1 (OPA1) on the IMM, while fission is regulated by recruitment of dynamin 1 like (Drp1) by OMM-related proteins including (FIS1, MFF, MiD49 and MiD51) (97, 98). In addition, mitochondrial mass is highly influenced by counteracting processes such as mitophagy and biogenesis.

Mitophagy refers to removal of damaged and depolarised mitochondria via ubiquitin- or receptor-mediated pathways (99). These target mitochondria to form autophagosomes that fuse with lysosomes where they are degraded. The ubiquitin-mediated pathway occurs when the MMP reduces, or in the presence of mitochondrial proteotoxicity. This involves aggregation of the kinase PINK1 on the OMM; with MMP reduction PINK1 accumulates and phosphorylates the ubiquitin E3 ligase, Parkin (100, 101). Parkin promotes ubiquitination and proteasomal degradation prior to engulfment by phagophores. This occurs during stresses such as hypoxia and involves direct recruitment of autophagosomes by interaction with mitophagy-related receptors. (97, 102). Removal of mitochondria is associated with increased biogenesis which is required for cell growth to produce increased metabolites and energy. This is coordinated by a combination of nuclear and mitochondrial encoded genes co-regulated by transcription factors NRF1/2, PPARγ, ERRα, β, γ, and PGC-1α.

1.3.3 Mitochondria in sepsis

Mitochondria are heavily implicated in the development of multi-organ failure in sepsis (103). Failing organs shift their energy expenditure from anabolic functions to maintenance of ionic biochemistry which helps preserve their histology and retain the potential for recovery; indeed, failed organs show little evidence of cellular injury/death (45, 104-107). The shift is evidenced by altered glucose metabolism with increased gluconeogenesis and hyperlactataemia; these changes result from overstimulation of Na^+/K^+ ATPase rather than tissue hypoxia (108, 109). There is also a metabolic switch from OxPHOS to aerobic glycolysis when immune cells are activated, further contributing to the rise in lactate. These findings suggest a metabolic shutdown rather than structural damage as a potential underlying mechanism.

Mitochondrial dysfunction may arise from multiple causes including tissue hypoxia, lack of substrate, oxidative damage, and a reduction in both expression and activity of mitochondrial complexes I, III and IV (110-113). Muscle biopsies from septic patients demonstrated reduced complex I activity and ATP production, which was worse in non-survivors (103). Another study of muscle biopsies from septic patients confirmed these findings and suggested that mitochondrial mass may additionally be reduced (114). This may be related to loss of mtDNA or mtDNA copy number in sepsis, which has been strongly associated with disease severity and increased mortality (115-117). Loss of mtDNA may be associated with a compensatory increase in mitochondrial biogenesis in an attempt to promote cell recovery and survival. This phenomenon is thought to be mediated by nuclear peroxisome proliferator activated receptors (PPARs), including PPAR-γ and PPAR-δ (110, 118-120). These induce specific transcriptional coactivators (peroxisome proliferator activated receptor-γ co-activator (PGC)-1α and -1β) which increase expression of nuclear respiratory factors (NRF)-1 and -2). These, in turn, upregulate downstream gene expression including mitochondrial transcription factor A (TFAM) which promotes replication of mtDNA-encoded genes (121). PGC-1α is also an inhibitor of mitophagy (122). In muscle biopsies from septic patients, survivors demonstrated an early increase in PGC-1α expression

(123). Another study of the muscle cell transcriptome demonstrated dysregulated mitochondrial genes and transcription factors and genes with impaired mitochondrial biogenesis (110).

Under physiological conditions dysfunctional mitochondria are selectively removed by mitophagy through encapsulation and lysosomal degradation. A study of PBMCs from septic patients showed an increase in PINK1 levels suggesting increased mitophagy (124). However, an increase in mitophagy has been associated with mitochondrial recovery in sepsis (125). In a study of non-surviving septic rabbits, there was reduced autophagy, increased organ damage and greater mitochondrial respiratory impairment (126). Similarly, accumulation of damaged mitochondria increases due to defective mitophagy and/or autophagy with persisting oxidative stress and release of mitochondrial DAMPs (127).

Mitochondrial DAMPs are features that the mitochondria retain from their bacterial ancestors. These include cardiolipin, formyl peptides, mitochondrial circular DNA and 70S ribosomes. When released into the circulation by damaged cells they behave like PAMPs, triggering immune reactions through activation of PRRs such as TLR-9 (128). They can promote formation of the NLRP3 inflammasome which causes release of IL-1β and IL-18, promoting inflammation (129, 130). Mitochondrial DAMPs have also been associated with multi-organ dysfunction and a higher risk of mortality (131). Longer exposure, however, has been associated with suppression of innate immune responses to subsequent inflammatory stimuli (tolerance) (132). This suggests that mitochondrial dysfunction may exacerbate both the initial over-exaggerated inflammatory response and, if sustained, cause immunosuppression.

Studies have also demonstrated that sepsis increases mitochondrial ROS production (133, 134) leading to decreased ATP production but also uncoupling (proton leak) and opening of the mitochondrial permeability transition pore (mPTP) (135-137). These features are associated with organ failure and worse patient outcomes.

1.3.4 Mitochondria in immunomodulation

Mitochondria are involved in regulation of immune cell functionality (e.g. activation, proliferation and differentiation) and survival (138). In terms of ATP provision, the proportion of oxidative and glycolytic-derived ATP varies between immune cells and with their activation status. For example, neutrophils with fewer mitochondria predominantly rely on glycolysis, whereas monocytes, macrophages and lymphocytes utilise oxidative ATP for housekeeping activities (139). Neutrophil activation, maturation and effector functions depend on glycolysis, the pentose phosphate pathway and, to a lesser degree, on oxidative ATP (140). Although glycolysis is not as efficient as OxPHOS at producing ATP (2 moles from one mole of glucose compared to 28-30 moles from mitochondrial respiration), it can nonetheless provide substrates for cell growth, phagocytosis & cytokine production (141).

In monocytes, metabolic pathways are influenced by specific stimuli. For example, TLR-4 activation upregulates glycolysis and reduces OxPHOS, while TLR-2 activation increases both glycolysis and OxPHOS (142). At sites of inflammation with low glucose availability, monocytes upregulate fatty acid oxidation and thus OxPHOS (143). These metabolic pathways cause an early rise in oxidative stress which is counteracted by a rise in antioxidant mechanisms and mitochondrial turnover (144). Such changes are frequently observed with immunoparalysis during sepsis (144). In macrophages, activation by endotoxin leads to M1-polarisation and upregulation of aerobic glycolysis, the pentose phosphate pathway and glutamine metabolism (145, 146). These increase HIF-1α which, in turn, increases production of the pro-inflammatory IL-1β. By contrast, the M2 phenotype depends on β-oxidation-driven OxPHOS (147). B- and T-lymphocytes upregulate OxPHOS and glycolysis on activation, thus increasing ROS and mitochondrial mass (148-152). T-regulatory cells require fatty acid oxidation-fuelled OxPHOS to differentiate and for effector function.

Evidence of mitochondrial dysfunction in immune cells is seen during sepsis. One study of leukocyte gene expression patterns in human volunteers injected

with endotoxin reported a reduction in genes involved in the generation of ETC complexes I-V, pyruvate dehydrogenase and the coenzymes required for ATP synthesis by OxPHOS (153). Other studies of septic PBMCs have shown significant impairment of O_2 consumption and ETC complex activities (154), Impairment of complex V was associated with an increased risk of organ failure and death (155).

These studies have shown that mitochondrial dysfunction is likely to be a key factor underlying patient outcomes in sepsis. However, what is less clear is the extent to which this dysfunction results from iatrogenic causes. Mitochondrial and/or cellular damage may result as an unintended target of therapies commonly used in intensive care, including antibiotics, catecholamines and sedatives. These may exacerbate immune dysfunction in sepsis, worsening clinical severity and patient outcome. Below is a summary of the clinical management of sepsis and how drugs used in the management may contribute to immune and mitochondrial dysfunction.

1.4 **Management of sepsis**

The management of sepsis is considered a medical emergency so early recognition and timely intervention are paramount. Despite decades of research, there is no cure for sepsis; our current management remains supportive and includes source isolation, appropriate antibiotics to eradicate the invading pathogen, intravenous fluids and/or vasoactive therapy to support the circulatory system and restore tissue perfusion, mechanical organ support such as mechanical ventilation and renal replacement therapy, and blood products to correct coagulopathy.

1.4.1 Fluid resuscitation

Although essential for the management of septic patients, there has been long-standing debates about the type, rate and volume of fluid needed, as well as indicators used to guide fluid resuscitation. Surviving Sepsis Campaign guidelines (156) suggest giving 30 mL/kg (ideal body weight) of intravenous

(IV) crystalloid fluid within the first 3 hours. However, liberal fluid administration with a positive balance in the first 24 hours has been associated with worse outcome (157). There is no compelling evidence for the use of one crystalloid over another however colloids, especially hydroxyethyl starches, have been associated with worse outcomes when given in large quantities.

There is also a lack of compelling evidence to indicate the optimal method for guiding fluid therapy, however in clinical practice mean arterial pressure (MAP), central venous O_2 saturation ($ScvO_2$) and lactate are commonly used. Three large randomised controlled trials (RCTs) demonstrated that early goal-directed therapy (GDT) aimed at achieving a target $ScvO_2$ >70% using a combination of IV fluids, vasoactive drugs and blood transfusion was no better than routine care (158). GDT was more costly and labour intensive. More information is required about how to optimise fluid resuscitation in sepsis and prevent iatrogenic harm.

1.4.2 Vasopressor/Inotrope therapy

Vasopressors and/or inotropes are often required to correct hypotension and restore tissue perfusion. These should be commenced promptly if the patient does not respond to fluid resuscitation as severity and duration of hypotension are associated with an increased mortality risk (159). Several vasoactive agents are available, e.g. catecholamines such as adrenaline (epinephrine) and noradrenaline (norepinephrine (NE)), vasopressin and angiotensin II. NE is currently considered first-line treatment for septic shock, while vasopressin is recommended to prevent NE dose escalation above moderate levels (160). NE has a rapid onset, short duration of action and is relatively easy to administer and titrate. It predominantly acts on α-adrenergic receptors to induce vasoconstriction, however the subsequent increase in peripheral vascular resistance may increase cardiac afterload and thereby decrease cardiac output. At high concentrations, NE can also act on β-adrenoreceptors to increase myocardial contractility but may cause ischaemia and arrhythmias. Vasopressin acts on vasopressin receptors in the vasculature (V_{1a}) and in renal tubules (V_2) to induce vasoconstriction and increased renal reabsorption

of water. Values of mean arterial pressure (MAP) ≥65 mmHg are usually targeted; lower values are associated with renal impairment while higher values increase vasopressor-related complications (161). Adrenoreceptor agonists also have immunomodulatory and bioenergetic effects which are discussed below. Vasopressin reduces the local circulation to many organs including the kidneys, liver and heart. These drugs, although often necessary in the short-term management of sepsis, can cause significant iatrogenic harm with lasting damage that may worsen prognosis.

1.4.3 Antibiotic therapy

SSC guidelines suggest early administration of antimicrobials, ideally within an hour of recognition of sepsis. However, this needs to be balanced against antibiotic-related risks (e.g. cutaneous skin reactions, hepatic and renal dysfunction, anaphylaxis, blood dyscrasias), induction of anti-microbial resistance (162), and effects on the gut microbiome (168)). Evidence indicates that antibiotics also have immunomodulatory effects and that mitochondria may mediate some of their side effects (discussed below). There is a large variability in course duration across Europe, however longer courses appear to provide no additional clinical benefit (163).

1.4.4 Additional therapies

Most critically ill patients with sepsis require oxygen delivered via non-invasive or invasive mechanical ventilation. The recommended ventilation strategy is to use low tidal volumes (6 mL/kg ideal body weight) and to maintain inspiratory plateau pressures below 30 cmH_2O to reduce the risk of barotrauma. Invasive ventilation however requires the use of analgesics (e.g. morphine, fentanyl) and sedatives (e.g. benzodiazepines, propofol). Neuromuscular blockade infusions may be required in severe cases of respiratory failure. Corticosteroids are often used when NE requirements are high as this helps to reverse hypotension related to endogenous critical illness-induced corticosteroid insufficiency

Additional drugs commonly received by critically ill septic patients include: stress ulcer prophylaxis (usually a proton pump inhibitor), venous thromboembolism prophylaxis (with low molecular weight heparin), insulin for blood glucose management, and nutritional support.

Multiple immunomodulatory therapies have been investigated to date in sepsis, but none have convincingly demonstrated mortality benefit. Such strategies include immunosuppressants used to counteract the deleterious effects of the hyper-inflammatory phase (e.g., TNF-α antagonists, IL-1 receptor antagonists) and agents that modulate inflammation and coagulopathy (e.g. activated protein C). In recent years attention has been turned to immune stimulants to reverse sepsis-induced immunosuppression (e.g., GM-CSF, IL-7, anti-PDL-1 antibody), however these have yet to be tested in large-scale studies. (84, 164, 165).

The number of drugs used in ICU patient management, often at high doses given for long durations, and with altered clearance resulting in potentially toxic levels, increase the likelihood of developing undesired drug interactions and causing iatrogenic harm. Improved survival is associated with doing less, including shorter durations of antibiotics, less fluid replacement, lower tidal volumes and avoidance of polypharmacy. Overuse of broad-spectrum antibiotics, catecholamines and sedatives have been implicated in iatrogenic harm (162, 163). These agents have effects on both the immune system and on mitochondria, as discussed below.

1.5 Commonly used drug classes in sepsis and their effect on the immune system and mitochondria

1.5.1 Antibiotics

Antibiotics are a cornerstone of sepsis management. Commonly used classes have differing mechanisms of action including inhibition of bacterial cell wall synthesis (e.g. β-lactams), nucleic acid synthesis (e.g. quinolones) or protein

synthesis (e.g. aminoglycosides, macrolides), or alterations of metabolic functions essential for survival (e.g. sulphonamides). Though antibiotics are life-saving, they carry a risk of harm including effects on mitochondria and immune cells (166-170). For mitochondria, this may relate to their bacterial origin as proposed by the endosymbiotic theory, making them an unintended target. Potential mechanisms include inhibition of nucleic acid and protein synthesis and/or transport mechanisms.

1.5.1.1 Aminoglycosides

Aminoglycosides are bactericidal antibiotics (e.g. gentamicin) that act against gram negative bacteria. This family of amino-modified sugars contain hydrophilic portions and cationic amine moieties that preferentially bind nucleic acids due to their negative charge. More specifically, they bind to the aminoacyl-tRNA site (A site) of 16S ribosomal RNA of the 30S ribosome where they cause translational errors and assembly of incorrect amino acid products, or premature termination of protein synthesis (171, 172).

Aminoglycosides-induced nephrotoxicity and ototoxicity may be partially mediated through mitochondria (173-175). Aminoglycosides act on the mitochondrial ribosomal A site to activate phosphatidylinositol phospholipase C and cause a proinflammatory response (176). In renal and sensory hair-cell mitochondria, aminoglycosides inhibit OxPHOS and ATP production (14), increase oxidative damage (177) and induce apoptosis (178-186).

Immunomodulatory effects are conflicting; some suggest an inhibitory effect on chemotaxis, phagocytosis and bacterial killing, others suggest no change (166-169). However, no study has yet investigated aminoglycoside effects on mitochondrial function in immune cells.

1.5.1.2 β-lactams

β-lactams are bactericidal antibiotics that consist of five categories: the penams (e.g. penicillins, ampicillin), penems (e.g. faropenem), carbapenems

(e.g. meropenem, imipenem), cephems (e.g. cephalosporins), and monobactams (e.g. aztreonam). All consist of an azetidinone nucleus with a cyclic amide and a carbonyl beta lactam, forming a four-membered beta-lactam ring (187). These inhibit bacterial cell wall synthesis by binding and acetylating transpeptidase enzymes to disrupt peptidoglycan cross-linking (188). As peptidoglycans surround and strengthen the bacterial cell membrane, their absence makes the bacterium susceptible to osmotic lysis.

β-lactams have immunomodulatory functions in hypersensitivity reactions (189-191), cancer (192, 193) and infection (194-200), though evidence for the latter is conflicting. β-lactams kill bacteria and release by-products into the circulation that can activate the immune system. The evidence base on their impact on chemotaxis, phagocytosis and cytokine production is conflicting. Increased, decreased and no effect are reported for chemotaxis (194-200). Similarly, phagocytosis may be upregulated (201-206), downregulated (196, 207, 208) or unaffected (198, 209, 210). Cytokine production has also been suggested to be increased (207, 211), decreased (196) or unaffected (212) depending on the study in question. β-lactams reduce neutrophil production and may variably affect the respiratory burst (213, 214). They reduce lymphocyte proliferation but do not affect effector functions (215).

There remains scarcity of evidence for β-lactam effects on immune cell mitochondria. Most studies have been undertaken in hepatic or renal mitochondria where β-lactams impair mitochondrial anionic substrate transport (e.g., glutamate and malate) (216, 217) and affect β-oxidation and the TCA cycle (218). In the liver, β-lactams increase oxidative damage, open the mPTP and induce apoptosis (219).

1.5.1.3 Glycopeptides

Glycopeptides are bactericidal antibiotics that are glycosylated cyclic or polycyclic peptides synthesized non-ribosomally, and were originally isolated from soil *Actinomyces*. Semi-synthetic, second-generation glycopeptides

include oritavancin and dalbavancin. These act by binding to the terminal D-Ala-D-Ala moiety of peptidoglycan precursors, sequestering the substrate and preventing penicillin-binding proteins from cross-linking them into mature peptidoglycans (220). This compromises cell wall integrity in a similar manner to ß-lactams, leading to osmotic lysis.

Glycopeptides cause adverse reactions via mast cell degranulation (221-226), neutropenia and destruction of gut microbiota (227-231). At therapeutic concentrations glycopeptides do not affect chemotaxis, adherence nor phagocytosis of neutrophils (168, 229, 232-235). At higher concentrations, however, they inhibit all three (236). Reports on cytokine release are conflicting with both an increase (15) and decrease (237) having been reported.

Effects of glycopeptide on immune cell mitochondria are unreported. Glycopeptides may however induce nephrotoxicity by causing mitochondrial dysfunction through oxidative damage, impairing complex activity and reducing ATP production (238-240).

1.5.1.4 Macrolides

These consist of a macrocyclic lactone ring with ≥14 members attached to one or more sugar moieties by glycosidic bonds (241-243). Erythromycin, the first clinically relevant macrolide, contains 14 members as does clarithromycin, whereas azithromycin is 15-membered (241). This antibiotic class acts by reversibly binding to domain V of 23S ribosomal RNA of the 50s subunit of the bacterial ribosome, blocking translocation of peptidyl tRNA and thereby inhibiting bacterial protein synthesis.

Macrolides are often considered anti-inflammatory, diminishing accumulation of leukocytes, particularly neutrophils, by reducing chemokine release (e.g. IL-8, LTB-4 and IL-β) (244-248). They also inhibit cytokines and neutrophil elastase production and NADPH oxidase activity (249-260). *In vitro* experiments show that macrolides blunt cytokine production (261-264) and reduce immune cells recruitment to the site of injury (265). However, contrary

data report that macrolides have no effect or increase chemotaxis (266-271), cytokine release and phagocytosis (272-276), or, alternatively, decrease phagocytosis (277-279) and the oxidative burst (280-282).

There are no studies on the effects of macrolides on mitochondria in immune cells, however in other tissues increases in complex activity (I and III), O_2 consumption and ATP synthesis have been reported (283, 284).

1.5.1.5 Quinolones

Quinolones target both Gram-positive and Gram-negative bacteria by inhibiting nucleic acid synthesis via inhibition of topoisomerase-II, topoisomerase-IV and DNA gyrase (285, 286). This induces DNA breakages resulting in bacterial death. The first quinolone was isolated in the 1960s and several generations have been developed since, e.g. oxolinic acid (first-generation), ciprofloxacin and ofloxacin (second-generation), and levofloxacin and moxifloxacin (third generation).

Quinolones can induce hypersensitivity reactions (287-290) and destroy the gut microbiota (291, 292). They suppress pro-inflammatory cytokines (11, 293-307), but not chemotaxis nor phagocytosis except at very high concentrations (308-311). Conflicting studies show that ciprofloxacin may increase phagocytosis and killing of microorganisms (312, 313). In lymphocytes, quinolones inhibit proliferation by upregulating Fas Ligand, caspases-8 and -3 activity (314, 315).

In mitochondria, quinolones target and inhibit topoisomerase enzymes which prevent mtDNA replication (316, 317). Ciprofloxacin inhibits the ETC and decreases mitochondrial membrane potential (318, 319). Beneficial effects of quinolones have been shown in several cancers (e.g. colorectal (320, 321), breast (322) and lung (323)) via actions on mitochondria. Precise mechanisms remain uncertain.

1.5.1.6 Oxazolidinones

The newest class of antibiotics that is used for skin and respiratory tract infections caused by Gram-positive organisms (324). They bind the 50S ribosomal subunit to inhibit formation of the 70S initiation complex, resulting in inhibition of bacterial protein synthesis. Oxazolidinones are associated with myelosuppression due to impaired mitochondrial protein synthesis and complex activity (325-328). Prolonged use may cause lactic acidosis by impairing mitochondrial bioenergetics (329-331). Data on oxazolidinone-mediated immune dysfunction are conflicting, either reducing (332-340) or having no effect on cytokine production and phagocytosis (341-343).

The variably reported immunomodulatory effects of each antibiotic classes may result from differences in research methodology (e.g., dosing, techniques, timing, cell types used and patient characteristics), and but also may be due to generational differences in the antibiotic class. There is limited evidence on the effects of antibiotics on immune cell mitochondria. However, as mitochondrial dysfunction is strongly linked to immune dysregulation in sepsis, this merits further investigation given the potential for significant iatrogenic harm if therapeutic agents are not used appropriately.

1.5.2 Catecholamines

Catecholamines noradrenaline (norepinephrine, NE) and adrenaline (epinephrine) are produced endogenously from phenylalanine via a series of hydroxylation and decarboxylation reactions (344). They are mainly released from chromaffin cells in the adrenal medulla or in post-ganglionic fibres in sympathetic nerves (345). Catecholamines variably activate α- and β-adrenoceptors and dopaminergic receptors, which collectively orchestrate a ‘fight or flight’ response to stress. The half-life of catecholamines is short due to re-uptake into nerve terminals, diffusion into extracellular fluid or metabolism by various enzymes (e.g. monoamine oxidases and catechol O-methyltransferases). Synthetic forms of norepinephrine and epinephrine are

routinely used in septic patients, however these drugs also impact on immune cell and mitochondrial functionality, as discussed below.

1.5.2.1 Catecholamines and immune function

The catecholamines have immunomodulatory effects mediated by adrenoceptors and dopaminergic receptors. β_2-adrenoceptors are most commonly expressed on immune cells (346-352) which can synthesise, store (353-356) and metabolise (357, 358) catecholamines. β_2-adrenoceptor activation via cAMP-PKA signal transduction reduces production of pro-inflammatory cytokines (e.g. TNF-α, IL-6, IL-1β) and increases anti-inflammatory cytokines (e.g. IL-10) (359-365). β_2-adrenoceptor activation also inhibits chemotaxis, phagocytosis and the respiratory burst in neutrophils, and phagocytosis in macrophages *in vitro* (366-369). β_2-adrenoreceptor activation supresses T-cell proliferation (354, 370) and shifts the T_H1/T_H2 balance towards T_H2 polarisation in monocytes and DCs (371-374). α-adrenoreceptors have pro-inflammatory actions by activating NF-kB, which increase cytokines in monocytes *in vitro* (375-377).

1.5.2.2 Catecholamines and mitochondria

The relationship between catecholamines and mitochondria is bidirectional. Mitochondria can influence the uptake (378), release (379, 380) and function of catecholamines (381), however data are conflicting. This may be explained by differences in methodology (e.g., source of sepsis, cells/tissues investigated, timings and concentrations of catecholamines used), limiting the applicability of the findings to clinical settings where lower concentrations of catecholamines are used.

There is limited evidence of the role of catecholamines on immune cell mitochondria in sepsis. Within the context of stress, catecholamines affect mitochondrial metabolism by promoting release of reserved energy substrates to increase OxPHOS (382). Catecholamines also increase lipolysis and

glycogenolysis and suppress glycolysis thereby modulating substrate availability to mitochondria (382). However, in primary human monocytes, NE (1 mM) reduced oxygen consumption and ROS production (383). In rat liver mitochondria, epinephrine (0.1 mg/100 g body weight for 6 days) increased maximal aerobic respiratory capacity and oxidative damage (384) while dopamine (0.01-1 ug/ml) and dobutamine (0.01-1 ug/ml) increased fatty acid oxidation (385). In some septic pig models, dopamine and NE increased mitochondrial respiratory efficiency (386, 387), while in another study NE had no effect (388). In rat brain mitochondria, dopamine (100 and 200uM, after 10 mins) reduced mitochondrial swelling, increased cytochrome c release and ROS production (389-391).

There are limited *in vivo* data on catecholamine-induced mitochondrial dysfunction in sepsis. In one study, skeletal muscle biopsies from NE-treated patients showed impaired complex I, ATP production and anti-oxidant capacity (103). Studies in PBMCs from septic patients treated with NE showed impaired ATP synthase activity (155), reduced O_2 consumption (392) and increased mitochondrial uncoupling (136). Thus, catecholamines likely influence immune cell and mitochondrial function, however more studies are required to fully elucidate these effects, particularly at pharmacologically relevant concentrations.

1.5.3 Sedatives

Sedatives are routinely used in ICU patients requiring mechanical ventilation to provide patient comfort and to facilitate synchronisation of breathing with the ventilator. Commonly used classes of drugs include opiates (e.g. morphine, fentanyl), benzodiazepines (e.g. midazolam) and propofol. All of these agents have immunomodulatory effects *in vitro,* however their impact in sepsis has not been specifically addressed (393). For the purpose of this thesis my focus will be on propofol as it is one of the most frequently used sedatives where relevant findings have the potential to significantly contribute to sepsis care.

Propofol (2,6 diisopropylphenyl) is an intravenous anaesthetic agent used commonly for induction and maintenance of anaesthesia due to its favourable pharmacokinetic profile, rapid onset and short duration of action. It is highly lipophilic and is found as an intralipid-based emulsion consisting of soybean oil (100 mg/mL), glycerol (22.5 mg/mL), egg lecithin (12 mg/mL) and EDTA 0.005% (394). Propofol potentiates $GABA_A$ receptors to increase chloride currents and hyperpolarise membranes.

1.5.3.1 Propofol and Immunomodulation

GABA receptors and components necessary for GABAnergic responses are variably present on immune cells depending on their activation states (395-397). Activation of GABA receptors has both pro- and anti-inflammatory activity in PBMCs (398). *In vivo* studies report propofol to have beneficial effects on infection rates, cancer recurrence and mortality (399, 400). However, these may be multifactorial as the effect of propofol has not been studied in isolation. The vast majority of conclusions are reached from *in vitro* studies which are often conflicting and not easily applicable to clinical settings.

At clinically relevant concentrations, propofol had minimal effect on PBMC proliferation (401, 402), but at high concentrations it suppresses T-cell proliferation *in vitro* (403). In elective surgery patients, propofol increased T- and B-lymphocyte counts but reduced NK cell counts (404, 405). In a murine MRSA-infected kidney model, propofol reduced immune effector cells at the infection site (406) and increased abscess formation (407). In murine liver, repeated propofol exposure reduced $CD4^+$ T-cell counts by increasing apoptosis but did not affect cytokine production (408). Reports of the effect of propofol on cytokine production are conflicting (409-412); most suggest that propofol reduces production (413, 414) by downregulation of p38 (415), ERK 1/2 (416) or NF-kB (417). Several *in vitro* studies have shown that propofol does not affect granulocyte recruitment or phagocytosis (418-421). Opposing studies have however shown that propofol reduces phagocytosis and the respiratory oxidative burst via $GABA_A$ receptor activation, mitochondrial dysfunction or by an effect on cell membranes (422-432). *In vivo* studies of

patients undergoing elective surgery all showed reduced phagocytosis and killing capacity after propofol exposure (433-435).

1.5.3.2 Propofol and mitochondria

Mitochondrial dysfunction may be the mechanism by which propofol causes immune dysregulation. In macrophages, but not in PBMCs, propofol increased mitochondrial ROS production, NLRP3 inflammasome activation and caspase-mediated apoptosis. (436). In a RAW 264.7 monocyte/macrophage cell line, propofol reduced mitochondrial membrane potential but did not affect complex activity (437). In lymphocytes, propofol did not affect mitochondrial ROS production nor glutathione stores (438).

In myocardium (439, 440), brain (441) and hepatocytes (442-444) propofol was protective against oxidative injury. In a human neuroblastoma cell line (SH-SY5Y) and in primary neurons, propofol attenuated mitochondrial oxidative damage by reducing ROS-mediated calcium release from the endoplasmic reticulum (445, 446). In a H9C2 cardiomyocyte cell line and in rat cardiomyocytes, propofol reduced the H_2O_2-related increase in caspase activity by reducing ROS production and restoring antioxidant stores (447-449). However, high doses of propofol in SH-SY5Y, HeLa and myoblast (C2C12) cells impaired complex (I-III) activity, increased ROS production and reduced mtDNA production (450, 451). In neuronal stem cells, propofol caused mitochondrial vacuolisation and swelling, and reduced mitochondrial membrane potential by downregulating Parkin1 protein (452). There is also some evidence in cardiomyocytes, human skeletal muscle, HeLa and T67 cell lines to suggest that propofol impairs mitochondrial complex activity (453-455).

Propofol also causes mitochondrial dysfunction *in vivo*. In the most extreme form, this manifests as the ‘propofol infusion syndrome’ where high doses lead to hyperlactataemia, metabolic acidosis, rhabdomyolysis, renal impairment, hepatomegaly, and cardiac arrhythmias (456). This is more common in patients with mitochondrial disorders (457). In animal studies, propofol affected neuronal mitochondrial membrane potential, mitochondrial DNA, intracellular

calcium homeostasis and caspase-3 and -9 activity (458-460). In studies of elective surgery, propofol impaired mitochondrial complex activity (461, 462).

1.5.4 Summary

Antibiotics, catecholamines and sedatives have immunomodulatory effects and can alter mitochondrial function, however this is not consistent across the drug groups. Further investigation is required, particularly in septic patient populations, so that the underlying mechanisms and preventative measures can be elucidated.

Table 1.3. The pharmacokinetics of ciprofloxacin, NE and propofol

	Ciprofloxacin	**Norepinephrine**	**Propofol**
Mechanism of action	Inhibits bacterial topoisomerases II and IV which inhibit DNA supercoiling and so replication.	Stimulates mainly α_1-AR but also β-Ars at higher concentration	Potentiates $GABA_A$ receptor to increase chloride current, and inhibits NMDA and mu opioid receptors.
Dose in humans (mg/kg)	200-750 mg BD, serum concentration is 6.3–6.5 mg/mL after 200 mg dose PO, and 0.87 and 0.1 mg/mL 1 and 2 h later respectively.	0.05-0.5 µg/kg/min.	1-2 mg/kg for anaesthesia and 0.3-4 mg/kg for sedation.
Molecular weight	331.34	169.18	178.27
pH	3-3-4.6	3.5-4	6-8.5
pKa	6-8.8	8.8-9.5	11
Protein binding (%)	20-30		98
Plasma half life	3-4 hours	2 minutes	2-10 minutes
Volume of distribution (L/kg)	2-3	0.09-0.4	4-15
Clearance (ml/kg/min)	4-9		30-60
Elimination half- life (h)	4-5		5-12
Metabolism	Hepatic metabolism but 40-70% may be excreted unchanged in urine	By mitochondrial monoamine oxidases in liver, kidney and brain, and cytoplasmic catechol O-methyl	Mostly hepatic; 40% conjugated to glucuronide and 60% metabolised to quinol

		transferase. 5% excreted unchanged	
Metabolites	Largely inactive, desethyleneciprofloxacin sulfociprofloxacin, oxociprofloxacin, N-formylciprofloxacin	Inactive, vanillylmandelic acid or 3-methoxy 4-hydroxymandelic acids	Inactive
References	(463-466)	(467)	(467-470)

1.6 Hypothesis and Aims

1.6.1 Hypothesis

Antibiotics, vasoactive drugs and sedatives have a cumulative effect on the suppression of immune cell functionality induced by sepsis. This is modulated through their effects on immune cell mitochondrial functionality.

1.6.2 Aims

To assess the impact, alone and in combination, of ciprofloxacin, norepinephrine and propofol on the following:

(i) Immune function in PBMCs from healthy volunteers in the presence and absence of a septic stimulus.

(ii) Mitochondrial function in PBMCs from healthy volunteers in the presence and absence of a septic stimulus.

(iii) Immune and mitochondrial dysfunction in PBMCs isolated from potentially septic and septic patients.

Chapter 2 Materials and Methods

This chapter describes the materials and methods used to generate the data presented in this thesis. Methods include flow cytometry, enzyme-linked immunosorbent assays, Seahorse respirometry and the NanoString nCounter gene expression system. Details of the suppliers of the reagents, consumables and equipment have been included. The chapter finishes with a description and rationale of the statistical tests used for analysis.

2.1 Human subjects

All human experiments were performed in accordance with the Declaration of Helsinki. Those involving patients were approved by the University College London (UCL)/UCL Hospitals Joint Research Ethics Committee (Gentian study, IRAS number: 266594). All subjects provided informed written consent, prior to sample sampling, or next-of-kin/representative agreement and retrospective consent if lacking competency. Whole blood and serum samples were obtained from patients presenting to the Emergency Department (ED) with potential infection or sepsis, and those being managed in the ICU with a diagnosis of sepsis. Patients were excluded if they were aged below 18 years, had severe anaemia, were treated with palliative intent, or had poor vascular access. A single sample of ~20 mL of blood was obtained at the same time as a clinically indicated blood culture, and collected into various pre-filled tubes. These include a BD Vacutainer® serum separating tube for a serum sample and two BD Vacutainer® CPT™ Cell Preparation Tubes (CPT).

Whole blood was also obtained from healthy control subjects recruited from staff and students at the Bloomsbury Institute of Intensive Care Medicine, with ethics approval obtained from UCL. Healthy control subjects were excluded if they had a medical history of any chronic inflammatory or immunosuppressive disease, recent immunisation, allergies, or were taking regular medications including steroids, paracetamol, non-steroidal anti-inflammatory medications or antibiotics. Volunteers were advised to avoid heavy exercise or alcohol

intake in the 48 hours prior. Twenty mL of whole blood was collected into a syringe that was heparinised (10 units/ml) to prevent sample coagulation prior to processing.

2.2 PBMC Isolation

Leukocytes can be separated into sub-populations using multiple techniques such as density gradient centrifugation or using cell surface markers. These techniques maximise yield, viability and functionality while ensuring high-throughput and consistency. Density gradient centrifugation uses the size and mass of molecules in a mixture to separate the sample into multiple layers with the heaviest molecules at the bottom and the lightest at the top. Cell surface markers can positively select for a sub-population of interest using fluorescence-activated cell sorting, or by negative selection of cells isolated after removal of other populations using antibody-bound magnetic selection beads.

In this work, I used density gradient centrifugation to isolate PBMCs as this was less likely to prematurely activate cells and produce off-target effects. This was performed using Ficoll® Paque Plus solution (Sigma Aldrich) or BD Vacutainer® CPT™ Cell Preparation Tubes (BD Bioscience); both methods were used with equal efficacy and are described below. PBMCs were chosen as the sub-population of interest as these consist of both innate and adaptive immune cells, they remain viable in tissue culture for over 24 hours, and enable study of relationship between cell populations. Neutrophils were excluded as they undergo apoptosis from 6 hours onwards and may release NETs which then activate other immune cells and which may confound results.

2.2.1 Density gradient centrifugation with Ficoll® Paque Plus solution

Peripheral blood samples from healthy controls were diluted with Dulbecco's Ca^{2+}/Mg^{2+} free phosphate buffered saline (PBS, Thermofisher) in a 1:2 ratio.

Thirty mL was layered on 15 mL of Ficoll® Paque Plus solution in a 50 mL Falcon tube and then centrifuged at 1400 rpm for 30 minutes at room temperature (RT) with no brake on deceleration. The resulting PBMC layer was transferred into a fresh 50 mL Falcon tube using a sterile pipette and washed three times with Ca^{2+}/Mg^{2+}-free PBS followed by centrifugation at 1400 rpm for 7 minutes at RT (Figure 2.1). Isolated PBMC live cell count and percentage cell viability were counted using the vital dye exclusion method. Cells were resuspended in media and 10 µL mixed in a 1:1 ratio with 0.4% trypan blue (Sigma) dye. This cell/dye mixture was added to a cell counting chamber and measured by a Countess™ automated cell counter (Invitrogen). This method was used for healthy volunteer samples as it is reliable and consistently produced a high yield of viable PBMCs. It was unsuitable for patient samples due to clinical delays which postponing sample processing.

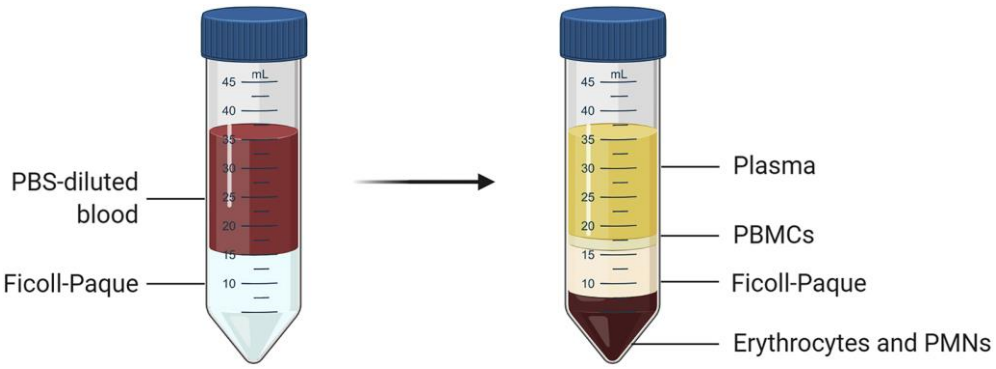


Figure 2.1. PBMC isolation by density gradient centrifugation with Ficoll® Paque Plus (471)**.**

2.2.2 Density gradient centrifugation with BD Vacutainer® CPT™ Tubes

Peripheral blood samples from patients were collected and transferred into BD Vacutainer® CPT™ tubes. PBMCs isolated were comparable in phenotype and function with those obtained using Ficoll® Paque Plus solution. Two CPT™ tubes were filled with 8 mL of whole blood and centrifuged at 1500*g* for 15 minutes at RT. The resulting PBMC layer was then transferred into a fresh 50 mL Falcon tube, washed three times and counted as above.

2.3 Tissue culture

PBMCs were cultured in a Containment Level 2 laboratory using an aseptic technique within a tissue culture hood with a laminar flow unit. All reagents and plasticware used for tissue culture were bought pre-sterilised and opened in the tissue culture hood. Media used for tissue culture were warmed to RT before use, while the incubator was set to 37°C with 5% CO_2. Dulbecco's Modified Eagle Medium (DMEM, Sigma Aldrich) with no phenol red, pyruvate or HEPES but containing L-glutamine and glucose (4.5 g/L) was used as the media of choice. All experimental conditions were supplemented with 10% Foetal Bovine Serum (FBS, Thermofisher); in patient samples from ICU and ED, 10% serum from the respective patient was additionally added. Separately, a set of healthy volunteer samples was also incubated with 10% pooled septic serum obtained from the ICU patients. The 10% value was chosen following extensive optimisation work carried out by my host lab.

Approximately 100 000 PBMCs in 100 μL per well were seeded in Nunc™ 96-well, Nunclon delta-treated, flat-bottom microplates (Thermofisher). The cells were then incubated with ciprofloxacin (100 μg/mL), propofol (50 μg/mL), NE (10 μg/mL), or all three drugs combined, in the presence or absence of *K. pneumoniae* endotoxin (LPS; Thermofisher) (100 ng/mL) for 6 or 24 hours. These drugs were chosen due to their common use ICUs, availability, relative stability in cell culture, and the relative consistency of their effects on the immune system and mitochondria compared to other drugs. Concentrations were chosen following a literature review of concentrations representative of therapeutic drug levels, and following preliminary optimisation experiments involving high, medium and low doses. These concentrations correspond to the higher end of normal doses used in clinical practice while remaining physiologically relevant.

At 6 and 24 hours, cytokines were measured in the supernatant using enzyme-linked immunosorbent assays (ELISAs). Phagocytosis and mitochondrial

functions were assessed using flow cytometry, and mitochondrial respiratory function was measured by respirometry (Seahorse XF96).

2.4 Single-analyte ELISAs

The concentration of the proinflammatory cytokines TNF-α and IL-6, and the anti-inflammatory IL-10 were measured in cell culture supernatants after 24 hours of incubation with the drugs of interest (alone or in combination) in the presence of LPS. Initial optimisation experiments showed minimal cytokine production after 6 hours' incubation. No cytokines were produced in the healthy samples with no LPS at either 6 or 24 hours. As a result, the 24-hour timepoint was chosen to allow maximal cytokine accumulation. All samples except control samples included LPS. Cytokines were determined using commercially available ELISA kits (e.g., Human Duoset® ELISA kits, R&D Systems). These were performed according to the manufacturer's protocol and validated by running standard curves in duplicates for each experiment.

Experiments were optimised by testing various dilutions of sample for each cytokine to identify a dilution that provided consistency of results and avoided the extremes of the standard curve (e.g., no dilution, 1:10 and 1:100). The dilution varied with the sample type; for healthy volunteers, the dilution used was 1:10 for TNF-α, and IL-6, and 1:1 for IL-10. For patient samples and for healthy volunteers with pooled septic serum, a dilution of 1:100 was used for TNF-α and IL-6, and 1:10 for IL-10. For septic patient serum samples, a dilution of 1:100 was used. The standard curve for each cytokine was produced by serial dilutions of the standard solution as per protocol (Table 2.1).

These kits use the 'sandwich' principle where Pierce™ 96 well ELISA plates (Thermofisher) were prepared by coating them with capture antibody overnight. These were then washed three times with wash buffer (0.05% Tween®20 in PBS). Non-specific binding was blocked by incubation with reagent diluent containing 1% BSA in PBS for a minimum of 1 hour. The plates were washed again three times with wash buffer and sample supernatants added in duplicates to the plates, along with standards of known concentration

and blank wells as per protocol. These were incubated for 2 hours and subsequently washed three times. Following this, detection antibody diluted in reagent diluent was added for 2 hours. After washing, a streptavidin horseradish peroxidase solution was added for 20 minutes in the dark, before a final wash and the addition of the 3,3',5,5'-tetramethylbenzidine substrate solution. This colorimetric reaction was stopped after 20 minutes by the addition of 2N sulphuric acid stop solution. All steps were carried out at RT.

A SpectroStar® Nano plate reader (BMG Labtech, Germany) was used to measure the optical density (OD) at 450 nm for each sample. MARS data analysis software was used to provide the OD values. GraphPad Prism (Version 9.3.1 for macOS, GraphPad Software, La Jolla, CA, USA, 2021) was used to produce a four-parameter logistic curve from the OD values of the reference standards (Table 1.4), which was subsequently used to determine the concentration of each cytokine.

Table 2.1. Summary of range of detectable concentrations for the Human Duoset® ELISA kits used to detect cytokines released by PBMCs.

Cytokine	**Range of detectable concentrations**	**Catalogue no.**
IL-6	9.38- 600 pg/ml	DY206
IL-10	31.3- 2000 pg/ml	DY217B
TNF-α	15.6- 1000 pg/ml	DY210-15

2.5 Flow cytometry

2.5.1 Principles of flow cytometry

Flow cytometry is a laser-based technique that enables rapid multi-parameter analysis of vast numbers of cells at a single-cell level (e.g. expression of cell surface and intracellular molecules, cell characterisation in a heterogeneous mixture or purity in isolated populations, as well as cell size, granularity and volume). Flow cytometry consists of three main components: fluidics which transport cells through the instrument, lasers to excite fluorochromes and

optics to detect fluorescence emission, and electronics to convert optical into digital signals that are processed and displayed by a computer. The flow cytometer used can simultaneously measure ~15-20 parameters.

2.5.1.1 Fluidics

This is responsible for transporting cells to the interrogation point in a single file so that the fluorochrome-conjugated antibodies on the cells can be optimally excited by the lasers. The single file of cells is produced by hydrodynamic focussing of two streams of fluid with the sample stream at higher pressure compared to the sheath fluid which is at lower pressure. This generates a laminar flow which, in turn, aligns cells singularly along the flow cell. Flow rates can be adjusted according to user needs, e.g. for immunophenotyping higher flow rates are required, while slower flow rates are used for analysis of DNA content. The fluidics system must be kept free of air bubbles and debris, and should be maintained at an optimum pressure.

2.5.1.2 Optics

The optics system consists of laser beams to scatter light in forward (FS) and side (SS) directions. The laser beam is coherent and monochromatic which allows excitation of the fluorochrome-conjugated antibodies on cells by specific wavelengths. Common excitation lasers include: ultraviolet (350 nm), violet (405 nm), blue (488 nm), yellow-green (561 nm) and red (640 nm). The system also has lenses, filters and mirrors that detect scattered and emitted fluorescent light, separates them into specific wavelengths, and directs them towards relevant detectors. Suitable filters (long, short or band pass filters) are positioned so that an optical detector can detect a specific fluorochrome.

2.5.1.3 Electronics

There are multiple photodetectors (e.g., photodiodes or photomultiplier tubes) which digitally convert the light scattered or fluorescence emitted into data points. This is amplified and displayed as flow dot plots on the computer.

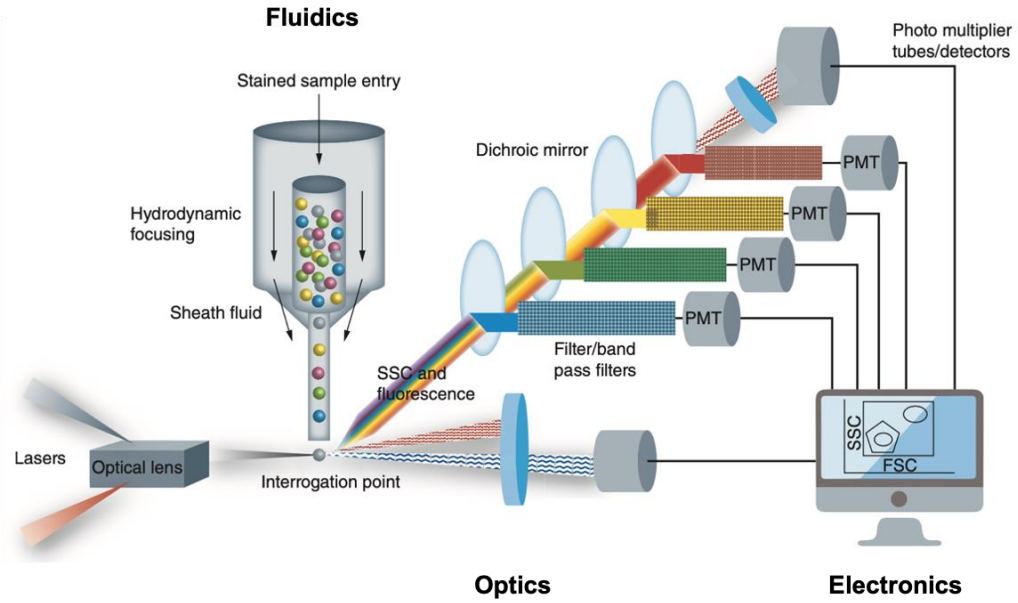


Figure 2.2. Main components of flow cytometry (472)**.**

2.5.2 Antibodies used

Working dilutions of each antibody were established by titration and assessment of expression of cell surface markers on the PBMCs. Information on specific antibodies used in this thesis is shown in Table 2.2. This includes the fluorescent conjugate, clone, manufacturer and final concentrations used. All antibodies used were directly conjugated. Panels were designed using FluoroFinder® software online, which considered the spectral overlap between the markers of interest and suggested the brightest fluorochrome depending on the relative expression of the molecule of interest on the target population.

2.5.2.1 Cell surface staining

Cells were stained after 6 or 24 hours of incubation with the drugs of interest ± LPS. Staining was carried out in flat-bottom 96-well culture plates for convenience and to prevent inadvertent stimulation of cells. A master mix of antibodies was produced at relevant concentrations and this was sequentially added to the appropriate wells. Single stains were prepared for CD14/CD16 for identification of monocytes (Miltenyi Biotech, Viogreen), CD3/CD19 (Biolegend, BV421) for identification of lymphocytes, and anti-HLA-DR

antibody (Biolegend, BV711) by the addition of 0.5 µL of each antibody to 100 µL of PBMC sample. Annexin V (Biolegend, APC) and LD Near InfraRed (NIR) (Thermofisher, APC Cy7) single stains were prepared as stated in the cell viability section below. These studies did not distinguish between subpopulations of monocytes and lymphocytes to avoid presence of large spectral overlap with mitochondrial dyes.

Cells were prepared by firstly adding L/D NIR in a 1/1000 dilution in Ca^{2+}/Mg^{2+} free PBS before incubating for 30 minutes at RT in the dark. Followed this, a 'master mix' containing anti-human CD14, CD16, CD3 and CD19 antibodies and annexin V (0.5 µL of each antibody/100 µL of sample) was produced. Samples were mixed with antibody solution by pipetting up and down and then incubated for 15 minutes at RT in the dark. They were protected from light throughout the staining process. Following staining with the master mix, cells were then stained for specific immune or mitochondrial markers of interest as below. Cells were not fixed as mitochondrial studies require fresh cells.

2.5.2.2 Cell viability using flow cytometry

Cell viability was determined using Live/Dead NIR and annexin V was used to exclude apoptotic and necrotic cells. Single stain controls were prepared by incubating PBMCs on a heat block at 60°C for 15 mins to induce cell death. These were mixed with cells that had not undergone cell death and were either stained with. Live/Dead NIR was added at 1/1000 dilution in Ca^{2+}/Mg^{2+} free PBS and incubated for 30 mins at RT in the dark. Similarly, 0.5 µL of annexin V was added to the 100 µL sample and incubated for 15 minutes at RT in the dark.

2.5.2.3 Phagocytosis by flow cytometry

Phagocytosis was determined using pHrodo™ Red *E Coli* BioParticles™ conjugate (Thermofisher). This is a pH sensitive dye that does not fluoresce outside cells where the pH is 7.4. Once immune cells recognise the conjugated

E. coli and endocytose and/or phagocytose the dye, the local pH changes to ~4 which brightly fluoresces the dye.

Negative controls were prepared by addition of cytochalasin D (Gibco) to the relevant sample at a final concentration of 10 µM, and then incubated at 37°C for 60 mins. Cytochalasin D is a cell-permeable fungal toxin that binds to actin filaments to inhibit actin polymerization; this is required for phagocytosis and endocytosis. After 60 mins, pHrodo™ was added to all relevant samples including negative controls, at a final concentration of 100 µg/ml and incubated at RT for 60-90 mins. All samples were then diluted in 300 µL of cell stain buffer prior to analysis by flow cytometry.

2.5.2.4 HLA-DR expression

HLA-DR, a component of the MHC II receptor is found on the cell surface of antigen-presenting cells and on T-lymphocytes. HLA-DR expression was demonstrated on cells using anti-HLA-DR antibody (Biolegend, BV711). Approximately 1 µL per 100 µL of sample was added to the master mix described above. Single stain controls were used to determine HLA-DR positive and negative populations in each experiment.

2.5.2.5 Mitochondrial membrane potential

This was measured using tetramethyl-rhodamine methyl ester (TMRM, Thermofisher), a lipophilic, cationic, fluorescent dye that accumulates in the negatively charged mitochondrial membrane. It distributes according to the mitochondrial membrane potential (MMP); a more negative potential accumulates more dye that is manifest by a higher mean fluorescence intensity (MFI). TMRM can be used for non-quenching (0.5 to 30 nM) and quenching (>50 nM to 1 µM) studies (473). In non-quenching mode, TMRM fluorescence is directly proportional to dye concentration, allowing estimation of the MMP. In quenching mode, TMRM accumulates in the mitochondrial membrane and forms aggregates which do not linearly relate to MMP.

Cells were firstly stained with L/D NIR and the master mix containing anti-human CD14/CD16 antibodies, anti-human CD3/CD19 antibodies, annexin V and HLA-DR. TMRM (25 nM) was then added and cells incubated for 15 minutes in a 5% CO_2 incubator at 37°C. Carbonyl cyanide-4-phenylhydrazone (FCCP, Sigma Aldrich), an inophore that uncouples mitochondrial OxPHOS, was used as a positive control for TMRM as this dissipates the MMP. Cells were incubated with FCCP (10 µM) for 15 minutes at 37°C in a 5% CO_2 incubator prior to the addition of TMRM as above.

2.5.2.6 Mitochondrial reactive oxygen species

This was measured using Mitosox Red (Thermofisher Scientific, PE CY5.5), a lipophilic, cationic fluorescent dye derived from hydroethidium. It has a triphenylphosphonium group that selectively targets mitochondria in which Mitosox accumulate. Within the mitochondria, it is rapidly oxidised by superoxide to firstly form Mito-HE free radical and then 2-OH Mito E^+. The latter increases its fluorescence in proportion to the superoxide concentration. Mito-HE can undergo non-specific oxidation, the result of which also produces an increase in fluorescence. 2-OH Mito E^+ also intercalates with nucleic acids.

Table 2.2. Summary of the antibodies used to delineate markers of interest.

Antibodies or markers of interest	**Fluorochrome**	**Clone**	**Company**	**Catalogue no**	**Volume per 100µL sample**
Anti-CD14 antibody	Viogreen	REA599	Miltenyi Biotec	130-110-525	0.5µL
Anti-CD16 antibody	Viogreen	REA423	Miltenyi Biotec	130-113-397	0.5µL
Anti-CD3 antibody	BV 421	OKT3	Biolegend	317344	0.5µL
Anti-CD19 antibody	BV 421	SJ25C1	Biolegend	363018	0.5µL
Anti-HLA-DR antibody	BV711	L243	BD Bioscience	307644	1µL
Annexin V	APC	N/A	BD Bioscience	640920	0.5µL
Live/Dead NIR (775)	APC Cy7	Polyclonal	Thermofisher	L10119	1µL

pHrodo™ (E-coli)	PE	N/A	Thermofisher	P35361	100µg/ml = 10µL (1mg/mL)*
TMRM	PE	N/A	Thermofisher		25 nM
Mitosox Red	PerCP-Cy 5.5	N/A	Thermofisher	M36008	5 µM

* = stock concentration

Cells were firstly stained with L/D NIR and the master mix containing anti-human CD14/CD16 antibodies, anti-human CD3/CD19 antibodies, annexin V and HLA-DR. These were then incubated with Mitosox (5 µM) for 15 mins at 37°C in a 5% CO_2 incubator. Antimycin A (Sigma Aldrich), a complex III inhibitor, was used as a positive control. Antimycin A 100 µM was added for 15 mins at 37°C in a 5% CO_2 incubator before adding Mitosox. Samples were then diluted in 300 µL of cell stain buffer prior to analysis by flow cytometry.

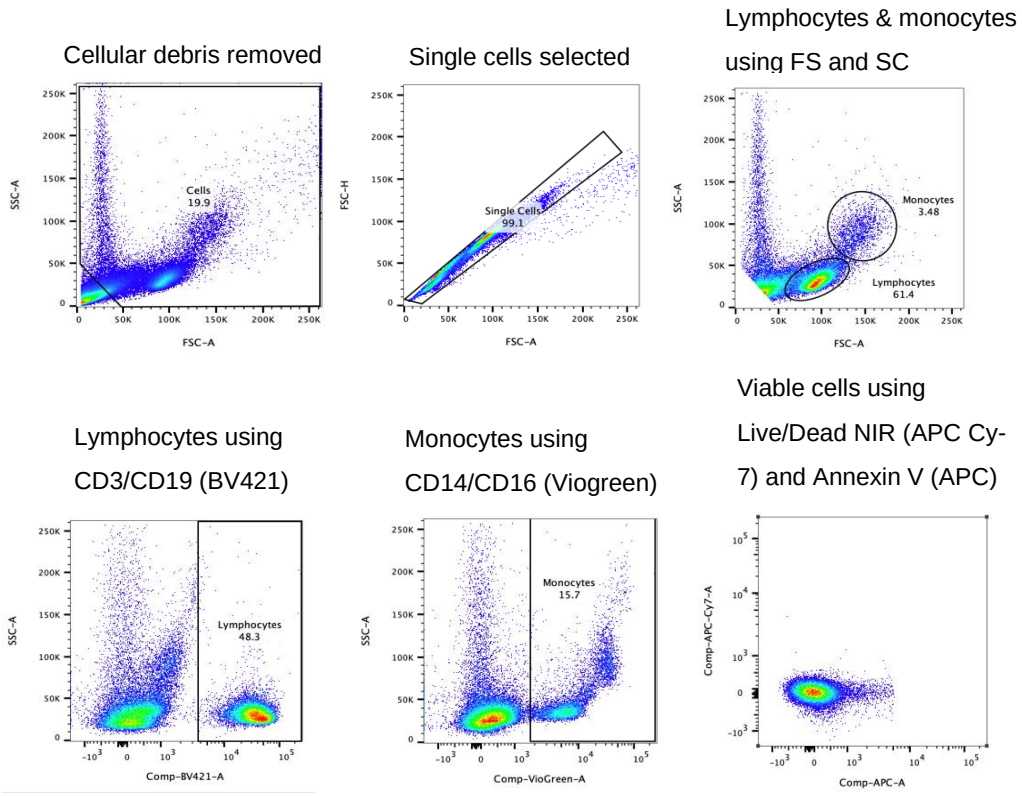
2.5.3 Quality control

Experiments were optimised by running concentration curves for each fluorophore to ensure consistency while avoiding waste. Positive and negative controls were run for each experiment for the following fluorochromes to ensure reproducibility; pHrodo™, TMRM, Mitosox, L/D NIR and annexin V. Unstained and single colour-stained controls were analysed prior to the experimental samples in order to set compensation values between antibodies with overlapping emission spectra in the master mix. Compensation between fluorochromes was calculated by BD FACS Diva software; <30% spectral overlap was permitted between fluorochromes. Isotype controls were initially used to identify and remove non-specific binding which would otherwise falsely be identified as positive for the surface markers. Where this was not possible due to sample availability and cost, pre-experimental optimisation with reagent titration, viability confirmation and Fc-block was utilised instead.

2.5.4 Data gathering and analysis

Samples were analysed using the LSR Fortessa™ flow cytometer (BD Bioscience, USA) and FlowJo™ Software (for Mac, v10.4. Becton Dickinson; USA, 2018) was used to analyse data. Firstly, cellular debris was excluded

and single cells were selected using dot plots based on size (forward scatter) and granularity (side scatter). Lymphocyte and monocyte populations were then selected using either CD3/CD19 and CD14/CD16 respectively. Live/dead NIR and annexin V were used to identify viable cells and exclude apoptotic and necrotic cells. These cells were then analysed for differences in mtROS and MMP. Phagocytosis was analysed in the viable PBMC population and HLA-DR in monocytes only. The gating strategy and the positive and negative controls are shown in Figure 2.3. The Mean Fluoresce Intensity (MFIs) obtained from FlowJo were summarized using Excel (Microsoft). All statistical analyses were performed using GraphPad Prism (Version 9.3.1 for macOS, GraphPad Software, La Jolla, CA, USA, 2021). Where parametric tests were carried out, the Shapiro-Wilk test was used *a priori* to determine if data were normally distributed. Two-way ANOVAs followed by post-hoc Dunnett's or Bonferroni's multiple comparisons test were used for data analysis unless otherwise stated.



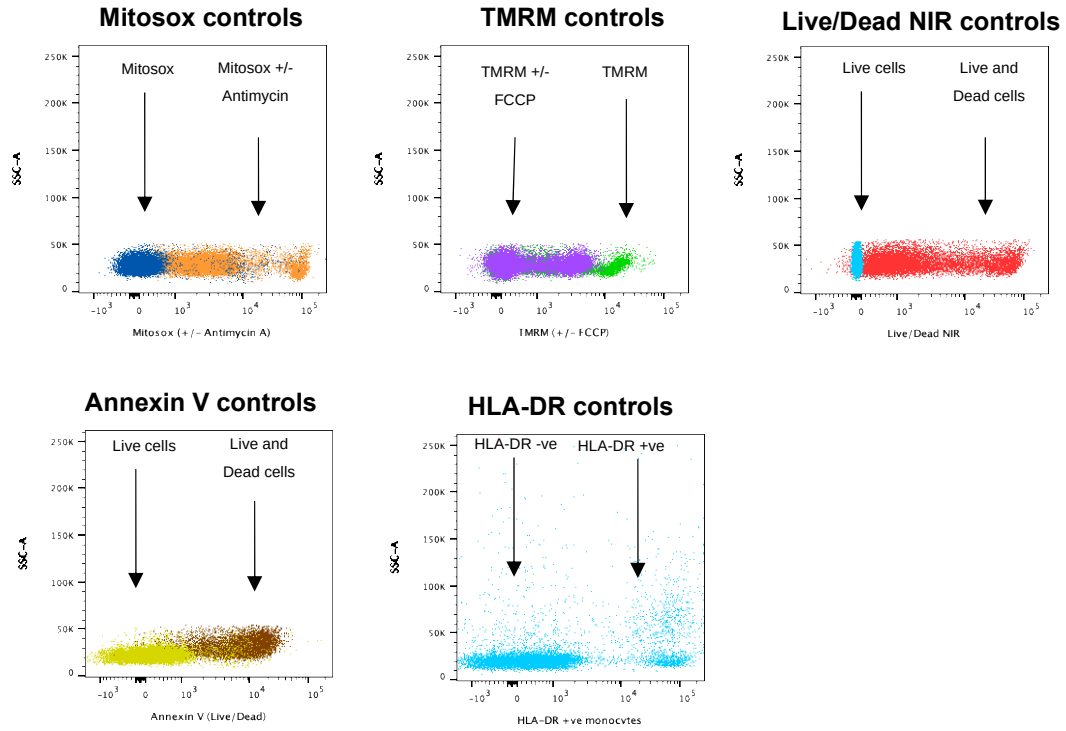


Figure 2.3. Gating strategy and control examples used for flow cytometry experiments.

2.6 Mitochondrial stress test using Seahorse XF96 Analyser

2.6.1 Principle of the Seahorse respirometry

Extracellular flux assays are used to assess the metabolic state of cells. These measure two major energy pathways: anaerobic glycolysis by measuring the extracellular acidification rate due to lactate production, and aerobic respiration by calculating the oxygen consumption rate (OCR). The mitochondrial stress test is an assay that directly measures the OCR of cells, more specifically: basal respiration, proton leak, maximum respiration rate, and non-mitochondrial respiration (Figure 2.4). Basal respiration is respiration required to meet endogenous cellular ATP demands. This can drive proton leak pathways because ATP production via OxPHOS is not completely coupled and protons may return from the inner membrane to the matrix without producing

ATP. ATP-linked respiration is synonymous with mitochondria-related ATP synthesis and is sensitive to the complex V inhibitor, oligomycin. Maximal respiration refers to the maximum to which respiration can be increased to counteract the reduction in MMP induced by the ionophore FCCP. Spare respiration capacity is the difference between basal and maximal respiration capacity and is the ability of a cell to meet an increased energy demand. Non-mitochondrial respiration is the respiration calculated after complex I and III are inhibited by rotenone and antimycin A, respectively.

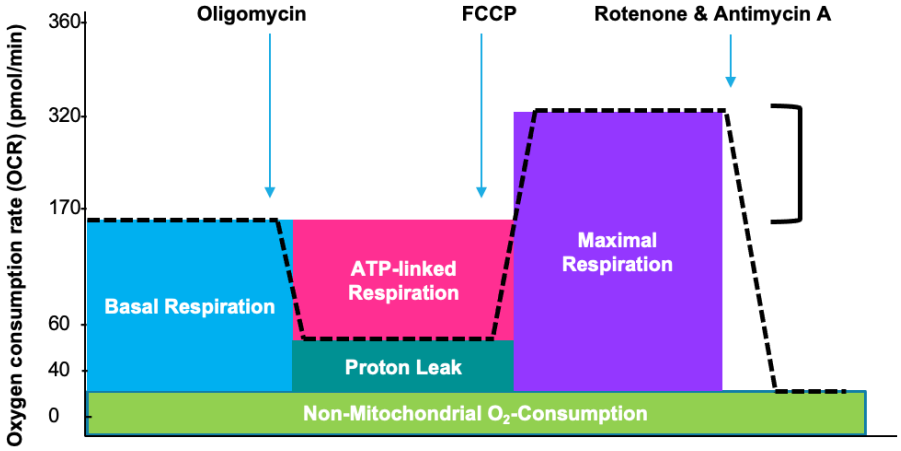


Figure 2.4. A typical mitochondrial stress profile of cells.

The test measures different aspects of mitochondrial respiration which are 'stressed' by sequential addition of various drugs: oligomycin, FCCP, antimycin A and rotenone. Oligomycin inhibits ATP synthase activity and reduces the oxygen required for ATP synthesis, thus giving a measure of ATP-linked respiration. Proton leak remains steady unless there is mitochondrial damage. FCCP disrupts the MMP, causing the cells to increase oxygen requirements to restore membrane potential for ETC activity; this is known as maximal respiration. The rise from basal and maximal respiration is the spare respiratory capacity. Antimycin A and rotenone reduce the oxygen consumption required for the maintenance of the ETC. This image has been adapted with permission from Agilent Technologies, Inc.

The mitochondrial stress test is achieved by sequential exposure of cells to oligomycin (ATP synthase inhibitor), FCCP (ionophore uncoupler), rotenone and antimycin A (complex I and III inhibitors respectively) (Figure 2.5).

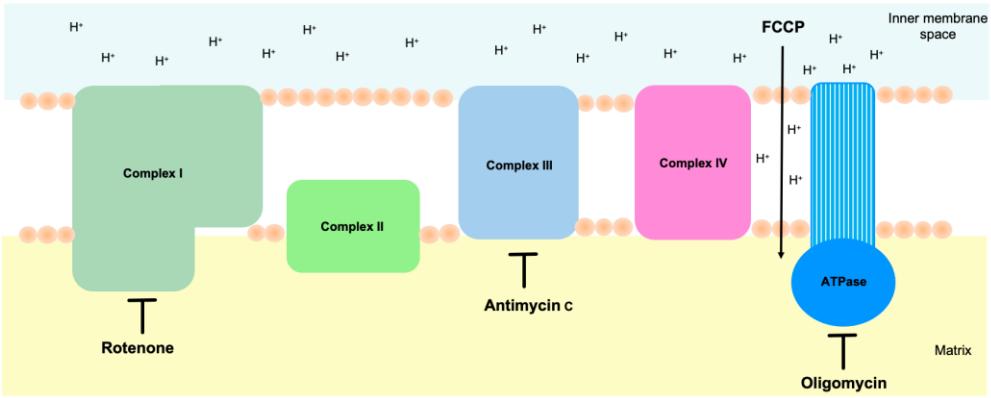


Figure 2.5. Drugs used in the mitochondrial stress test.

Rotenone and antimycin A target complexes I and III, respectively. Oligomycin inhibits complex V while FCCP disrupts the inner mitochondrial membrane.

The Seahorse XF96 analyser (Agilent Technologies, Santa Clara, CA, USA) allows real-time measurement of OCR via the mitochondrial stress test assay. It consists of microchambers, sensor probes and an injection port system. The microchamber isolates ~2 µL of medium above a monolayer of adherent cells. This allows measurements of transient changes in dissolved oxygen concentration by the sensor probes sited 200 µm above the cell monolayer. The sensory probes consist of resin-based oxygen and pH sensors. The oxygen sensor is a phosphorescent porphyrin which has a slow emission decay following a pulse of light. As this is quenched by oxygen, oxygen can be measured by the rate of decay or from a reduced steady state emission. Changes are measured every 2-5 minutes from which OCR is calculated. The probes then lift, allowing the media above the monolayer to mix with the larger volume of media above; this restores the microenvironment around the cells to their baseline. OCR is measured 3-4 times during the course of an experiment. The injection port system allows sequential addition of up to four drugs per well at defined concentrations and intervals (474).

2.6.2 Mitochondrial stress test assay

The workflow of mitochondrial stress test spanned over two days. On Day 1 the sensor cartridge (Agilent Technologies) was hydrated by separating and

filling the utility plate with 200 µL of sterile water per well. The sensor cartridge was then replaced, submerging the sensors in the sterile water; both were placed in a non-CO_2 incubator at 37°C overnight. The following morning, 1 hour prior to running the assay, sterile water was discarded and replaced with 200 µL of pre-warmed Seahorse XF Calibrant (Agilent). The sensor cartridge and utility plate were placed back in the non-CO_2 incubator at 37°C for 45-60 mins prior to loading of the injection ports with assay-specific drugs.

PBMCs were isolated and counted as above. Approximately 300,000 cells were seeded per well. As PBMCs are non-adherent, Corning™ Cell-Tak cell and tissue adhesive (Thermofisher) was required to adhere PBMCs to the XF cell culture microplate (Agilent). Cell-Tak is a polyphenolic protein solution that enables cells to adhere to solid surfaces. For a 96-well XF cell culture microplate with a surface area of ~0.75cm^2 per well, 22.4 µg/mL of Cell-Tak was required to cover the plate. This was achieved by diluting 32 µL of Cell-Tak in 2.5 mL of sterile water and distributing 25 µL per well. The microplate was incubated for 20 mins at RT on a shaker and subsequently washed twice with 200µL sterile water per well. PBMCs were suspended in 100 µL of DMEM for cell culture as above (no phenol red, pyruvate or HEPES but with added L-glutamine, glucose (4.5 g/L) and either 10% FBS, 10% serum or 10% pooled septic serum). Drugs of interest were also added: ciprofloxacin (100 µg/mL), propofol (50 µg/mL), NE (10 µg/mL) or all three drugs combined ± LPS (100 ng/mL). The microplate was then incubated in a 5% CO_2 incubator for 6 hours. Background wells were chosen and no cells were added to these wells.

The number of cells and incubation time period were chosen after optimisation experiments. Cell counts <100 000 or >300 000 per well led to wide variations between replicates, the latter due to a large overlap between adhered cells. 300 000 cells per well was chosen as this provided accurate results. A time course of 3, 6 and 24 hours showed that cells were less responsive to assay-related drugs beyond 6 hours and little difference was seen at 3 and 6 hours. A 6-hour incubation was therefore chosen as this was consistent with the early timepoint used in flow cytometry experiments and because this would allow the PBMCs to incubate with the drugs of interest for an adequate period of

time. Each condition was replicated four times to ensure accuracy and reliability of results (Figure 2.6).

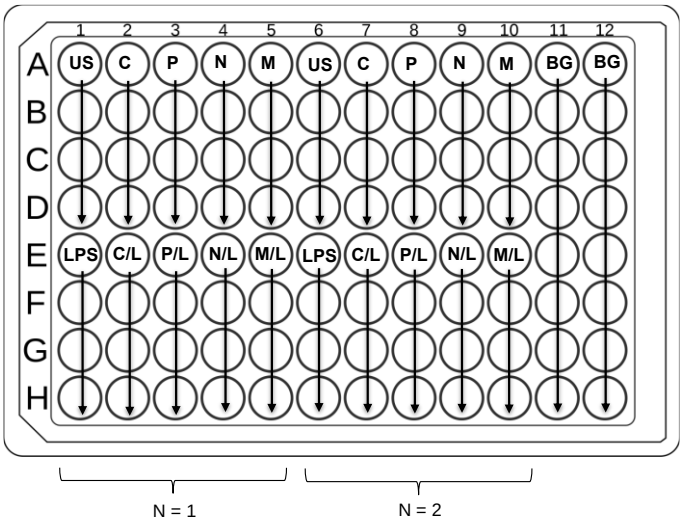


Figure 2.6. Well map used for the XF Seahorse culture microplate.

US = wells containing unstimulated cells, LPS = wells containing LPS, C = wells containing ciprofloxacin, C/L = wells containing ciprofloxacin and LPS, P = wells with propofol, P/L = wells with propofol and LPS, N = wells containing NE, N/L = wells containing NE and LPS, M = wells containing mixture of drugs, M/L = wells containing mixture of drugs and LPS and BG indicating empty background wells.

After 6 hours, cell culture media was removed carefully using a pipette, ensuring that a 20 µL residual volume was left behind per well. Cells were washed in 180 µL of Seahorse bicarbonate-free DMEM medium (Agilent), pre-warmed to 37^{o}C and supplemented with L-glutamine 2 µM, sodium pyruvate 1 µM and glucose 5 µM (all Agilent). These concentrations were used following a comprehensive literature search. Cells were checked under a microscope to ensure they were evenly distributed in a monolayer and had not been washed away. PBMCs were then resuspended in 180 µL of Seahorse media and incubated in a CO_2-free incubator for 20-25 mins and no longer than 60 mins.

In the meantime, oligomycin, FCCP, antimycin A and rotenone were diluted to 10x the final experimental concentration and loaded into injection ports on the sensor cartridge in Ports A, B and C respectively. Port D was loaded with 25

µL of PBS. These concentrations were chosen following optimisation by undertaking concentration curve experiments.

Following this, XF Wave software was set up using the Assay Wizard. This includes setting temperature to 37ºC, defining groups, determining the plate map, labelling the assay media, background buffers, injections to be given and the experiment protocol (including the number and duration of cycles, mixing, wait period and measurement). Once defined, the template was saved and the sensor cartridge and the utility plate were loaded onto the Seahorse XF analyser and calibrated. This typically took ~30 mins. Once complete, the microplate with PBMCs was loaded onto the analyser and the assay commenced. Total assay time was ~60-90 mins, after which the cell culture microplate and the sensor cartridge could be removed from the machine. Following this, cells were counted as below for normalisation.

Table 2.3. Summary of drugs and concentrations used in the mitochondria stress test assay.

	[Stock]	**[In each port] x10 the [Final]**	**[Final] at the end**	**Recommended volume at each injection port**	**Dilution and volume of drug to get the [x10]**
Oligomycin	5 mM	35 µM	3.5 µM	Port A 25 µL (25 x 96 = 2.4 mL)	1/143, 14 µL of oligomycin in 4 mL of Seahorse media
FCCP	10 mM	25 µM	2.5 µM	Port B 25 µL	1/400, 8 µL of FCCP in 4 mL of Seahorse media
Rotenone and antimycin A	10 mM (Rot and Ant)	50 µM	5 uM	Port C 25 µL	1/200, 20 µL of rotenone and antimycin in 4 mL of Seahorse media

2.6.3 Normalisation of cells

Cells can be normalised by cell count, genomic DNA content, or total cellular protein content. Here, cell counts were used to normalise the mitochondrial stress test assay. This involved removing the Seahorse media using a pipette and washing the wells twice with 180 µL of PBS. Cells were then carefully re-

diluted in Hoechst (2'-[4-ethoxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5'-bi-1H-benzimidazole trihydrochloride trihydrate) 33342 solution (Thermofisher) in a dilution of 1/1000 and incubated for 15 mins at RT in the dark. Hoechst 33342 is a cell-permeable DNA stain excited by ultraviolet light and which emits blue fluorescence at 460 to 490 nm. It binds adenine-thymine (A-T) regions of DNA and exhibits distinct fluorescence emission spectra that are dependent on dye: base pair ratios. The cells were placed and analysed using an Image Xpress Imaging System (Molecular Devices, San Jose, CA, USA).

2.6.4 Data analysis

Normalisation data and results from the Seahorse XF analyser were uploaded onto Wave 2.6 Desktop software (Agilent Seahorse Analytics). Once uploaded, the following quality control steps were carried out prior to obtaining results: the accuracy of the plate map was confirmed, the group results were checked individually and any obvious outliers excluded, both OCR rate (pmol/min) and the levels (mmHg O_2) were checked for consistency, the data was normalised to cell counts and background correction was applied. Following this, mean and standard deviations were obtained for basal respiration, protein leak, maximal respiration, spare respiration capacity, non-mitochondrial respiration and ATP-production (all OCR values given as pmol/L), coupling efficiency in percentage (%) and spare respiratory capacity percentage (%). One-way ANOVA analysis with either Kruskal-Wallis or Dunn's multiple comparison test were used to compare drug groups against unstimulated control, or drug/LPS combinations against LPS control.

2.7 Nanostring nCounter gene expression system

2.7.1 Principle of Nanostring gene expression system

This high-throughput, sensitive and reproducible method detects expression of up to 800 RNA, DNA or protein targets in a single reaction. This includes genomic mutations, copy number variation, single nucleotide variation,

chimeric RNAs and miRNA expression. Only 100 ng total RNA or an equivalent amount of tissue lysate is required. The main advantage of this system compared to next-generation sequencing is the speed and methodological simplicity; no conversion of mRNA to cDNA by reverse transcription or amplification of cDNA by polymerase chain reaction is needed.

The nCounter system consists of a multiplexed probe library with two sequence specific probes for each target mRNA, collectively known as CodeSets (Figure 2.7). The CodeSets contain a pair of capture and reporter probes with 35- to 50-base target-specific sequences complementary to the target mRNA. The capture probes additionally have a short common sequence labelled with biotin at the 3' end, which provides the site of attachment for the target mRNA. The reporter probes also consist of a single stranded DNA backbone attached to complementary RNA segments labelled with specific fluorophores. These unique colour-coded RNA segments at the 5' end enable barcoding and detection of target mRNA.

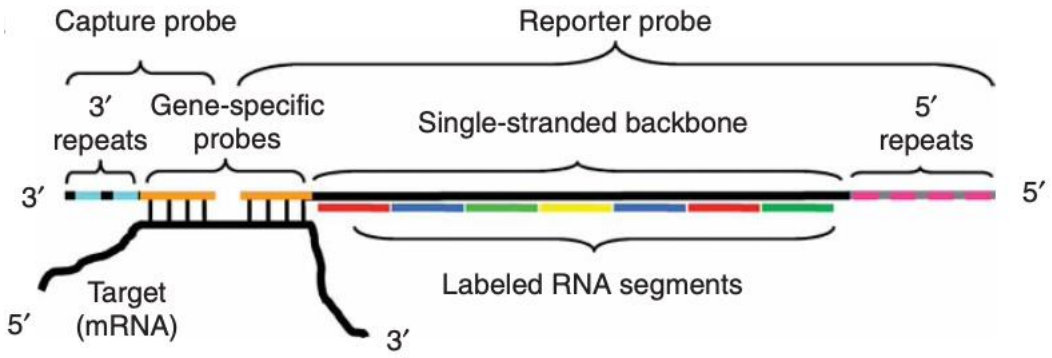


Figure 2.7. CodeSet with capture probe, target mRNA and reporter probe (475).

2.7.2 Preparation of samples

The Nanostring nCounter system is designed around gene expression. The 'Human Immunology V2' and 'Metabolic Pathways' panels, which consisted of 579 and 748 genes respectively, were used with 15-20 internal reference genes for data normalization. These genes cover core pathways and processes of the immune response (major classes of cytokines and their

receptors, chemokine ligands and receptors, interferons and receptors, the TNF-receptor superfamily, and KIR family genes) and metabolic pathways (biosynthetic pathways, nutrient capture and catabolic pathways, cell stress, metabolic signalling and transcriptional regulation) in humans. The focus of these experiments was on overall immune function and mitochondrial respiration.

Nanostring nCounter panels were carried out on PBMCs from four healthy volunteers incubated with pooled septic serum for 6 hours across two panels as above. 200 000 PBMCs per well were used to ensure a reliable mRNA yield. The sample combination was rationalised to: unstimulated, LPS, ciprofloxacin with LPS, propofol with LPS, NE with LPS, and a mixture of all three drugs with LPS. These were chosen as a compromise between obtaining reliable and informative results and convenience, time required and cost.

2.7.3 mRNA Extraction

mRNA was extracted using the Qiagen RNeasy Plus Mini Kit (Qiagen) which can extract up to 0.5 μg total RNA from unstimulated lymphocytes of up to 1 $x10^6$ per sample. This provided an adequate range to isolate 100 ng of total RNA from PBMCs for the two panels. Cells were harvested and homogenized using 350 μL of Buffer RLT plus and vortexed for 30 secs. This buffer consists of highly denaturing guanidine-thiocyanate containing buffer, which inactivates RNAses to ensure purification of intact RNA. Samples were transferred to gDNA eliminator spin columns in a 2 mL collection tube and centrifuged for 30s at >8000g. The follow through was kept and 350 μL 70% ethanol was added and mixed to provide appropriate binding conditions. The sample (700 μL total) was then applied to the Rneasy Mini spin column and centrifuged for 15 seconds at >8000g. Buffer RW1 (700 μL) and Buffer RPE (500 μL) were added twice with centrifugation of 15s at >8000g after each, the last centrifugation was for 2 mins at >8000g. This ensured that total RNA was bound to the membrane and contaminants were washed away. The final step involved the addition of 30 μL water to the Rneasy spin column and keeping

the eluted RNA and immediately freezing in liquid nitrogen and storing at -80ºC.

2.7.4 mRNA Quantity and Purity

When appropriate, an aliquot of these samples was taken to the Frances Crick Institute and checked for quantity and purity using a Qubit 4.0 fluorometer (Thermofisher) and a 2200 TapeStation (Agilent). Qubit fluorometers quantify nucleic acids or proteins in a sample by determining fluorescence intensity on binding to these molecules. Samples are mixed with the Qubit working solution, incubated at RT for 2 min and fluorescence read. The TapeStation system determines the size, quantity, and integrity of a sample using an automated electrophoresis solution. This involves loading the samples, loading tips and screen tape onto the TapeStation and using the associated software to obtain the results. Here, RINe values were used to determine total RNA integrity as shown in Table 2.4. Once obtained, samples were diluted to 20ng/µL, and a total of 400ng RNA per sample was prepared.

2.7.5 Immunology and Metabolic Pathway panels

These samples were then transferred to the UCL Nanostring facility where the two panels (Immunology V2 and Metabolic Pathway) were performed and raw data provided in the form of RCC files. These were uploaded onto nSolver 4.0 (NanoString Technologies, Washington, USA) and ROSALIND® analysis software (ROSALIND, San Diego, CA) for data quality checks, background thresholding and normalization.

2.7.6 Data analysis

Quality control measurements including imaging quality, binding density and positive control linearity were obtained.

Imaging quality was determined by comparing the ratio between fields of view (FOV) scanned successfully against those attempted to be scanned. For the

MAX system, 555 FOVs were attempted to be scanned for each sample. If the ratio between the two was <0.75 then the sample was flagged. Binding density is an indication of cartridge saturation and is given by the square of the number of barcodes per µm. The recommended range is 0.05- 2.25 /µm^2 for the MAX instruments. Values <0.05 indicate potential problems with the amount of sample, expression level of targets in the code set, or the size of the code set. A higher value indicates potential overlap between samples. Positive controls were used to check for hybridisation success as well as processing and binding efficiency. The overall assay efficiency was assessed by assessing the geometric mean of positive controls, if >3 fold different from the means of all samples, a flag was raised. Positive controls were also used to assess linearity; six known concentrations (ranging from 0.125-128 fM) of positive controls were used to determine a titration curve, where an R^2 value >0.95 was considered adequate. The limit of detection was also assessed by expecting the counts of positive E control to be higher than the mean of negative controls plus two standard deviations. Gene expression normalization was carried out using the geNorm algorithm, based on the best housekeeping genes. This removes sources of technical and input variability.

Samples that passed QC checks were then normalised and used for individual gene counts. The summary was presented on unsupervised clustered heatmaps. Data variability was screened using principal component analyses. Differential expression was graphed as a volcano plot with individual genes −log10 (p-value) and log2 fold change compared to the control group. ROSALIND® software (ROSALIND, Inc. San Diego, CA) was subsequently used to confirm findings in nSolver and for enrichment analysis compared to a given control using WikiPathways, Bioplanet, PANTHER and REACTOME. This process was repeated for both codeset panels.

Table 2.4. Summary of samples used in this study, the concentration of total RNA and the RNA integrity per sample.

Sample	**Quantity of total RNA (ng)**	**Concentration of total RNA (ng/ul)**	**RNA Integrity**
n=1 US	400ng	20ng/ul	RINe 9
n=1 LPS	400ng	20ng/ul	RINe 2.8
n=1 Cipro/LPS	400ng	20ng/ul	RINe 9.1
n=1 Prop/LPS	400ng	20ng/ul	RINe 9.1
n=1 NA/LPS	400ng	20ng/ul	RINe 9
n=1 Mix/LPS	400ng	20ng/ul	RINe 9.2
n=2 US	400ng	20ng/ul	RINe 9
n=2 LPS	400ng	20ng/ul	RINe 9.4
n=2 Cipro/LPS	400ng	20ng/ul	RINe 9.1
n=2 Prop/LPS	400ng	20ng/ul	RINe 8.9
n=2 NA/LPS	400ng	20ng/ul	RINe 8.3
n=2 Mix/LPS	400ng	20ng/ul	RINe 9.2
n=3 US	400ng	20ng/ul	RINe 8.8
n=3 LPS	400ng	20ng/ul	RINe 8.9
n=3 Cipro/LPS	400ng	20ng/ul	RINe 9.2
n=3 Prop/LPS	400ng	20ng/ul	RINe 8.9
n=3 NA/LPS	400ng	20ng/ul	RINe 8.6
n=3 Mix/LPS	400ng	20ng/ul	RINe 8.9
n=4 US	400ng	20ng/ul	RINe 9.1
n=4 LPS	400ng	20ng/ul	RINe 9
n=4 Cipro/LPS	400ng	20ng/ul	RINe 8.9
n=4 Prop/LPS	400ng	20ng/ul	RINe 8.9
n=4 NA/LPS	400ng	20ng/ul	RINe 9
n=4 Mix/LPS	400ng	20ng/ul	RINe 9.2

Chapter 3 Examining the impact of antibiotics, catecholamines and sedatives on immune cell function

3.1 Introduction

Sepsis leads to exaggerated pro- and anti-inflammatory responses that ultimately cause immune cells to reprogram and undergo anergy, exhaustion and apoptosis. This renders the immune system less effective at eradicating pathogens, increasing susceptibility to secondary infection and mortality risk. Underlying mechanisms and the potential contribution of iatrogenic factors remain poorly characterised.

Medications commonly used to support septic patients (e.g., antibiotics, catecholamines and sedatives) may further compromise the immune dysfunction already occurring in sepsis and, as a consequence, may worsen disease severity and prognosis. Studies to date are mostly *in vitro* using different immune cell types, with differing timing and infectious or inflammatory stimuli. Medications have only been studied in isolation and there are likely to be synergistic or competing effects from polypharmacy combinations frequently used in critically ill patients. The literature unfortunately shows a wide heterogeneity of results for each drug class. This likely represents methodological issues as most are performed in *in-vitro* models and often using supra-pharmacological drug concentrations. As a result, a clear understanding of medication-induced immune modulation is lacking, and further investigation is required to elucidate these differences.

The aim of this chapter is to determine if ciprofloxacin, propofol, norepinephrine (NE) or the combination of these drugs alters the immune cell function of PBMCs. This was studied in cell culture in the presence and absence of endotoxin (lipopolysaccharide, LPS) after 6- and 24-hours'

incubation. This chapter also aims to explore how these effects may vary under a number of experimental conditions:

- PBMCs from healthy volunteers,
- PBMCs from healthy volunteers incubated with pooled septic serum,
- PBMCs from ED patients suspected of sepsis without prior exposure to these medications,
- PBMCs from ICU patients with confirmed sepsis.

This was tested by comparing the following:

1) Production of pro-inflammatory cytokines IL-6 and TNF-α, and the anti-inflammatory cytokine IL-10.
2) Ability of cells to phagocytose *E. coli.*
3) Expression of HLA-DR.

3.2 Healthy volunteers and patient characteristics

Immune studies were carried out on whole blood samples donated by seven healthy volunteers. The healthy volunteers consisted of five male and two female participants, aged between 25-64 years, with no significant medical or drug history and no allergies. All participants avoided excessive alcohol consumption and strenuous exercise in the preceding 48 hours.

In addition, five patients with suspected sepsis were recruited from the Emergency Department (ED) and seven septic patients were recruited from the ICU. Patient characteristics are summarised in Tables 3.1 and 3.2. Additional samples from other healthy volunteers were taken for initial optimisation studies; these have not been included in the results.

Table 3.1. Characteristics of patients.

Characteristics	ED patients	ICU patients
Age (median, range) years	62.5 (24-90)	52 (42-80)
Sex	1 male, 4 female	6 male, 1 female
SOFA Score (median, range)	5.5 (4-6)	9 (5-12)
Elective surgery	0 (0%)	4 (57%)
Medical	4 (80%)	2 (29%)
Emergency Surgery	0 (0%)	1 (14%)
Trauma	1 (20%)	0 (0%)
Reason for admission		
Respiratory	0 (0%)	6 (86%)
Cardiovascular	0 (0%)	3 (43%)
Neurological	0 (0%)	3 (43%)
Digestive/Liver	0 (0%)	3 (43%)
Monitoring	2 (40%)	1 (14%)
Other	3 (60%)	0 (0%)
Source of admission		
Theatre/Recovery	0 (0%)	5 (71%)
ED/ambulance	5 (100%)	2 (28%)
Comorbid conditions		
COPD/Asthma	4 (80%)	0 (0%)
IHD, Heart failure	2 (40%)	4 (57%)
Diabetes	2 (40%)	3 (43%)
Chronic renal failure	2 (40%)	0 (0%)
Chronic liver disease	0 (0%)	0 (0%)
Immunosuppression	1 (20%)	2 (29%)
Other	2 (40%)	4 (57%)
No. of comorbidities		
0	1 (20%)	1 (14%)
1	1 (20%)	1 (14%)
2	0 (0%)	1 (14%)
≥3	3 (60%)	4 (57%)
Acute organ dysfunction (systems)		
0	5 (100%)	2 (28%)
1	0 (0%)	2 (28%)
2	0 (0%)	3 (43%)
≥3	0 (0%)	0 (0%)

Table 3.2. Type of infection, treatment characteristics and outcome of ED and ICU patients.

Treatment characteristics	ED patients	ICU patients
Site of infection		
Respiratory	2 (40%)	5 (71%)
Abdominal	1 (20%)	4 (57%)
Bloodstream	0 (0%)	3 (43%)
Renal/urinary tract	0 (0%)	0 (0%)
Skin	1 (20%	2 (28%)
CNS	0 (0%)	0 (0%)
Other/none	1 (20%)	0 (0%)
Micro-organism found during admission		
Gram positive bacteria	1 (20%)	3 (43%)
Gram negative bacteria	1 (20%)	4 (57%)
Anaerobes	1 (20%)	2 (28%)
Fungal	0 (0%)	2 (28%)
Viral	0 (0%)	1 (14%)
No growth	3 (60%)	0 (0%)
Drug classes exposed to at time of sampling		
Antibiotics	0 (0%)	7 (100%)
Inotropes (Noradrenaline)	0 (0%)	4 (57%)
Sedative (Propofol)	0 (0%)	4 (57%)
Length of stay in ICU (days)	0 (0%)	37 (5-87)
Outcome		
Alive	5 (100%)	6 (76%)
Dead	0 (0%)	1 (14%)

3.3 Cytokine Production

An important effector function of immune cells is the production of cytokines to direct the immune response. In initial optimisation experiments, I found no detectable cytokines were produced by PBMCs in the absence of LPS. This is consistent with previous literature which demonstrated undetectable cytokines in healthy volunteers in the absence of a septic stimulus (e.g. TNF-α ≤13 pg/ml, IL-6 ≤2.6 pg/ml and IL-10 ≤4 pg/ml)(476). Addition of ciprofloxacin, propofol and NE in the absence of LPS also did not produce any detectable cytokines. The implications of these results are discussed in the discussion chapter.

3.3.1 IL-6 production

IL-6 production increased in PBMCs in all participant groups following LPS stimulation after 24 hours (Figure 3.1). This was most marked in septic ICU patients followed by healthy volunteers with septic serum, healthy volunteers and ED patients.

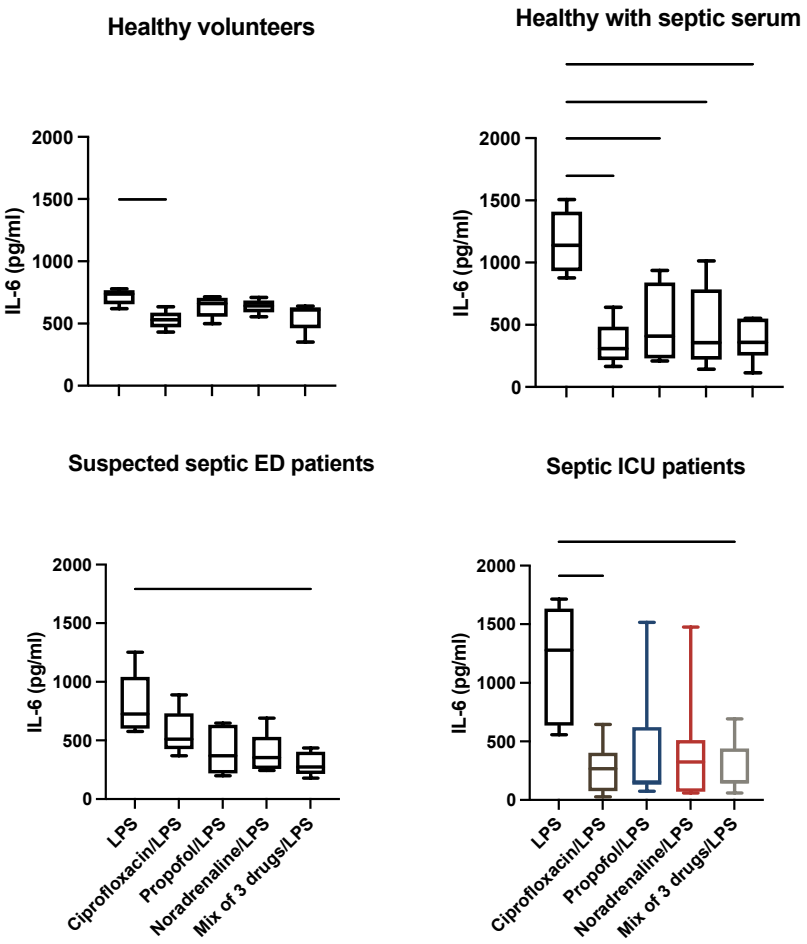


Figure 3.1. IL-6 release by PBMCs at 24 hours.

PBMCs were treated with LPS and ciprofloxacin, propofol, NE and a mix of the three drugs. After a 24-hour incubation period, IL-6 release was measured and compared to that seen with LPS alone. Data are shown as median ± interquartile ranges. Results were analysed using one-way ANOVA with Kruskal-Wallis multiple comparisons test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. n=5 in healthy volunteer and ED PBMC groups, n=7 in healthy volunteers PBMC co-incubated with septic serum group and septic ICU PBMCs.

Compared to LPS alone, IL-6 production showed a reducing trend in all drug groups co-incubated with LPS. This difference was statistically significant across all drug groups in PBMCs from healthy volunteers co-incubated with septic serum.

Of the drug/LPS combinations, ciprofloxacin/LPS reduced IL-6 in all PBMC groups; this achieved statistical significance in PBMCs from healthy volunteers ± co-incubated with septic serum and in PBMCs from septic ICU patients. The mix of three drugs and LPS combined reduced IL-6 significantly in PBMCs from healthy volunteers incubated with septic serum, and from ED and ICU patients. The mix of three drugs did not exert a cumulative effect on IL-6 production compared to the individual drug groups. Propofol and NE only produced a significant reduction in IL-6 in PBMCs in healthy volunteers co-incubated with septic serum.

3.3.2 TNF-α production

After 24 hours, TNF-α production increased in PBMCs stimulated by LPS (Figure 3.2). As with IL-6, this was most marked in the PBMCs from septic ICU patient group followed by healthy volunteers co-incubated with septic serum, healthy volunteers and then ED patients.

Of the experimental conditions, the mix of three drugs combined with LPS significantly reduced TNF-α in all participant groups while the ciprofloxacin/LPS combination significantly reduced TNF-α in PBMCs from healthy volunteers and co-incubated with septic serum. In the PBMCs from healthy volunteers co-incubated with septic serum, there was a reduction in TNF-α across all drug/LPS combinations compared to LPS alone. There was no cumulative effect in the mix of three drugs/LPS combination compared to individual drugs/LPS.

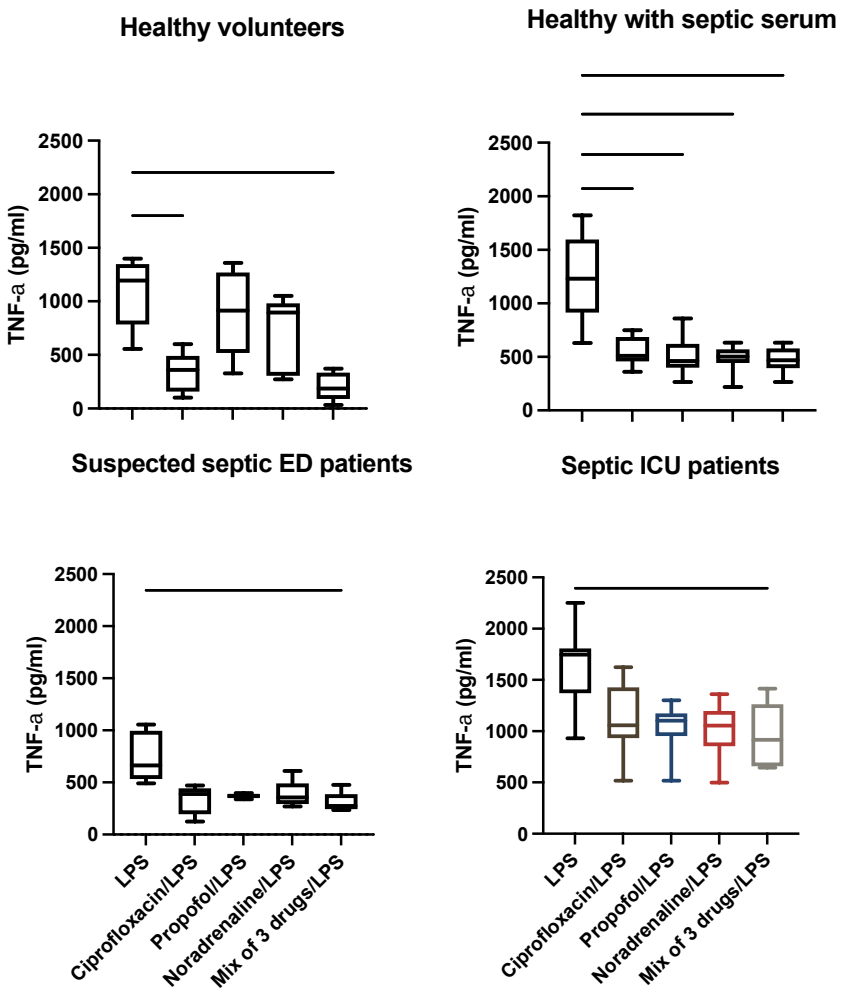


Figure 3.2. TNF-α release by PBMCs at 24 hours

PBMCs were treated with ciprofloxacin, propofol and NE and LPS for 24 hours. TNF-α release was measured and compared to that seen with LPS alone. Data are shown as median ± interquartile ranges. Results were analysed using one-way ANOVA with post-hoc Kruskal-Wallis multiple comparisons test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. n=5 in healthy volunteer and ED PBMC groups, n=7 in healthy volunteers PBMC co-incubated with septic serum group and septic ICU PBMCs.

3.3.3 IL-10 production

IL-10 production also increased in PBMCs following LPS stimulation after 24 hours incubation (Figure 3.3). This was most marked in healthy volunteer PBMCs co-incubated with septic serum, followed by PBMCs from septic ICU patients, healthy volunteers and then ED patients. Though values generally trended lower across all conditions for each drug, significant falls were only

seen in samples taken from septic ICU patients. Again, no synergistic effect was seen with the combination of drugs.

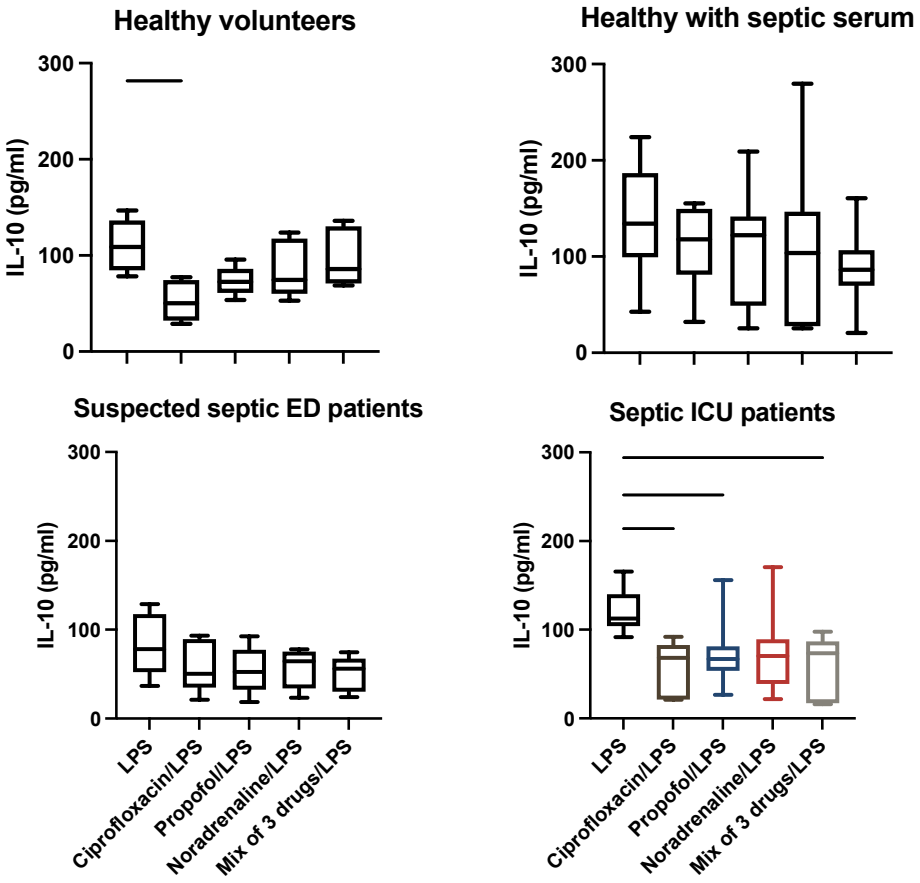


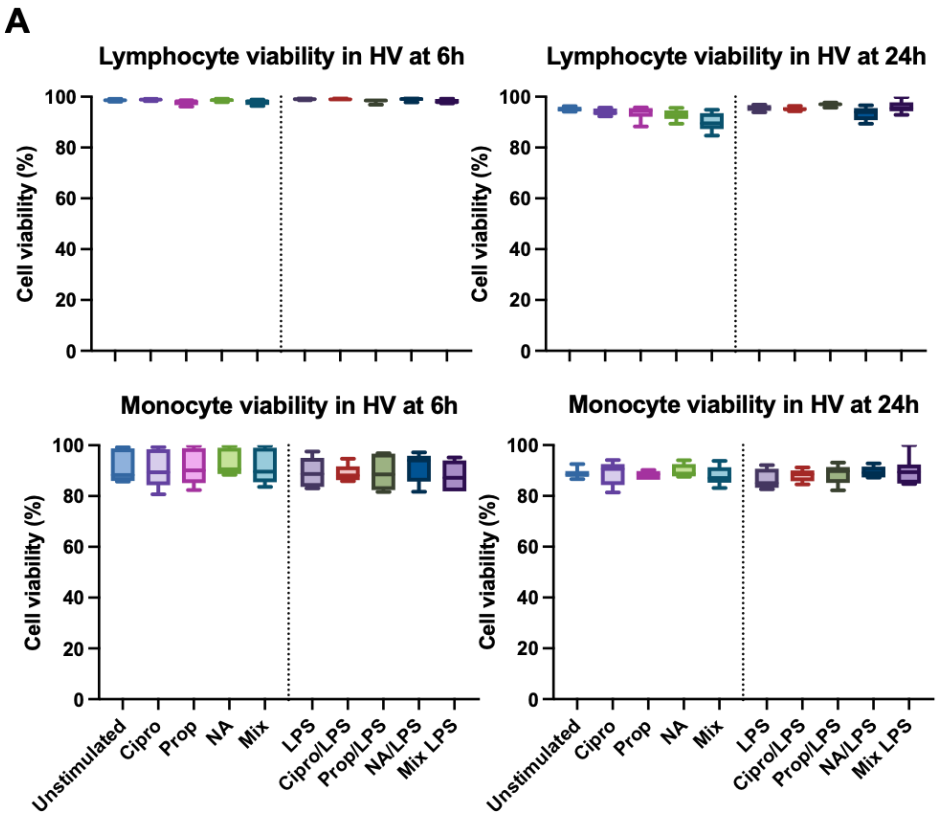
Figure 3.3. IL-10 release by PBMCs after 24 hours incubation.

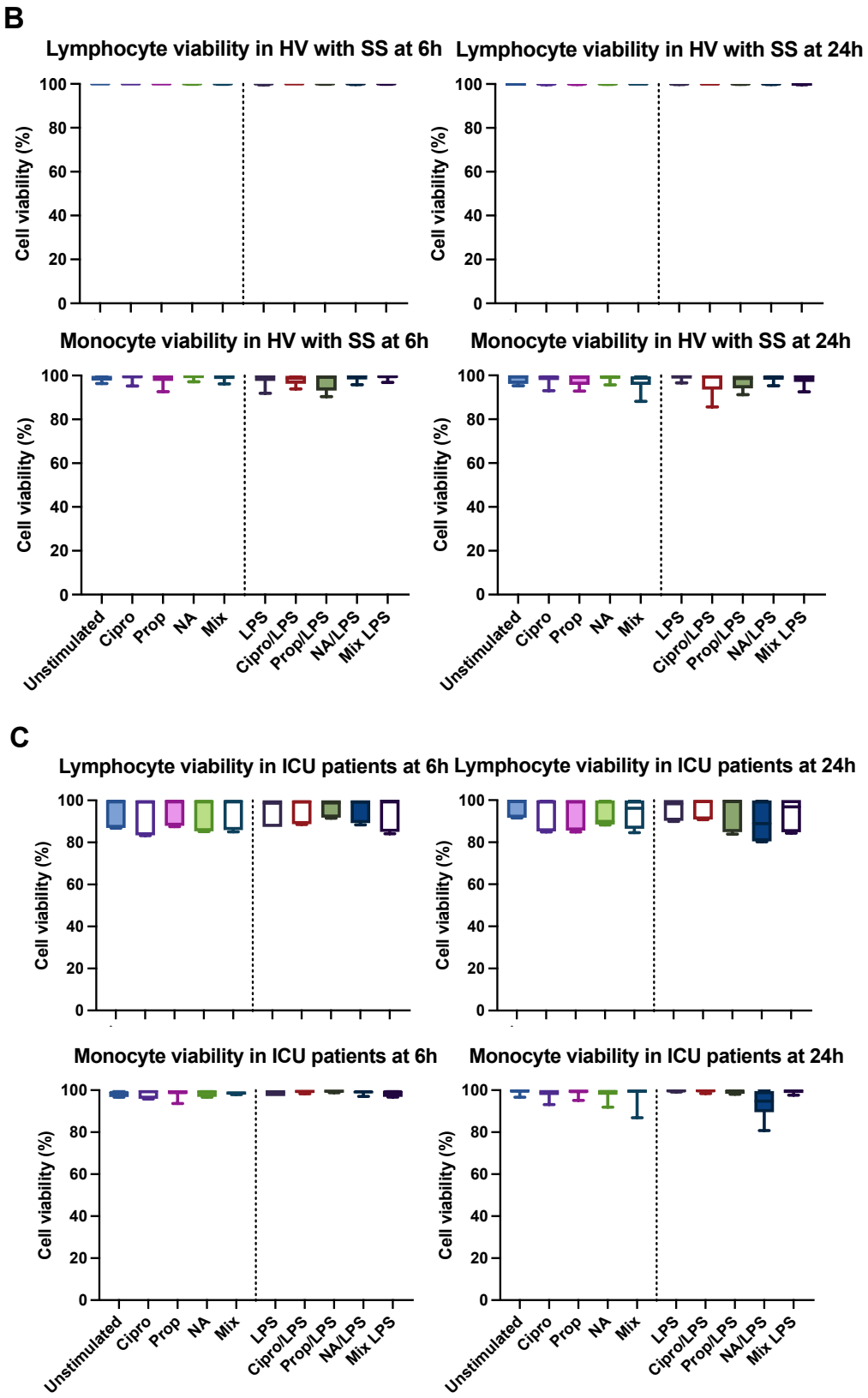
PBMCs were treated with ciprofloxacin, propofol and NE and LPS for 24 hours. IL-10 release was measured and compared to that seen with LPS alone. Data are shown as median ± interquartile range. Results were analysed using one-way ANOVA with post-hoc Kruskal-Wallis multiple comparisons test. *p< 0.05, **p<0.01, ***p<0.001, ****p<0.0001. n=5 in healthy volunteer and ED PBMC groups, n=7 in healthy volunteers PBMC co-incubated with septic serum group and septic ICU PBMCs.

3.4 Cell Viability

Viable cells were selected using a combination of Live/Dead NIR fluorophore which excludes dead cells, and annexin V which excludes necrotic and apoptotic cells. The proportion of viable cells remaining were then compared to the parent cell populations and given as a percentage for each participant

group. There were no significant differences in percentage cell viability between participant groups, incubation with drugs of interest, nor septic stimuli at 6 or 24 hours. Cell viability for all groups ranged from approximately 80-100%, these differences are likely due to differences in cell handling e.g. cell isolation, culture and pipetting (Figure 3.4). Monocytes appear more sensitive to cell handling compared to lymphocytes.




B
Lymphocyte viability in HV with SS at 6h
Lymphocyte viability in HV with SS at 24h
Monocyte viability in HV with SS at 6h
Monocyte viability in HV with SS at 24h
Cell viability (%)
0
20
40
60
80
100
Unstimulated
Cipro
Prop
NA
Mix
LPS
Cipro/LPS
Prop/LPS
NA/LPS
Mix LPS
C
Lymphocyte viability in ICU patients at 6h
Lymphocyte viability in ICU patients at 24h
Monocyte viability in ICU patients at 6h
Monocyte viability in ICU patients at 24h

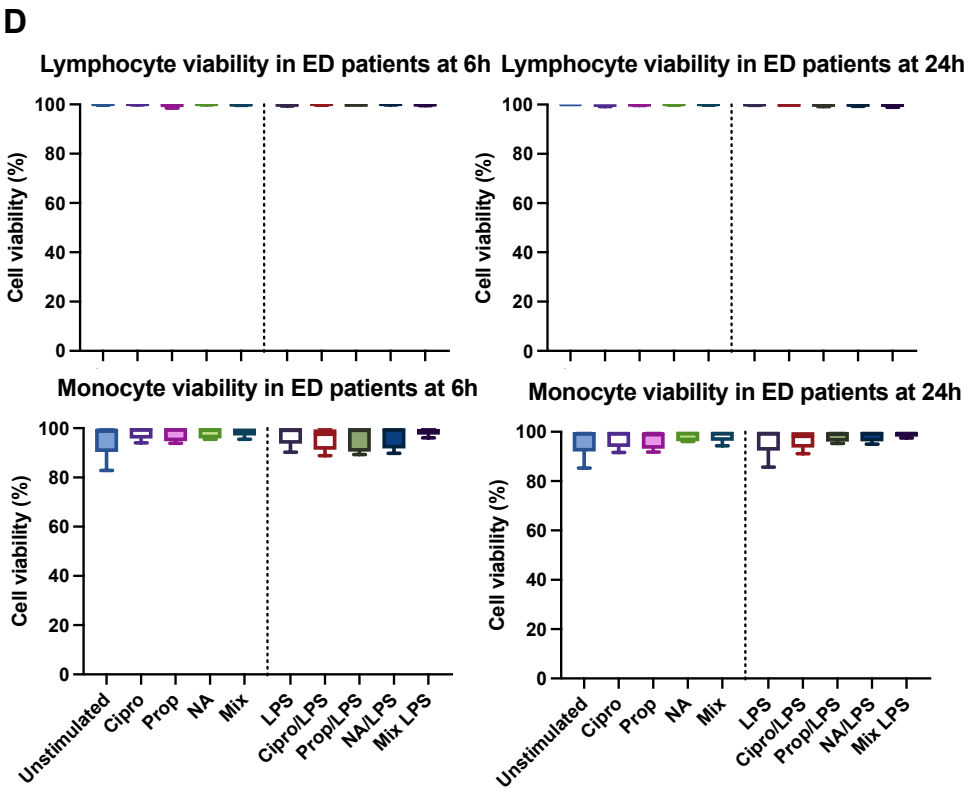


Figure 3.4 Cell viability

PBMCs were treated with ciprofloxacin, propofol, NE and a mix of three drugs in the presence and absence of LPS for 6 and 24h. Cell viability was determined using Live/Dead NIR and Annexin V fluorophores. Dead, necrotic and apoptotic cells were excluded by gating around the viable cells and comparing the percentage to the parent population of cells. Data are shown as median ± interquartile ranges of the viable cell percentages. Results were analysed using one-way ANOVA with Kruskal-Wallis multiple comparisons test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. A demonstrates these in healthy volunteers (n=5), B demonstrates in healthy volunteer co-incubated with septic serum (n=7), C demonstrates cell viability in ICU patients (n=7) and D in ED patients (n=5).

3.5 Phagocytosis

Phagocytosis was measured using the fluorophore pHrodo Red which is conjugated with *E. coli*. When this bacterium is phagocytosed, there is a reduction in the pH and a subsequent increase in the fluorescence. Cytochalasin D was used as negative control as this inhibits actin polymerisation and thus phagocytosis. Firstly, concentration of pHrodo was optimised using concentration curve with the following concentrations: 50, 100

and 200 µg/ml. The concentration of cytochalasin D remained at 10 µM for each experiment (Figure 3.5). PBMCs were initially incubated with cytochalasin D for 60 minutes at RT, then all samples had the relevant concentrations of pHrodo added for another 60-90 minutes.

All three concentrations of pHrodo could clearly differentiate between pHrodo and its negative control with cytochalasin D. The biggest difference was demonstrated at a concentration of 100 µg/ml. This concentration was therefore used was for all subsequent experiments (Figure 3.5).

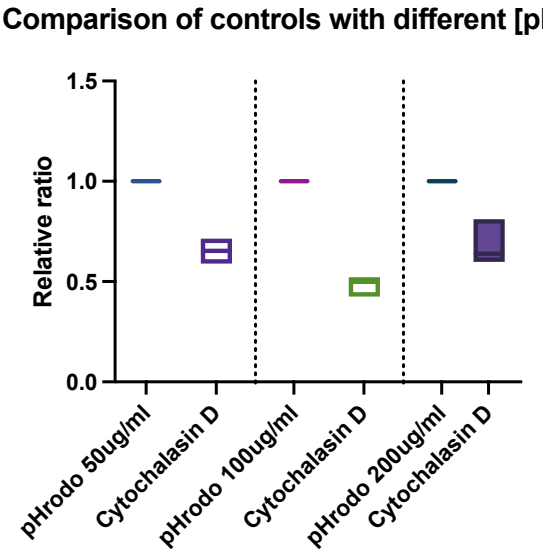


Figure 3.5. Comparison of phagocytosis controls using different concentrations of pHrodo.

PBMCs were incubated with pHrodo of differing concentrations to enable the distinction between baseline phagocytosis and the negative control. PBMCs for the negative controls were incubated with cytochalasin D for 60 minutes (10 µM) to inhibit phagocytosis; then all samples had pHrodo added to enable phagocytosis to occur for 60-90 minutes (50, 100, 200 µg/ml). Data shown as ratios relative to phagocytosis (pHrodo). The values are shown as box and whisker plot and datapoints are median and IQR of the relative ratios (n=3).

PBMCs were then incubated with ciprofloxacin, propofol, NE ± LPS for either 6 or 24 hours. At 90 minutes before the end of the incubation period, pHrodo was added to each sample and incubated for up to 90 minutes. Samples were analysed by flow cytometry to obtain Mean Fluorescent Intensities (MFIs).

These were log_{10} transformed and analysed using two-way ANOVA and *post-hoc* Bonferroni's multiple comparisons test. This test was chosen as it provides insight into the contribution of variables measured such as the effect of incubation time, presence and absence of LPS, differences in participant groups (e.g. PBMCs from healthy volunteers versus septic ICU patients or those from suspected sepsis ED patients)

For each drug, the incubation time of 6 versus 24 hours had no significant effect on phagocytosis (Figure 3.6). There was also no statistically significant difference in phagocytosis when drug groups were combined with LPS.

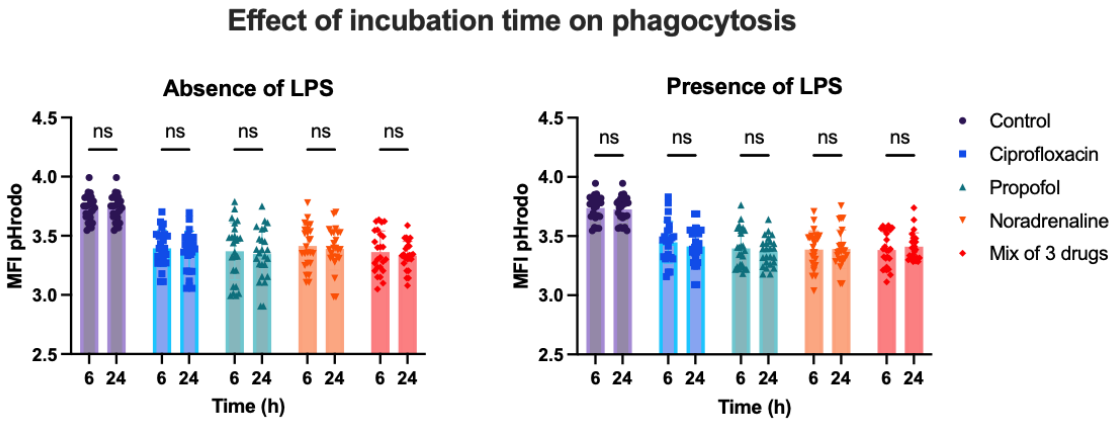


Figure 3.6. Effect of incubation time on phagocytosis.

PBMCs were treated with ciprofloxacin, propofol and NE in the presence or absence of LPS for 6 and 24 hours. Phagocytosis of E. coli was measured as MFI using flow cytometry. These were log_{10} transformed and data for each drug group were combined across the patient participant groups (totalling 5 from ED patients, 6 from healthy volunteers, 7 from healthy volunteers incubated with septic serum, and 7 from PBMCs taken from septic ICU patients). Results were then analysed using two-way ANOVA with Bonferroni's multiple comparisons test. *p< 0.05, **p<0.01, ***p<0.001, ****p<0.0001. Data are shown as mean and SD.

The impact of the different participant groups on phagocytosis was then investigated using two-way ANOVAs and Dunnett's multiple comparison tests. The participant groups had significantly different effects on phagocytosis, though some of this effect was also attributable to factors such as time of incubation and the presence/absence of LPS (Table 3.3). More specifically,

participant groups demonstrated a statistically significant effect on phagocytosis at 6 hours in the presence and absence of LPS but not at 24 hours.

Table 3.3. F-test value and corresponding p-values for the effect of participant groups on phagocytosis at 6 and 24 hours in the presence and absence of LPS.

Participant group	F-value	p-value
At 6 hours, no LPS	F (3, 21) = 21.16	P<0.0001
At 6 hours, LPS	F (3, 21) = 8.195	P=0.0008
At 24 hours, no LPS	F (3, 21) = 5.351	P=0.0068
At 24 hours, LPS	F (3, 21) = 2.361	P=0.1004

The effect of phagocytosis for a given drug was then compared between PBMCs from individual participant groups, such as those from healthy volunteers versus healthy volunteers co-incubated with septic serum, or versus PBMCs from ED patients, or versus PBMCs from septic ICU patients. Significant differences were identified between groups (Figure 3.7), most notably at 6 hours in the absence of LPS, with a significant increase in phagocytosis by PBMCs from both healthy volunteers incubated with septic serum and from ED patients compared to those from healthy volunteers. Of note, septic ICU patients did not show any significant difference compared to healthy volunteers.

Finally, two-way ANOVA and Dunnett's multiple comparison tests were used to investigate the effect of individual drug groups on phagocytosis within each participant group. The drug groups had significant effects on phagocytosis at all time points both in the presence and absence of LPS (Table 3.4). There was a significant reduction in phagocytosis following incubation with each drug group compared to control (Figure 3.8). Those conditions where statistical significance was not reached still showed a strong trend towards reduction of phagocytosis, suggesting a Type II error. There was, however, no additive effect following incubation with the combination of all three drugs compared to

individual drugs. Details of mean differences, 95% confidence intervals and p-values are given in Appendix A.

Table 3.4. F-test value and corresponding p-values for the effect of drug class on phagocytosis at 6 and 24 hours in the presence and absence of LPS.

Drug class	F-value	p-value
At 6 hours, no LPS	F (3.230, 67.82) = 42.13	P<0.0001
At 6 hours, LPS	F (3.550, 74.55) = 44.47	P<0.0001
At 24 hours, no LPS	F (2.899, 60.89) = 41.55	P<0.0001
At 24 hours, LPS	F (2.749, 57.73) = 57.88	P<0.0001

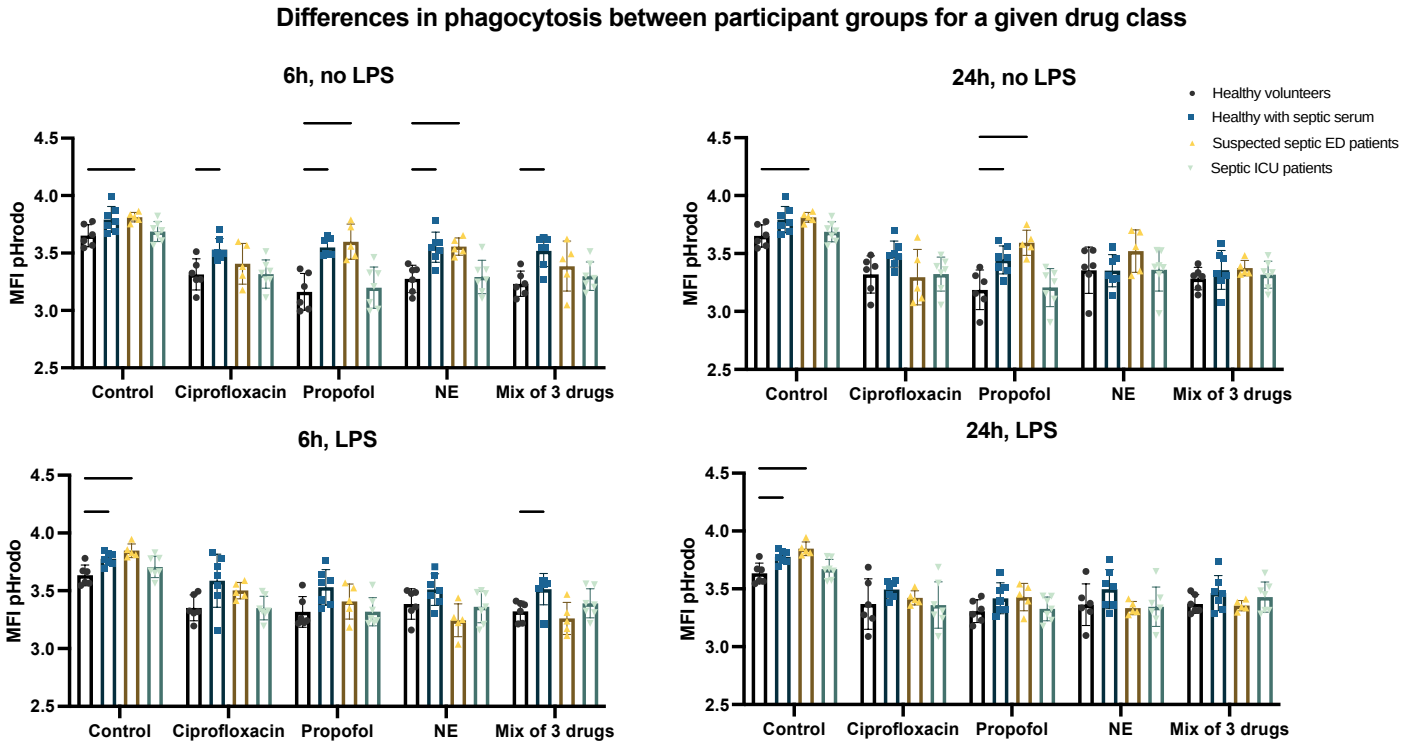


Figure 3.7. Differences in phagocytosis between different subject groups at 6 and 24 hours ± LPS.

PBMCs were treated with ciprofloxacin, propofol and/or NE for 6 and 24 hours in the presence and absence of LPS. Phagocytosis was measured and compared between each subject group at 6 and 24 hours. Results were analysed using two-way ANOVA with Dunnett's multiple comparisons test. *p< 0.05, **p<0.01, ***p<0.001, ****p<0.0001. Data are shown as mean ± SD combining PBMCs from 5 healthy volunteers, 5 ED patients, 7 from healthy volunteers co-incubated with septic serum and 7 from septic ICU patients.

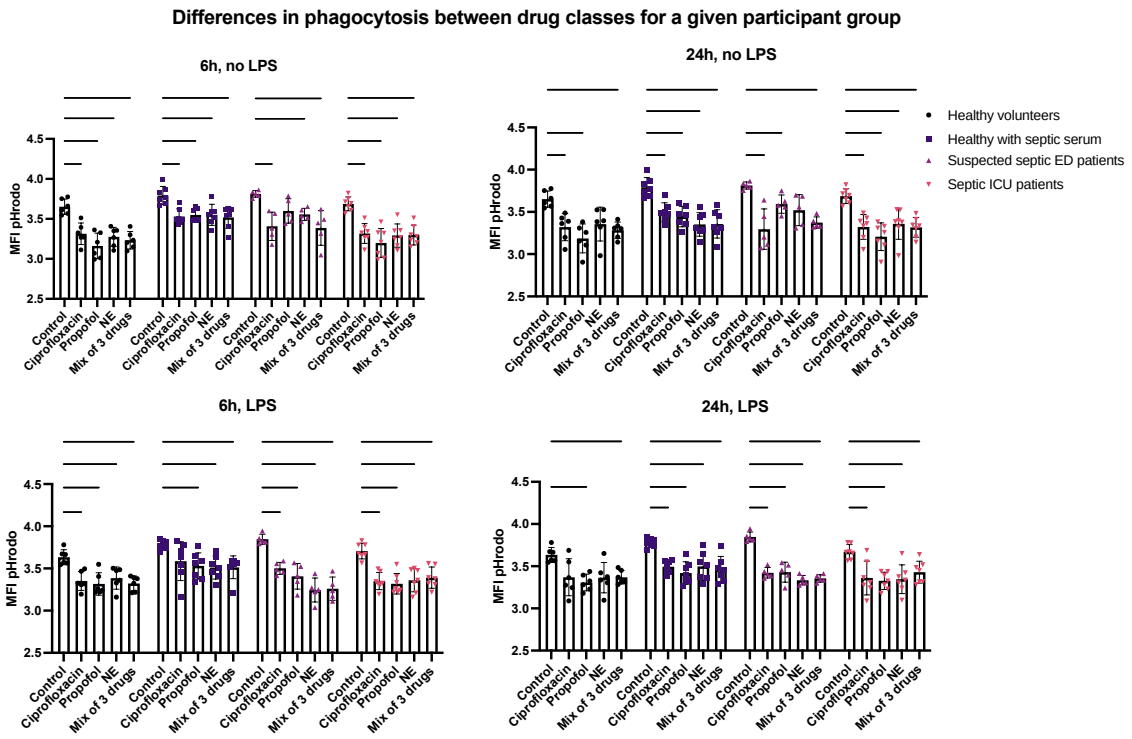


Figure 3.8. Differences in phagocytosis between different drug classes for a given participant group at 6 and 24 hours ± LPS.

PBMCs were treated with ciprofloxacin, propofol and/or NE in the presence and absence of LPS for 6 and 24 hours. Phagocytosis was measured and compared between each drug group. Results were analysed using two-way ANOVA with Dunnett's multiple comparisons test. *p< 0.05, **p<0.01, ***p<0.001, ****p<0.0001. Data are shown as mean ± SD combining PBMCs from 5 healthy volunteers, 5 ED patients, 7 from healthy volunteers co-incubated with septic serum and 7 from septic ICU patients.

3.6 HLA-DR Expression

HLA-DR, an MHC class II cell surface receptor on antigen presenting cells, is involved in peptide antigen presentation to T-cells. It activates T-cells to directly remove the source of the antigens, and B-cells to produce antibodies against the same antigen. HLA-DR is initially upregulated in response to stimulation. However, it is often reduced on monocytes from septic patients (79, 80). This is used to indicate immunosuppression and correlates with mortality (81, 82). The phenotype changes with prolonged stimulation from pro-inflammatory to immune suppressive.

HLA-DR expression was measured on monocytes after incubation with ciprofloxacin, propofol and/or NE in the presence and absence of LPS for 6 or 24 hours. MFIs for HLA-DR were obtained following flow cytometry and log10 transformation. The impact of incubation for 6 and 24 hours on expression of HLA-DR was assessed using two-way ANOVA and post-hoc Bonferroni tests. For each drug group, the incubation time of 6 versus 24 hours had no significant effect on HLA-DR (Figure 3.9). Of note, there was no statistically significant difference in HLA-DR expression when LPS was added to cell culture in addition to drug groups.

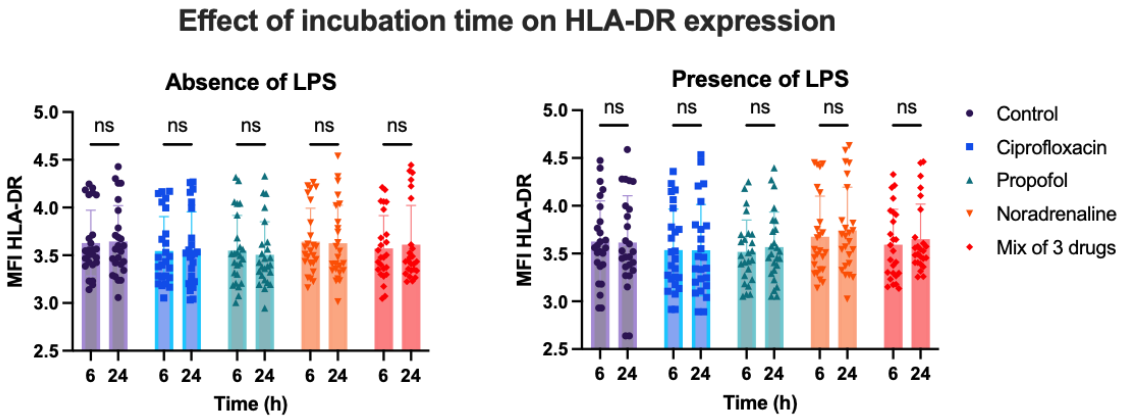


Figure 3.9. Effect of time on HLA-DR expression

PBMCs were treated with ciprofloxacin, propofol and NE in the presence and absence of LPS for 6 and 24 hours. HLA-DR MFIs were obtained using flow cytometry and log_{10} transformed. The effect of incubation time was then analysed using two-way ANOVA with Bonferroni's multiple comparisons test. *p< 0.05, **p<0.01, ***p<0.001, ****p<0.0001. Data are

shown as mean ± SD combining PBMCs from 5 healthy volunteers, 5 ED patients, 7 healthy volunteers co-incubated with septic serum and 7 septic ICU patients.

The effect of participant groups on HLA-DR expression was examined using two-way ANOVAs and Dunnett's multiple comparisons. The participant groups significantly affected HLA-DR expression with some of the effect attributable to the drug group (Table 3.5).

Table 3.5. F-test value and corresponding p-values for the effect of participant groups on HLA-DR expression at 6 and 24 hours in the presence and absence of LPS.

Participant group	F-value	p-value
At 6 hours, no LPS	F (3, 21) = 40.37	P<0.0001
At 6 hours, LPS	F (3, 21) = 24.11	P<0.0001
At 24 hours, no LPS	F (3, 21) = 34.03	P<0.0001
At 24 hours, LPS	F (3, 21) = 24.63	P<0.0001

The effect of HLA-DR expression for a given drug was then compared between PBMCs from individual participant groups, e.g. healthy volunteers versus healthy volunteers co-incubated with septic serum. HLA-DR was reduced in all participant groups when compared to healthy volunteers (Figure 3.10). This was the case at both 6 and 24 hours in the presence and absence of LPS.

Following this, the effect of individual drugs within each participant group was investigated using two-way ANOVA and Dunnett's tests. The drug groups had a significant effect on HLA-DR expression at all time points in the presence and absence of LPS. (Table 3.6). However, analysis of HLA-DR expression in individual drug groups compared to controls for each participant group showed less consistent statistically significant differences (Figure 3.11). There was also no cumulative effect from the mix of three drugs.

Table 3.6. F-test value and corresponding p-values for the effect of drug groups on HLA-DR expression at 6 and 24 hours in the presence and absence of LPS.

Drug class	F-value	p-value
At 6 hours, no LPS	F (3.022, 63.47) = 6.706	P=0.0005
At 6 hours, LPS	F (2.788, 58.55) = 5.958	P=0.0017
At 24 hours, no LPS	F (3.242, 76.99) = 5.357	P<0.0001
At 24 hours, LPS	F (2.724, 64.69) = 5.953	P<0.0001

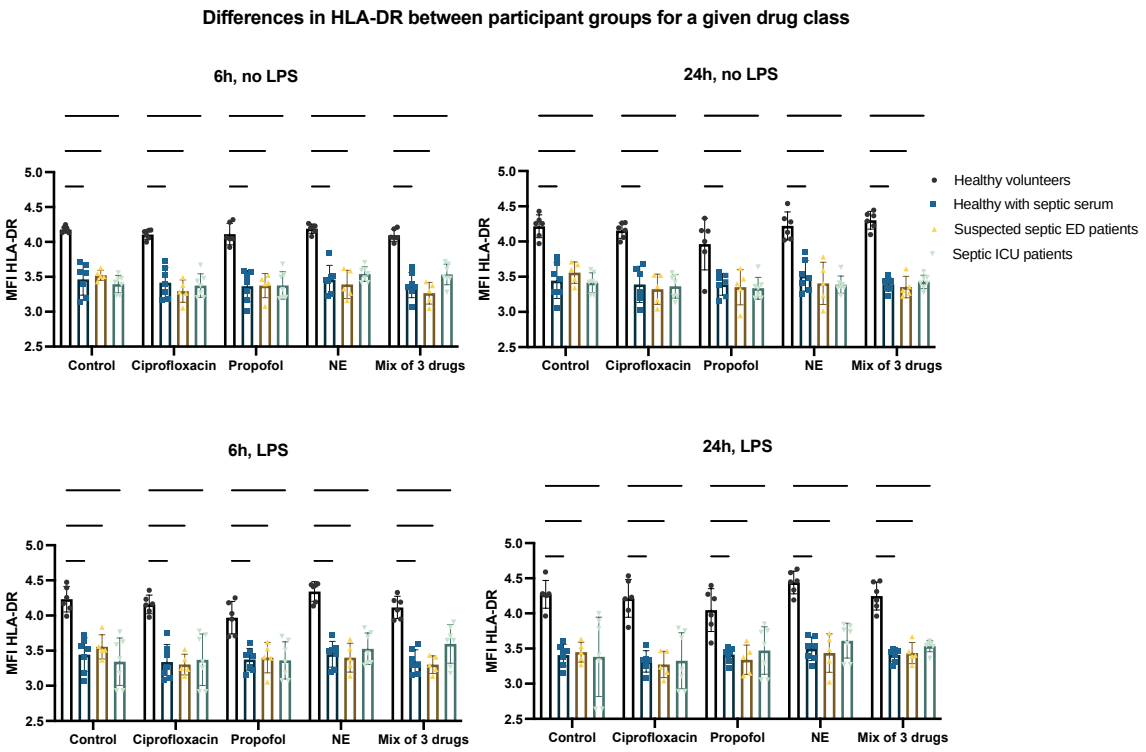


Figure 3.10. Differences in HLA-DR expression between different participant groups at 6 and 24 hours ± LPS.

PBMCs were treated with ciprofloxacin, propofol and NE for 6 and 24 hours in the presence and absence of LPS. HLA-DR was measured and compared between each participant group. Results were analysed using two-way ANOVA with Dunnett's multiple comparisons test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Data are shown as mean ± SD combining PBMCs from 5 healthy volunteers, 5 ED patients, 7 from healthy volunteers co-incubated with septic serum and 7 from septic ICU patients.

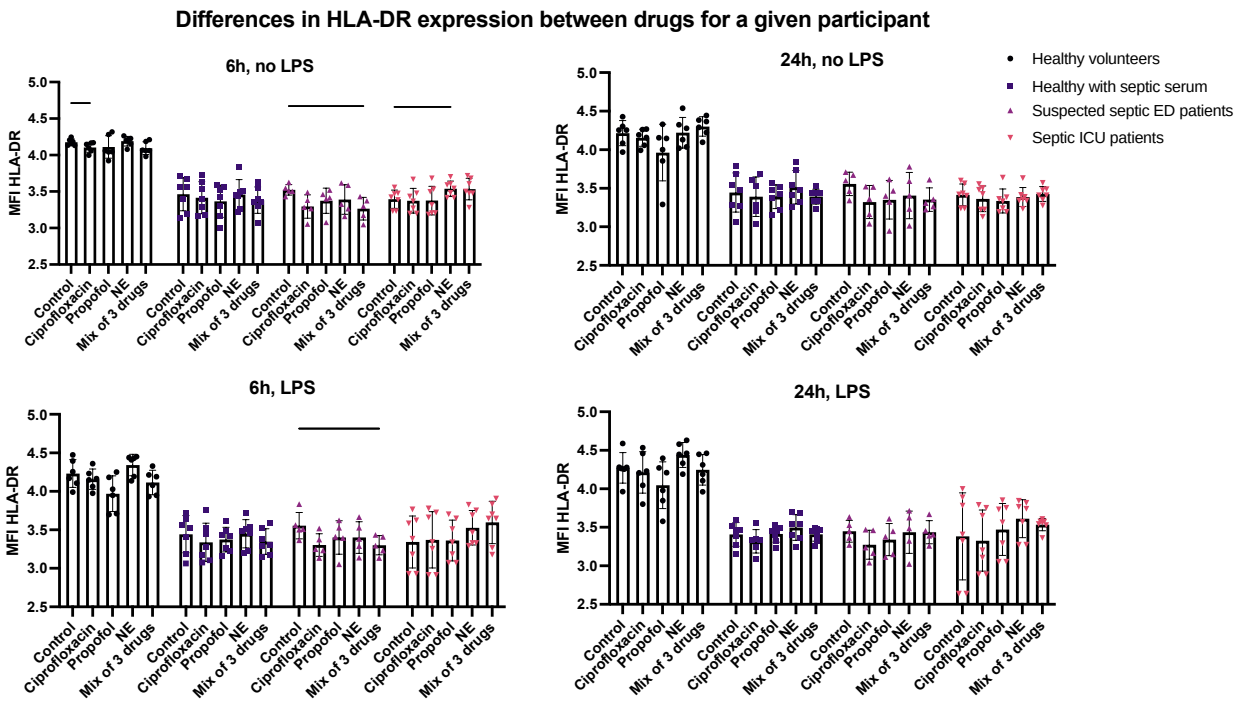


Figure 3.11. Differences in HLA-DR expression between different drugs for a given participant group at 6 and 24 hours ± LPS

PBMCs were treated with ciprofloxacin, propofol and NE for 6 and 24 hours in the presence and absence of LPS. HLA-DR expression was measured and compared between each drug class at 6 and 24 hours. Results were analysed using two-way ANOVA with Dunnett's multiple comparisons test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Data are shown as mean ± SD combining PBMCs from 5 healthy volunteers, 5 ED patients, 7 from healthy volunteers co-incubated with septic serum and 7 from septic ICU patients.

3.7 Summary of immune studies

This chapter presents the immune findings when PBMCs are incubated with various drugs in the presence and absence of LPS after 6 and 24h. The cytokine data shows a significant rise in the production of IL-6, TNF-α and IL-10 after 24 hours' incubation with LPS. This was most marked in PBMCs from healthy volunteers with septic serum and in septic ICU patient populations. Ciprofloxacin/LPS and the mix of three drugs reduced both pro (TNF-α, IL-6) and, to a lesser extent, anti-inflammatory (IL-10) cytokine production, particularly in PBMCs from healthy volunteers co-incubated with septic serum. No cumulative effect was seen with the mix of three drugs compared to their individual use.

The flow cytometry results demonstrated that both phagocytosis and HLA-DR expression were not affected by incubation time, however both were affected by the participant group and by exposure to the different drugs. This effect was more consistently seen with phagocytosis, where drug groups showed a significant reduction in phagocytosis compared to control within each subject population. This was not the case with HLA-DR, where the participant populations showed a consistent reduction in HLA-DR expression compared to healthy volunteers.

Chapter 4 Examining the impact of antibiotics, catecholamines and sedatives on mitochondrial function

4.1 Introduction

Mitochondria are heavily implicated in the development of multiorgan failure in sepsis (103). Failed organs have preserved oxygen delivery but decreased utilisation, preserved histology with limited cell death and recover fully in surviving patients (34-38). These findings suggest a metabolic shutdown rather than structural damage as a crucial underlying pathophysiological mechanism.

There is considerable evidence to suggest mitochondrial dysfunction arises in immune cells in sepsis. Mitochondria are involved in the regulation of immune cell functionality (e.g., activation, proliferation and differentiation) and survival (138). ATP provision by OxPHOS and glycolysis varies between immune cells and their activation status. For example, neutrophils predominantly rely on glycolysis, while PBMCs utilise oxidative ATP for housekeeping activities (139). At sites of inflammation with low glucose availability, monocytes upregulate fatty acid oxidation and thus OxPHOS (143). This is associated with increased oxidative stress and antioxidants and mitochondrial mass; the latter occur as physiological response to increased oxidative stress (144). These changes are frequently observed in immune paralysis during sepsis (144).

Mitochondrial dysfunction in sepsis may arise from multiple causes including tissue hypoxia, substrate non-availability, inflammation-induced gene downregulation, and oxidative damage (39-42). These may also result as an unintended consequence of therapies commonly used in ICU, particularly antibiotics, catecholamines and sedatives, thereby worsening disease severity and patient outcomes.

The aim of this chapter is to determine if mitochondrial function in PBMCs is altered by incubation with ciprofloxacin, propofol, norepinephrine (NE) and/or a mix of these three drugs in the presence and absence of LPS after 6 and 24 hours. This chapter also aims to explore how these effects vary under different experimental conditions:

- PBMCs from healthy volunteers,
- PBMCs from healthy volunteers co-incubated with pooled septic serum,
- PBMCs from ED patients with presumed infection but without prior exposure to these medications, and,
- PBMCs from ICU patients with sepsis.

These were investigated by:

1) Mitochondrial membrane potential (MMP), as measured by TMRM.
2) Mitochondrial reactive oxygen species (mtROS) production, as measured by Mitosox Red.
3) Mitochondrial oxygen consumption rate (OCR) and respiratory parameters, including basal respiration, proton leak, maximum respiration rate and non-mitochondrial respiration, measured using Seahorse extracellular flux assays.

4.2 Optimisation experiments

Prior to the above investigations, a series of experiments were carried out to optimise the septic stimuli required for the mitochondrial experiments. This was done by trialling LPS from three different sources (*Klebsiella pneumoniae*, *E. coli* and *Staphylococcus aureus*) and PMA. A concentration curve was carried out in PBMCs for each LPS type (50ng/ml, 100ng/ml and 1mg/ml). These were compared to unstimulated samples in terms of mtROS production and changes in MMP. I also tried PMA/ionomycin concentrations equivalent to 25ng/ml, 50ng/ml and 100ng/ml for PMA and 0.5, 1 and 2µg/ml for ionomycin as an alternative stimulus. All results were analysed in both lymphocyte and monocyte populations and positive controls with antimycin A were carried out.

These experiments were also repeated at various time points (30 minutes, 1, 3, 6, and 24 hours) to ensure that that the effect of incubation time was also investigated. All results are shown as ratios to make a comparison between the groups easier to carry out.

Cell viability across all LPS groups was similar with no significant decrease at the higher concentration nor at longer incubation times. Of the LPS types, *Klebsiella pneumoniae* and *Salmonella*-derived LPS demonstrated a consistent increase in mtROS in lymphocytes and monocytes at each time point compared to *E. coli*-derived LPS (Figures 4.1 and 4.2). However, mtROS production with all LPS subtypes did not increase proportionally with increasing concentrations or incubation time.

Klebsiella pneumoniae-derived LPS demonstrated the most consistent reduction in MMP across both lymphocytes and monocytes compared to *Salmonella* and *E. coli*-derived LPS (Figures 4.3 and 4.4). The latter two demonstrated both an increase and decrease in MMP but this did not correlate with concentration or incubation time.

PMA/Ionomycin led to a variable increase in mtROS production in lymphocytes and monocytes. There was an increased correlation with PMA/Ionomycin concentration at 3 hours in lymphocytes and 24 hours in both cell populations. PMA/Ionomycin led to a consistent reduction in MMP at 3, 6 and 24 hours for both cell populations but not at 30 minutes or 1 hour (Figure 4.5 and 4.6). At 3, 6 and 24 hours, the reduction in MMP correlated with increasing concentration of PMA/Ionomycin. The overall ratios of increase in mtROS or reduction in MMP were similar between the various LPS subtypes and PMA/Ionomycin.

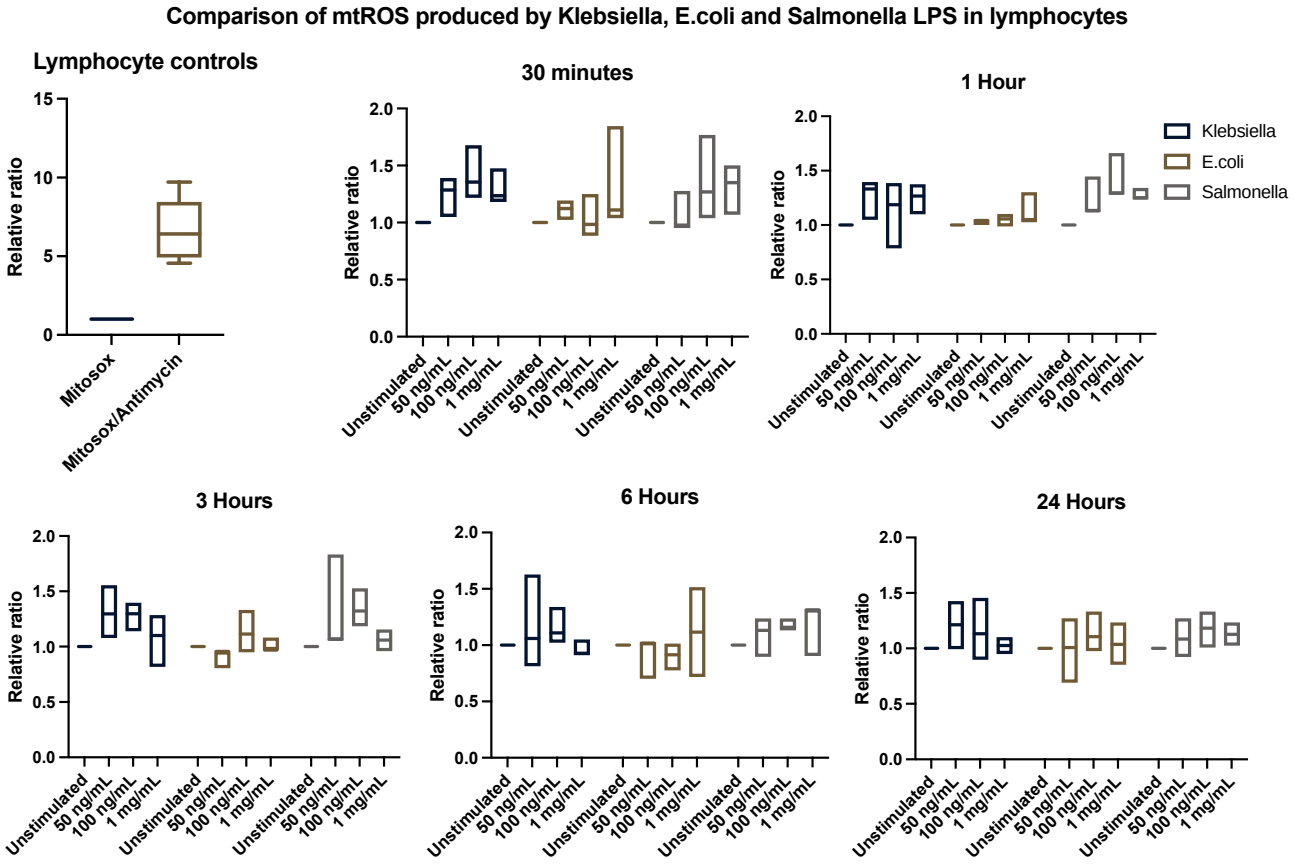


Figure 4.1. Comparison of mtROS production by Klebsiella, E.coli and Salmonella derived LPS in lymphocytes.

PBMCs were incubated with three concentrations of LPS (50ng/ml, 100ng/ml and 1mg/ml) for 30 minutes, 1, 3, 6 and 24 hours and the mtROS production was compared. This was then repeated for three different types of LPS (Klebsiella, E. coli and Salmonella). Data shown as a box and whisker plot with median and IQR values. n=3.

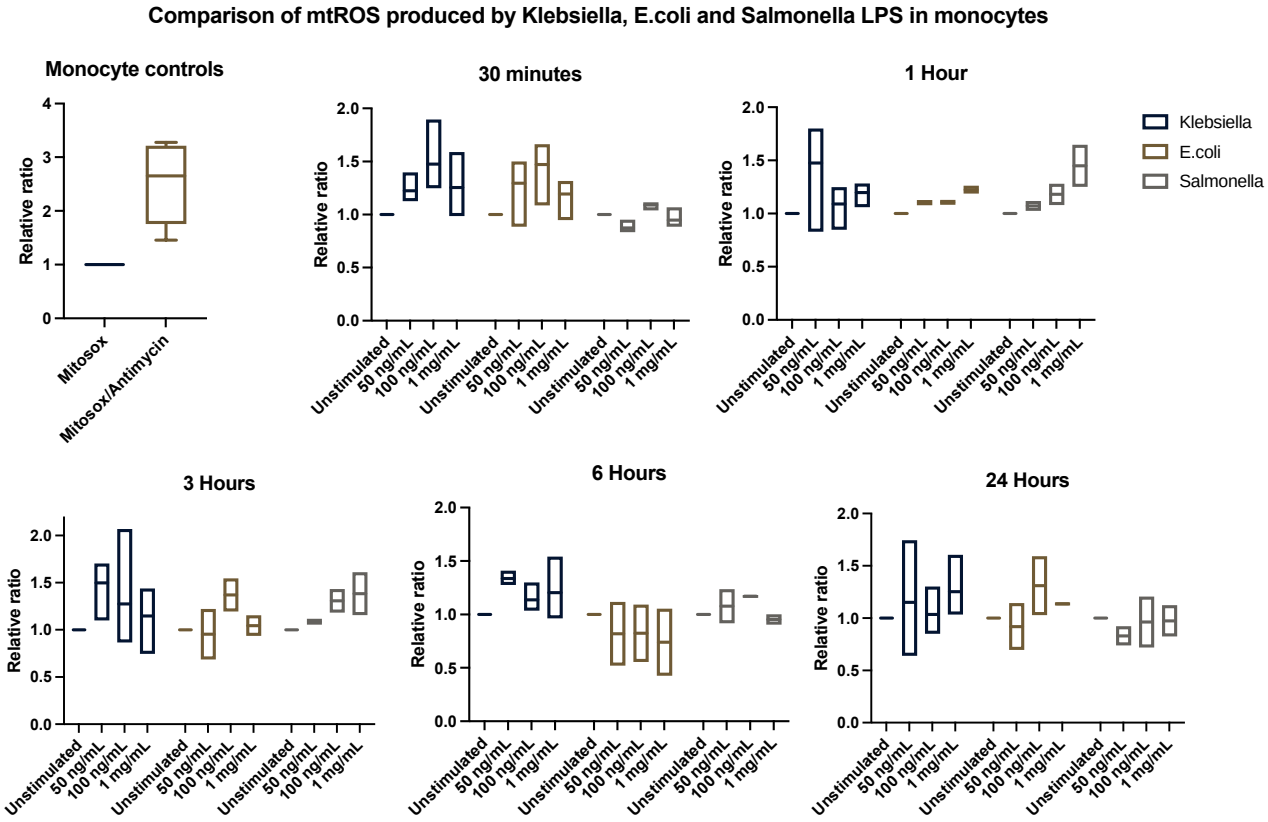


Figure 4.2. Comparison of mtROS production by Klebsiella, E. coli and Salmonella derived LPS in monocytes.

PBMCs were incubated with three concentrations of LPS (50ng/ml, 100ng/ml and 1mg/ml) for 30 minutes, 1, 3, 6 and 24 hours and the mtROS production was compared. This was then repeated for three different types of LPS (Klebsiella, E.coli and Salmonella). Data shown as a box and whisker plot with median and IQR values. n=3.

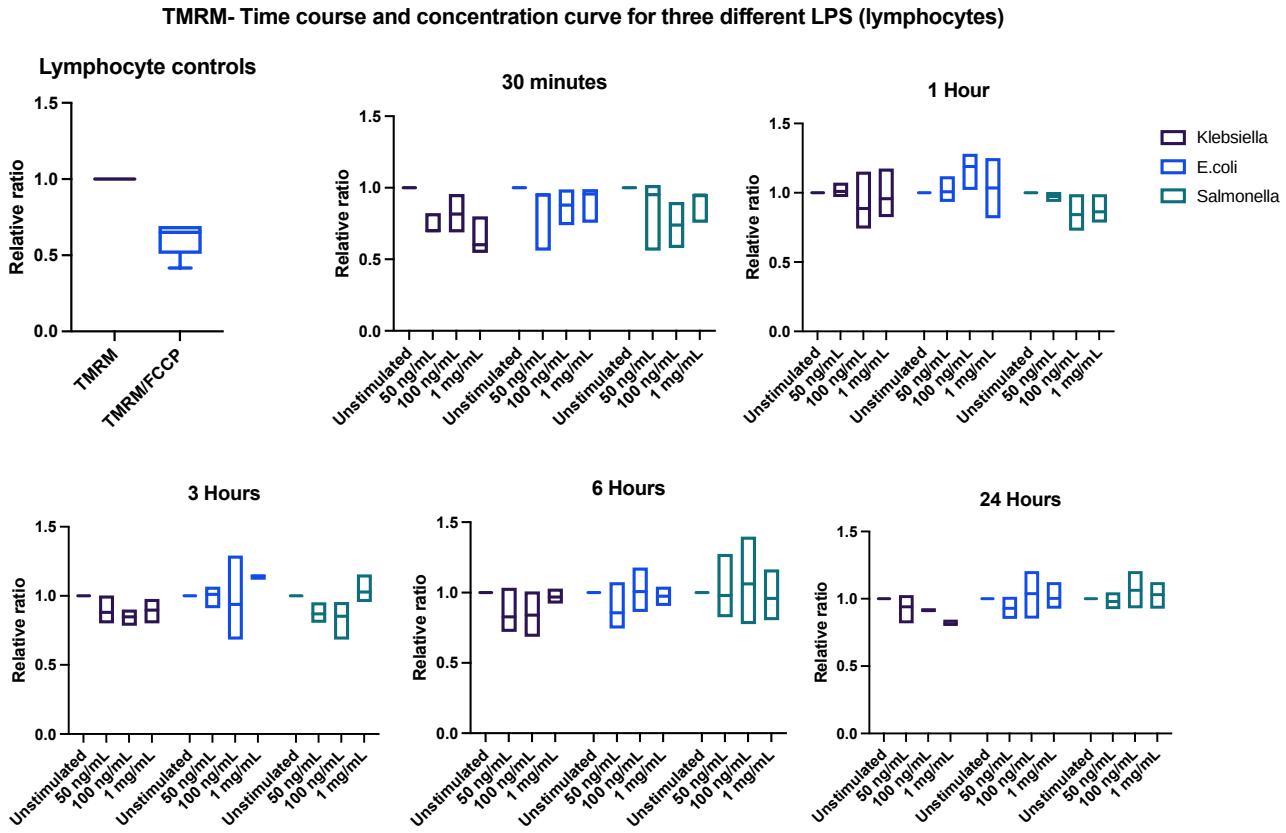


Figure 4.3. Comparison of changes in MMP by Klebsiella, E. coli and Salmonella derived LPS in lymphocytes.

PBMCs were incubated with three concentrations of LPS (50ng/ml, 100ng/ml and 1mg/ml) for 30 minutes, 1, 3, 6 and 24 hours and the changes in MMP were compared. This was then repeated for three different types of LPS (Klebsiella, E.coli and Salmonella). Data shown as a box and whisker plot with median and IQR values. n=3.

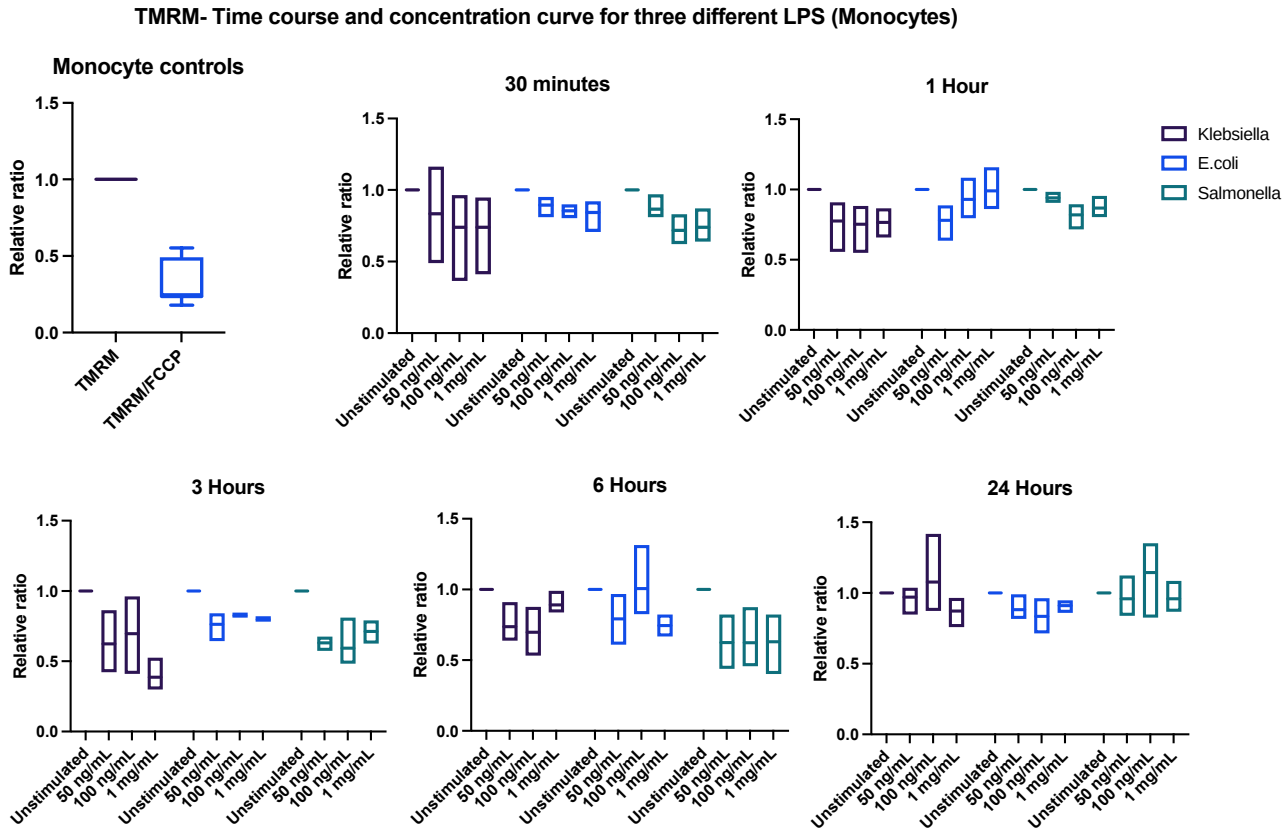


Figure 4.4. Comparison of changes in MMP by Klebsiella, E. coli and Salmonella derived LPS in monocytes.

PBMCs were incubated with three concentrations of LPS (50ng/ml, 100ng/ml and 1mg/ml) for 30 minutes, 1, 3, 6 and 24 hours and the changes in MMP were compared. This was then repeated for three different types of LPS (Klebsiella, E. coli and Salmonella). Data shown as a box and whisker plot with median and IQR values. n=3.

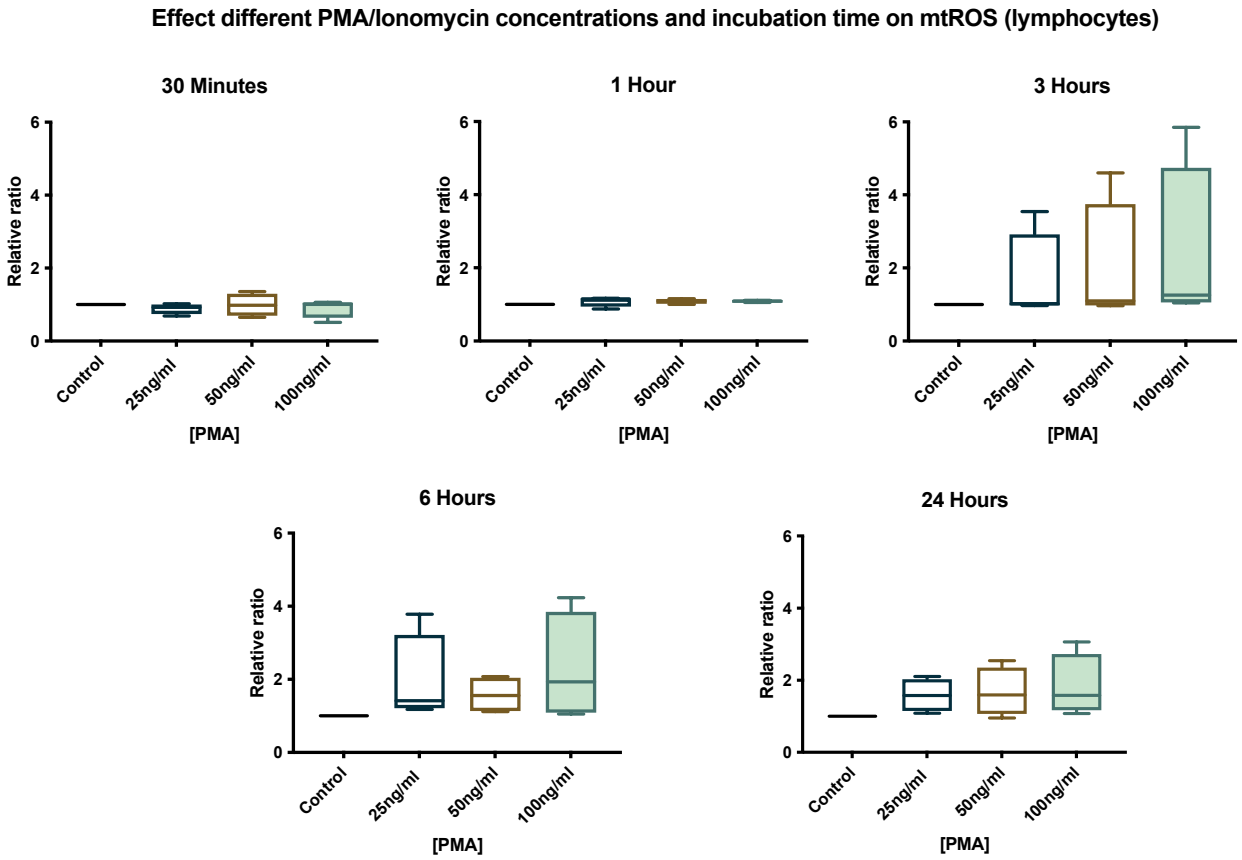


Figure 4.5. Comparison of mtROS production by different PMA/Ionomycin concentrations and incubation times (lymphocytes).

PBMCs were incubated with three concentrations of PMA/Ionomycin (25, 50 and 100ng/ml PMA and 0.5, 1 and 2µg/ml Ionomycin) for 30 minutes, 1, 3, 6 and 24 hours. mtROS production was measured and compared in lymphocytes and data shown as box and whisker plots with median and IQR values. n=4.

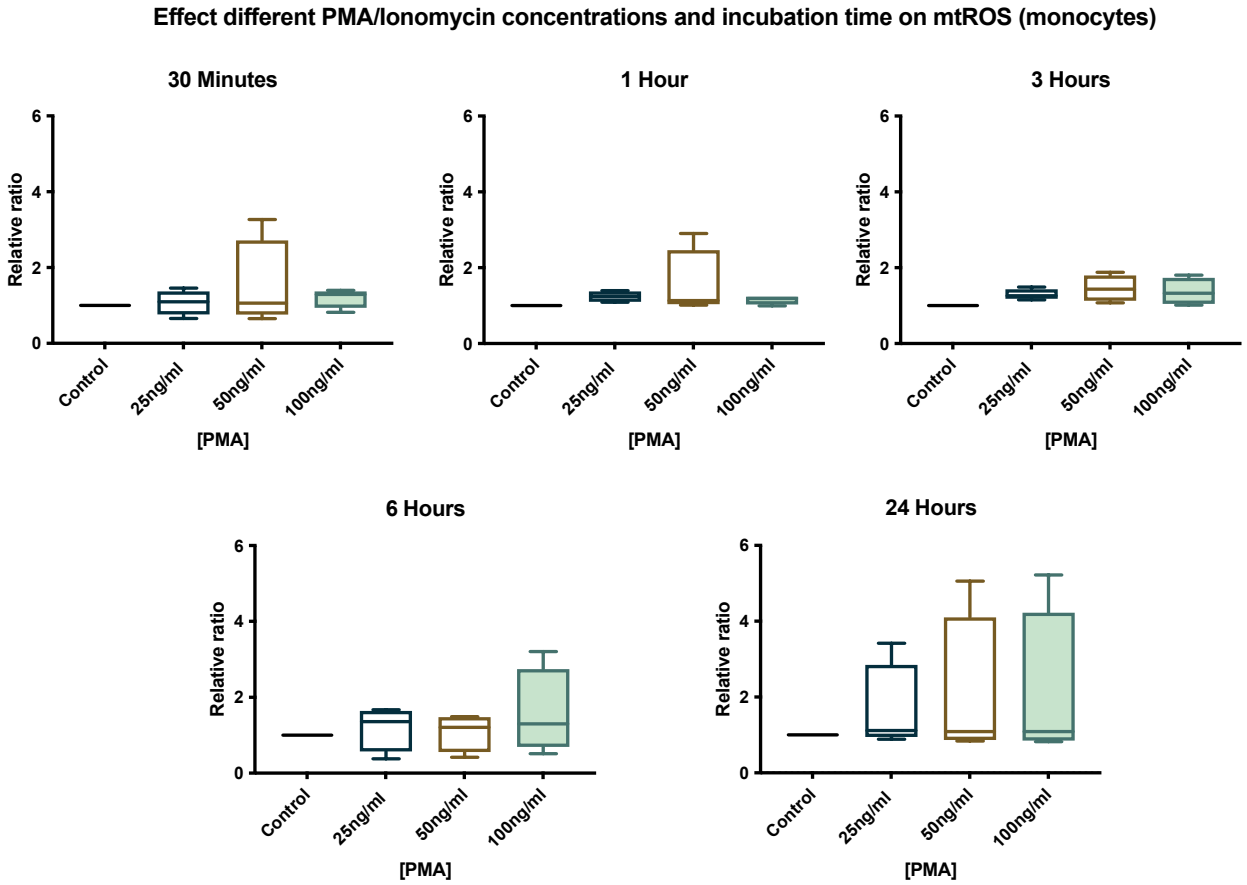


Figure 4.6. Comparison of mtROS production by different PMA/Ionomycin concentrations and incubation times (monocytes).

PBMCs were incubated with three concentrations of PMA/Ionomycin (25, 50 and 100ng/ml PMA and 0.5, 1 and 2µg/ml Ionomycin) for 30 minutes, 1, 3, 6 and 24 hours. mtROS production was measured and compared in monocytes and data shown as box and whisker plots with median and IQR values. n=4

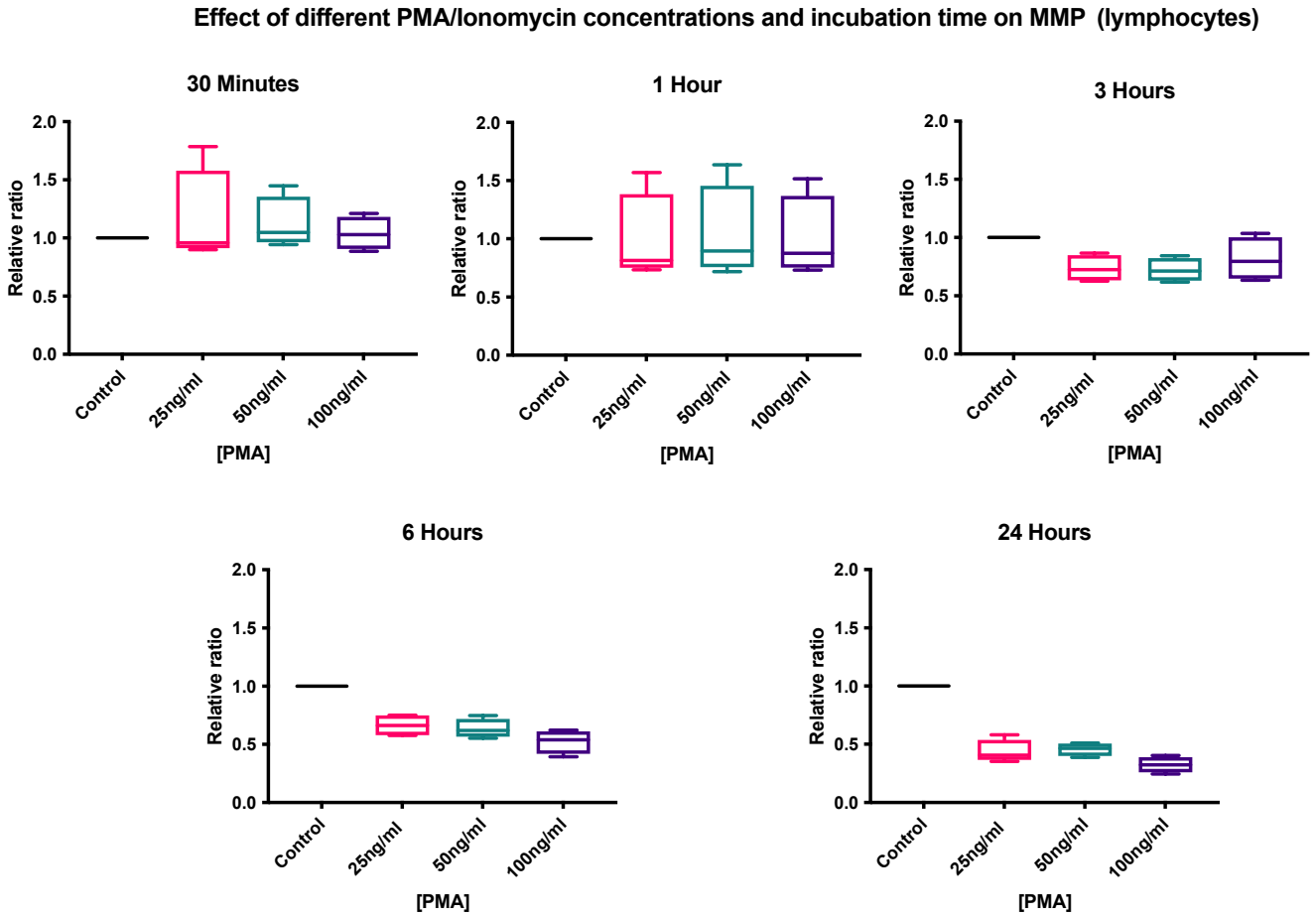


Figure 4.7. Comparison of changes in MMP by different PMA/Ionomycin concentrations and incubation times (lymphocytes).

PBMCs were incubated with three concentrations of PMA/Ionomycin (25, 50 and 100ng/ml PMA and 0.5, 1 and 2µg/ml Ionomycin) for 30 minutes, 1, 3, 6 and 24 hours. Changes in MMP were measured and compared in lymphocytes and data shown as box and whisker plots with median and IQR values. n=4

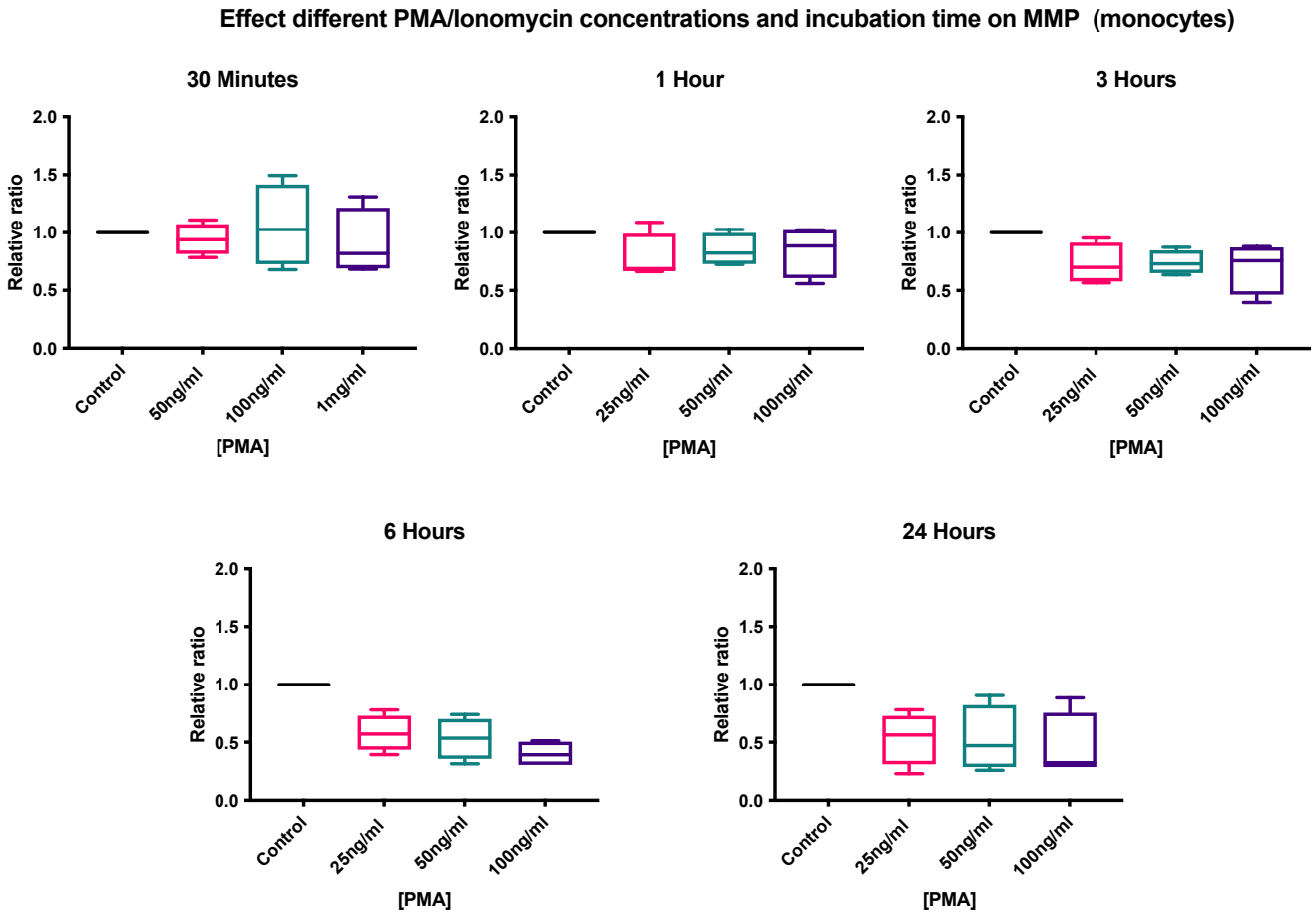


Figure 4.8. Comparison of changes in MMP by different PMA/Ionomycin concentrations and incubation times (monocytes).

PBMCs were incubated with three concentrations of PMA/Ionomycin (25, 50 and 100ng/ml PMA and 0.5, 1 and 2µg/ml Ionomycin) for 30 minutes, 1, 3, 6 and 24 hours. Changes in MMP were measured and compared in monocytes and data shown as box and whisker plots with median and IQR values. n=4

4.3 Mitochondrial membrane potential

Mitochondrial membrane potential (MMP) was determined using the MFI of TMRM. PBMCs were firstly incubated with ciprofloxacin, propofol and/or NE in the presence and absence of LPS for either 6 or 24 hours. MFIs were obtained for lymphocyte and monocyte cell populations using flow cytometry. These were firstly log10 transformed and then analysed using two-way ANOVA and either Bonferroni's or Dunnett's multiple comparisons tests to investigate the effect of incubation time, patient participant population and drug groups on MMP. Controls were established using the protonophore FCCP which uncouples mitochondrial oxidative phosphorylation. It disrupts ATP synthesis by transporting protons across the inner membrane of the mitochondria, decreasing the proton gradient and reducing MMP. These studies were undertaken in lymphocytes and monocytes as part of quality control Effect of incubation time on MMP in lymphocyte and monocyte populations

Two-way ANOVA and Bonferroni's multiple comparison tests were used to investigate the effect of incubation time on MMP in both lymphocyte and monocyte populations. For each drug group, the incubation time of 6 versus 24 hours significantly affected MMP in both cell populations (Table 4.1). Bonferroni's tests in both cases showed a significant increase in MMP at 24 hours compared to 6 hours in the absence of LPS. There was also a significant increase in MMP in monocytes at 24 hours compared to 6 hours in the presence of LPS (Figure 4.9). This may be an indication of either an increase in ATP demand increasing H^+ in the inner mitochondrial membrane space, or possibly due to increased cell stress prior to cell death.

Table 4.1. F-test value and corresponding p-values for the effect of incubation of 6 versus 24h on MMP in lymphocyte and monocyte population in the presence and absence of LPS.

Lymphocytes	F-value	p-value
No LPS	F (1, 119) = 15.83	P=0.0001
LPS	F (1, 119) = 4.590	P=0.0342

Monocytes	F-value	p-value
No LPS	F (1, 119) = 13.81	P=0.0003
LPS	F (1, 119) = 13.44	P=0.0004

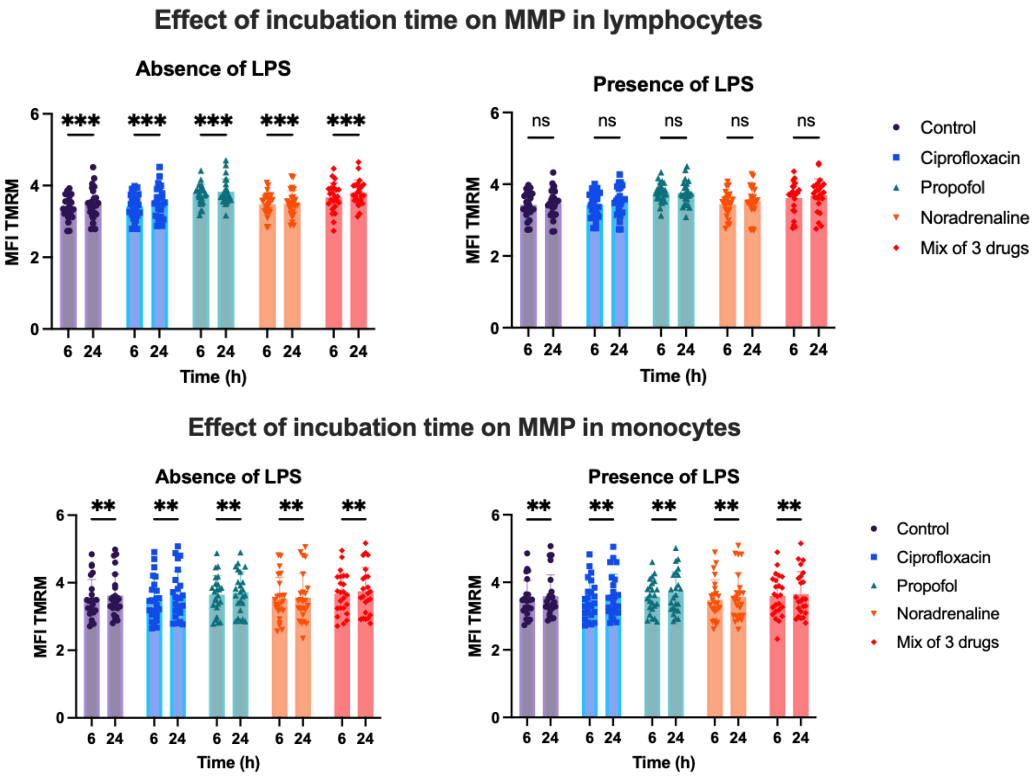


Figure 4.9. Effect of incubation time on MMP in lymphocyte and monocyte populations in the presence and absence of LPS.

PBMCs were treated with ciprofloxacin, propofol and/or NE in the presence and absence of LPS for 6 and 24 hours. MMP was measured as MFI of TMRM and $\log_{10}$ transformed. These were then compared using two-way ANOVA with Bonferroni's multiple comparisons test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Data are shown as mean ± SD. n=5 in healthy volunteer and ED groups, n=7 in healthy volunteers with septic serum group and septic ICU patient groups.

4.3.1 Effect of participant groups on MMP in lymphocytes and monocytes.

The effect of participant groups on MMP was then investigated in lymphocytes and monocytes using two-way ANOVA and Dunnett's multiple comparison tests. These demonstrated that the participant groups significantly affected

MMP in both cell populations at all time points in both the presence and absence of LPS (Table 4.2).

Table 4.2. F-test value and corresponding p-values for the effect of participant groups on MMP in lymphocytes and monocytes at 6 and 24 hours in the presence and absence of LPS.

Lymphocytes	F-value	p-value
At 6 hours, no LPS	F (3, 20) = 10.26	P=0.0003
At 6 hours, LPS	F (3, 20) = 11.14	P=0.0002
At 24 hours, no LPS	F (3, 20) = 8.553	P=0.0007
At 24 hours, LPS	F (3, 20) = 8.308	P=0.0009

Monocytes	F-value	p-value
At 6 hours, no LPS	F (3, 20) = 21.23	P<0.0001
At 6 hours, LPS	F (3, 20) = 17.30	P<0.0001
At 24 hours, no LPS	F (3, 20) = 30.19	P<0.0001
At 24 hours, LPS	F (3, 20) = 44.90	P<0.0001

The effect of MMP for a given drug was then compared between individual participant groups, such as PBMCs from healthy volunteers versus those from healthy volunteers co-incubated with septic serum, or versus PBMCs from infected ED patients, or versus those from septic ICU patients. In both immune cell types, there was a consistent reduction in MMP in PBMCs from healthy volunteers co-incubated with septic serum, or from septic ICU patients compared to healthy volunteers. This was seen at both 6 and 24h and in the presence and absence of LPS (Figures 4.10 and 4.11).

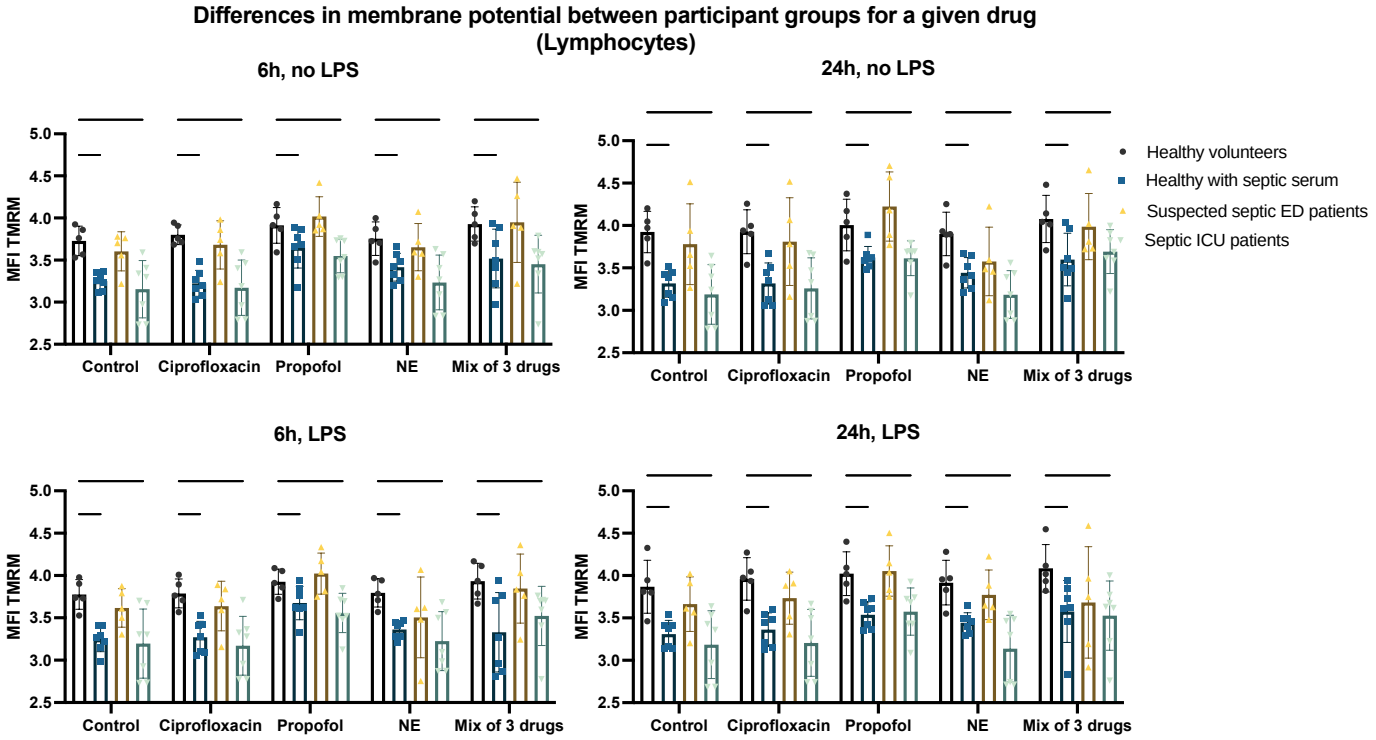


Figure 4.10. Differences in MMP between different participant groups at 6 and 24 hours in the presence and absence of LPS in lymphocytes.

Blood samples from each participant group were treated with ciprofloxacin, propofol and/or NE ± LPS for 6 and 24 hours. Lymphocytes MMP was measured as TMRM MFI, log10 transformed and compared between each participant group at 6 and 24 hours in the presence and absence of LPS. Results were analysed using two-way ANOVA with Dunnett's multiple comparisons test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Data are shown as mean ± SD combining lymphocytes from 5 healthy volunteers, 5 ED patients, 7 from healthy volunteers co-incubated with septic serum and 7 from septic ICU patients.

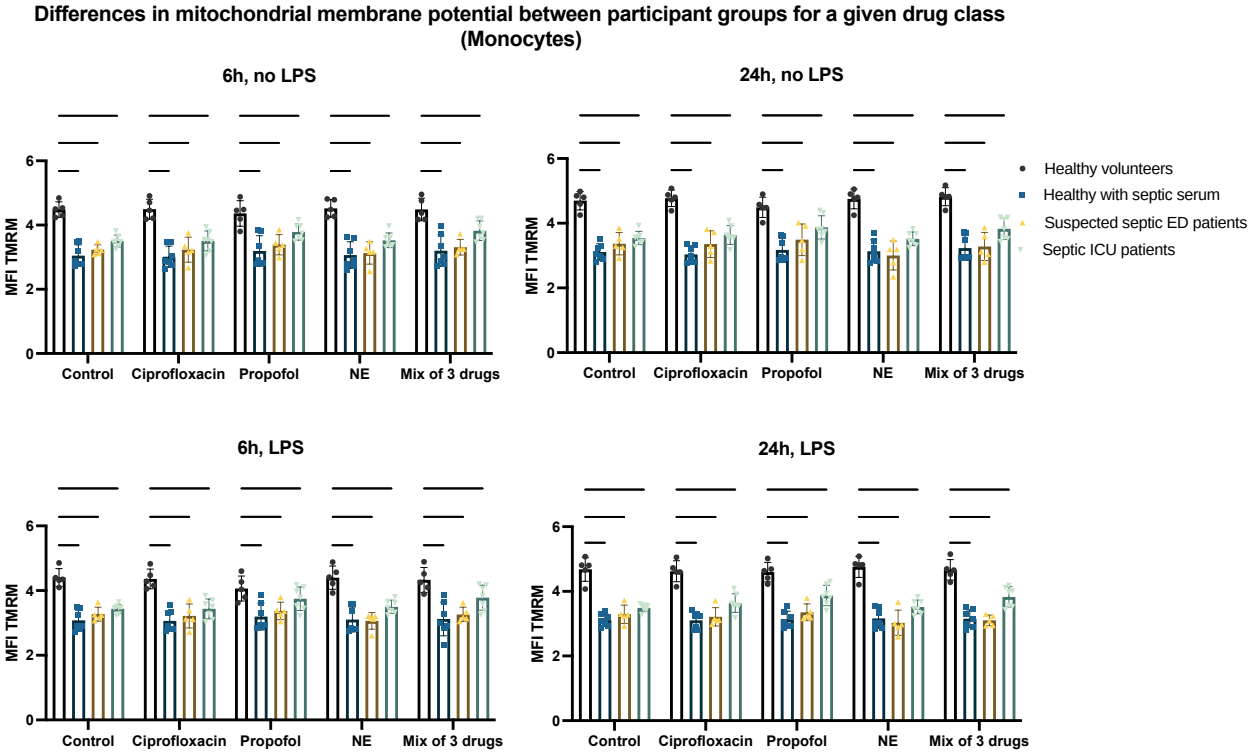


Figure 4.11. Differences in MMP between different participant groups at 6 and 24 hours in the presence and absence of LPS in monocytes.

Blood samples from each participant group were treated with ciprofloxacin, propofol and/or NE ± LPS for 6 and 24 hours. Monocyte MMP was measured as TMRM MFI, log10 transformed and compared between each participant group at 6 and 24 hours in the presence and absence of LPS. Results were analysed using two-way ANOVA with Dunnett's multiple comparisons test. *p< 0.05, **p<0.01, ***p<0.001, ****p<0.0001. Data are shown as mean ± SD combining monocytes from 5 healthy volunteers, 5 ED patients, 7 from healthy volunteers co-incubated with septic serum and 7 from septic ICU patients.

4.2.3 Effect of drug groups on MMP in lymphocytes and monocytes within the same participant group.

Two-way ANOVA and Dunnett's multiple comparison tests were used to investigate the effect of individual drug groups on MMP within each participant group in both cell populations. Drug groups significantly affected MMP at all time points ± LPS compared to non-treated controls in both lymphocytes and monocytes (Table 4.3).

Table 4.3. F-test value and corresponding p-values for the effect of drug groups on MMP at 6 and 24 hours in the presence and absence of LPS in lymphocytes and monocytes.

Lymphocytes	F-value	p-value
At 6 hours, no LPS	F (1.769, 40.68) = 15.86	P=0.001
At 6 hours, LPS	F (2.273, 52.27) = 9.158	P=0.001
At 24 hours, no LPS	F (2.064, 47.47) = 14.21	P=0.001
At 24 hours, LPS	F (1.587, 36.49) = 7.140	P=0.004

Monocytes	F-value	p-value
At 6 hours, no LPS	F (3.056, 70.29) = 6.924	P=0.001
At 6 hours, LPS	F (2.787, 64.10) = 2.026	P=0.123
At 24 hours, no LPS	F (2.759, 63.47) = 3.862	P=0.016
At 24 hours, LPS	F (2.898, 66.66) = 2.083	P=0.113

There were significant changes in MMP in lymphocytes following incubation with each drug group compared to control (Figure 4.12). These were not consistent across all participant groups. Monocytes however showed minimal changes in MMP following incubation with each drug group compared to control (Figure 4.13). There was also no additive effect following incubation with the combination of all three drugs compared to individual drugs.

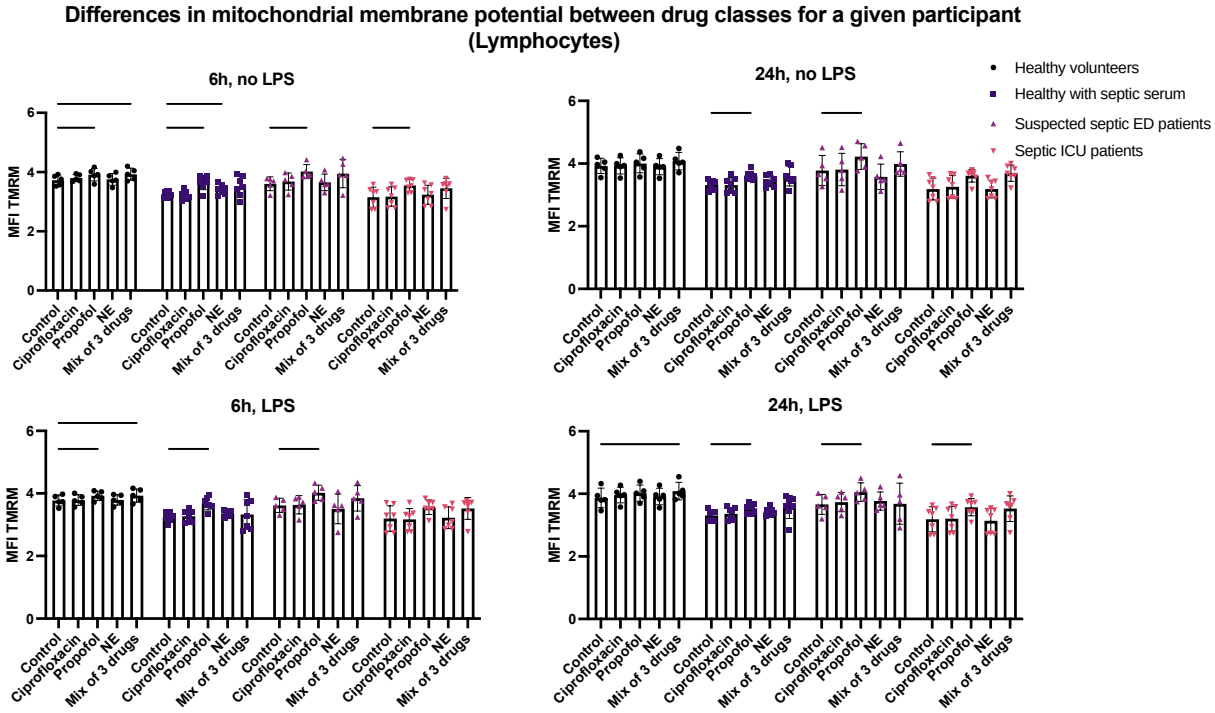


Figure 4.12. Differences in lymphocyte MMP between different drug classes for a given participant group, at 6 and 24 hours ± LPS.

Blood samples from each participant group were treated with ciprofloxacin, propofol and/or NE ± LPS for 6 and 24 hours. Lymphocyte MMP was measured as MFI of TMRM and compared between each drug group at 6 and 24 hours ± LPS. Results were analysed using two-way ANOVA with Dunnett's multiple comparisons test *p< 0.05, **p<0.01, ***p<0.001, ****p<0.0001. Data are shown as mean ± SD combining lymphocytes from 5 healthy volunteers, 5 ED patients, 7 from healthy volunteers co-incubated with septic serum and 7 from septic ICU patients.

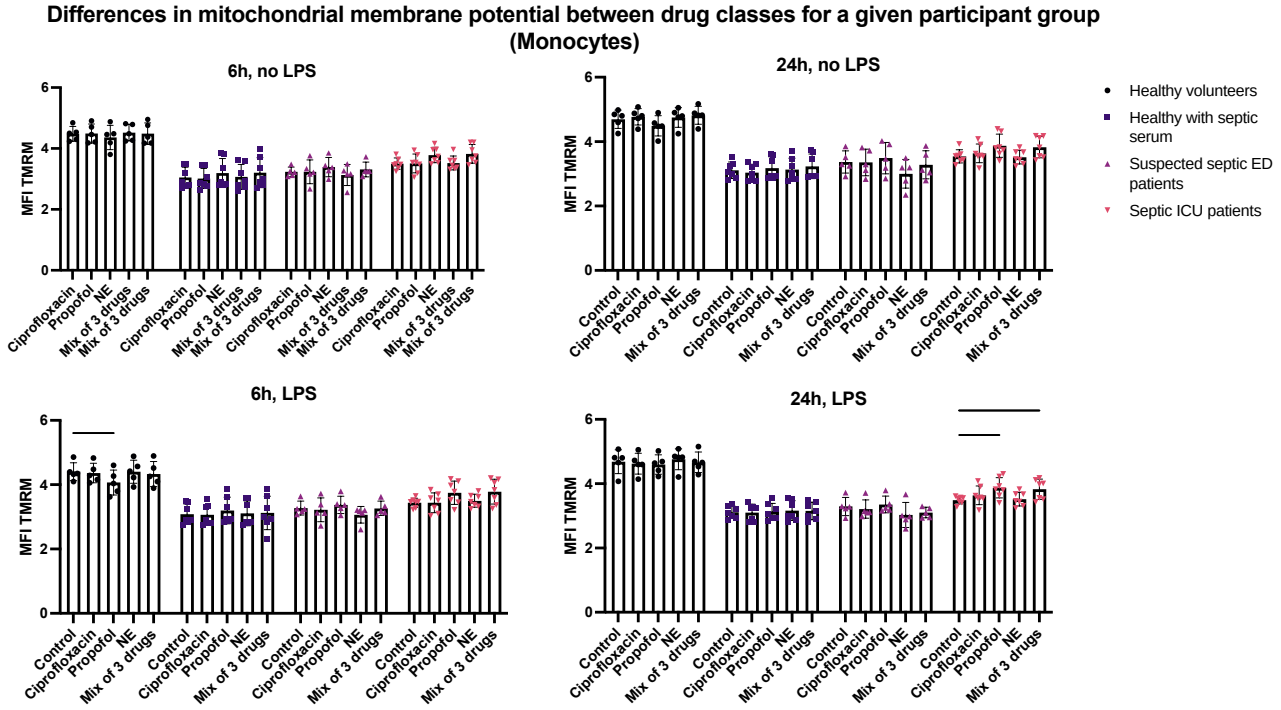


Figure 4.13. Differences in monocyte MMP between different drug classes for a given participant group, at 6 and 24 hours ± LPS.

Blood samples from each participant group were treated with ciprofloxacin, propofol and/or NE ± LPS for 6 and 24 hours. Monocytes MMP was measured as MFI of TMRM and compared between each drug group at 6 and 24 hours ± LPS. Results were analysed using two-way ANOVA with Dunnett's multiple comparisons test. *p< 0.05, **p<0.01, ***p<0.001, ****p<0.0001. Data are shown as mean ± SD combining monocytes from 5 healthy volunteers, 5 ED patients, 7 from healthy volunteers co-incubated with septic serum and 7 from septic ICU patients.

4.4 Mitochondrial reactive oxygen species production

Mitochondrial ROS (mtROS) production was determined using the MFI of Mitosox Red. PBMCs were firstly incubated with ciprofloxacin, propofol and/or NE in the presence and absence of LPS for either 6 or 24 hours. MFIs were obtained for lymphocyte and monocyte cell populations using flow cytometry. These were firstly log10 transformed and then analysed using two-way ANOVA and either Bonferroni's or Dunnett's multiple comparison tests to investigate the effect of incubation time, participant population and drug groups on MMP.

Controls were established using the mitochondrial electron transport chain inhibitor, antimycin. By inhibiting Complex III, antimycin increases production of mtROS and thus increases the MFI of Mitosox Red. These studies were undertaken as part of quality control. Effect of incubation time on mtROS in lymphocyte and monocyte populations

Firstly, two-way ANOVA and Bonferroni's multiple comparison tests were used to investigate the effect of incubation time on mtROS in both lymphocytes and monocytes.

For each drug group, the incubation time of 6 versus 24 hours significantly affected mtROS in both cell populations but only in the absence of LPS (Table 4.4).

Table 4.4. F-test value and corresponding p-values for the effect of time on mtROS in lymphocytes and monocytes in the presence and absence of LPS.

Lymphocytes	F-value	p-value
No LPS	F (1, 124) = 9.301	P=0.003
LPS	F (1, 124) = 3.149	P=0.078

Monocytes	F-value	p-value
No LPS	F (1, 124) = 7.631	P=0.007
LPS	F (1, 123) = 0.050	P= 0.823

Bonferroni's tests showed a significant increase in mtROS in both lymphocytes and monocytes at 24 hours compared to 6 hours (Figure 4.8).

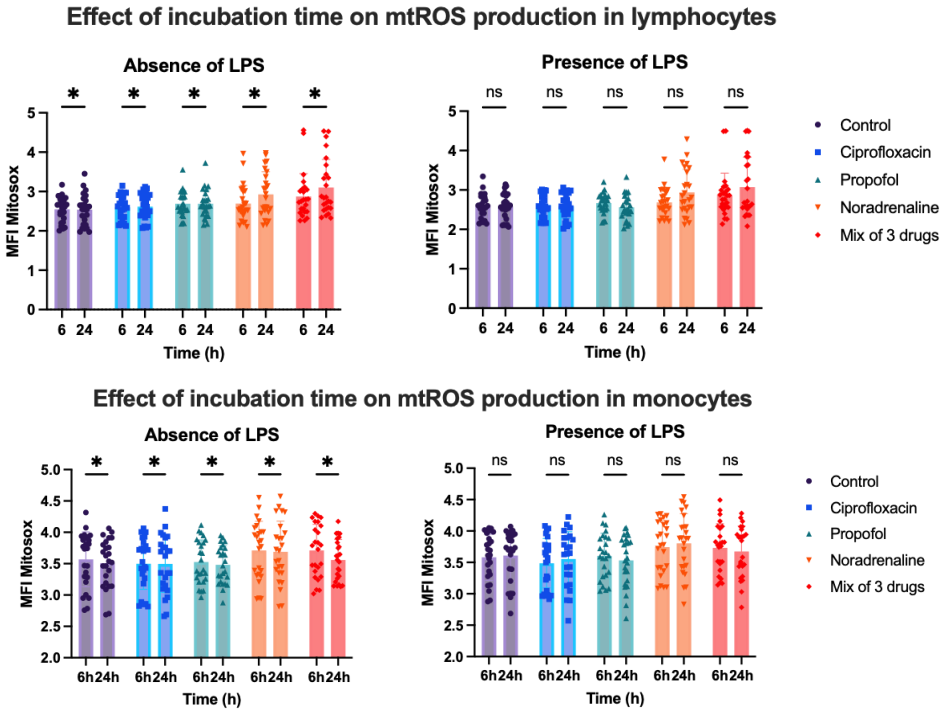


Figure 4.14. Effect of incubation time on mtROS production in lymphocytes and monocytes at 6 and 24 hours in the presence and absence of LPS.

PBMCs were treated with ciprofloxacin, propofol and/or NE in the presence and absence of LPS for 6 and 24 hours. mtROS production was measured as MFI of Mitosox Red. These were $\log_{10}$ transformed and compared using two-way ANOVA with Bonferroni's multiple comparisons test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Data are shown as mean ± SD combining lymphocytes or monocytes from 5 healthy volunteers, 5 ED patients, 7 from healthy volunteers co-incubated with septic serum and 7 from septic ICU patients.

4.4.1 Effect of participant groups on mtROS in lymphocytes and monocytes.

The effect of participant groups on mtROS was then investigated in lymphocytes and monocytes using two-way ANOVA and Dunnett's multiple comparison tests. The participant groups significantly differed in mtROS production in lymphocytes at 6 hours in the absence of LPS, and in monocytes at both timepoints in both the presence and absence of LPS (Table 4.5).

Table 4.5. F-test value and corresponding p-values for the effect of participant groups on mtROS in lymphocytes and monocytes at 6 and 24 hours in the presence and absence of LPS.

Lymphocytes	F-value	p-value
At 6 hours, no LPS	F (3, 21) = 5.260	P=0.007
At 6 hours, LPS	F (3, 21) = 10.81	P=0.001
At 24 hours, no LPS	F (3, 21) = 3.760	P=0.026
At 24 hours, LPS	F (3, 21) = 2.712	P=0.071

Monocytes	F-value	p-value
At 6 hours, no LPS	F (3, 21) = 15.17	P=0.001
At 6 hours, LPS	F (4, 96) = 5.746	P=0.001
At 24 hours, no LPS	F (4, 96) = 3.912	P=0.006
At 24 hours, LPS	F (4, 96) = 7.141	P=0.001

The effect of mtROS for a given drug was compared between PBMCs from individual participant groups, such as healthy volunteers versus healthy volunteers co-incubated with septic serum, or versus ED patients, or versus septic ICU patients. There was a consistent increase in mtROS compared to healthy volunteer cells in both cell types, however this was not consistently present for a single participant population under all experimental conditions.

In lymphocytes, there was an increase in mtROS across all participant groups compared to healthy volunteers at 6 hours in the presence of LPS, and at 24 hours in the absence of LPS (Figure 4.16). In monocytes, the increase in mtROS was not consistent across participant groups nor experimental conditions. mtROS increased in healthy volunteers co-incubated with septic serum compared to healthy volunteers at 6 hours in both the presence and absence of LPS, and at 24 hours in LPS-treated groups. In ED patients, mtROS increased at 6 hours with LPS, and at 24 hours in the absence and presence of LPS compared to healthy volunteers. In septic ICU patients, mtROS increased at 6 hours in the presence and absence of LPS but not at 24 hours (Figure 4.17).

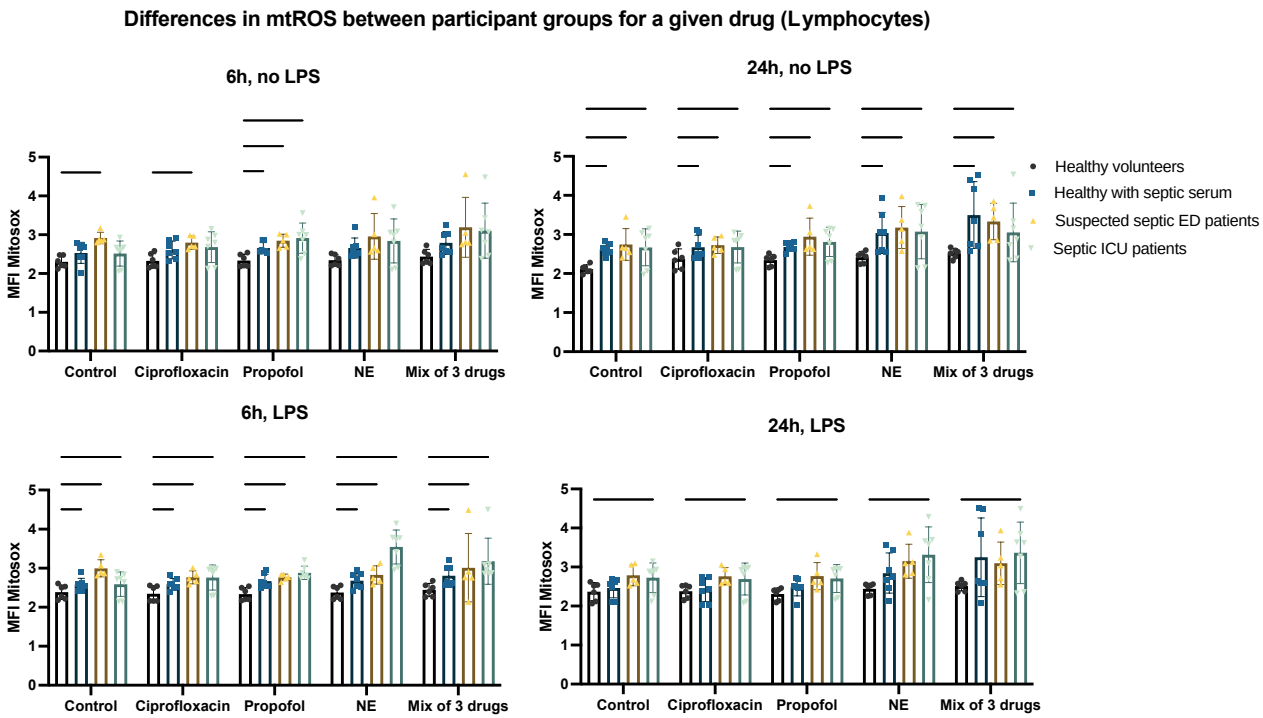


Figure 4.15. Differences in mtROS between different participant groups at 6 and 24 hours ± LPS (Lymphocytes

Blood samples from each participant group were treated with ciprofloxacin, propofol and/or NE in the presence and absence of LPS for 6 and 24 hours. Lymphocytes mtROS was measured as MFI of Mitosox Red, log10 transformed and compared between each participant group at 6 and 24 hours in the presence and absence of LPS. Results were analysed using two-way ANOVA with Dunnett's multiple comparisons test. *p< 0.05, **p<0.01, ***p<0.001, ****p<0.0001. Data are shown as mean ± SD combining lymphocytes from 5 healthy volunteers, 5 ED patients, 7 healthy volunteers co-incubated with septic serum, and 7 septic ICU patients.

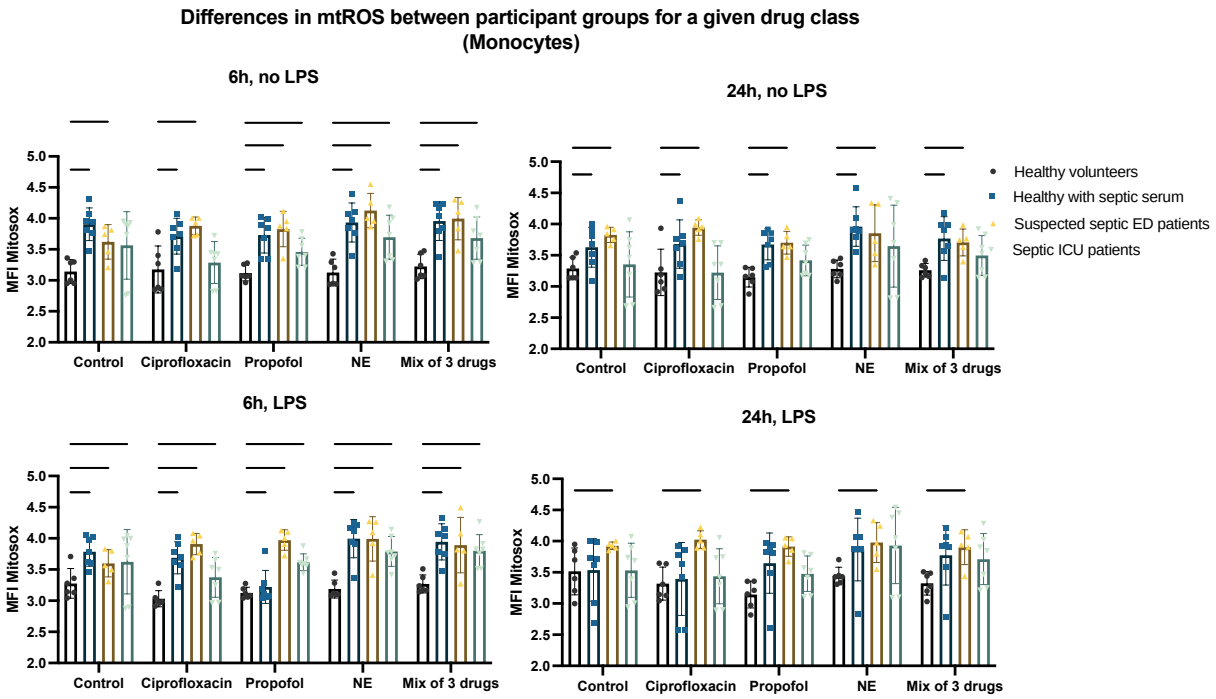


Figure 4.16. Differences in mtROS between different participant groups at 6 and 24 hours ± LPS (Monocytes).

Blood samples from each participant group were treated with ciprofloxacin, propofol and/or NE in the presence and absence of LPS for 6 and 24 hours. Monocytes mtROS was measured as MFI of Mitosox Red, log10 transformed and compared between each participant group at 6 and 24 hours in the presence and absence of LPS. Results were analysed using two-way ANOVA with Dunnett's multiple comparisons test. *p< 0.05, **p<0.01, ***p<0.001, ****p<0.0001. Data are shown as mean ± SD combining monocytes from 5 healthy volunteers, 5 ED patients, 7 healthy volunteers co-incubated with septic serum, and 7 septic ICU patients.

4.4.2 Effect of drug groups on mtROS in lymphocytes and monocytes within the same participant group.

Finally, two-way ANOVA and Dunnett's multiple comparison tests were used to investigate the effect of individual drug groups on mtROS production within each participant group. Drug groups significantly affected mtROS production in both cell populations under all experimental conditions (Table 4.6).

Table 4.6. F-test values and corresponding p-values for the effect of drug class on mtROS production in lymphocytes and monocytes at 6 and 24 hours in the absence and presence of LPS.

Drug class	F-value	p-value
At 6 hours, no LPS	F (1.884, 45.21) = 5.169	P=0.011
At 6 hours, LPS	F (2.691, 64.59) = 4.919	P=0.005
At 24 hours, no LPS	F (1.691, 40.57) = 15.47	P=0.001
At 24 hours, LPS	F (1.599, 38.39) = 17.68	P=0.001

Drug class	F-value	p-value
At 6 hours, no LPS	F (1.997, 47.93) = 4.233	P=0.020
At 6 hours, LPS	F (2.681, 64.34) = 5.746	P=0.002
At 24 hours, no LPS	F (3.329, 79.89) = 3.912	P=0.009
At 24 hours, LPS	F (2.958, 71.00) = 7.141	P=0.001

While there were some significant differences in mtROS production in both cell populations, these were not present uniformly in different participant groups (Figure 4.18 and 4.19).

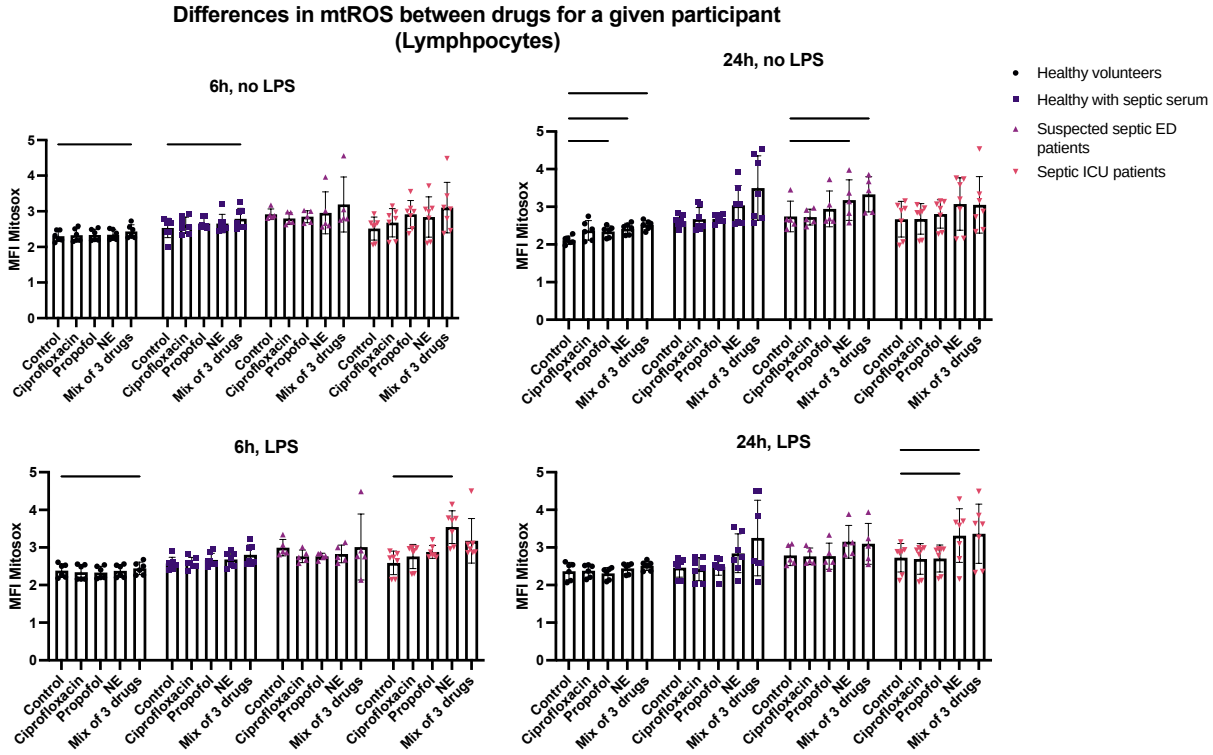


Figure 4.17. Differences in lymphocyte mtROS between different drug classes for a given participant group, at 6 and 24 hours in the presence and absence of LPS.

Blood samples from each participant group were treated with ciprofloxacin, propofol and/or NE in the presence and absence of LPS for 6 and 24 hours. MtROS production was measured as MFI of Mitosox and compared between each drug group at 6 and 24 hours in the presence and absence of LPS. Results were analysed using two-way ANOVA with Dunnett's multiple comparisons test. *p< 0.05, **p<0.01, ***p<0.001, ****p<0.0001. Data are shown as mean ± SD combining lymphocytes from 5 healthy volunteers, 5 ED patients, 7 healthy volunteers co-incubated with septic serum, and 7 septic ICU patients.

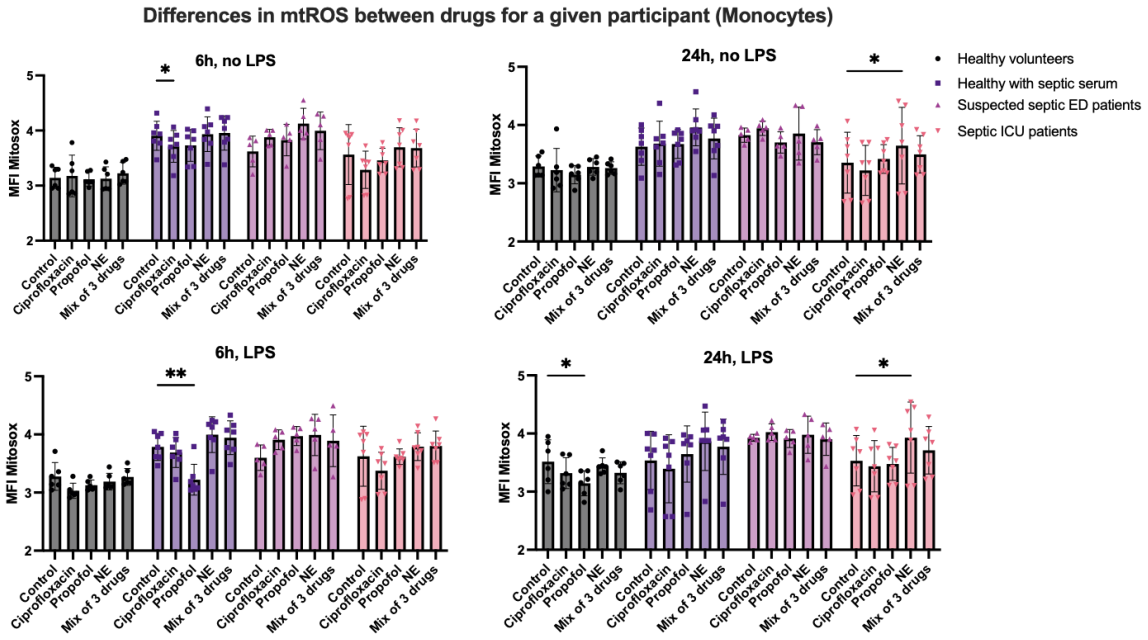


Figure 4.18. Differences in mtROS between different drug classes for a given participant group, at 6 and 24 hours in the presence and absence of LPS (monocytes).

Blood samples from each participant group were treated with ciprofloxacin, propofol and/or NE in the presence and absence of LPS for 6 and 24 hours. MtROS production was measured as MFI of Mitosox and compared between each drug group at 6 and 24 hours in the presence and absence of LPS. Results were analysed using two-way ANOVA with Dunnett's multiple comparisons test. *p< 0.05, **p<0.01, ***p<0.001, ****p<0.0001. Data are shown as mean ± SD combining monocytes from 5 healthy volunteers, 5 ED patients, 7 healthy volunteers co-incubated with septic serum, and 7 septic ICU patients.

4.5 Mitochondrial stress test using Seahorse XF96 Analyser

The mitochondrial stress test was used to measure oxygen consumption rate (OCR) and other respiratory variables including basal respiration, proton leak, maximum respiration rate, and non-mitochondrial respiration. ~300,000 PBMCs from healthy volunteers were suspended in DMEM media containing 10% pooled septic serum and FBS. Cells were plated on the XF cell culture microplate following adhesion with Cell-Tak as per protocol. Ciprofloxacin (100 µg/mL), propofol (50 µg/mL) and/or NE (10 µg/mL) ± LPS (100 ng/mL) were added to the relevant wells in replicates of four and incubated for 6 hours.

After 6 hours, cells were suspended in Seahorse media consisting of DMEM with L-glutamine (2 µM), sodium pyruvate (1 µM), and glucose (5 µM). After 25-30 minutes, cells were loaded to an already calibrated seahorse XF96 analyser and oligomycin (3.5 µM), The uncoupler FCCP (2.5 µM), the Complex III inhibitor antimycin A and the Complex I inhibitor rotenone (5 uM) were sequentially added. OCR and the other mitochondrial parameters were measured at specific intervals. PBMCs were then washed twice with 200 µL of PBS and stained with 1/1000 concentration of Hoechst. These were then counted using the ImageXpress Imaging System.

The results from the Seahorse XF96 analyser were uploaded onto Agilent Wave 2.6 Desktop software for Mac. Cell counts were used to normalise the data. Data were then checked for quality control (including removal of outliers). Replicate data were then averaged and exported to GraphPad Prism for further analysis.

The OCRs were averaged for the six participants and the drug/LPS combinations. Line graphs were used to ensure that the trace shape was as expected. The specific mitochondrial respiratory variables were analysed using one-way ANOVA and either Kruskal-Wallis test or Dunn’s multiple comparisons tests.

4.5.1 OCR curves

Healthy PBMCs were incubated with pooled septic serum and treated with ciprofloxacin, propofol and/or NE in the presence and absence of LPS for 6 or 24 hours. Mean OCRs were calculated for replicates after removal of outliers and quality control checks. OCRs were then averaged for PBMCs from the six healthy volunteers and visually compared to controls to confirm whether the response to the various mitochondrial inhibitors or uncoupler was as expected.

For each experimental condition, the response to mitochondrial drugs (oligomycin, FCCP and antimycin/rotenone) was as expected. Oligomycin disrupts ATP synthase activity by binding to the F_0 component of the complex V; this reduced the OCR required for ATP synthesis. Proton leak remained steady for each experiment. FCCP then disrupted the MMP causing cells to increase their oxygen requirements to attempt to restore the membrane potential for ETC activity. This generated the value for maximal respiration. The difference between basal and maximal respiration equals the spare respiratory capacity. Finally, antimycin A and rotenone were added, which reduced oxygen consumption by inhibiting OxPHOS. Non-mitochondrial respiration was then calculated by deduction from the basal and maximal respiration values (Figure 4.20).

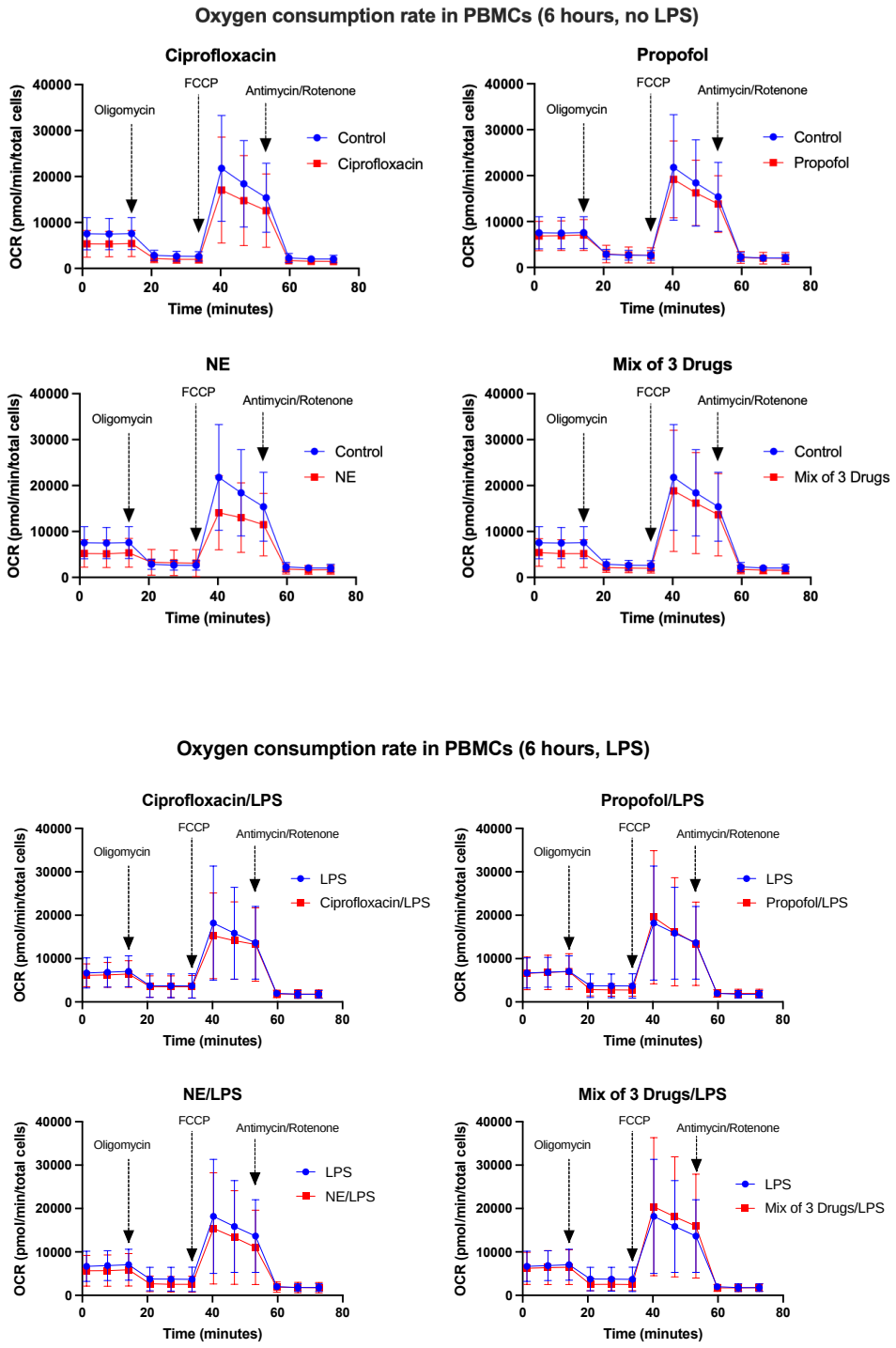


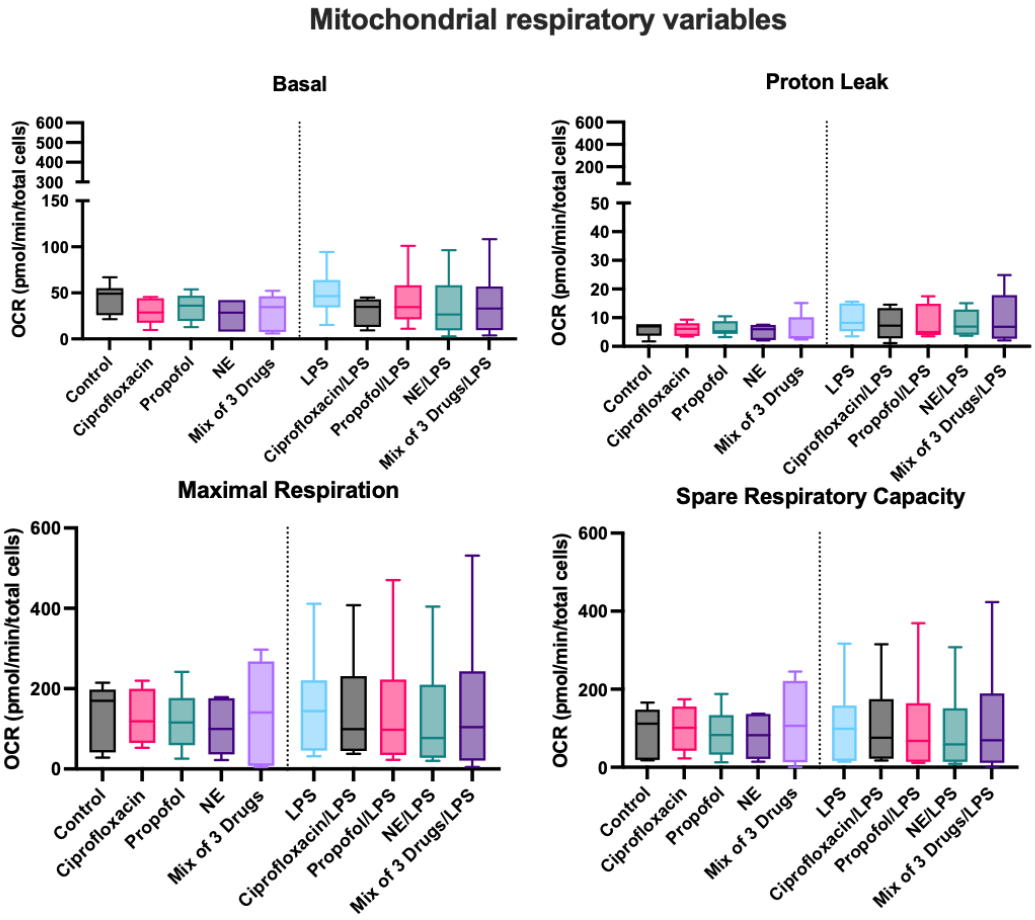
Figure 4.19. OCR of healthy PBMCs co-incubated with septic serum, drugs of interest and LPS for 6 hours.

Healthy PBMCs were incubated with pooled septic serum/FBS and treated with ciprofloxacin, propofol and/or NE ± LPS for 6 hours. Mean OCRs were calculated and visually compared to controls to check for whether the response to the various drugs was as expected. Data are shown as mean ± SD. n=6.

4.5.2 Mitochondrial variables

Basal respiration, proton leak, maximal respiration, spare respiratory capacity, non-mitochondrial respiration and ATP production were compared to the relevant controls using one-way ANOVA and either Kruskal-Wallis or Dunn's multiple comparison tests.

These showed no statistically significant differences between unstimulated controls and drug groups, nor between LPS controls and drug/LPS combinations. There was no additive effect from the mixture of drugs compared to the individual drugs (Figure 4.21).



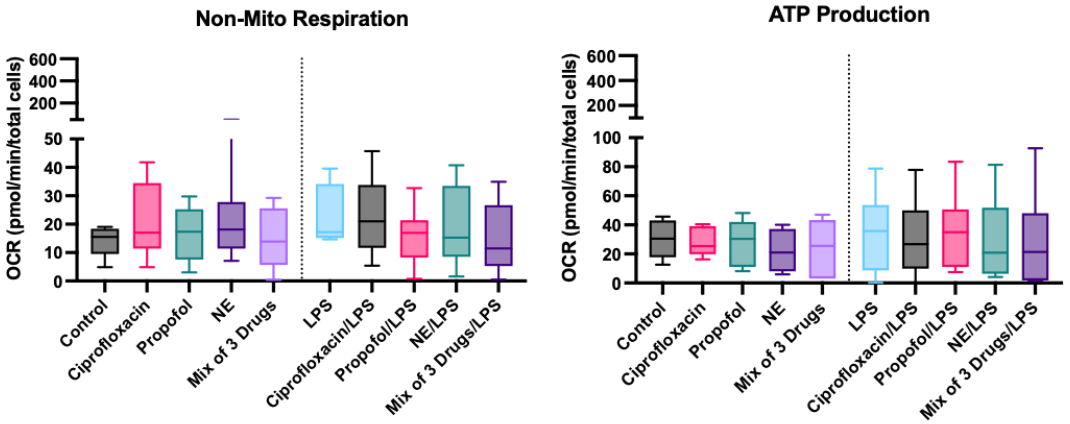


Figure 4.20. Mitochondrial respiratory variables.

Healthy PBMCs were incubated with septic serum/FBS and treated with ciprofloxacin, propofol and/or NE ± LPS for 6 and 24 hours. Means of mitochondrial respiratory values were calculated for each replicate after removal of outliers and quality control checks. One-way ANOVA analysis with either Kruskal-Wallis or Dunn's multiple comparison test were used to compare drug groups against unstimulated control, or drug/LPS combinations against LPS control. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Data are shown as box and whisker plots with median ± interquartile range. n=6.

4.6 Summary of mitochondrial studies

For each drug group, incubation time significantly increased MMP and mtROS for both cell types. However, this was not consistent across all experimental conditions. Similarly, participant groups and individual drug groups significantly affected MMP and mtROS production in both lymphocytes and monocytes. This was also not consistent under all experimental conditions. There was no cumulative effect following incubation with combination of all three drugs compared to each drug alone.

Mitochondrial variables including basal respiration, proton leak, maximal respiration, spare respiratory capacity, non-mitochondrial respiration and ATP production showed no statistically significant difference between unstimulated control and drug groups, nor between LPS controls and drug group/LPS combinations. There was also no additive effect from the mixture of drugs for any of these parameters.

Chapter 5 Examining immunology and metabolic gene expression profiles of PBMCs incubated with ciprofloxacin, propofol, norepinephrine and LPS.

5.1 Introduction

Sepsis has a complex and multi-faceted pathophysiological mechanism which, despite decades of research using traditional methods, still remains elusive. With advances in molecular biology, novel methods can be used to review this age-old syndrome with a fresh approach. As with cancer biology, the focus of genetic studies in sepsis is to investigate individual host responses, develop clinically relevant point-of-care diagnostics, and identify disease clusters to help guide therapeutic decisions. However, unlike cancer, studies in sepsis are more challenging to conduct and interpret. This is partially explained by the dynamic natural history of the condition, difficulty with identifying and sampling infected tissues, pathogen-related factors (e.g. source, location, virulence and resistance patterns), host-related factors (e.g. age, gender, ethnicity, co-morbidities and prior exposure to pathogens), and therapeutic interventions that often modulate the host response.

Studies of the innate immune system indicate that >3700 genes may be dysregulated in response to a bacterial infection, notably with downregulated gene expression affecting the majority (153). Sepsis can be distinguished from sterile SIRS using gene expression profiles (477, 478), though more information may be required to distinguish between the genetic profiles of sepsis arising from Gram-positive or Gram-negative bacteria (479). Numerous studies have identified genetic variations that increase the risk of developing sepsis, while other studies have classified septic patients into specific biological signatures (e.g. into inflamed or immunosuppressed subtypes) to

identify patients at high risk of mortality (480-483). However, such studies in sepsis are still in their relative infancy and require further in-depth investigation before widespread utilisation in clinical practice (484).

I used Nanostring technology to investigate the impact of ciprofloxacin, propofol and/or NE on immunological and metabolic pathways in PBMCs in the presence of LPS. Nanostring technology is a robust, high-throughput method that can detect RNA from a small sample of 100ng. I chose the 'Human Immunology V2' and 'Metabolic Pathways' panels, which consisted of 579 and 748 genes respectively, with 15-20 internal reference genes. This was because collectively these pathways covered genes involved in major immune pathways (e.g. production of cytokines, chemokines and their receptors, and signalling in major pathways including the TNF-receptor superfamily and KIR family) and metabolic pathways (biosynthetic pathways, nutrient capture and catabolic pathways, cell stress, metabolic signalling and transcriptional regulation) in humans. The focus of my experiment was on overall immune function and mitochondrial respiration.

Total RNA was extracted from 200 000 PBMCs per well from four healthy individuals co-incubated in septic serum after six hours. Qiagen RNeasy Plus mini kit was used for RNA extraction. The quality and quantity of RNA obtained was determined using a Qubit 4.0 fluorometer and TapeStation. These samples were then transferred to a local Nanostring facility where gene expression was determined. RCC files were uploaded onto nSolver 4.0 (NanoString Technologies, Washington, USA) and ROSALIND® analysis software (ROSALIND, San Diego, CA) for data quality checks, background thresholding and normalization as outlined in the methods section.

5.2 Quality control

Firstly, quality control checks were carried out. All samples passed the imaging quality controls as they had field of view of >95% when the barcodes were immobilised onto the cartridge surface. The binding density of all samples was in the expected range of 0.1- 2.25 μm^{-2} as I used the Max system. The positive

control linearity for all samples (except one) was > 0.95 when the R^2 of controls was performed. An unstimulated sample in the metabolic pathway was excluded as its positive linearity was 0.92. The mean negative controls were in the region of 20 as expected, this indicated an appropriate level of background noise. Positive E control which indicates the system's limit of detection was significantly above the negative controls as expected. Normalization of samples was then carried out using the GeNorm algorithm, which included all the housekeeping genes in the immunology and metabolic pathway panels. If the housekeeping genes are not as expected, they are excluded and may indicate a problem with the assay. This was not the case in our experiments (Table 5.1 and 5.2).

Table 5.1. Quality control parameters for all samples in the immunology panel.

Sample name	Imaging quality (% FOV captured)	Binding density	Positive control linearity	Mean (Neg controls)	SD (Neg controls)	Positive control E
Unstimulated1	0.990991	0.58	0.98	20.12	14.92	128
LPS1	0.994595	0.75	0.98	20.12	13.44	115
Ciprofloxacin/LPS1	0.990991	0.92	0.98	20.75	13.13	100
Propofol/LPS1	0.990991	0.73	0.98	18.75	13.63	93
NE/LPS1	0.987387	0.68	0.98	15.88	11.52	102
Mix/LPS1	0.992793	0.65	0.98	17.88	18.12	100
Unstimulated2	0.996396	1.18	0.98	16.75	12.27	101
LPS2	0.994595	0.83	0.98	20.25	14.34	98
Ciprofloxacin/LPS2	0.990991	0.73	0.98	17.75	11.52	128
Propofol/LPS2	0.996396	0.67	0.98	17.62	11.65	116
NE/LPS2	0.983784	0.95	0.98	21.38	13.82	108
Mix/LPS2	0.989189	0.76	0.98	18.38	12.55	103
Unstimulated3	0.989189	0.47	0.98	13.75	10.21	103
LPS3	0.990991	0.77	0.98	19	11.86	107
Ciprofloxacin/LPS3	0.989189	0.69	0.98	19.25	12.44	83
Propofol/LPS3	0.994595	0.65	0.99	19	11.35	122
NE/LPS3	0.994595	0.76	0.98	19.5	12.09	93
Mix/LPS3	0.974775	0.8	0.98	21.62	14.53	94
Unstimulated3	0.990991	0.44	0.98	17.38	10.94	95
LPS3	0.994595	0.77	0.98	19.5	8.83	92
Ciprofloxacin/LPS3	0.990991	0.84	0.98	23.62	12.16	108

Propofol/LPS3	0.990991	0.74	0.98	20.5	13.21	104
NE/LPS3	0.989189	0.65	0.99	17.88	12.26	118
Mix/LPS3	0.987387	0.67	0.97	15.25	10.61	65

Table 5.2. Quality control parameters for all samples in the metabolic panel

Sample name	Imaging quality (% FOV captured)	Binding density	Positive control linearity	Mean (Neg controls)	SD (Neg controls)	Positive control E
Unstimulated1	0.994595	0.37	0.98	15.62	4.5	102
LPS1	0.989189	0.44	0.98	17.25	6.96	112
Ciprofloxacin/LPS1	0.987387	0.37	0.98	17.25	5.18	126
Propofol/LPS1	0.992793	0.43	0.98	16.75	2.31	99
NE/LPS1	0.989189	0.38	0.98	13.75	6.23	96
Mix/LPS1	0.987387	0.77	0.98	13.75	5.8	88
Unstimulated2	0.956757	0.38	0.99	14.38	4.66	127
LPS2	0.990991	0.42	0.99	16.5	4.44	130
Ciprofloxacin/LPS2	0.996396	0.45	0.98	16.38	8.25	118
Propofol/LPS2	0.992793	0.48	0.99	17	8.02	127
NE/LPS2	0.992793	0.44	0.99	17.12	5.67	139
Mix/LPS2	0.994595	0.44	0.99	16.38	6.39	142
Unstimulated3	0.996396	0.41	0.98	12	5.32	87
LPS3	0.996396	0.39	0.98	16.88	6.51	111
Ciprofloxacin/LPS3	0.998198	0.43	0.98	17.75	5.15	111
Propofol/LPS3	0.998198	0.45	0.98	16.88	6.92	95
NE/LPS3	0.998198	0.5	0.99	14.38	7.25	135
Mix/LPS3	0.998198	0.41	0.99	18	8.16	116
Unstimulated3	0.992793	0.45	0.92	9.12	7.85	73
LPS3	0.998198	0.4	0.99	14.25	5.34	116
Ciprofloxacin/LPS3	0.996396	0.4	0.98	16	4.63	119
Propofol/LPS3	0.998198	0.49	0.98	17.25	4.37	111
NE/LPS3	0.998198	0.54	0.99	15.5	3.02	134
Mix/LPS3	0.96036	0.44	0.99	13.75	6.04	131

5.3 Overview of both panels

Unsupervised hierarchical clustering of all samples in the immunology and metabolic pathways showed clear clustering by unstimulated samples, suggesting these samples were the most similar to each other, while the other

experimental conditions clustered more randomly. This indicates variation in differential gene expression under the different experimental conditions.

In total, there were only 60 and 29 genes with a fold change >1.5 or <-1.5 and an adjusted p-value <0.05 in the immunology and metabolic pathways, respectively. The fold change value of 1.5 was chosen arbitrarily by ROSALIND® and can be adjusted according to genes of interest, (e.g. some genes that may not be as highly expressed but have important metabolic functions) (Figure 5.1). Data showing differential gene expression for all samples across both panels has been included in the appendix.

The volcano plot shows the breakdown of 60 genes in the immunology panel (46 upregulated and 14 downregulated) and 29 genes in the metabolic panel (23 upregulated and 6 downregulated) when samples containing drugs and LPS were compared to unstimulated and LPS samples (Figure 5.1).

Gene set analyses were performed to rationalise the significance of the gene expression changes with respect to specific pathways. This was determined automatically by nSolver and ROSALIND® software, where a global significance score was given according to Nanostring annotations of pathways. The score signifies the relevance of the level of gene expression in a given pathway, with a higher score indicating more relevance. It does not indicate the direction of the gene change (e.g. up- versus down-regulation). These panels were then further analysed using detailed pathway analysis with Wikipathways, Bioplanet, PANTHER and REACTOME.

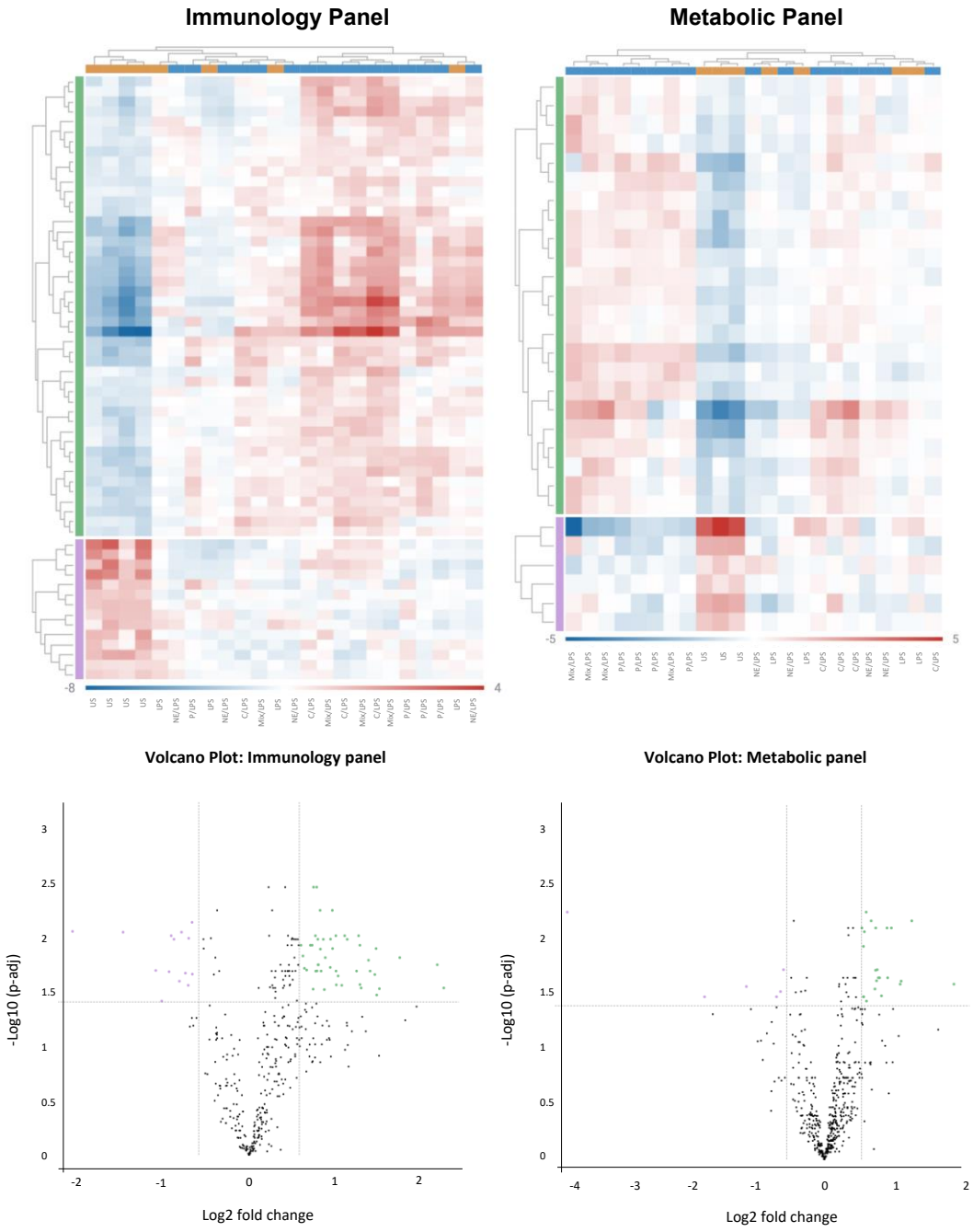


Figure 5.1. Overview data in the immunology and metabolic pathways demonstrated as heatmaps and Volcano plots.

Heatmaps and Volcano plots show a comparison of gene expression profiles of PBMCs from 4 healthy volunteers incubated with 10% pooled septic serum, drugs of interest and LPS, compared to unstimulated and LPS samples. The heatmaps show samples clustered by sample types (x-axis) and by gene type (y-axis). Red demonstrates gene upregulation, while blue shows downregulation. The volcano plot shows log ratio of fold change on the x-axis and the negative log of the adjusted p-value on the y-axis. Each dot represents a gene; genes on the right side above the line at p= 1.5 are upregulated and on the left are downregulated.

Table 5.3. Significance scores following gene set analysis in the immunology and metabolic pathways.

Immunology pathway

Terms	Significance Score
Inflammasomes	3.5615
Th2 differentiation	3.0278
Transcriptional regulation	2.7985
NF-kB signalling	2.6909
Immunometabolism	2.67
Th1 differentiation	2.6156
TNF family signalling	2.5456
Apoptosis	2.4956
NLR signalling	2.4943
Chemokine signalling	2.4792

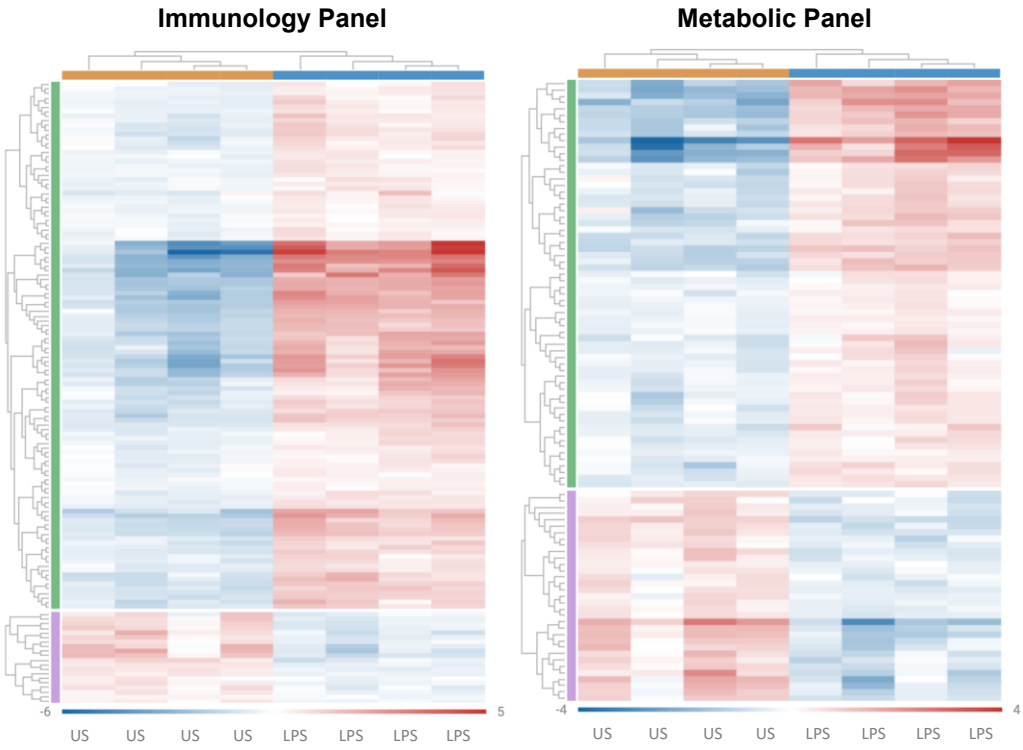
Metabolic pathway

Terms	Significance Score
NF-kB signalling	2.8018
Tryptophan kynurenine metabolism	2.4993
Myc	2.4347
Nucleotide salvage	2.427
Hypoxia	2.3636
MAPK	2.3071
Nucleotide synthesis	2.143
Cytokine & chemokine signalling	2.1289
Transcriptional regulation	2.0874
TLR signalling	2.0719

5.4 Comparison of unstimulated and LPS samples

In the immunology panel, LPS upregulated 116 genes and downregulated 20 genes with a fold change >1.5 or <-1.5 when compared to unstimulated samples (both with an adjusted p-value <0.05) (Figure 5.2). Gene set analysis demonstrated these were mostly genes belonging to pro-inflammatory pathways, particularly type I and II interferon, NLR and TLR signalling pathways. Gene set analysis scores ranged from 5.66 to 9.56 (Table 5.4). This score suggests significant and consistent involvement of these genes in the pathways mentioned. Detailed analysis with Wikipathways, Bioplanet, PANTHER and REACTOME demonstrated significant differential gene expression involving interferon signalling, which has been demonstrated on a Wikipathway illustration in Figure 5.3. Downregulated genes included: CLEC7A, TGFBI, CD14, CLEC4A, FCGR2A/C, FCGR2A, IL16, ITGAX, IL6R, CLEC5A, SYK, CFP, TCF7, CFD, BST1, CSF3R and CD27. When analysed using pathways, these genes did not consistently belong to specific pathways.

Likewise, in the metabolic pathway, 64 genes were upregulated and 33 downregulated with a fold change of >1.5 or <-1.5 and an adjusted p-value of <0.05. Gene set analyses showed that these genes mostly belonged to immune cell functional pathways that were also available on the metabolic panels. These markers varied slightly from the immunology panel so the panels highlighted include antigen presentation, TLR signalling, cytokine and chemokine signalling, MAPK, PI3K-Akt signalling and lysosomal degradation. Those belonging to mitochondrial function included genes important in hypoxia and reactive oxygen responses. When these were analysed further using pathways, no metabolic pathway involving mitochondria was specifically highlighted.



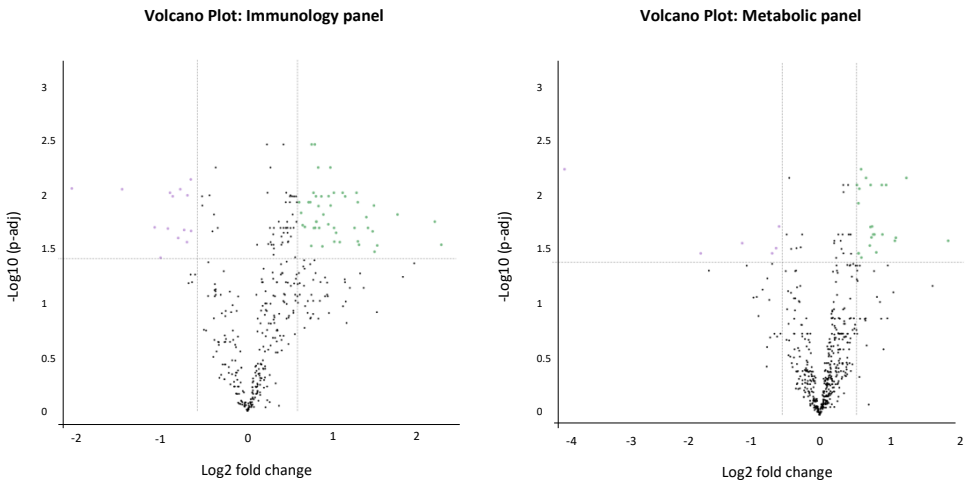


Figure 5.2. Heatmaps and Volcano plots of the immunology and metabolic pathways comparing unstimulated samples with those containing LPS alone.

Heatmaps and Volcano plots for comparison between gene expression profiles of PBMCs from 24 healthy volunteer samples incubated with 10% pooled septic serum or LPS, or control (unstimulated).

Table 5.4. Significance scores following gene set analysis of immunology and metabolic panels comparing unstimulated samples with those containing LPS alone.

Immunology pathway

Terms	Significance Score
Type I Interferon signalling	9.4567
Type II Interferon signalling	6.8128
MHC class I antigen presentation	6.6687
NLR signalling	6.4679
Th1 differentiation	6.0813
TLR signalling	5.9139
Inflammasomes	5.77
Cytokine signalling	5.7089
NF-kB signalling	5.6751
Immunometabolism	5.6688

Metabolic pathway

Terms	Significance Score
Hypoxia	4.4652
NF-kB signalling	4.4428
Antigen presentation	4.1049
TLR signalling	4.0566
Cytokine & chemokine signalling	3.9677
MAPK	3.935
Lysosomal degradation	3.3144
PI3K	3.258
TCR & co-stimulatory signalling	3.1416
Reactive oxygen response	3.0841

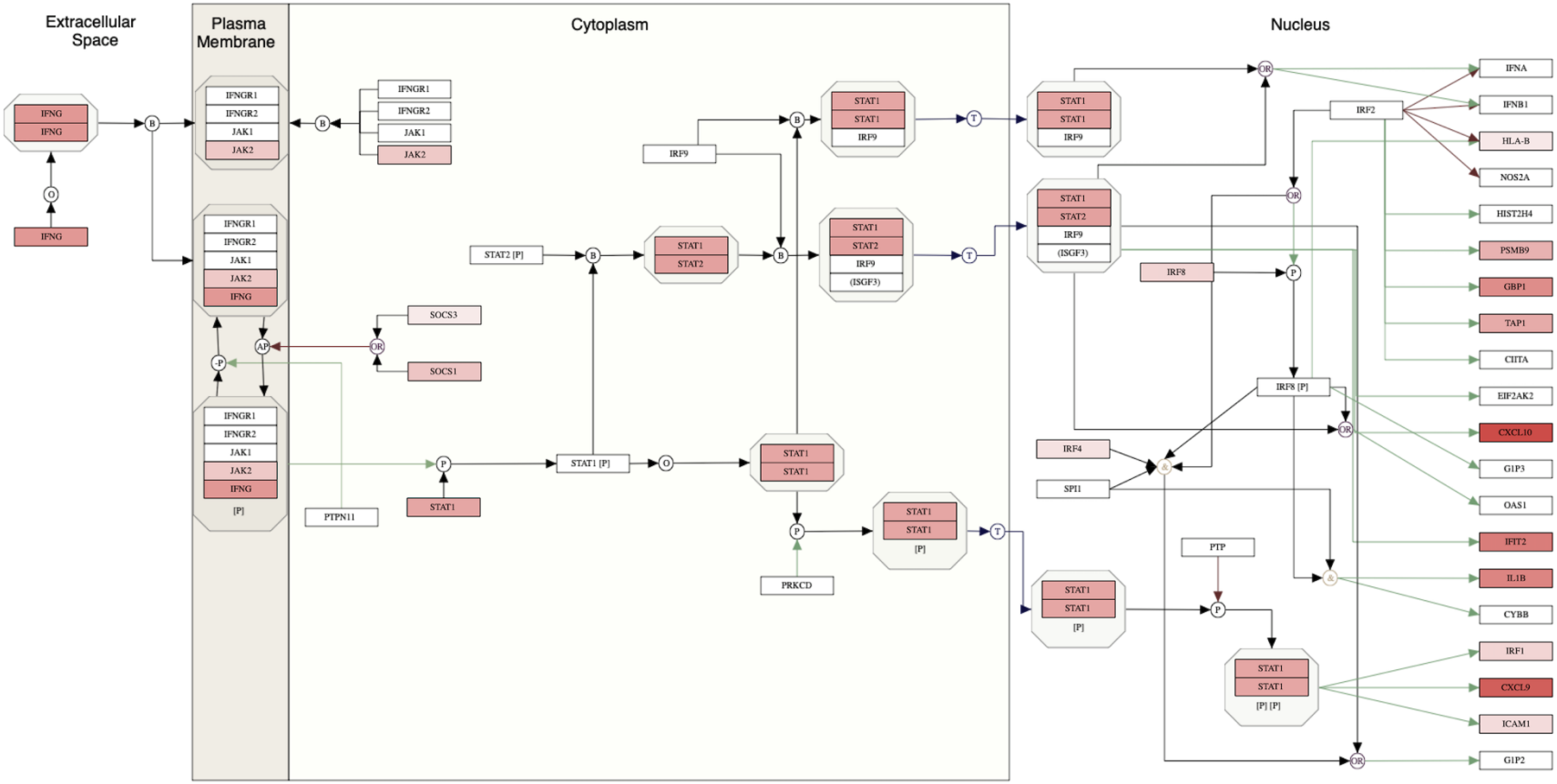


Figure 5.3. Wikipathway of Interferon II signalling.

This illustration demonstrates the upregulated genes in the interferon II signalling pathway when comparing unstimulated samples with LPS containing samples. The gene expression profile was from 4 healthy volunteers where PBMCs were incubated with or without 10% pooled septic serum. Total RNA was then isolated and NanoString immunology and metabolic pathway panels performed.

5.5 Comparison of ciprofloxacin/LPS with LPS alone

When comparing samples containing ciprofloxacin and LPS with those containing LPS, in the immunology panel 26 genes were upregulated (SERPING1, IL1RN, S100A8, CLEC4E, LILRB2, LILRA5, CCRL2, S100A9, CFD, FCGRT, TLR2, CCR5, GZMA, BST1, CCR1, CSF2RB, ADA, CSF3R, CYBB, CCR2, TLR4, IRAK2, LCP2, PECAM1 and LILRB1) and 2 were downregulated (TNFRSF13C, ICOSLG) with a fold change of >1.5 or <-1.5 and an adjusted p-value <0.05 (Figure 5.3). Gene set analysis demonstrated that these genes belonged to the following pathways: immunometabolism, apoptosis, complement system, inflammasomes, oxidative stress, MHC class I antigen presentation, lymphocyte trafficking, haemostasis, TLR signalling and B-cell receptor signalling (Table 5.5). However, the significance score ranged from 1.39 to 1.62, suggesting low biological relevance. This is also demonstrated within the detailed analysis of the pathways using Wikipathways where no specific pathway was significantly involved.

In the metabolic panel, 14 genes were upregulated (BCL2A1, TKT, CAT, S100A12, TLR2, GZMA, KYNU, CSF3R, CYBB, ALDH2, TLR4, ADA, SOD2 and GLRX) and 1 gene downregulated (ACSF3) with the fold change and adjusted p-values mentioned above. GSA showed the following pathways: pentose phosphate pathway, TLR signalling, tryptophan/kynurenine metabolism, NF-kB, nucleotide salvage, mitochondrial respiration, fatty acid synthesis, antigen presentation, glycolysis, lysosomal degradation. The specific scores in this pathway were also low ranging from 1.22 to 1.74. On more detailed review of specific genes using Wikipathways, while there was some differential expression of genes such as CAT and SOD2, these changes were often isolated, with limited evidence of consistent involvement throughout whole pathways. This is illustrated in the example below (Figure 5.4).

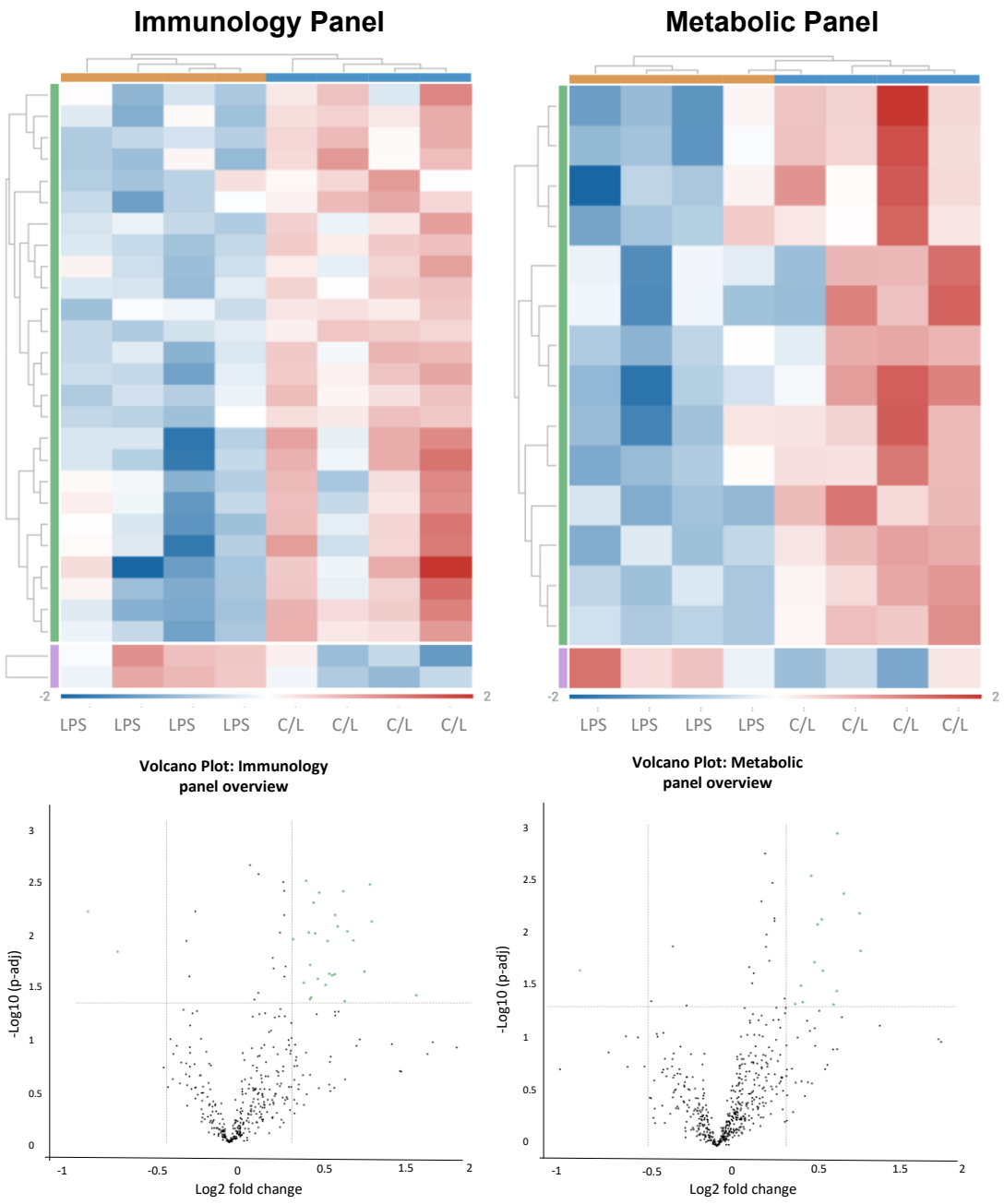


Figure 5.4. Heatmaps and Volcano plots of the immunology and metabolic pathways comparing ciprofloxacin/LPS samples with those containing LPS

The heatmaps and Volcano plots show a comparison in the gene expression profiles of 24 healthy volunteer samples incubated with 10% pooled septic serum, and are comparing samples containing ciprofloxacin/LPS with LPS alone.

Table 5.5. Significance scores following gene set analysis of immunology and metabolic panels comparing ciprofloxacin/LPS samples with those containing LPS alone.

Immunology pathway

Terms	Significance Score
Immunometabolism	1.6229
Apoptosis	1.5538
Complement system	1.548
Inflammasome	1.5449
Oxidative stress	1.5168
MHC class I antigen presentation	1.5123
Lymphocyte trafficking	1.4837
Haemostasis	1.4531
TRL signalling	1.3979
B cell receptor signalling	1.3939

Metabolic pathway

Terms	Significance Score
Pentose Phosphate Pathway	1.736
TLR signalling	1.6046
Tryptophan/Kynurenine metabolism	1.5938
NF-kB signalling	1.3921
Nucleotide salvage	1.3456
Mitochondrial respiration	1.3114
Fatty acid synthesis	1.2985
Antigen presentation	1.2944
Glycolysis	1.2351
Lysosomal degradation	1.2208

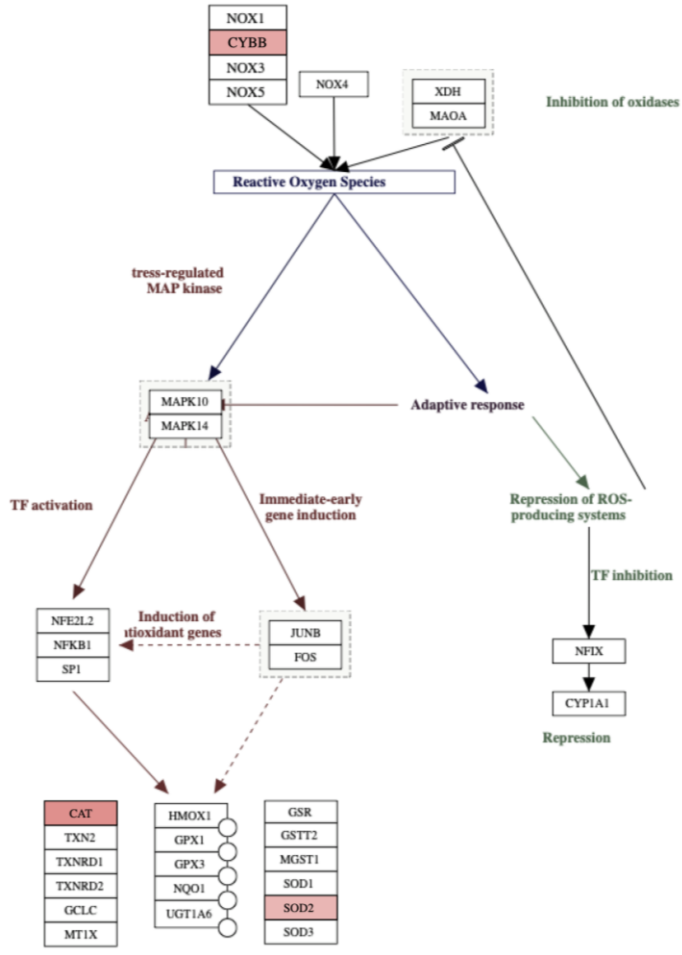
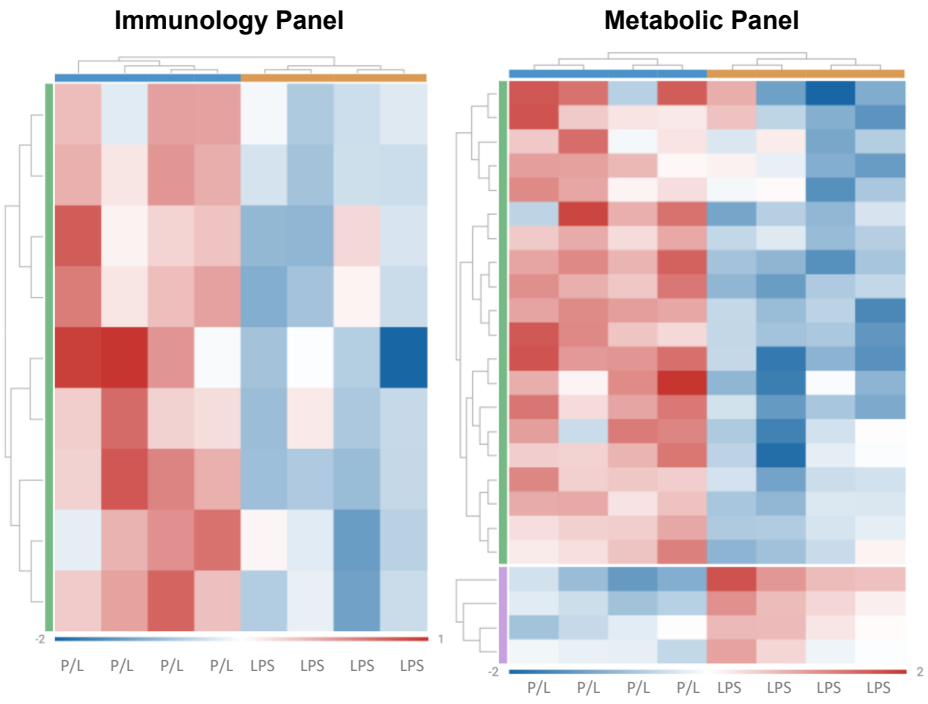


Figure 5.5. Wikipathway of oxidative stress. Source Wikipedia: Oxidative_stress.

5.6 Comparison of propofol/LPS with LPS alone

When comparing samples containing propofol and LPS with LPS alone, in the immunology panel 9 genes were upregulated (CD22, IRF4, PTPN6, CD40LG, GFI1, TGFBR2, FOXP3, STAT6 and SLAMF1) and none downregulated with a fold change of >1.5 or <-1.5 and an adjusted p-value <0.05 (Figure 5.5). Gene set analysis demonstrated these genes belonged to several pathways (Table 5.6). However, significance scores were low and the actual involvement of the genes per pathway was varied and ranged from 1-5 per pathway.

Similarly in the metabolic panel, 20 genes were upregulated (MYC, MYB, ODC1, BUB1, NME1, CD180, SLC16A6, IRF4, SLC3A2, ZNF682, PCK2, PSAT1, MSRB2, PYCR2, LTB, SHMT2, CD84, STAT6, GRAP2 and IDNK) and 4 genes downregulated (SLC16A1, EFNA4, SLC25A1 and OAT) with the fold change and adjusted p-values as above. GSA also demonstrated low relevance in terms of pathways (Table 5.6). For both pathways, more detailed review of specific genes in pathways using various platforms including Wikipathways demonstrated isolated changes in gene expression, with limited evidence of consistent involvement throughout whole pathways.



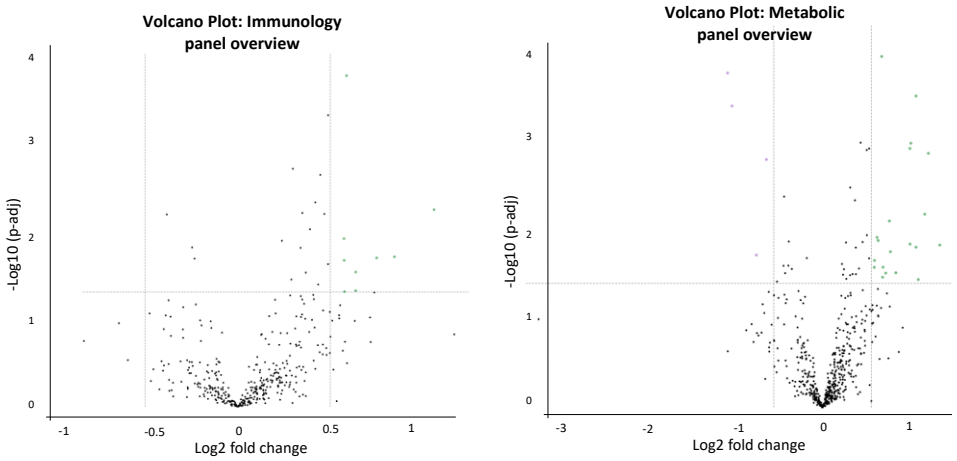


Figure 5.6. Heatmaps and Volcano plots of the immunology and metabolic pathways comparing propofol/LPS samples with those containing LPS.

The heatmaps and Volcano plots show a comparison in the gene expression profiles of 24 healthy volunteer samples incubated with 10% pooled septic serum, and are comparing samples containing propofol/LPS with LPS alone.

Table 5.6. Significance scores following gene set analysis of immunology and metabolic panels comparing propofol/LPS samples with those containing LPS alone.

Immunology pathway

Terms	Significance Score
TGF-b signalling	1.7972
Treg differentiation	1.7897
Th17 differentiation	1.486
Th2 differentiation	1.4208
Autophagy	1.3924
Transcriptional regulation	1.3333
B cell Receptor signalling	1.2834
Lymphocyte trafficking	1.2799
Complement system	1.2292
Lymphocyte activation	1.1957

Metabolic pathway

Terms	Significance Score
Amino acid transporters	2.0743
Vitamin & cofactor metabolism	1.8645
Myc	1.7572
Glutamine metabolism	1.6328
Amino acid synthesis	1.4699
Nucleotide synthesis	1.4111
Glycolysis	1.4078
Pentose Phosphate Pathway	1.3901
Transcriptional regulation	1.2608
PI3K	1.258

5.7 Comparison of NE/LPS with LPS alone

When comparing samples containing NE and LPS with LPS alone, in the immunology panel only 2 genes were upregulated (NT5E and ABL1) and 2 downregulated (CD40LG and LEF1) with a fold change of >1.5 or <-1.5 and an adjusted p-value <0.05. Similarly in the metabolic pathway, only 6 genes were upregulated (NT5E, ABL1, MAT2A, ASL, PIK3R1 and PGD) and 3 downregulated (RPTOR, MAPKAP1 and PIK3CD) (Figure 5.6). The results of the gene set analysis are shown in Table 5.7; significance scores and involvement in pathways using detailed analysis were however relatively low.

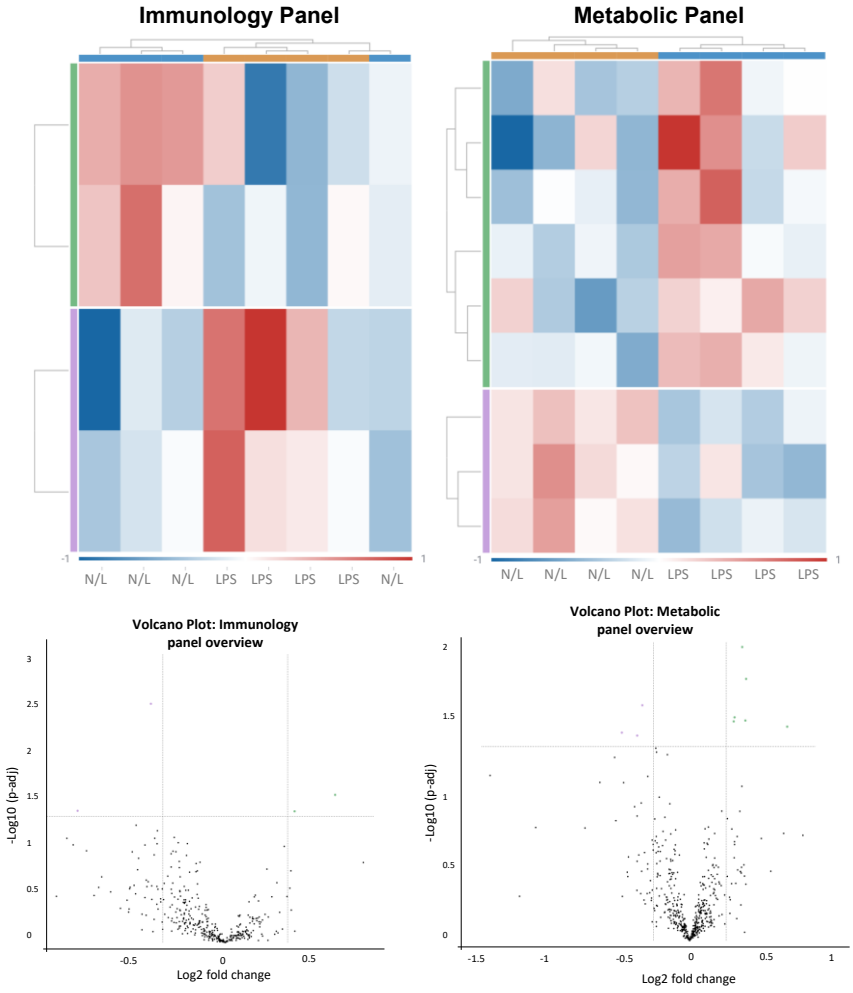


Figure 5.7. Heatmaps and Volcano plots of the immunology and metabolic pathways comparing NE/LPS samples with those containing LPS.

The heatmaps and Volcano plots show a comparison in the gene expression profiles of 24 healthy volunteer samples incubated with 10% pooled septic serum, and are comparing samples containing NE/LPS with LPS alone.

Table 5.7. Significance scores following gene set analysis of immunology and metabolic panels comparing NE/LPS samples with those containing LPS alone.

Immunology pathway

Terms	Significance Score
Transcriptional regulation	0.966
Autophagy	0.9369
Immunometabolism	0.8928
Treg differentiation	0.8472
B cell receptor signalling	0.8346
T cell receptor signalling	0.8268
TGF-b signalling	0.8194
Th1 differentiation	0.8087
Lymphocyte trafficking	0.8072
Lymphocyte activation	0.7973

Metabolic pathway

Terms	Significance Score
Arginine metabolism	1.0938
IDH1/2 activity	1.0793
Epigenetic regulation	1.0777
Autophagy	1.0118
AMPK	0.9746
mTOR	0.9411
PI3K	0.8882
DNA damage repair	0.8527
Vitamin & cofactor metabolism	0.8492
Endocytosis	0.8372

5.8 Comparison of mix of drugs/LPS with LPS alone

When comparing samples containing a mix of the 3 drugs and LPS versus LPS alone, 3 genes in the immunology panel were downregulated (RUNX1, NFATC3, ATG12) with a fold change of <-1.5 and an adjusted p-value <0.05. Similarly in the metabolic pathway, 8 genes were upregulated (ODC1, TLR2, FNIP2, SLC3A2, PDHA1, PGM2, GPX4, PGD) and 2 downregulated (GSK3B, RUNX1) (Figure 5.7). The results of the gene set analysis are shown below but, as mentioned above, the significance scores and involvement of any specific pathway using detailed analysis is likely to be low.

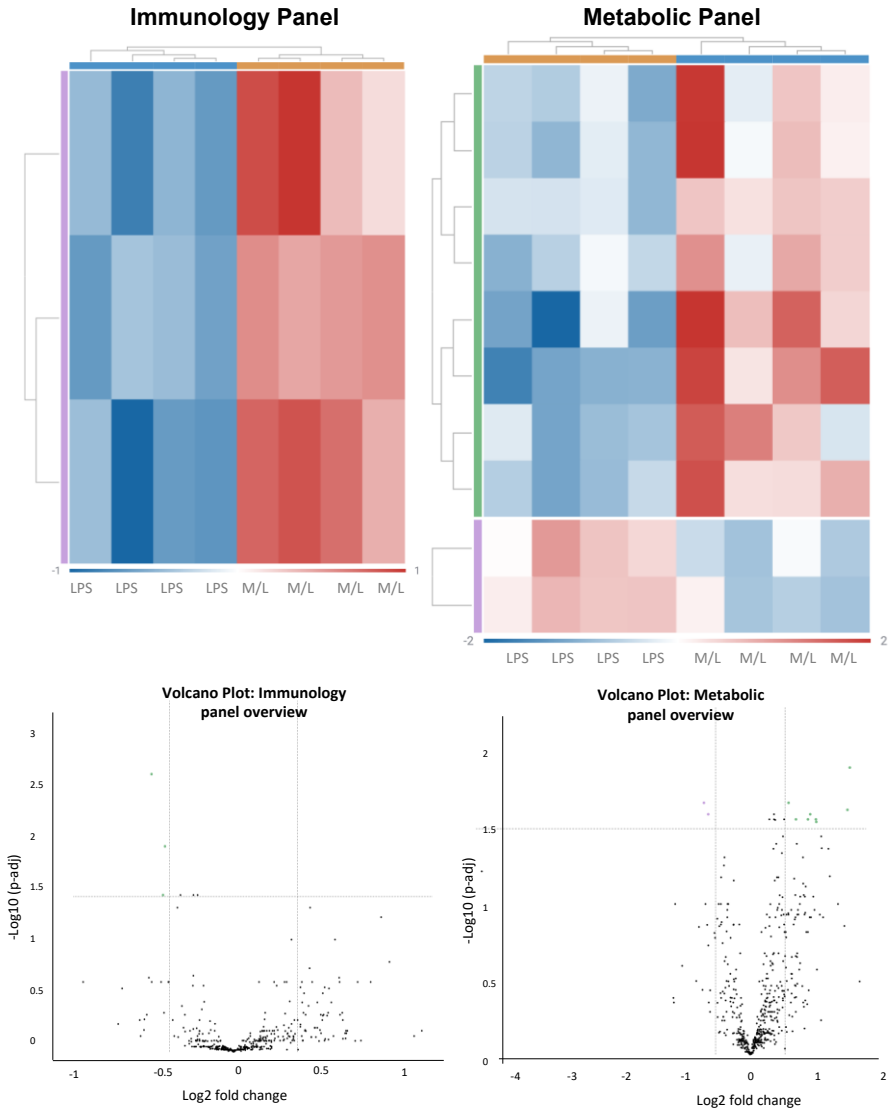


Figure 5.8. Heatmaps and Volcano plots of the immunology and metabolic pathways comparing mix of drugs/LPS samples with those containing LPS.

The heatmaps and Volcano plots show a comparison in the gene expression profiles of 24 healthy volunteer samples incubated with 10% pooled septic serum, and are comparing samples containing mix of drugs/LPS with LPS alone.

Table 5.8. Significance scores following gene set analysis of immunology and metabolic panels comparing mix of drugs/LPS samples with those containing LPS alone.

Immunology pathway

Terms	Significance Score
Autophagy	2.4464
Immunometabolism	1.7676
Transcriptional regulation	1.6778
B cell Receptor signalling	1.6101
Inflammasomes	1.6022
Complement system	1.5959
Phagocytosis and degradation	1.4629
Lymphocyte trafficking	1.448
Hemostasis	1.4453
MHC class I antigen presentation	1.4267

Metabolic pathway

Terms	Significance Score
Pentose Phosphate Pathway	2.5008
Myc	2.0483
Lysosomal degradation	2.044
Glycolysis	2.0256
Epigenetic regulation	2.0146
KEAP1/NRF2 pathway	1.9273
Transcriptional regulation	1.906
Tryptophan/Kynurenine metabolism	1.8564
Nucleotide synthesis	1.8323
Amino acid transporters	1.8285

5.9 Summary of Nanostring results

These results demonstrate that LPS upregulates pro-inflammatory pathways as expected when compared to unstimulated samples. However, little significant change in differential gene expression in either the immunology or metabolic pathway panels was seen when samples containing ciprofloxacin, propofol and/or NE in the presence of LPS were compared to LPS alone. The significance of these findings will be discussed in Chapter 6.

In addition, I repeated the above analyses using a fold change of 1.5 and a p value of <0.1 as an attempt to investigate whether Type II error due to low sample size may be affecting my results. This demonstrated a higher overall number of differentially expressed genes, however the global significance scores were the same suggesting no specific pathways were substantially affected.

Chapter 6 Discussion

6.1 Introduction

Sepsis is defined as life-threatening organ dysfunction caused by a dysregulated host response to infection (20). This is associated with both exaggerated pro- and anti-inflammatory responses. The latter may however persist for months, resulting in immunosuppression which increases the risk of nosocomial infections and mortality (84, 485, 486). Additional factors contributing to mortality are patient characteristics such as age and underlying co-morbidities (27), pathogen characteristics including type of microorganism, virulence and resistance to treatment (29) and, most importantly, the response to infection and treatment (e.g. organ dysfunction) (28).

Sepsis-induced immunosuppression is common and results from immune reprogramming that renders immune cells less capable of eradicating unwanted microorganisms. This includes increased apoptosis, impaired migration (60, 69, 70), and phagocytosis of innate immune cells (65). Antigen presentation and activation of adaptive immune cells are also impaired (72-74). There is, in addition, an increase in apoptosis of adaptive cells and impaired effector functions such as proliferation, antibody production and cytotoxicity. Both arms of the immune system have an increased tendency towards an anti-inflammatory phenotype (76-78). Collectively, these reduce the ability of the immune cells to fight off the initial pathogens and increase the risk of secondary infections with additional pathogens. The underlying mechanism is still unclear despite decades of research.

Mitochondrial dysfunction has been implicated in organ dysfunction during sepsis and this includes immune cell dysfunction (487-489). Bioenergetic failure induced by various sepsis-related factors (e.g. tissue hypoxia, excess ROS production, reduced antioxidant capacity and suppressed mitochondrial biogenesis) deplete the energy substrates necessary to fuel normal immune cell function and protect them from oxidative damage. There is also evidence

to suggest that mitochondrial damage, with release of cardiolipin and mtDNA, can perpetuate an inflammatory response increasing the severity of sepsis and risk of mortality (115-117).

Crucially, mitochondrial and/or cellular damage may result as an unintended target of therapies commonly used in intensive care, including antibiotics, catecholamines and sedatives. This iatrogenic contribution towards mitochondrial dysfunction and immunosuppression is often largely overlooked. Multiple *in-vitro* and *ex-vivo* studies have shown that these agents have some immunosuppressant function and can affect mitochondrial function.

Antibiotics, in particular quinolones such as ciprofloxacin, have been shown to suppress cytokines and phagocytosis at high concentrations in the context of sepsis (11, 293-307), and to downregulate adaptive immune effector functions such as proliferation and cytotoxicity (314, 315). The findings are however not consistent (312, 313). Quinolones may also target mitochondrial DNA replication and, as such, can affect electron transport chain activity and mitochondrial membrane potential (MMP) (318, 319).

Similarly, catecholamines also have immunomodulatory activities in sepsis, mostly mediated by actions on the β_2-adrenoceptor. This includes reduced cytokine production (359-365), inhibition of chemotaxis, phagocytosis and killing capacity of immune cells (366-369), and suppressed T-cell proliferation (354, 370). On the other hand, there are reports of upregulation of pro-inflammatory activity, mediated by α-adrenoreceptors (375-377). Catecholamines additionally affect mitochondrial structure and function e.g. by promoting energy substrate availability and O_2 consumption for OxPHOS, thereby improving respiratory efficiency and reducing mitochondrial swelling and damage (382, 389-391). There are also conflicting studies that suggest catecholamines impair ATP synthase activity (155), reduce O_2 consumption (392) and increase mitochondrial uncoupling (136).

Propofol also has immune and mitochondrial effects, especially at high concentrations (401, 402). Propofol can increase lymphocyte apoptosis (408), reduce proliferation (403) and reduce cytokine production (409-412). As above, there are also conflicting studies regarding cytokine production (413, 414) and phagocytosis with some suggesting no change (418-421) while others suggest a propofol-induced reduction in phagocytosis (422-432). Propofol may also affect mitochondrial ROS production (436) and MMP (437). A few *in-vivo* studies demonstrate an impact of propofol on mitochondrial function as potentially mediating propofol infusion syndrome (458-460).

Apart from the inconsistency described above, these drugs are generally studied in isolation; data on any synergistic effects are missing. This, however, is the reality of clinical practice and merits further investigation, particularly in immunosuppressed septic patients.

The aim of this project was to investigate the functional and mitochondrial effects on immune cells of three commonly used ICU drugs (ciprofloxacin, propofol and norepinephrine), both individually and in combination. The specific immune functions tested were cytokine production, phagocytosis and monocyte HLA-DR expression as a surrogate of cell activation status. The mitochondrial functions tested were mitochondrial ROS production, MMP and oxygen consumption. These were investigated using a range of techniques including ELISA, flow cytometry, seahorse respirometry with extracellular flux assays and Nanostring technology. Importantly, I incubated the cells with drug concentrations representative of those measured *ex-vivo* in patients, which has not often been the case with previous studies using supra-therapeutic doses.

This chapter summaries the principal findings and discusses the potential implications, main strengths and limitations of the research, and future work.

6.2 Summary and implications of immune studies

Chapter 3 described how 24 hours' incubation with LPS stimulated PBMCs to increase release of both pro- and anti-inflammatory cytokines (TNF-α, IL-6, IL-10) compared to unstimulated samples. Production was most marked in PBMCs taken from septic ICU patients followed by healthy volunteers co-incubated with septic serum. The results in PBMCs from healthy volunteers were similar to previous studies which reported TNF-α release of 1960 ± 1365 pg/ml at 4 hours and 790 pg/ml at 24 hours, IL-6 production of 460 ± 270 pg/ml and IL-10 production of 60 ± 30 pg/ml after 24 hours incubation with LPS *P. Aeruginosa* (10ng/ml) (490). A review summarising papers investigating the cytokine release after a 2 ng/kg infusion of LPS in healthy volunteers, showed an increase in TNF-α (peak at 7000 pg/ml at 1-2 hours), IL-6 (peak at under 30 000 pg/ml at 2-3 hours) and IL-10 levels (peak at 200 pg/ml at 2-3 hours); these returned to negligible levels at 24 hours(491). The studies on the production of cytokines in infected and septic patients have shown variable results over a 48-hour period (492). One study in critically ill surgical patients with a documented infection showed that TNF-α levels were <100 pg/ml in most of the patients except during septic shock when they reached 735.9 ± 873 pg/ml for a few hours. IL-6 levels on the other hand were higher in the presence of a bacterial infection with the range in most patients being 10-1000 pg/ml and extreme peak levels reaching 500,000 pg/ml (493). Several other reports have also suggested that levels of IL-6, IL-10 and TNF-α are higher in patients with septic shock compared to severe sepsis where they may not be significantly raised and there may be an association between changes in these cytokines and survival (494).

My results showed an augmented cytokine release in PBMCs derived from septic patients; this may be because these PBMCs have been primed with septic stimuli prior to LPS exposure. In addition, the marked activation seen in naïve immune cells from healthy volunteers exposed to septic serum indicates the presence of activating circulating factors such as cytokines, reactive species (e.g. nitric oxide bound to albumin and haemoglobin) and bacterial

components that can behave as PAMPs or DAMPs, also augmenting the inflammatory response (495). Septic patients have additional factors that contribute to an augmented or suppressed response; these include: age, co-morbidities and prior drug exposure. A large cytokine response – both pro-and anti-inflammatory – can co-exist with declining immune function in septic animals (496-498). This suggests cytokine production may not be informative of immune cell function in isolation and should be studied with other markers of functionality such as phagocytosis, migration, T-cell activation, proliferation and cytotoxicity.

All drug/LPS combinations (ciprofloxacin, propofol, NE and the mix of all three drugs) variably reduced IL-6 and TNF-α production in PBMCs from all four participant groups (healthy volunteers ± septic serum, ED patients, septic ICU patients) when compared to LPS alone. However, this was only statistically significant for ciprofloxacin/LPS and the mix of the three drugs with LPS. There was no cumulative effect seen with the drug combination. Ciprofloxacin has been previously reported to reduce IL-6 and TNF-α and increase IL-10 production, driven by activation of β_2-adrenoceptor-mediated cAMP-PKA signal transduction (359-365). Ciprofloxacin downregulates cAMP, a potent modulator of immune-triggered cytokine production (499). As neither propofol nor NE significantly affected cytokine production except in healthy volunteers with septic serum, ciprofloxacin may be the predominant driver of depressed cytokine production. While NE and propofol have previously been shown to reduce cytokine production (413, 414, 500, 501), reports are conflicting (375, 376, 409-412), so there was no previous clear final consensus. The specific cytokines induced by these drugs is difficult to report due to differences in methodology (e.g. type and doses of septic stimuli used, cell numbers used in the assays, timing of incubation and dose differences in the various drugs used). An example is a study of PBMCs incubated with LPS (10 μg/ml) and Ciprofloxacin (25-100 μg/ml) for 24 hours, where Ciprofloxacin was shown to reduce TNF-α production by 3-6 ng/10^3 cells (293). Another in-vivo study in mice receiving a peritoneal injection of LPS (5mg/kg) and Ciprofloxacin (50-100mg/kg) demonstrated a reduction in TNF-α to 63.6% of control ($P < 0.05$)

and serum IL-1β levels to 60% of control (not significant) and increased IL-6 to 151.3% of control. These differences are difficult to interpret and further investigations are required to clarify these findings.

Focussing on the participant groups, PBMCs taken from healthy volunteers co-incubated with septic serum showed a significant reduction in IL-6 and TNF-α production with all drug/LPS combinations, and this exceeded the response seen in PBMCs taken from septic patients. This may relate to a pre-existing degree of response downregulation related to the prior exposure to DAMPs, PAMPs, antibiotics and sedatives and raised levels of catecholamines, both exogenous and endogenous.

Of note, the rise in IL-10 production induced by LPS was suppressed by co-incubation with each of the three drugs, and this occurred in PBMCs from all participant groups. Although a consistent finding, it only reached statistical significance in PBMCs from septic ICU patients; this may however represent a Type II error as sample sizes were relatively small. This reduction may be mediated by downregulation of ERK1/2, p38 and NF-κB signalling, which are known to increase IL-10 (502). β_2- receptor activation can also reduce IL-10 (503). IL-10 production is raised in septic patients, and is associated with worse outcomes (504). It was however surprising that IL-10 levels were not higher in the septic ICU patient group.

Similarly, when the effect of these drugs was tested on PBMC phagocytosis, there was a marked reduction seen across all drugs studied compared to unstimulated and LPS controls. There was also no additive effect from the mixture of drugs compared to individual drugs. Previous studies of ciprofloxacin on phagocytosis report either no effect, except at very high concentrations (308-311), or that it may even enhance phagocytosis (312, 313). There was no clear mechanism stated for either. Catecholamines inhibit phagocytosis by β_2-adrenoceptor activation (366-369), whereas propofol also shows conflicting results on phagocytosis with either no effect (418-421) or inhibition via $GABA_A$ receptor activation, mitochondrial dysfunction, or through an effect on cell membranes (422-432).

There were statistically significant differences in phagocytosis across the different participant groups, though these were not present across all experimental conditions. Changes in phagocytosis were most clearly demonstrated at 6 hours in the absence of LPS. Compared to healthy volunteers, phagocytosis increased in PBMCs taken from healthy volunteers co-incubated with septic serum and ED patients whereas PBMCs taken from septic ICU patients tended towards a reduction. This lends supports to the earlier postulate that the immune response in healthy volunteers and ED patients is more appropriate to a septic stimulus whereas cells taken from septic ICU patients may be more deranged or exhausted (65).

HLA-DR is a component of the MHC class II system which plays an important role in antigen presentation and is often used as a marker of monocyte activation state. Monocyte HLA-DR expression reduces in sepsis, and this is associated with an increased risk of secondary infection and mortality (81, 82). I found statistically significant reductions in monocyte HLA-DR across all participant groups compared to healthy volunteers, indicating that a septic stimulus can cause a relatively early impairment of monocyte function and induce anergy. The mean HLA-DR MFI from my results were 15080.5 (SD 1589.94) in healthy monocytes, 2805 (SD 1402.02) in healthy volunteers with septic serum, 2001 (SD 503.97) in ICU patients and 3572 (SD 421.50) in ED patients. My results are difficult to compare to previously reported studies as these are either reported as MFI of HLA-DR, mean HLA-DR per monocyte, number of HLA-DR positive monocytes, or antibodies per cell. The comparability of these different techniques is uncertain. In addition, there is no agreed consensus regarding the threshold at which antibodies per cell offer prognostic value in sepsis (505, 506). One such study showed that PBMCs expressing HLA-DR in healthy donors >85%, the mean HLA-DR per monocyte, expressed as MFI, was 52.3 ± 20 in healthy controls. This was reduced significantly in patients with severe sepsis ($P < 0.01$) (476).

I could not find any impact of any drug/LPS combination on HLA-DR expression. Further investigation is required to discern the role of HLA-DR in

acute illness and the effects of the different drug groups on HLA-DR expression.

Collectively, I found that drugs commonly used in ICU variably affect immune cell function, however effects varied between drug groups and participant groups. Apart from methodological factors previously described, other causes of potential heterogeneity include:

1. Differences in participant groups from which PBMCs were derived. PBMCs were obtained from healthy volunteers, patients from emergency departments (ED) with suspected sepsis, and from ICU with sepsis. Standardisation of samples is clearly important and was applied as far as possible, however, this has to be balanced against pragmatic considerations such as availability of suitable patients; ease, volume and timing of blood sampling; processing times of samples; and ethical considerations (e.g. consent). Confounders include variations in age; the onset, duration and severity of sepsis; co-morbidities, chronic medications, the infecting pathogen and site of infection; and the clinical management received (24, 25, 27, 507). These will influence results, particularly when comparisons are made across groups. In order to reduce the effect of these potential confounders, each group was compared to internal controls. However, another critical difference between the groups is the prior exposure to my drugs of interest; healthy volunteers and ED patients are unlikely to have received these, while septic ICU patients would have had exposure to at least one or more of these drugs.

2. Heterogenous cell populations. The study of PBMCs provides extremely useful insights into overall immune response and function. This is invaluable when assessing how the broader immune system responds to a septic stimulus. However, the lack of distinction between certain cell types may mean that some synergistic or opposing effector functions may be either exaggerated or obscured. Cell heterogeneity however carries important methodological implications, for example in the potential of macrophages to remain adherent to the bottom of cell culture plates, and therefore to be omitted when monocytes are harvested for analysis if they are not actively collected.

The obvious solution to the issue of cell heterogeneity would be to repeat the above experiments with individually sorted cell populations, for example using MACS beads. This could be done for a subset of experiments using FACS filters during the analysis stage, however the additional required fluorophores are likely to make already overcrowded mitochondrial panels difficult to both compensate and analyse reliably. A further case for this could be made if more significant differences had been found with drug treatments, as it would then be important to identify which cell subsets were most responsible. However, given the findings above and the corresponding financial and time restrictions, performing an in-depth analysis of all cell subsets was not judged to be an appropriate use of limited resources.

2. Sample size. My experiments were carried out on relatively small sample sizes that may introduce Type II error if the experimental power is insufficient to detect real biological differences. As results from individual subjects are variable, larger sample sizes may be required to demonstrate additional biological differences and reduce the effect of inter-individual variability where results are inconclusive. While increasing numbers would be ideal, there were often significant practical considerations such as project disruption due to COVID-19. However, despite some experiments being performed with limited numbers, clear trends were often seen in the data, allowing conclusions to be drawn where appropriate.

3. Dose, timing and source of the septic stimulus. The primary septic stimulus used in the experiments was *Klebsiella pneumoniae* LPS at a concentration of 100 ng/ml. This dose was chosen following literature review (508, 509) and initial optimisation experiments. Lipopolysaccharide was used as it is a cell wall component of Gram-negative bacteria. It consists of three structural domains: lipid A, the core oligosaccharide, and the O antigen. Of these lipid A determines the virulence. In *E.coli* and *Salmonella*-derived LPS, the lipid A is hexacylated and bisphosphorylated which makes it highly immunogenic (510). I used *K. pneumoniae* derived LPS as it was readily available, it worked reliably and when compared to *E. coli*-derived LPS,

mitochondrial results were similar. I did not compare immunogenicity of different types of LPS in this thesis.

I used fresh PBMCs and incubated them concurrently with LPS and drugs of interest. I could also have primed PBMCs with LPS for 24-72 hours prior to the addition of drugs, so that the cells were 'stressed' and resembled septic cells more accurately. This was not undertaken as PBMCs change their phenotype and function if in cell culture for prolonged periods; this may also confound results and make data interpretation difficult. There is also the additional challenge of choosing an appropriate stimulus to prime the cells. *In-vivo* studies have shown that organisms causing the first and second infection can influence outcome (511).

Finally, although LPS is highly immunogenic, in a cell culture environment it requires co-factors present in fetal bovine serum (FBS) to induce a robust response (495). FBS consists of carbohydrates, lipids, proteins as well as electrolytes, hormones and enzymes that are required for cell growth. The FBS concentration can influence LPS-induced stimulation of immune cells; by convention a concentration of 10% is used in most studies (495) as was the case in my studies. I also used 10% pooled septic serum which provides an additional septic stimulus potentiating immune stimulation. It is worth noting that both a single exposure to a sterile stimulus in the form of LPS or the additional presence of septic serum in culture does not resemble the sustained exposure to multiple inflammatory stimuli that occurs during sepsis.

4. Dose, timing, formulation of drugs. Ciprofloxacin, propofol and NE were chosen as these are commonly used in clinical practice, are readily available, and have a supportive literature to suggest effects on immune cell function and mitochondrial activity. Doses were chosen following a literature review and initial optimisation experiments and reflect clinically relevant doses. The formulations were based on clinical availability and use. Final concentrations after 6- or 24-hours' incubation are expected to be similar to the starting concentration as no enzymes were identified in cell culture that could metabolise the drugs. The only exception was NE where some metabolism

may have been carried out by mitochondrial monoamine oxidases. This is not well characterised in the literature so the exact impact is unknown. Future work could involve accurate measurement of drug concentrations using methods such as High-Performance Liquid Chromatography (HPLC) or mass spectrometry.

5. Immune parameters studied. The immune parameters studied were cytokine production, phagocytosis and HLA-DR expression as these are informative of both innate and adaptive functions, and are well reported in the literature. However, future work may include studying other immune functions including cell migration, antigen presentation, T-cell activation, T-cell cytotoxicity and B-cell antibody production. In addition, immune cell fractions can be studied in isolation rather than together in cell culture to outline specific functions carried out by subsets of immune cells.

6. Sensitivity and specificity of ELISA kits used. Although kits are extensively validated, there are still batch variations which contribute to differences in cytokines measured. This was mitigated by producing appropriate reference standard curves with each experiment and demonstrating differences between positive and negative controls (e.g. with LPS).

7. Timing of ELISA assays. Cytokines were only measured at 24 hours. This provided a cumulative measure of the three cytokines as these are not known to break down in cell culture. IL-6 and TNF-α tend to rise after 2-4 hours and IL-10 after 8 hours following LPS stimulation (490). Future work may involve measuring a larger number of cytokines that are more specific for each cell subset. Cytokines could also be measured after longer incubation periods though, as mentioned previously, the cell phenotype may change over time.

8. Effects of cell culture environment on fluorophores in flow cytometry. Several cell culture components such as phenol red, glutamate, high glucose doses and FBS concentration may influence cell function and/or interact with fluorophores thus confounding results (512-514). I used media that did not

contain phenol red as this can falsely increase or decrease the MFI of fluorophores. Appropriate positive and negative controls were also undertaken during each experiment to mitigate this.

9. Analysis using FlowJo software. There are several known methodological approaches to analysing data using FlowJo which can lead to significant user-dependent variations and inaccuracies (515). This was mitigated by firstly ensuring that appropriate quality controls measures were undertaken during data acquisition. Gating strategies were kept consistent between experimental conditions and appropriate compensation settings were applied. Despite this, inaccuracies may have arisen as experimental conditions led to some shift in cell populations requiring the gating to be adapted. Finally, statistical tests were also kept constant for each experimental condition.

10. Effect of log_{10} transformation of data. On advice from a UCL statistician (Dr Gareth Ambler), I used log_{10} transformation to reduce variability in data. As variability was present between different participant groups, this made comparisons difficult (516). Log_{10} transformation provides the additional benefit of reducing skewness of data so parametric tests can be used for analysis. A potential disadvantage is interpretation of log transformed factors.

6.3 Summary and implications of mitochondrial results

The aim of these experiments was to determine the effects of commonly used drugs in ICU on immune cell functionality and to determine whether changes in mitochondrial function could contribute.

The results in chapter 4 showed that MMP and mtROS production increase significantly at 24 hours compared to 6 hours, in both monocyte and lymphocyte populations. This may be due to mitochondrial stress, increased cellular signalling via mtROS and increased ATP demand to maintain OxPHOS and other homeostatic mechanisms. However, as MMP and mtROS

are positively correlated, the simultaneous increase in both parameters may be physiologically explained as below. Reduced NADH and $FADH_2$ from the Krebs cycle transfer their electrons to complexes I and II respectively. These electrons are transferred via several redox centres along the ETC to complex IV where they react with oxygen to form water. Simultaneously, at complexes I, III and IV, the electron motive force is converted to a proton motive force which drives H^+ into the intermembrane space against their concentration gradient. This generates a transmembrane potential which is utilised by complex V to produce ATP. mtROS production increases when either the flow of electrons slows or the concentration of O_2 increases (517, 518). The flow of electrons slows when there is increased ATP demand with reduced substrate or O_2 availability, or when ETC complex activity becomes impaired (519). In the former instance, the increase in ATP demand drives more H^+ into the intermembrane space, however due to lack of substrate, no ATP is generated and the MMP stays high, while the slowing of electrons increases mtROS.

When studying the effect of different participant groups on MMP and mtROS, both showed a significant change; MMP in PBMCs from healthy volunteers with septic serum and septic ICU patients was reduced compared to healthy volunteers. This was associated with an increase in mtROS in PBMCs from all participant groups compared to healthy volunteers. These significant findings did not consistently occur across every experimental condition e.g. in PBMCs from healthy volunteers co-incubated with septic serum and in septic ICU patients compared to healthy volunteers, mtROS increased at 6 hours in the presence and absence of LPS, but not at 24 hours. These may suggest that immune cells upregulate mtROS production and thus proton uncoupling and reducing ATP production. This may be necessary for immune cell activation and cell signalling while limiting cellular damage. These may also indicate ETC impairment (520). A potential mechanism in sepsis may be increase in nitric oxide which inhibits mitochondrial respiration by blocking the ETC (112). Interestingly, participant groups with exposure to septic serum that demonstrated marked increase in cytokine production and a decrease in phagocytosis and HLA-DR expression, also showed an increase in mtROS and a reduction in MMP. This is consistent with current studies of sepsis.

The drugs/LPS combinations showed some significant changes in MMP and mtROS, however the specific nature of this effect varied across experimental conditions and participant groups, with no uniform findings for any individual drug. This made interpretation of data difficult. These findings were also supported by the mito stress test, which showed no statistically significant differences in basal respiration, proton leak, maximal respiration, spare respiratory capacity, non-mitochondrial respiration and ATP production between unstimulated controls and drug groups, and between LPS controls and drug/LPS combinations. There was also no additive effect from the mixture of drugs for any of these parameters. All samples generated the appropriate responses to mito stress related drugs suggesting that the above findings were accurate.

Potential reasons for the lack of consistent changes in mitochondrial function may be related to the transiency of mitochondrial changes and the robustness of relevant compensatory mechanisms (such as anti-oxidant availability, and changes in ATP in order to maintain baseline mitochondrial functions) which should be studied simultaneously to understand the fuller picture. These results may also be related to the small sample size leading to Type II error or specific methodological factors, which are discussed below:

1. Cell stimulation- Although initial optimisation experiments included stimulation of PBMCs with LPS from *K. pneumoniae* at concentration of 10 ng/ml to 1 µg/ml, and a time course from 30 minutes to 24 hours, neither dose nor duration of incubation demonstrated any statistically significant differences in MMP or mtROS in PBMCs from healthy individuals. However, when PBMCs from different participant groups were compared to healthy volunteers, there were clear differences in MMP and mtROS. This may suggest that LPS *alone* at a final dose of 100ng/ml may not be sufficient to demonstrate mitochondrial changes, however the addition of septic serum may help unmask these changes. Previous studies in endothelial cells (521), fibroblasts (522) and in muscle cells (154) have shown that human septic serum reduces mitochondrial respiration, complex activity and O_2 consumption, and increase ROS production. In PBMCs, septic serum increased O_2 consumption by

uncoupling from ATP production (135). Future work may require characterisation of the effect of septic serum on mitochondria of immune cells.

2. Fluorophore properties in flow cytometry- Tetramethyl-rhodamine methyl ester (TMRM) and Mitosox Red are commonly used fluorophores for the investigation of MMP and mtROS. However, there are major recognised concerns associated with their use (523-525). Firstly, it is not feasible to outline the kinetics of either of these fluorophores using flow cytometry as both are affected by mitochondrial membrane potential and density. I attempted to investigate the effect of mitochondrial density in this context using Mitotracker Green, as suggested by the literature, however in my experiments this fluorophore was also affected by mitochondrial membrane potential so did not accurately indicate mitochondrial density (526). Additionally, it is uncertain how rapidly mitochondrial density would change under septic conditions.

TMRM, which is a lipophilic, cationic, fluorescent dye that accumulates in the negatively charged mitochondrial membrane according to the MMP (more negative MMP accumulates more dye and leads to higher MFI). TMRM can be used in non-quenching (0.5 to 30 nM) and quenching (>50 nM to 1 μM) modes (171). In non-quenching mode, TMRM fluorescence is directly proportional to dye concentration allowing estimation of the MMP. In quenching mode, TMRM accumulates in the mitochondrial membrane and forms aggregates which do not linearly relate to MMP. For accuracy I used TMRM in the non-quenching mode and ensured each experiment had appropriate controls.

Mitosox Red is a lipophilic, cationic fluorescent dye derived from hydroethidium. Mitosox has a triphenylphosphonium group that selectively targets mitochondria in which it accumulates. Within the mitochondria, it is rapidly oxidised by superoxide to firstly form Mito-HE free radical. and then 2-OH Mito E^+. The latter increases its fluorescence in proportion to the superoxide concentration. Mito-HE can undergo non-specific oxidation, the result of which also produces an increase in fluorescence. 2-OH Mito E^+ also intercalates with nucleic acids. Mitosox accumulated intracellularly if concentrations >2 μM were used; this reduced mitochondrial superoxide

specificity as cellular superoxides can also oxidise it (527). This is also known to cause cellular toxicity (523). Mitosox also undergoes non-specific oxidation to intermediates that have the same emission spectra on flow cytometry as superoxide specific products (528). Thus, mtROS cannot be accurately determined with this method. Unfortunately, there are limited mtROS-specific alternative fluorophores, so its use continues in most laboratories.

3. MFI from flow cytometry were not normalised against mitochondrial quantity/density- It was assumed that all samples from a particular individual consisted of similar quantity of mitochondria as the number of cells in each experimental condition were equal. However, there may be variations in mitochondrial density between participant groups that may affect results. I attempted to measure mitochondrial density using mitotracker green with flow cytometry, however found inaccuracies arising from the effect of MMP on the mitotracker fluorophore, as a result I no longer included this in the protocol (529). There are additional methods that can be used including mass spectrometry and isolation of mitochondria and measurement of protein content. However, these need to be carried out simultaneously to flow cytometry for accuracy, which presents additional challenges.

4. Effects of other components in cell culture on the experiment- As mentioned above several components in cell culture medium may affect interact with fluorophores thus confounding the results. In mitochondrial studies, phenol red was avoided as it falsely increased background fluorescence and interfered with measurements. In addition, controls were run with each experiment to ensure that the protocols worked as expected.

5. Variation in processing time- There were variations in processing time and acquisition of data on flow cytometer which may also led to changes in results. This is partially because PBMCs could not be fixed for mitochondrial studies as per manufacturer's protocols. This meant that despite best effort, logistical delays may have contributed to differences in results between samples.

6. Use of gating to outline positive and negative populations vs. MFI of the entire population for mitochondrial dyes in FlowJo software. Unlike antibodies which produce two distinct positive and negative populations, mitochondrial dyes often shift cell populations making gating more challenging. I have not found a general consensus in the literature about the most suitable methods of data analysis in the context of mitochondrial dyes. This again may have contributed to in consistencies in results related to these dyes.

7. Wash steps in extracellular flux assay work flow and impact on cell count, normalisation and on drug concentrations. There are multiple wash steps in the extracellular flux assay workflow as specified by the manufacturers that cannot be altered for optimal functioning of the assay. These can cause loss of adherent cells from the culture plate, which causes large variations in results from replicates. This can be partially mitigated by removing wells with significant losses from final analysis, however this approach although recommended, leads to large inter-individual variability in analysis of results. In addition, when used for normalisation (either as cell counts, protein content, DNA content etc), this again causes gross inaccuracies in results. Finally, the wash steps also occur after the addition of drugs of interest (ciprofloxacin, propofol and/or NE in combination with LPS) and septic serum. It is therefore not possible to determine if removal of these drugs and septic serum relatively early in the assay workflow affects final results or whether PBMCs retain mitochondrial changes which are later demonstrated. I did not add these drugs not the septic serum after wash steps due to possibility of interference with the assay conditions. The assay requires strict control of pH, temperature, CO_2 content which may be altered by these. I used the assay as oppose to other methods because it provided simultaneous information on the O_2 consumption rate (an indicator of OXPHOS) from low numbers of cells and in real-time. Other methods such as Clark electrode or Oroboros provide may be associated with additional challenges e.g. use of permeabilised cells or isolated mitochondria rather than intact cells, larger number of cells required per assay, measurement of replicates or multiple samples is not easily feasible due to the availability of the number of chambers, and effect of stirring etc which may be required for the assays (530).

8. Cell adherence and effects on results- Extracellular flux assay with Seahorse requires cells to be immobilised with cell-adherents such as CellTak. However, even at the recommended dose this can activate cells and affect cell normalisation by DNA content. The degree of activation can be monitored but not removed using unstimulated control samples. I carried out normalisation by cell count which is not affected by CellTak.

9. Time of incubation on mitochondrial function- During extracellular assay preoptimization stage, I carried out a time course of 3, 6 and 24 hours to identify which timepoints were most suitable for this assay. This showed that mitochondria in PBMCs became less responsive to the assay-related drugs (oligomycin, FCCP, rotenone and antimycin) beyond 6 hours and there was little difference observed between 3 and 6 hours. As a compromise between incubation period of cells with drugs of interest (Ciprofloxacin, propofol and/or NE and LPS) and mitochondrial responsiveness, I carried out the extracellular assay at 6 hours. However, the PBMCs used in the experiment were from healthy individuals co-incubated with septic serum. Future work may involve a comparison of septic patients with exposure to specific drugs of interest with healthy volunteers, as this groups of participants may be more representative of sepsis *in-vivo.* I chose PBMCs healthy volunteers co-incubated with septic serum as this group demonstrated overall consistent immune changes and were easier to obtain in terms of samples/timing.

There is currently a lack of literature on the effects of ciprofloxacin, propofol and NE on mitochondria from immune cells. Two *in-vitro* studies in T-lymphocytes and Jurkat T-cells showed that ciprofloxacin inhibited electron transport chain activity and decreased MMP (318, 319). In monocytes *in-vitro,* NE reduced O_2 consumption and ROS production (383) and in macrophages, but not in PBMCs, propofol increased mtROS production, NLRP3 inflammasome activation and caspase-mediated apoptosis. (436). In RAW 264.7 monocyte/macrophage cell line, propofol reduced MMP without affecting complex activity (437) while in lymphocytes, it did not affect mtROS production nor glutathione stores (438). The underlying mechanism for these limited findings is also unclear and further studies are required to elucidate this further.

6.4 Future work

This project has provided significant further insights into the potential effects of commonly used intensive care drugs on immune and mitochondrial function. Given the scale of the topic there are necessarily many questions which remain unanswered. The next steps of this project would ideally focus on further immune cell profiling in order to identify any aberrant effector functions that have not been covered, e.g. immune cell migration, lymphocyte activation, proliferation, effector functions. Once this has been achieved, further work would be required to unpick alternative mechanisms underlying the immune cell dysfunction outlined in this thesis, given that mitochondrial alterations do not appear to be chiefly responsible. This will hopefully lead to a better understanding of the cellular consequences of inappropriate medication prescribing in the intensive care setting. As with any project studying iatrogenic medication effects, the final goal of this work is the establishment of clinical correlates of laboratory data in order to provide reliable evidence to inform best practice and improve overall patient outcomes.

Appendix A- Immune Results

Table A1. Pairwise differences in phagocytosis between PBMCs from different participant groups at 6 hours with no LPS.

Dunnett's test	Mean diff.	95.00% CI of diff.	p-values
Control			
Healthy volunteers vs. Healthy with septic serum	-0.142	-0.299 to 0.016	0.080
Healthy volunteers vs. Suspected septic ED patients	-0.161	-0.289 to -0.032	0.018
Healthy volunteers vs. Septic ICU patients	-0.036	-0.176 to 0.104	0.821
Ciprofloxacin			
Healthy volunteers vs. Healthy with septic serum	-0.216	-0.402 to -0.032	0.024
Healthy volunteers vs. Suspected septic ED patients	-0.093	-0.376 to 0.189	0.665
Healthy volunteers vs. Septic ICU patients	-0.004	-0.202 to 0.194	>0.999
Propofol			
Healthy volunteers vs. Healthy with septic serum	-0.387	-0.599 to -0.174	0.003
Healthy volunteers vs. Suspected septic ED patients	-0.436	-0.704 to -0.169	0.004
Healthy volunteers vs. Septic ICU patients	-0.037	-0.293 to 0.219	0.959
NE			
Healthy volunteers vs. Healthy with septic serum	-0.276	-0.465 to -0.086	0.006
Healthy volunteers vs. Suspected septic ED patients	-0.281	-0.449 to -0.112	0.003
Healthy volunteers vs. Septic ICU patients	-0.016	-0.216 to 0.183	0.991
Mix of 3 drugs			
Healthy volunteers vs. Healthy with septic serum	-0.288	-0.473 to -0.102	0.004
Healthy volunteers vs. Suspected septic ED patients	-0.154	-0.492 to 0.183	0.408
Healthy volunteers vs. Septic ICU patients	-0.066	-0.242 to 0.110	0.624

Table A2. Pairwise differences in phagocytosis between PBMCs from different participant groups at 6 hours in the presence of LPS.

Dunnett's test	Mean diff.	95.00% CI of diff.	p-values
Control			
Healthy volunteers vs. Healthy with septic serum	-0.143	-0.263 to -0.022	0.022
Healthy volunteers vs. Suspected septic ED patients	-0.212	-0.341 to -0.084	0.003
Healthy volunteers vs. Septic ICU patients	-0.072	-0.209 to 0.064	0.376
Ciprofloxacin			
Healthy volunteers vs. Healthy with septic serum	-0.235	-0.511 to 0.039	0.095
Healthy volunteers vs. Suspected septic ED patients	-0.150	-0.307 to 0.007	0.061
Healthy volunteers vs. Septic ICU patients	0.001	-0.162 to 0.164	>0.999
Propofol			
Healthy volunteers vs. Healthy with septic serum	-0.213	-0.429 to 0.003	0.053
Healthy volunteers vs. Suspected septic ED patients	-0.091	-0.340 to 0.158	0.612
Healthy volunteers vs. Septic ICU patients	-0.002	-0.197 to 0.193	>0.999
NE			
Healthy volunteers vs. Healthy with septic serum	-0.125	-0.328 to 0.078	0.271
Healthy volunteers vs. Suspected septic ED patients	0.141	-0.098 to 0.379	0.280
Healthy volunteers vs. Septic ICU patients	0.025	-0.178 to 0.227	0.974
Mix of 3 drugs			
Healthy volunteers vs. Healthy with septic serum	-0.191	-0.362 to -0.021	0.029
Healthy volunteers vs. Suspected septic ED patients	0.062	-0.154 to 0.28	0.728
Healthy volunteers vs. Septic ICU patients	-0.068	-0.231 to 0.094	0.544

Table A3. Pairwise differences in phagocytosis between PBMCs from different participant groups at 24 hours in the absence of LPS.

Dunnett's test	Mean diff.	95.00% CI of diff.	p-values
Control			
Healthy volunteers vs. Healthy with septic serum	-0.142	-0.299 to 0.016	0.080
Healthy volunteers vs. Suspected septic ED patients	-0.161	-0.289 to -0.032	0.018
Healthy volunteers vs. Septic ICU patients	-0.036	-0.176 to 0.104	0.820
Ciprofloxacin			
Healthy volunteers vs. Healthy with septic serum	-0.175	-0.395 to 0.046	0.123
Healthy volunteers vs. Suspected septic ED patients	0.024	-0.352 to 0.399	0.995
Healthy volunteers vs. Septic ICU patients	-0.002	-0.238 to 0.234	>0.999
Propofol			
Healthy volunteers vs. Healthy with septic serum	-0.259	-0.493 to -0.024	0.032
Healthy volunteers vs. Suspected septic ED patients	-0.4067	-0.647 to -0.166	0.003
Healthy volunteers vs. Septic ICU patients	-0.0204	-0.275 to 0.235	0.992
NE			
Healthy volunteers vs. Healthy with septic serum	0.004	-0.271 to 0.278	>0.999
Healthy volunteers vs. Suspected septic ED patients	-0.165	-0.491 to 0.160	0.392
Healthy volunteers vs. Septic ICU patients	-0.003	-0.297 to 0.290	>0.999
Mix of 3 drugs			
Healthy volunteers vs. Healthy with septic serum	-0.074	-0.279 to 0.132	0.645
Healthy volunteers vs. Suspected septic ED patients	-0.089	-0.228 to 0.050	0.233
Healthy volunteers vs. Septic ICU patients	-0.033	-0.193 to 0.127	0.895

Table A4. Pairwise differences in phagocytosis between PBMCs from different participant groups at 24 hours in the presence of LPS.

Dunnett's test	Mean Diff.	95.00% CI of diff.	p-value
Control			
Healthy volunteers vs. Healthy with septic serum	-0.143	-0.263 to -0.023	0.022
Healthy volunteers vs. Suspected septic ED patients	-0.212	-0.341 to -0.084	0.003
Healthy volunteers vs. Septic ICU patients	-0.036	-0.169 to 0.097	0.801
Ciprofloxacin			
Healthy volunteers vs. Healthy with septic serum	-0.125	-0.415 to 0.163	0.449
Healthy volunteers vs. Suspected septic ED patients	-0.053	-0.342 to 0.236	0.891
Healthy volunteers vs. Septic ICU patients	0.0085	-0.311 to 0.328	0.999
Propofol			
Healthy volunteers vs. Healthy with septic serum	-0.112	-0.286 to 0.063	0.249
Healthy volunteers vs. Suspected septic ED patients	-0.122	-0.313 to 0.070	0.232
Healthy volunteers vs. Septic ICU patients	-0.018	-0.171 to 0.133	0.973
NE			
Healthy volunteers vs. Healthy with septic serum	-0.133	-0.399 to 0.132	0.412
Healthy volunteers vs. Suspected septic ED patients	0.030	-0.207 to 0.268	0.958
Healthy volunteers vs. Septic ICU patients	0.017	-0.248 to 0.284	0.995
Mix of 3 drugs			
Healthy volunteers vs. Healthy with septic serum	-0.095	-0.279 to 0.089	0.385
Healthy volunteers vs. Suspected septic ED patients	0.015	-0.096 to 0.125	0.960
Healthy volunteers vs. Septic ICU patients	-0.059	-0.222 to 0.103	0.636

Table A5. Pairwise differences in phagocytosis between different drug classes at 6 hours in the absence of LPS.

Dunnett's test	Mean Diff.	95.00% CI of diff.	p-value
Healthy volunteers			
Control vs. Ciprofloxacin	0.338	0.143 to 0.532	0.005
Control vs. Propofol	0.489	0.264 to 0.713	0.002
Control vs. NE	0.376	0.159 to 0.593	0.005
Control vs. Mix of 3 drugs	0.419	0.184 to 0.654	0.004
Healthy with septic serum			
Control vs. Ciprofloxacin	0.262	0.096 to 0.429	0.006
Control vs. Propofol	0.243	0.143 to 0.344	0.001
Control vs. NE	0.242	0.063 to 0.420	0.013
Control vs. Mix of 3 drugs	0.273	0.030 to 0.516	0.031
Suspected septic ED patients			
Control vs. Ciprofloxacin	0.405	0.122 to 0.689	0.015
Control vs. Propofol	0.213	-0.047 to 0.475	0.092
Control vs. NE	0.256	0.082 to 0.430	0.013
Control vs. Mix of 3 drugs	0.426	0.064 to 0.787	0.029
Septic ICU patients			
Control vs. Ciprofloxacin	0.370	0.181 to 0.559	0.002
Control vs. Propofol	0.488	0.275 to 0.702	0.001
Control vs. NE	0.395	0.152 to 0.638	0.006
Control vs. Mix of 3 drugs	0.389	0.211 to 0.567	0.001

Table A6. Pairwise differences in phagocytosis between different drug classes at 6 hours in the presence of LPS.

Dunnett's test	Mean Diff.	95.00% CI of diff.	p-value
Healthy volunteers			
Control vs. Ciprofloxacin	0.282	0.125 to 0.439	0.004
Control vs. Propofol	0.317	0.166to 0.468	0.002
Control vs. NE	0.248	0.042 to 0.453	0.025
Control vs. Mix of 3 drugs	0.311	0.168 to 0.454	0.002
Healthy with septic serum			
Control vs. Ciprofloxacin	0.189	-0.127 to 0.505	0.255
Control vs. Propofol	0.246	0.056 to 0.436	0.016
Control vs. NE	0.266	0.097 to 0.435	0.006
Control vs. Mix of 3 drugs	0.263	0.060 to 0.464	0.016
Suspected septic ED patients			
Control vs. Ciprofloxacin	0.345	0.186 to 0.503	0.003
Control vs. Propofol	0.439	0.185 to 0.692	0.007
Control vs. NE	0.602	0.415 to 0.787	0.001
Control vs. Mix of 3 drugs	0.586	0.294 to 0.877	0.004
Septic ICU patients			
Control vs. Ciprofloxacin	0.3556	0.198 to 0.512	0.001
Control vs. Propofol	0.3876	0.216 to 0.558	0.001
Control vs. NE	0.3458	0.170 to 0.521	0.002
Control vs. Mix of 3 drugs	0.3155	0.144 to 0.486	0.003

Table A7. Pairwise differences in phagocytosis between different drug classes at 24 hours in the absence of LPS.

Dunnett's test	Mean Diff.	95.00% CI of diff.	p-value
Healthy volunteers			
Control vs. Ciprofloxacin	0.331	0.048 to 0.614	0.027
Control vs. Propofol	0.465	0.158 to 0.772	0.009
Control vs. NE	0.296	-0.065 to 0.658	0.098
Control vs. Mix of 3 drugs	0.367	0.204 to 0.529	0.002
Healthy with septic serum			
Control vs. Ciprofloxacin	0.297	0.126 to 0.469	0.004
Control vs. Propofol	0.348	0.210 to 0.486	0.001
Control vs. NE	0.441	0.272 to 0.609	0.001
Control vs. Mix of 3 drugs	0.435	0.177 to 0.692	0.005
Suspected septic ED patients			
Control vs. Ciprofloxacin	0.516	0.082 to 0.949	0.028
Control vs. Propofol	0.219	0.002 to 0.436	0.048
Control vs. NE	0.292	-0.082 to 0.666	0.106
Control vs. Mix of 3 drugs	0.438	0.345 to 0.532	<0.001
Septic ICU patients			
Control vs. Ciprofloxacin	0.365	0.124 to 0.606	0.008
Control vs. Propofol	0.481	0.250 to 0.711	0.002
Control vs. NE	0.328	0.052 to 0.605	0.024
Control vs. Mix of 3 drugs	0.370	0.247 to 0.492	<0.001

Table A8. Pairwise differences in phagocytosis between different drug classes at 24 hours in the presence of LPS.

Dunnett's test	Mean Diff.	95.00% CI of diff.	p-value
Healthy volunteers			
Control vs. Ciprofloxacin	0.265	-0.035 to 0.565	0.077
Control vs. Propofol	0.325	0.129 to 0.521	0.006
Control vs. NE	0.269	-0.041 to 0.58	0.081
Control vs. Mix of 3 drugs	0.264	0.192 to 0.335	<0.001
Healthy with septic serum			
Control vs. Ciprofloxacin	0.282	0.147 to 0.416	0.002
Control vs. Propofol	0.357	0.229 to 0.485	<0.001
Control vs. NE	0.279	0.094 to 0.464	0.008
Control vs. Mix of 3 drugs	0.312	0.140 to 0.483	0.003
Suspected septic ED patients			
Control vs. Ciprofloxacin	0.424	0.262 to 0.587	0.002
Control vs. Propofol	0.416	0.181 to 0.652	0.007
Control vs. NE	0.512	0.352 to 0.673	0.001
Control vs. Mix of 3 drugs	0.491	0.432 to 0.550	<0.001
Septic ICU patients			
Control vs. Ciprofloxacin	0.309	0.051 to 0.568	0.024
Control vs. Propofol	0.343	0.181 to 0.504	0.001
Control vs. NE	0.323	0.048 to 0.598	0.025
Control vs. Mix of 3 drugs	0.240	0.110 to 0.371	0.003

Table A9. Pairwise differences in HLA-DR between PBMCs from different participant groups at 6 hours in the absence of LPS.

Dunnett's test	Mean Diff.	95.00% CI of diff.	p-value
Control			
Healthy volunteers vs. Healthy with septic serum	0.713	0.448 to 0.978	<0.001
Healthy volunteers vs. Suspected septic ED patients	0.653	0.541 to 0.765	<0.001
Healthy volunteers vs. Septic ICU patients	0.781	0.634 to 0.927	<0.001
Ciprofloxacin			
Healthy volunteers vs. Healthy with septic serum	0.690	0.433 to 0.946	<0.001
Healthy volunteers vs. Suspected septic ED patients	0.811	0.561 to 1.061	<0.001
Healthy volunteers vs. Septic ICU patients	0.731	0.531 to 0.930	<0.001
Propofol			
Healthy volunteers vs. Healthy with septic serum	0.745	0.460 to 1.029	<0.001
Healthy volunteers vs. Suspected septic ED patients	0.737	0.451 to 1.024	<0.001
Healthy volunteers vs. Septic ICU patients	0.735	0.471 to 0.999	<0.001
NE			
Healthy volunteers vs. Healthy with septic serum	0.731	0.492 to 0.969	<0.001
Healthy volunteers vs. Suspected septic ED patients	0.800	0.482 to 1.117	<0.001
Healthy volunteers vs. Septic ICU patients	0.652	0.520 to 0.783	<0.001
Mix of 3 drugs			
Healthy volunteers vs. Healthy with septic serum	0.712	0.493 to 0.930	<0.001
Healthy volunteers vs. Suspected septic ED patients	0.829	0.588 to 1.070	<0.001
Healthy volunteers vs. Septic ICU patients	0.559	0.376 to 0.742	<0.001

Table A10. Pairwise differences in HLA-DR between PBMCs from different participant groups at 6 hours in the presence of LPS.

Dunnett's test	Mean Diff.	95.00% CI of diff.	p-value
Control			
Healthy volunteers vs. Healthy with septic serum	0.790	0.471 to 1.109	<0.001
Healthy volunteers vs. Suspected septic ED patients	0.676	0.376 to 0.977	<0.001
Healthy volunteers vs. Septic ICU patients	0.889	0.478 to 1.300	<0.001
Ciprofloxacin			
Healthy volunteers vs. Healthy with septic serum	0.821	0.519 to 1.124	<0.001
Healthy volunteers vs. Suspected septic ED patients	0.855	0.612 to 1.099	<0.001
Healthy volunteers vs. Septic ICU patients	0.787	0.356 to 1.218	0.002
Propofol			
Healthy volunteers vs. Healthy with septic serum	0.598	0.281 to 0.914	0.001
Healthy volunteers vs. Suspected septic ED patients	0.568	0.187 to 0.949	0.006
Healthy volunteers vs. Septic ICU patients	0.607	0.234 to 0.981	0.003
NE			
Healthy volunteers vs. Healthy with septic serum	0.895	0.652 to 1.139	<0.001
Healthy volunteers vs. Suspected septic ED patients	0.942	0.620 to 1.264	<0.001
Healthy volunteers vs. Septic ICU patients	0.816	0.535 to 1.097	<0.001
Mix of 3 drugs			
Healthy volunteers vs. Healthy with septic serum	0.770	0.526 to 1.014	<0.001
Healthy volunteers vs. Suspected septic ED patients	0.814	0.575 to 1.054	<0.001
Healthy volunteers vs. Septic ICU patients	0.518	0.180 to 0.856	0.004

Table A11. Pairwise differences in HLA-DR between PBMCs from different participant groups at 24 hours in the absence of LPS.

Dunnett's test	Mean Diff.	95.00% CI of diff.	p-value
Control			
Healthy volunteers vs. Healthy with septic serum	0.771	0.451 to 1.093	0.0002
Healthy volunteers vs. Suspected septic ED patients	0.658	0.391 to 0.9264	0.0002
Healthy volunteers vs. Septic ICU patients	0.801	0.566 to 1.036	<0.0001
Ciprofloxacin			
Healthy volunteers vs. Healthy with septic serum	0.765	0.424 to 1.106	0.0008
Healthy volunteers vs. Suspected septic ED patients	0.834	0.501 to 1.167	0.0007
Healthy volunteers vs. Septic ICU patients	0.791	0.579 to 1.005	<0.0001
Propofol			
Healthy volunteers vs. Healthy with septic serum	0.572	0.086 to 1.059	0.0250
Healthy volunteers vs. Suspected septic ED patients	0.612	0.084 to 1.140	0.0249
Healthy volunteers vs. Septic ICU patients	0.627	0.141 to 1.113	0.0165
NE			
Healthy volunteers vs. Healthy with septic serum	0.708	0.395 to 1.022	0.0002
Healthy volunteers vs. Suspected septic ED patients	0.816	0.346 to 1.285	0.0035
Healthy volunteers vs. Septic ICU patients	0.834	0.566 to 1.103	<0.0001
Mix of 3 drugs			
Healthy volunteers vs. Healthy with septic serum	0.908	0.732 to 1.085	<0.0001
Healthy volunteers vs. Suspected septic ED patients	0.948	0.701 to 1.196	<0.0001
Healthy volunteers vs. Septic ICU patients	0.875	0.699 to 1.051	<0.0001

Table A12. Pairwise differences in HLA-DR between PBMCs from different participant groups at 24 hours in the presence of LPS.

Dunnett's test	Mean Diff.	95.00% CI of diff.	p-value
Control			
Healthy volunteers vs. Healthy with septic serum	0.864	0.586 to 1.142	<0.001
Healthy volunteers vs. Suspected septic ED patients	0.823	0.533 to 1.112	<0.001
Healthy volunteers vs. Septic ICU patients	0.888	0.226 to 1.551	0.012
Ciprofloxacin			
Healthy volunteers vs. Healthy with septic serum	0.894	0.530 to 1.259	<0.001
Healthy volunteers vs. Suspected septic ED patients	0.938	0.547 to 1.329	<0.001
Healthy volunteers vs. Septic ICU patients	0.886	0.377 to 1.395	0.002
Propofol			
Healthy volunteers vs. Healthy with septic serum	0.634	0.230 to 1.038	0.007
Healthy volunteers vs. Suspected septic ED patients	0.706	0.267 to 1.146	0.004
Healthy volunteers vs. Septic ICU patients	0.575	0.0918 to 1.060	0.021
NE			
Healthy volunteers vs. Healthy with septic serum	0.943	0.695 to 1.191	<0.001
Healthy volunteers vs. Suspected septic ED patients	1.004	0.579 to 1.429	<0.001
Healthy volunteers vs. Septic ICU patients	0.827	0.515 to 1.141	<0.001
Mix of 3 drugs			
Healthy volunteers vs. Healthy with septic serum	0.836	0.573 to 1.099	<0.001
Healthy volunteers vs. Suspected septic ED patients	0.810	0.515 to 1.105	<0.001
Healthy volunteers vs. Septic ICU patients	0.711	0.449 to 0.974	0.0003

Table A13. Pairwise differences in HLA-DR between different drug classes at 6 hours in the absence of LPS.

Dunnett's test	Mean Diff.	95.00% CI of diff.	p-value
Healthy volunteers			
Control vs. Ciprofloxacin	0.071	0.009 to 0.132	0.029
Control vs. Propofol	0.064	-0.140 to 0.270	0.669
Control vs. NE	-0.013	-0.076 to 0.049	0.862
Control vs. Mix of 3 drugs	0.082	-0.029 to 0.194	0.135
Healthy with septic serum			
Control vs. Ciprofloxacin	0.047	-0.102 to 0.197	0.701
Control vs. Propofol	0.096	-0.069 to 0.262	0.275
Control vs. NE	0.004	-0.233 to 0.241	>0.999
Control vs. Mix of 3 drugs	0.081	-0.166 to 0.329	0.683
Suspected septic ED patients			
Control vs. Ciprofloxacin	0.228	-0.015 to 0.473	0.061
Control vs. Propofol	0.148	-0.096 to 0.395	0.203
Control vs. NE	0.133	-0.151 to 0.417	0.348
Control vs. Mix of 3 drugs	0.258	0.065 to 0.450	0.018
Septic ICU patients			
Control vs. Ciprofloxacin	0.021	-0.071 to 0.113	0.867
Control vs. Propofol	0.019	-0.127 to 0.166	0.975
Control vs. NE	-0.142	-0.186 to -0.098	0.0002
Control vs. Mix of 3 drugs	-0.139	-0.325 to 0.047	0.140

Table A14. Pairwise differences in HLA-DR between different drug classes at 6 hours in the presence of LPS.

Dunnett's test	Mean Diff.	95.00% CI of diff.	p-value
Healthy volunteers			
Control vs. Ciprofloxacin	0.074	-0.015 to 0.163	0.094
Control vs. Propofol	0.262	-0.028 to 0.553	0.072
Control vs. NE	-0.111	-0.272 to 0.049	0.161
Control vs. Mix of 3 drugs	0.116	-0.175 to 0.407	0.509
Healthy with septic serum			
Control vs. Ciprofloxacin	0.105	-0.135 to 0.345	0.478
Control vs. Propofol	0.069	-0.148 to 0.287	0.696
Control vs. NE	-0.006	-0.219 to 0.207	0.999
Control vs. Mix of 3 drugs	0.096	-0.123 to 0.315	0.476
Suspected septic ED patients			
Control vs. Ciprofloxacin	0.252	-0.131 to 0.636	0.166
Control vs. Propofol	0.153	-0.062 to 0.370	0.136
Control vs. NE	0.154	-0.043 to 0.351	0.104
Control vs. Mix of 3 drugs	0.253	0.0353 to 0.472	0.030
Septic ICU patients			
Control vs. Ciprofloxacin	-0.027	-0.096 to 0.040	0.531
Control vs. Propofol	-0.019	-0.149 to 0.110	0.964
Control vs. NE	-0.184	-0.435 to 0.066	0.147
Control vs. Mix of 3 drugs	-0.254	-0.685 to 0.176	0.264

Table A15. Pairwise differences in HLA-DR between different drug classes at 24 hours in the absence of LPS.

Dunnett's test	Mean Diff.	95.00% CI of diff.	p-value
Healthy volunteers			
Control vs. Ciprofloxacin	0.060	-0.041 to 0.162	0.240
Control vs. Propofol	0.253	-0.140 to 0.646	0.197
Control vs. NE	-0.005	-0.199 to 0.188	0.999
Control vs. Mix of 3 drugs	-0.085	-0.214 to 0.043	0.184
Healthy with septic serum			
Control vs. Ciprofloxacin	0.054	-0.096 to 0.204	0.582
Control vs. Propofol	0.054	-0.197 to 0.305	0.886
Control vs. NE	-0.068	-0.232 to 0.096	0.517
Control vs. Mix of 3 drugs	0.052	-0.219 to 0.323	0.918
Suspected septic ED patients			
Control vs. Ciprofloxacin	0.236	-0.080 to 0.552	0.119
Control vs. Propofol	0.206	-0.238 to 0.651	0.352
Control vs. NE	0.152	-0.342 to 0.647	0.628
Control vs. Mix of 3 drugs	0.204	-0.026 to 0.435	0.072
Septic ICU patients			
Control vs. Ciprofloxacin	0.051	-0.085 to 0.188	0.590
Control vs. Propofol	0.079	-0.034 to 0.193	0.170
Control vs. NE	0.028	-0.112 to 0.169	0.904
Control vs. Mix of 3 drugs	-0.010	-0.193 to 0.171	0.998

Table A16. Pairwise differences in HLA-DR between different drug classes at 24 hours in the presence of LPS.

Dunnett's test	Mean Diff.	95.00% CI of diff.	p-value
Healthy volunteers	0.058	-0.345 to 0.462	0.957
Control vs. Ciprofloxacin	0.224	-0.021 to 0.470	0.069
Control vs. Propofol	-0.168	-0.572 to 0.234	0.474
Control vs. NE	0.025	-0.374 to 0.424	0.997
Control vs. Mix of 3 drugs			
Healthy with septic serum	0.089	-0.142 to 0.320	0.535
Control vs. Ciprofloxacin	-0.005	-0.167 to 0.156	0.999
Control vs. Propofol	-0.090	-0.306 to 0.125	0.513
Control vs. NE	-0.003	-0.180 to 0.174	>0.999
Control vs. Mix of 3 drugs			
Suspected septic ED patients	0.174	-0.060 to 0.409	0.121
Control vs. Ciprofloxacin	0.108	-0.065 to 0.281	0.190
Control vs. Propofol	0.012	-0.301 to 0.326	0.999
Control vs. NE	0.012	-0.078 to 0.102	0.954
Control vs. Mix of 3 drugs			
Septic ICU patients	0.055	-0.207 to 0.319	0.890
Control vs. Ciprofloxacin	-0.088	-0.401 to 0.224	0.767
Control vs. Propofol	-0.229	-0.653 to 0.194	0.321
Control vs. NE	-0.152	-0.854 to 0.549	0.882

Appendix B- Mitochondrial results

Table B1 Pairwise differences in Mitosox in lymphocytes between different participant groups at 6 hours in the absence of LPS.

Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	p-value
Control			
Healthy volunteers vs. Healthy with septic serum	-0.228	-0.559 to 0.104	0.1967
Healthy volunteers vs. Suspected septic ED patients	-0.615	-0.871 to -0.359	<0.001
Healthy volunteers vs. Septic ICU patients	-0.214	-0.602 to 0.173	0.328
Ciprofloxacin			
Healthy volunteers vs. Healthy with septic serum	-0.280	-0.589 to 0.028	0.076
Healthy volunteers vs. Suspected septic ED patients	-0.468	-0.768 to -0.169	0.005
Healthy volunteers vs. Septic ICU patients	-0.353	-0.829 to 0.123	0.153
Propofol			
Healthy volunteers vs. Healthy with septic serum	-0.319	-0.554 to -0.085	0.010
Healthy volunteers vs. Suspected septic ED patients	-0.511	-0.797 to -0.226	0.002
Healthy volunteers vs. Septic ICU patients	-0.579	-1.040 to -0.118	0.017
NE			
Healthy volunteers vs. Healthy with septic serum	-0.311	-0.627 to 0.004	0.053
Healthy volunteers vs. Suspected septic ED patients	-0.615	-1.548 to 0.3183	0.169
Healthy volunteers vs. Septic ICU patients	-0.496	-1.158 to 0.1648	0.139
Mix of 3 drugs			
Healthy volunteers vs. Healthy with septic serum	-0.354	-0.720 to 0.012	0.058
Healthy volunteers vs. Suspected septic ED patients	-0.756	-1.983 to 0.471	0.199
Healthy volunteers vs. Septic ICU patients	-0.668	-1.498 to 0.162	0.111

Table B2 Pairwise differences in Mitosox in lymphocytes between different participant groups at 6 hours in the presence of LPS.

Dunnett's multiple comparisons test	Mean diff.	95.00% CI of diff.	P value
Control			
Healthy volunteers vs. Healthy with septic serum	-0.287	-0.484 to -0.091	0.002
Healthy volunteers vs. Suspected septic ED patients	-0.496	-0.710 to -0.282	<0.001
Healthy volunteers vs. Septic ICU patients	-0.614	-0.810 to -0.412	<0.001
Ciprofloxacin			
Healthy volunteers vs. Healthy with septic serum	-0.287	-0.484 to -0.091	0.002
Healthy volunteers vs. Suspected septic ED patients	-0.496	-0.710 to -0.282	<0.001
Healthy volunteers vs. Septic ICU patients	-0.614	-0.810 to -0.413	<0.001
Propofol			
Healthy volunteers vs. Healthy with septic serum	-0.287	-0.484 to -0.091	0.002
Healthy volunteers vs. Suspected septic ED patients	-0.496	-0.710 to -0.282	<0.001
Healthy volunteers vs. Septic ICU patients	-0.614	-0.810 to -0.417	<0.001
NE			
Healthy volunteers vs. Healthy with septic serum	-0.287	-0.484 to -0.091	0.002
Healthy volunteers vs. Suspected septic ED patients	-0.496	-0.710 to -0.282	<0.001
Healthy volunteers vs. Septic ICU patients	-0.614	-0.810 to -0.417	<0.001
Mix of 3 drugs			
Healthy volunteers vs. Healthy with septic serum	-0.287	-0.484 to -0.091	0.002
Healthy volunteers vs. Suspected septic ED patients	-0.496	-0.710 to -0.282	<0.001
Healthy volunteers vs. Septic ICU patients	-0.614	-0.810 to -0.417	<0.001

Table B3 Pairwise differences in Mitosox in lymphocytes between different participant groups at 24 hours in the absence of LPS.

Dunnett's multiple comparisons test	Mean diff.	95.00% CI of diff.	P value
Control			
Healthy volunteers vs. Healthy with septic serum	-0.549	-0.805 to -0.292	<0.001
Healthy volunteers vs. Suspected septic ED patients	-0.631	-0.911 to -0.352	<0.001
Healthy volunteers vs. Septic ICU patients	-0.503	-0.760 to -0.246	<0.001
Ciprofloxacin			
Healthy volunteers vs. Healthy with septic serum	-0.549	-0.805 to -0.292	<0.001
Healthy volunteers vs. Suspected septic ED patients	-0.631	-0.911 to -0.352	<0.001
Healthy volunteers vs. Septic ICU patients	-0.503	-0.761 to -0.246	<0.001
Propofol			
Healthy volunteers vs. Healthy with septic serum	-0.549	-0.805 to -0.292	<0.001
Healthy volunteers vs. Suspected septic ED patients	-0.631	-0.911 to -0.352	<0.001
Healthy volunteers vs. Septic ICU patients	-0.503	-0.760 to -0.246	<0.001
NE			
Healthy volunteers vs. Healthy with septic serum	-0.549	-0.805 to -0.292	<0.001
Healthy volunteers vs. Suspected septic ED patients	-0.631	-0.911 to -0.352	<0.001
Healthy volunteers vs. Septic ICU patients	-0.503	-0.760 to -0.246	<0.001
Mix of 3 drugs			
Healthy volunteers vs. Healthy with septic serum	-0.549	-0.805 to -0.292	<0.001
Healthy volunteers vs. Suspected septic ED patients	-0.631	-0.911 to -0.352	<0.001
Healthy volunteers vs. Septic ICU patients	-0.503	-0.760 to -0.246	<0.001

Table B4 Pairwise differences in Mitosox in lymphocytes between different participant groups at 24 hours in the presence of LPS.

Dunnett's multiple comparisons test	Mean diff.	95.00% CI of diff.	P value
Control			
Healthy volunteers vs. Healthy with septic serum	-0.291	-0.556 to -0.025	0.0283
Healthy volunteers vs. Suspected septic ED patients	-0.511	-0.799 to -0.221	0.0002
Healthy volunteers vs. Septic ICU patients	-0.558	-0.823 to -0.292	<0.0001
Ciprofloxacin			
Healthy volunteers vs. Healthy with septic serum	-0.291	-0.556 to -0.025	0.028
Healthy volunteers vs. Suspected septic ED patients	-0.511	-0.799 to -0.221	<0.001
Healthy volunteers vs. Septic ICU patients	-0.558	-0.824 to -0.292	<0.001
Propofol			
Healthy volunteers vs. Healthy with septic serum	-0.291	-0.556 to -0.025	0.028
Healthy volunteers vs. Suspected septic ED patients	-0.511	-0.799 to -0.221	<0.001
Healthy volunteers vs. Septic ICU patients	-0.558	-0.824 to -0.292	<0.001
NE			
Healthy volunteers vs. Healthy with septic serum	-0.291	-0.556 to -0.025	0.028
Healthy volunteers vs. Suspected septic ED patients	-0.511	-0.799 to -0.221	<0.001
Healthy volunteers vs. Septic ICU patients	-0.558	-0.824 to -0.292	<0.001
Mix of 3 drugs			
Healthy volunteers vs. Healthy with septic serum	-0.291	-0.556 to -0.025	0.028
Healthy volunteers vs. Suspected septic ED patients	-0.511	-0.799 to -0.221	<0.001
Healthy volunteers vs. Septic ICU patients	-0.558	-0.824 to -0.292	<0.001

Table B5 Pairwise differences in Mitosox in lymphocytes between different drug groups at 6 hours in the absence of LPS.

Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	P Value
Healthy volunteers			
Control vs. Ciprofloxacin	-0.026	-0.095 to 0.042	0.529
Control vs. Propofol	-0.033	-0.097 to 0.029	0.312
Control vs. NE	-0.042	-0.109 to 0.024	0.205
Control vs. Mix of 3 drugs	-0.134	-0.252 to -0.017	0.029
Healthy with septic serum			
Control vs. Ciprofloxacin	-0.079	-0.397 to 0.241	0.835
Control vs. Propofol	-0.125	-0.410 to 0.160	0.477
Control vs. NE	-0.125	-0.493 to 0.242	0.656
Control vs. Mix of 3 drugs	-0.261	-0.508 to -0.014	0.040
Suspected septic ED patients			
Control vs. Ciprofloxacin	0.120	-0.155 to 0.396	0.393
Control vs. Propofol	0.071	-0.249 to 0.391	0.813
Control vs. NE	-0.042	-1.114 to 1.031	0.999
Control vs. Mix of 3 drugs	-0.276	-1.695 to 1.144	0.864
Septic ICU patients			
Control vs. Ciprofloxacin	-0.165	-0.957 to 0.628	0.896
Control vs. Propofol	-0.398	-1.189 to 0.393	0.374
Control vs. NE	-0.324	-1.267 to 0.619	0.651
Control vs. Mix of 3 drugs	-0.588	-1.611 to 0.435	0.282

Table B6 Pairwise differences in Mitosox in lymphocytes between different drug groups at 6 hours in the presence of LPS.

Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	P Value
Healthy volunteers			
Control vs. Ciprofloxacin	0.041	-0.033 to 0.115	0.277
Control vs. Propofol	0.048	-0.003to 0.099	0.060
Control vs. NE	0.007	-0.048 to 0.063	0.967
Control vs. Mix of 3 drugs	-0.059	-0.116 to -0.003	0.041
Healthy with septic serum			
Control vs. Ciprofloxacin	-0.014	-0.219 to 0.192	0.998
Control vs. Propofol	-0.094	-0.246 to 0.057	0.228
Control vs. NE	-0.106	-0.305 to 0.094	0.337
Control vs. Mix of 3 drugs	-0.229	-0.477 to 0.0184	0.067
Suspected septic ED patients			
Control vs. Ciprofloxacin	0.230	-0.287 to 0.747	0.380
Control vs. Propofol	0.231	-0.194 to 0.656	0.258
Control vs. NE	0.169	-0.307 to 0.646	0.534
Control vs. Mix of 3 drugs	-0.017	-1.572 to 1.538	>0.999
Septic ICU patients			
Control vs. Ciprofloxacin	-0.172	-0.836 to 0.492	0.813
Control vs. Propofol	-0.292	-0.822 to 0.238	0.310
Control vs. NE	-0.953	-1.190 to -0.715	<0.001
Control vs. Mix of 3 drugs	-0.589	-1.317 to 0.139	0.108

Table B7 Pairwise differences in Mitosox in lymphocytes between different drug groups at 24 hours in the absence of LPS.

Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	P Value
Healthy volunteers			
Control vs. Ciprofloxacin	-0.256	-0.591 to 0.077	0.119
Control vs. Propofol	-0.229	-0.402 to -0.056	0.017
Control vs. NE	-0.305	-0.474 to -0.136	0.005
Control vs. Mix of 3 drugs	-0.403	-0.573 to -0.233	0.001
Healthy with septic serum			
Control vs. Ciprofloxacin	-0.052	-0.488 to 0.384	0.983
Control vs. Propofol	-0.061	-0.297 to 0.176	0.815
Control vs. NE	-0.421	-0.984 to 0.141	0.138
Control vs. Mix of 3 drugs	-0.878	-1.866 to 0.110	0.078
Suspected septic ED patients			
Control vs. Ciprofloxacin	0.019	-0.565 to 0.603	0.999
Control vs. Propofol	-0.198	-0.465 to 0.068	0.120
Control vs. NE	-0.434	-0.793 to -0.074	0.027
Control vs. Mix of 3 drugs	-0.586	-1.097 to -0.075	0.032
Septic ICU patients			
Control vs. Ciprofloxacin	-0.008	-0.174 to 0.157	0.999
Control vs. Propofol	-0.139	-0.425 to 0.147	0.398
Control vs. NE	-0.403	-0.871 to 0.065	0.087
Control vs. Mix of 3 drugs	-0.382	-1.119 to 0.356	0.354

Table B8 Pairwise differences in Mitosox in lymphocytes between different drug groups at 24 hours in the presence of LPS.

Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	P Value
Healthy volunteers	-0.009	-0.119 to 0.099	0.992
Control vs. Ciprofloxacin	0.061	-0.087 to 0.209	0.489
Control vs. Propofol	-0.075	-0.219 to 0.069	0.323
Control vs. NE	-0.143	-0.324 to 0.039	0.111
Control vs. Mix of 3 drugs			
Healthy with septic serum	0.046	-0.264 to 0.356	0.963
Control vs. Ciprofloxacin	-0.022	-0.214 to 0.171	0.986
Control vs. Propofol	-0.379	-0.808 to 0.049	0.079
Control vs. NE	-0.784	-1.859 to 0.291	0.149
Control vs. Mix of 3 drugs			
Suspected septic ED patients	0.026	-0.154 to 0.205	0.944
Control vs. Ciprofloxacin	0.022	-0.257 to 0.303	0.992
Control vs. Propofol	-0.361	-0.825 to 0.102	0.106
Control vs. NE	-0.308	-0.878 to 0.262	0.263
Control vs. Mix of 3 drugs			
Septic ICU patients	0.033	-0.103 to 0.168	0.841
Control vs. Ciprofloxacin	0.019	-0.174 to 0.213	0.991
Control vs. Propofol	-0.586	-1.143 to -0.029	0.041
Control vs. NE	-0.638	-1.251 to -0.025	0.043

Table B9 Pairwise differences in TMRM in lymphocytes between different participant groups at 6 hours in the absence of LPS.

Dunnett's multiple comparisons test	Mean diff.	95.00% CI of diff.	p-value
Control			
Healthy volunteers vs. Healthy with septic serum	0.413	0.255 to 0.571	<0.001
Healthy volunteers vs. Suspected septic ED patients	0.044	-0.127 to 0.215	0.865
Healthy volunteers vs. Septic ICU patients	0.512	0.354 to 0.671	<0.001
Ciprofloxacin			
Healthy volunteers vs. Healthy with septic serum	0.413	0.255 to 0.571	<0.001
Healthy volunteers vs. Suspected septic ED patients	0.044	-0.127 to 0.215	0.865
Healthy volunteers vs. Septic ICU patients	0.512	0.354 to 0.671	<0.001
Propofol			
Healthy volunteers vs. Healthy with septic serum	0.413	0.255 to 0.571	<0.001
Healthy volunteers vs. Suspected septic ED patients	0.044	-0.127 to 0.215	0.865
Healthy volunteers vs. Septic ICU patients	0.512	0.354 to 0.671	<0.001
NE			
Healthy volunteers vs. Healthy with septic serum	0.413	0.255 to 0.571	<0.001
Healthy volunteers vs. Suspected septic ED patients	0.044	-0.127 to 0.215	0.865
Healthy volunteers vs. Septic ICU patients	0.512	0.354 to 0.671	<0.001
Mix of 3 drugs			
Healthy volunteers vs. Healthy with septic serum	0.413	0.255 to 0.571	<0.001
Healthy volunteers vs. Suspected septic ED patients	0.044	-0.127 to 0.215	0.865
Healthy volunteers vs. Septic ICU patients	0.512	0.354 to 0.671	<0.001

Table B10 Pairwise differences in TMRM in lymphocytes between different participant groups at 6 hours in the presence of LPS.

Dunnett's multiple comparisons test	Mean diff.	95.00% CI of diff.	p-value
Control			
Healthy volunteers vs. Healthy with septic serum	0.465	0.290 to 0.641	<0.001
Healthy volunteers vs. Suspected septic ED patients	0.117	-0.072 to 0.307	0.319
Healthy volunteers vs. Septic ICU patients	0.508	0.333 to 0.684	<0.001
Ciprofloxacin			
Healthy volunteers vs. Healthy with septic serum	0.465	0.290 to 0.641	<0.001
Healthy volunteers vs. Suspected septic ED patients	0.117	-0.072 to 0.307	0.319
Healthy volunteers vs. Septic ICU patients	0.508	0.333 to 0.684	<0.001
Propofol			
Healthy volunteers vs. Healthy with septic serum	0.465	0.290 to 0.641	<0.001
Healthy volunteers vs. Suspected septic ED patients	0.117	-0.072 to 0.307	0.319
Healthy volunteers vs. Septic ICU patients	0.508	0.333 to 0.684	<0.001
NE			
Healthy volunteers vs. Healthy with septic serum	0.465	0.290 to 0.641	<0.001
Healthy volunteers vs. Suspected septic ED patients	0.117	-0.072 to 0.307	0.319
Healthy volunteers vs. Septic ICU patients	0.508	0.333 to 0.684	<0.001
Mix of 3 drugs			
Healthy volunteers vs. Healthy with septic serum	0.465	0.290 to 0.641	<0.001
Healthy volunteers vs. Suspected septic ED patients	0.117	-0.072 to 0.307	0.319
Healthy volunteers vs. Septic ICU patients	0.508	0.333 to 0.684	<0.001

Table B11 Pairwise differences in TMRM in lymphocytes between different participant groups at 24 hours in the absence of LPS.

Dunnett's multiple comparisons test	Mean diff.	95.00% CI of diff.	p-value
Control			
Healthy volunteers vs. Healthy with septic serum	0.506	0.319 to 0.694	<0.001
Healthy volunteers vs. Suspected septic ED patients	0.091	-0.111 to 0.294	0.563
Healthy volunteers vs. Septic ICU patients	0.578	0.391 to 0.766	<0.001
Ciprofloxacin			
Healthy volunteers vs. Healthy with septic serum	0.506	0.319 to 0.694	<0.001
Healthy volunteers vs. Suspected septic ED patients	0.091	-0.111 to 0.294	0.563
Healthy volunteers vs. Septic ICU patients	0.578	0.391 to 0.766	<0.001
Propofol			
Healthy volunteers vs. Healthy with septic serum	0.506	0.319 to 0.694	<0.001
Healthy volunteers vs. Suspected septic ED patients	0.091	-0.111 to 0.294	0.563
Healthy volunteers vs. Septic ICU patients	0.578	0.391 to 0.766	<0.001
NE			
Healthy volunteers vs. Healthy with septic serum	0.506	0.319 to 0.694	<0.001
Healthy volunteers vs. Suspected septic ED patients	0.091	-0.111 to 0.294	0.563
Healthy volunteers vs. Septic ICU patients	0.578	0.391 to 0.766	<0.001
Mix of 3 drugs			
Healthy volunteers vs. Healthy with septic serum	0.506	0.319 to 0.694	<0.001
Healthy volunteers vs. Suspected septic ED patients	0.091	-0.111 to 0.294	0.563
Healthy volunteers vs. Septic ICU patients	0.578	0.391 to 0.766	<0.001

Table B12 Pairwise differences in TMRM in lymphocytes between different participant groups at 24 hours in the presence of LPS.

Dunnett's multiple comparisons test	Mean diff.	95.00% CI of diff.	p-value

Control			
Healthy volunteers vs. Healthy with septic serum	0.525	0.329 to 0.720	<0.001
Healthy volunteers vs. Suspected septic ED patients	0.189	-0.021 to 0.408	0.088
Healthy volunteers vs. Septic ICU patients	0.644	0.449 to 0.840	<0.001
Ciprofloxacin			
Healthy volunteers vs. Healthy with septic serum	0.525	0.329 to 0.720	<0.001
Healthy volunteers vs. Suspected septic ED patients	0.189	-0.021 to 0.408	0.088
Healthy volunteers vs. Septic ICU patients	0.644	0.449 to 0.840	<0.001
Propofol			
Healthy volunteers vs. Healthy with septic serum	0.525	0.329 to 0.720	<0.001
Healthy volunteers vs. Suspected septic ED patients	0.189	-0.021 to 0.408	0.088
Healthy volunteers vs. Septic ICU patients	0.644	0.449 to 0.840	<0.001
NE			
Healthy volunteers vs. Healthy with septic serum	0.525	0.329 to 0.720	<0.001
Healthy volunteers vs. Suspected septic ED patients	0.189	-0.021 to 0.408	0.088
Healthy volunteers vs. Septic ICU patients	0.644	0.449 to 0.840	<0.001
Mix of 3 drugs			
Healthy volunteers vs. Healthy with septic serum	0.525	0.329 to 0.720	<0.001
Healthy volunteers vs. Suspected septic ED patients	0.189	-0.021 to 0.408	0.088
Healthy volunteers vs. Septic ICU patients	0.644	0.449 to 0.840	<0.001

Table B13 Pairwise differences in TMRM in lymphocytes between different drug groups at 6 hours in the absence of LPS.

Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	p-value
Healthy volunteers			
Control vs. Ciprofloxacin	-0.072	-0.205 to 0.059	0.250
Control vs. Propofol	-0.184	-0.340 to -0.027	0.029
Control vs. NE	-0.025	-0.184 to 0.132	0.917
Control vs. Mix of 3 drugs	-0.200	-0.276 to -0.123	0.001
Healthy with septic serum			
Control vs. Ciprofloxacin	0.027	-0.079 to 0.135	0.811
Control vs. Propofol	-0.397	-0.604 to -0.190	0.002
Control vs. NE	-0.164	-0.318 to -0.010	0.038
Control vs. Mix of 3 drugs	-0.267	-0.638 to 0.104	0.156
Suspected septic ED patients			
Control vs. Ciprofloxacin	-0.077	-0.207 to 0.052	0.210
Control vs. Propofol	-0.414	-0.790 to -0.038	0.036
Control vs. NE	-0.049	-0.575 to 0.475	0.985
Control vs. Mix of 3 drugs	-0.345	-1.23 to 0.541	0.470
Septic ICU patients			
Control vs. Ciprofloxacin	-0.017	-0.118 to 0.083	0.941
Control vs. Propofol	-0.394	-0.686 to -0.103	0.013
Control vs. NE	-0.080	-0.412 to 0.251	0.839
Control vs. Mix of 3 drugs	-0.297	-1.016 to 0.420	0.517

Table B14 Pairwise differences in TMRM in lymphocytes between different drug groups at 6 hours in the presence of LPS.

Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	P Value
Healthy volunteers			
Control vs. Ciprofloxacin	-0.002	-0.115 to 0.112	>0.999
Control vs. Propofol	-0.080	-0.194 to 0.033	0.139
Control vs. NE	0.023	-0.113 to 0.160	0.905
Control vs. Mix of 3 drugs	-0.153	-0.325 to 0.019	0.072
Healthy with septic serum			
Control vs. Ciprofloxacin	0.001	-0.1545 to 0.156	>0.999
Control vs. Propofol	-0.304	-0.5129 to -0.096	0.009
Control vs. NE	-0.124	-0.2793 to 0.030	0.110
Control vs. Mix of 3 drugs	-0.280	-0.6702 to 0.108	0.155
Suspected septic ED patients			
Control vs. Ciprofloxacin	-0.030	-0.200 to 0.139	0.889
Control vs. Propofol	-0.444	-0.735 to -0.153	0.011
Control vs. NE	0.203	-0.982 to 1.389	0.903
Control vs. Mix of 3 drugs	-0.207	-1.204 to 0.789	0.837
Septic ICU patients			
Control vs. Ciprofloxacin	-0.071	-0.192 to 0.050	0.267
Control vs. Propofol	-0.426	-0.880 to 0.027	0.063
Control vs. NE	0.0027	-0.249 to 0.255	>0.999
Healthy volunteers	-0.504	-1.050 to 0.039	0.066

Table B!5 Pairwise differences in TMRM in lymphocytes between different drug groups at 24 hours in the absence of LPS.

Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	p-value
Healthy volunteers			
Control vs. Ciprofloxacin	-0.002	-0.115 to 0.112	>0.999
Control vs. Propofol	-0.080	-0.194 to 0.033	0.139
Control vs. NE	0.023	-0.113 to 0.160	0.905
Control vs. Mix of 3 drugs	-0.153	-0.325 to 0.019	0.072
Healthy with septic serum			
Control vs. Ciprofloxacin	0.001	-0.154 to 0.156	>0.999
Control vs. Propofol	-0.305	-0.512 to -0.096	0.009
Control vs. NE	-0.124	-0.279 to 0.030	0.110
Control vs. Mix of 3 drugs	-0.280	-0.670 to 0.108	0.155
Suspected septic ED patients			
Control vs. Ciprofloxacin	-0.030	-0.200 to 0.139	0.889
Control vs. Propofol	-0.444	-0.735 to -0.153	0.011
Control vs. NE	0.203	-0.982 to 1.389	0.903
Control vs. Mix of 3 drugs	-0.207	-1.204 to 0.789	0.837
Septic ICU patients			
Control vs. Ciprofloxacin	-0.071	-0.192 to 0.050	0.267
Control vs. Propofol	-0.426	-0.880 to 0.027	0.063
Control vs. NE	0.0027	-0.249 to 0.255	>0.999
Control vs. Mix of 3 drugs	-0.504	-1.050 to 0.039	0.066

Table B16 Pairwise differences in TMRM in lymphocytes between different drug groups at 24 hours in the presence of LPS.

Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	p-value
Healthy volunteers			
Control vs. Ciprofloxacin	-0.092	-0.305 to 0.119	0.392
Control vs. Propofol	-0.153	-0.320 to 0.014	0.066
Control vs. NE	-0.048	-0.181 to 0.084	0.517
Control vs. Mix of 3 drugs	-0.218	-0.363 to -0.072	0.012
Healthy with septic serum			
Control vs. Ciprofloxacin	-0.052	-0.252 to 0.146	0.802
Control vs. Propofol	-0.228	-0.321 to -0.134	0.001
Control vs. NE	-0.133	-0.276 to 0.010	0.067
Control vs. Mix of 3 drugs	-0.267	-0.773 to 0.238	0.338
Suspected septic ED patients			
Control vs. Ciprofloxacin	-0.0706	-0.359 to 0.217	0.763
Control vs. Propofol	-0.391	-0.743 to -0.038	0.035
Control vs. NE	-0.109	-0.509 to 0.290	0.701
Control vs. Mix of 3 drugs	-0.020	-1.130 to 1.089	>0.999
Septic ICU patients			
Control vs. Ciprofloxacin	-0.020	-0.156 to 0.115	0.9604
Control vs. Propofol	-0.389	-0.744 to -0.034	0.0343
Control vs. NE	0.047	-0.081 to 0.176	0.6013
Control vs. Mix of 3 drugs	-0.341	-1.092 to 0.409	0.4485

Table B17 Pairwise differences in Mitosox in monocytes between different participant groups at 6 hours in the absence of LPS.

Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	P Value
Control			
Healthy volunteers vs. Healthy with septic serum	-0.764	-1.109 to -0.418	<0.001
Healthy volunteers vs. Suspected septic ED patients	-0.478	-0.917 to -0.040	0.035
Healthy volunteers vs. Septic ICU patients	-0.421	-1.061 to 0.218	0.208
Ciprofloxacin			
Healthy volunteers vs. Healthy with septic serum	-0.537	-1.065 to -0.009	0.046
Healthy volunteers vs. Suspected septic ED patients	-0.704	-1.207 to -0.201	0.011
Healthy volunteers vs. Septic ICU patients	-0.112	-0.664 to 0.439	0.897
Propofol			
Healthy volunteers vs. Healthy with septic serum	-0.613	-0.955 to -0.272	0.002
Healthy volunteers vs. Suspected septic ED patients	-0.709	-1.155 to -0.264	0.008
Healthy volunteers vs. Septic ICU patients	-0.342	-0.612 to -0.073	0.015
NE			
Healthy volunteers vs. Healthy with septic serum	-0.807	-1.212 to -0.403	0.001
Healthy volunteers vs. Suspected septic ED patients	-1.000	-1.444 to -0.556	0.001
Healthy volunteers vs. Septic ICU patients	-0.569	-1.011 to -0.128	0.013
Mix of 3 drugs			
Healthy volunteers vs. Healthy with septic serum	-0.731	-1.124 to -0.340	0.001
Healthy volunteers vs. Suspected septic ED patients	-0.772	-1.302 to -0.242	0.009
Healthy volunteers vs. Septic ICU patients	-0.456	-0.878 to -0.034	0.034

Table B18 Pairwise differences in Mitosox in monocytes between different participant groups at 6 hours in the presence of LPS.

Dunnett's multiple comparisons test	Mean diff.	95.00% CI of diff.	P Value
Control			
Healthy volunteers vs. Healthy with septic serum	-0.549	-0.722 to -0.375	<0.001
Healthy volunteers vs. Suspected septic ED patients	-0.694	-0.883 to -0.505	<0.001
Healthy volunteers vs. Septic ICU patients	-0.463	-0.637 to -0.289	<0.001
Ciprofloxacin			
Healthy volunteers vs. Healthy with septic serum	-0.549	-0.722 to -0.375	<0.001
Healthy volunteers vs. Suspected septic ED patients	-0.694	-0.883 to -0.505	<0.001
Healthy volunteers vs. Septic ICU patients	-0.463	-0.637 to -0.289	<0.001
Propofol			
Healthy volunteers vs. Healthy with septic serum	-0.549	-0.722 to -0.375	<0.001
Healthy volunteers vs. Suspected septic ED patients	-0.694	-0.883 to -0.505	<0.001
Healthy volunteers vs. Septic ICU patients	-0.463	-0.637 to -0.289	<0.001
NE			
Healthy volunteers vs. Healthy with septic serum	-0.549	-0.723 to -0.375	<0.001
Healthy volunteers vs. Suspected septic ED patients	-0.694	-0.883 to -0.505	<0.001
Healthy volunteers vs. Septic ICU patients	-0.463	-0.637 to -0.289	<0.001
Mix of 3 drugs			
Healthy volunteers vs. Healthy with septic serum	-0.549	-0.722 to -0.375	<0.001
Healthy volunteers vs. Suspected septic ED patients	-0.694	-0.883 to -0.505	<0.001
Healthy volunteers vs. Septic ICU patients	-0.463	-0.637 to -0.289	<0.001

Table B19 Pairwise differences in Mitosox in monocytes between different participant groups at 24 hours in the absence of LPS.

Dunnett's multiple comparisons test	Mean diff.	95.00% CI of diff.	P Value
Control			
Healthy volunteers vs. Healthy with septic serum	-0.501	-0.696 to -0.306	<0.001
Healthy volunteers vs. Suspected septic ED patients	-0.565	-0.777 to -0.352	<0.001
Healthy volunteers vs. Septic ICU patients	-0.185	-0.380 to 0.009	0.065
Ciprofloxacin			
Healthy volunteers vs. Healthy with septic serum	-0.501	-0.696 to -0.306	<0.001
Healthy volunteers vs. Suspected septic ED patients	-0.565	-0.777 to -0.352	<0.001
Healthy volunteers vs. Septic ICU patients	-0.185	-0.380 to 0.009	0.065
Propofol			
Healthy volunteers vs. Healthy with septic serum	-0.501	-0.696 to -0.306	<0.001
Healthy volunteers vs. Suspected septic ED patients	-0.565	-0.777 to -0.352	<0.001
Healthy volunteers vs. Septic ICU patients	-0.185	-0.381 to 0.009	0.065
NE			
Healthy volunteers vs. Healthy with septic serum	-0.501	-0.696 to -0.306	<0.001
Healthy volunteers vs. Suspected septic ED patients	-0.565	-0.777 to -0.352	<0.001
Healthy volunteers vs. Septic ICU patients	-0.185	-0.381 to 0.009	0.065
Mix of 3 drugs			
Healthy volunteers vs. Healthy with septic serum	-0.501	-0.696 to -0.306	<0.001
Healthy volunteers vs. Suspected septic ED patients	-0.565	-0.777 to -0.352	<0.001
Healthy volunteers vs. Septic ICU patients	-0.185	-0.381 to 0.009	0.065

Table B20 Pairwise differences in Mitosox in monocytes between different participant groups at 24 hours in the presence of LPS.

Dunnett's multiple comparisons test	Mean diff.	95.00% CI of diff.	P Value
Control			
Healthy volunteers vs. Healthy with septic serum	-0.294	-0.523 to -0.065	0.008
Healthy volunteers vs. Suspected septic ED patients	-0.599	-0.849 to -0.350	<0.001
Healthy volunteers vs. Septic ICU patients	-0.268	-0.498 to -0.039	0.017
Ciprofloxacin			
Healthy volunteers vs. Healthy with septic serum	-0.294	-0.523 to -0.065	0.008
Healthy volunteers vs. Suspected septic ED patients	-0.599	-0.849 to -0.350	<0.001
Healthy volunteers vs. Septic ICU patients	-0.268	-0.498 to -0.039	0.017
Propofol			
Healthy volunteers vs. Healthy with septic serum	-0.294	-0.523 to -0.065	0.008
Healthy volunteers vs. Suspected septic ED patients	-0.599	-0.849 to -0.350	<0.001
Healthy volunteers vs. Septic ICU patients	-0.268	-0.498 to -0.039	0.017
NE			
Healthy volunteers vs. Healthy with septic serum	-0.294	-0.523 to -0.065	0.008
Healthy volunteers vs. Suspected septic ED patients	-0.599	-0.849 to -0.350	<0.001
Healthy volunteers vs. Septic ICU patients	-0.268	-0.498 to -0.039	0.017
Mix of 3 drugs			
Healthy volunteers vs. Healthy with septic serum	-0.294	-0.523 to -0.065	0.008
Healthy volunteers vs. Suspected septic ED patients	-0.599	-0.849 to -0.350	<0.001
Healthy volunteers vs. Septic ICU patients	-0.268	-0.498 to -0.039	0.017

Table B21 Pairwise differences in Mitosox in monocytes between different drug groups at 6 hours in the absence of LPS.

Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	P Value
Healthy volunteers			
Control vs. Ciprofloxacin	-0.033	-0.366 to 0.299	0.988
Control vs. Propofol	0.024	-0.143 to 0.192	0.956
Control vs. NE	0.015	-0.123 to 0.153	0.982
Control vs. Mix of 3 drugs	-0.082	-0.237 to 0.073	0.307
Healthy with septic serum			
Control vs. Ciprofloxacin	0.192	0.052 to 0.333	0.012
Control vs. Propofol	0.175	-0.016 to 0.366	0.070
Control vs. NE	-0.028	-0.261 to 0.204	0.980
Control vs. Mix of 3 drugs	-0.050	-0.296 to 0.195	0.900
Suspected septic ED patients			
Control vs. Ciprofloxacin	-0.258	-0.925 to 0.407	0.472
Control vs. Propofol	-0.206	-1.063 to 0.650	0.771
Control vs. NE	-0.505	-1.213 to 0.200	0.133
Control vs. Mix of 3 drugs	-0.375	-1.350 to 0.599	0.477
Septic ICU patients			
Control vs. Ciprofloxacin	0.275	-0.710 to 1.262	0.774
Control vs. Propofol	0.103	-0.803 to 1.011	0.985
Control vs. NE	-0.132	-1.120 to 0.854	0.973
Control vs. Mix of 3 drugs	-0.117	-1.056 to 0.822	0.979

Table B22 Pairwise differences in Mitosox in monocytes between different drug groups at 6 hours in the presence of LPS.

Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	P Value
Healthy volunteers			
Control vs. Ciprofloxacin	0.246	-0.087 to 0.580	0.135
Control vs. Propofol	0.150	-0.117 to 0.417	0.272
Control vs. NE	0.088	-0.278 to 0.455	0.817
Control vs. Mix of 3 drugs	0.008	-0.335 to 0.353	0.999
Healthy with septic serum			
Control vs. Ciprofloxacin	0.096	-0.110 to 0.303	0.429
Control vs. Propofol	0.563	0.216 to 0.910	0.005
Control vs. NE	-0.211	-0.478 to 0.056	0.117
Control vs. Mix of 3 drugs	-0.157	-0.459 to 0.143	0.345
Suspected septic ED patients			
Control vs. Ciprofloxacin	-0.309	-0.858 to 0.240	0.240
Control vs. Propofol	-0.372	-0.976 to 0.229	0.193
Control vs. NE	-0.391	-1.195 to 0.411	0.322
Control vs. Mix of 3 drugs	-0.291	-1.349 to 0.766	0.697
Septic ICU patients			
Control vs. Ciprofloxacin	0.248	-0.712 to 1.209	0.814
Control vs. Propofol	0.007	-0.770 to 0.784	>0.999
Control vs. NE	-0.165	-0.950 to 0.619	0.892
Control vs. Mix of 3 drugs	-0.178	-0.970 to 0.614	0.870

Table B23 Pairwise differences in Mitosox in monocytes between different drug groups at 24 hours in the absence of LPS.

Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	P Value
Healthy volunteers			
Control vs. Ciprofloxacin	0.061	-0.319 to 0.442	0.939
Control vs. Propofol	0.139	-0.205 to 0.483	0.499
Control vs. NE	0.006	-0.160 to 0.173	0.999
Control vs. Mix of 3 drugs	0.026	-0.155 to 0.208	0.955
Healthy with septic serum			
Control vs. Ciprofloxacin	-0.051	-0.696 to 0.594	0.996
Control vs. Propofol	-0.045	-0.521 to 0.430	0.992
Control vs. NE	-0.330	-0.889 to 0.228	0.264
Control vs. Mix of 3 drugs	-0.139	-0.588 to 0.308	0.713
Suspected septic ED patients			
Control vs. Ciprofloxacin	-0.116	-0.331 to 0.098	0.262
Control vs. Propofol	0.125	-0.198 to 0.449	0.472
Control vs. NE	-0.026	-0.842 to 0.789	0.999
Control vs. Mix of 3 drugs	0.120	-0.331 to 0.572	0.717
Septic ICU patients			
Control vs. Ciprofloxacin	0.132	-0.033 to 0.299	0.112
Control vs. Propofol	-0.063	-0.449 to 0.323	0.949
Control vs. NE	-0.291	-0.568 to -0.013	0.041
Control vs. Mix of 3 drugs	-0.141	-0.556 to 0.273	0.656

Table B24 Pairwise differences in Mitosox in monocytes between different drug groups at 24 hours in the presence of LPS.

Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	P Value
Healthy volunteers			
Control vs. Ciprofloxacin	0.197	-0.086 to 0.481	0.160
Control vs. Propofol	0.371	0.101 to 0.641	0.014
Control vs. NE	0.073	-0.393 to 0.539	0.945
Control vs. Mix of 3 drugs	0.191	-0.135 to 0.518	0.247
Healthy with septic serum			
Control vs. Ciprofloxacin	0.143	-0.456 to 0.742	0.847
Control vs. Propofol	-0.110	-0.396 to 0.175	0.568
Control vs. NE	-0.329	-0.809 to 0.150	0.179
Control vs. Mix of 3 drugs	-0.236	-0.568 to 0.095	0.161
Suspected septic ED patients			
Control vs. Ciprofloxacin	-0.097	-0.399 to 0.204	0.597
Control vs. Propofol	0.012	-0.220 to 0.246	0.997
Control vs. NE	-0.053	-0.635 to 0.528	0.987
Control vs. Mix of 3 drugs	0.023	-0.402 to 0.450	0.997
Septic ICU patients			
Control vs. Ciprofloxacin	0.095	-0.081 to 0.272	0.326
Control vs. Propofol	0.054	-0.240 to 0.350	0.925
Control vs. NE	-0.397	-0.704 to -0.089	0.016
Control vs. Mix of 3 drugs	-0.180	-0.643 to 0.283	0.564

Table B25 Pairwise differences in TMRM in monocytes between different participant groups at 6 hours in the absence of LPS.

Dunnett's multiple comparisons test	Mean diff.	95.00% CI of diff.	P Value
Control			
Healthy volunteers vs. Healthy with septic serum	1.362	1.163 to 1.562	<0.001
Healthy volunteers vs. Suspected septic ED patients	1.210	0.994 to 1.426	<0.001
Healthy volunteers vs. Septic ICU patients	0.840	0.640 to 1.040	<0.001
Ciprofloxacin			
Healthy volunteers vs. Healthy with septic serum	1.362	1.163 to 1.562	<0.001
Healthy volunteers vs. Suspected septic ED patients	1.210	0.994 to 1.426	<0.001
Healthy volunteers vs. Septic ICU patients	0.840	0.640 to 1.040	<0.001
Propofol			
Healthy volunteers vs. Healthy with septic serum	1.362	1.163 to 1.562	<0.001
Healthy volunteers vs. Suspected septic ED patients	1.210	0.994 to 1.426	<0.001
Healthy volunteers vs. Septic ICU patients	0.840	0.640 to 1.040	<0.001
NE			
Healthy volunteers vs. Healthy with septic serum	1.362	1.163 to 1.562	<0.001
Healthy volunteers vs. Suspected septic ED patients	1.210	0.994 to 1.426	<0.001
Healthy volunteers vs. Septic ICU patients	0.840	0.640 to 1.040	<0.001
Mix of 3 drugs			
Healthy volunteers vs. Healthy with septic serum	1.362	1.163 to 1.562	<0.001
Healthy volunteers vs. Suspected septic ED patients	1.210	0.994 to 1.426	<0.001
Healthy volunteers vs. Septic ICU patients	0.840	0.640 to 1.040	<0.001

Table B26 Pairwise differences in TMRM in monocytes between different participant groups at 6 hours in the presence of LPS.

Dunnett's multiple comparisons test	Mean diff.	95.00% CI of diff.	P Value
Control			
Healthy volunteers vs. Healthy with septic serum	1.199	0.991 to 1.406	<0.001
Healthy volunteers vs. Suspected septic ED patients	1.075	0.850 to 1.299	<0.001
Healthy volunteers vs. Septic ICU patients	0.732	0.525 to 0.940	<0.001
Ciprofloxacin			
Healthy volunteers vs. Healthy with septic serum	1.199	0.991 to 1.406	<0.001
Healthy volunteers vs. Suspected septic ED patients	1.075	0.850 to 1.299	<0.001
Healthy volunteers vs. Septic ICU patients	0.732	0.525 to 0.940	<0.001
Propofol			
Healthy volunteers vs. Healthy with septic serum	1.199	0.991 to 1.406	<0.001
Healthy volunteers vs. Suspected septic ED patients	1.075	0.850 to 1.299	<0.001
Healthy volunteers vs. Septic ICU patients	0.732	0.525 to 0.940	<0.001
NE			
Healthy volunteers vs. Healthy with septic serum	1.199	0.991 to 1.406	<0.001
Healthy volunteers vs. Suspected septic ED patients	1.075	0.850 to 1.299	<0.001
Healthy volunteers vs. Septic ICU patients	0.732	0.525 to 0.940	<0.001
Mix of 3 drugs			
Healthy volunteers vs. Healthy with septic serum	1.199	0.991 to 1.406	<0.001
Healthy volunteers vs. Suspected septic ED patients	1.075	0.850 to 1.299	<0.001
Healthy volunteers vs. Septic ICU patients	0.732	0.525 to 0.940	<0.001

Table B27 Pairwise differences in TMRM in monocytes between different participant groups at 24 hours in the absence of LPS.

Dunnett's multiple comparisons test	Mean diff.	95.00% CI of diff.	P Value
Control			
Healthy volunteers vs. Healthy with septic serum	1.568	1.362 to 1.773	<0.001
Healthy volunteers vs. Suspected septic ED patients	1.406	1.184 to 1.628	<0.001
Healthy volunteers vs. Septic ICU patients	1.026	0.820 to 1.231	<0.001
Ciprofloxacin			
Healthy volunteers vs. Healthy with septic serum	1.568	1.362 to 1.773	<0.001
Healthy volunteers vs. Suspected septic ED patients	1.406	1.184 to 1.628	<0.001
Healthy volunteers vs. Septic ICU patients	1.026	0.820 to 1.231	<0.001
Propofol			
Healthy volunteers vs. Healthy with septic serum	1.568	1.362 to 1.773	<0.001
Healthy volunteers vs. Suspected septic ED patients	1.406	1.184 to 1.628	<0.001
Healthy volunteers vs. Septic ICU patients	1.026	0.820 to 1.231	<0.001
NE			
Healthy volunteers vs. Healthy with septic serum	1.568	1.362 to 1.773	<0.001
Healthy volunteers vs. Suspected septic ED patients	1.406	1.184 to 1.628	<0.001
Healthy volunteers vs. Septic ICU patients	1.026	0.820 to 1.231	<0.001
Mix of 3 drugs			
Healthy volunteers vs. Healthy with septic serum	1.568	1.362 to 1.773	<0.001
Healthy volunteers vs. Suspected septic ED patients	1.406	1.184 to 1.628	<0.001
Healthy volunteers vs. Septic ICU patients	1.026	0.820 to 1.231	<0.001

Table B28 Pairwise differences in TMRM in monocytes between different participant groups at 24 hours in the presence of LPS.

Dunnett's multiple comparisons test	Mean diff.	95.00% CI of diff.	P Value
Control			
Healthy volunteers vs. Healthy with septic serum	1.533	1.357 to 1.708	<0.001
Healthy volunteers vs. Suspected septic ED patients	1.467	1.278 to 1.656	<0.001
Healthy volunteers vs. Septic ICU patients	0.995	0.819 to 1.170	<0.001
Ciprofloxacin			
Healthy volunteers vs. Healthy with septic serum	1.533	1.357 to 1.708	<0.001
Healthy volunteers vs. Suspected septic ED patients	1.467	1.278 to 1.656	<0.001
Healthy volunteers vs. Septic ICU patients	0.995	0.819 to 1.170	<0.001
Propofol			
Healthy volunteers vs. Healthy with septic serum	1.533	1.357 to 1.708	<0.001
Healthy volunteers vs. Suspected septic ED patients	1.467	1.278 to 1.656	<0.001
Healthy volunteers vs. Septic ICU patients	0.995	0.819 to 1.170	<0.001
NE			
Healthy volunteers vs. Healthy with septic serum	1.533	1.357 to 1.708	<0.001
Healthy volunteers vs. Suspected septic ED patients	1.467	1.278 to 1.656	<0.001
Healthy volunteers vs. Septic ICU patients	0.995	0.819 to 1.170	<0.001
Mix of 3 drugs			
Healthy volunteers vs. Healthy with septic serum	1.533	1.357 to 1.708	<0.001
Healthy volunteers vs. Suspected septic ED patients	1.467	1.278 to 1.656	<0.001
Healthy volunteers vs. Septic ICU patients	0.995	0.819 to 1.170	<0.001

Table B29 Pairwise differences in TMRM in monocytes between different drug groups at 6 hours in the absence of LPS.

Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	p-value
Healthy volunteers			
Ciprofloxacin vs. Propofol	-0.012	-0.221 to 0.197	0.997
Ciprofloxacin vs. NE	0.120	-0.191 to 0.431	0.476
Ciprofloxacin vs. Mix of 3 drugs	-0.039	-0.284 to 0.207	0.922
Ciprofloxacin vs. Mix of 3 drugs	-0.006	-0.303 to 0.292	>0.999
Healthy with septic serum			
Ciprofloxacin vs. Propofol	0.034	-0.072 to 0.142	0.688
Ciprofloxacin vs. NE	-0.142	-0.340 to 0.055	0.155
Ciprofloxacin vs. Mix of 3 drugs	-0.016	-0.119 to 0.086	0.953
Ciprofloxacin vs. Mix of 3 drugs	-0.155	-0.366 to 0.055	0.144
Suspected septic ED patients			
Ciprofloxacin vs. Propofol	-<0.001	-0.434 to 0.433	>0. 999
Ciprofloxacin vs. NE	-0.1563	-0.481 to 0.168	0.330
Ciprofloxacin vs. Mix of 3 drugs	0.101	-0.339 to 0.541	0.795
Ciprofloxacin vs. Mix of 3 drugs	-0.081	-0.260 to 0.098	0.371
Septic ICU patients			
Ciprofloxacin vs. Propofol	-0.005	-0.448 to 0.438	>0.999
Ciprofloxacin vs. NE	-0.283	-0.592 to 0.026	0.069
Ciprofloxacin vs. Mix of 3 drugs	-0.023	-0.313 to 0.266	0.995
Ciprofloxacin vs. Mix of 3 drugs	-0.322	-0.735 to 0.091	0.121

Table B30 Pairwise differences in TMRM in monocytes between different drug groups at 6 hours in the presence of LPS.

Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	p-value
Healthy volunteers			
Control vs. Ciprofloxacin	0.042	-0.121 to 0.207	0.728
Control vs. Propofol	0.340	0.049 to 0.631	0.029
Control vs. NE	0.002	-0.275 to 0.279	>0.999
Control vs. Mix of 3 drugs	0.074	-0.345 to 0.494	0.893
Healthy with septic serum			
Control vs. Ciprofloxacin	0.018	-0.090 to 0.127	0.944
Control vs. Propofol	-0.114	-0.305 to 0.077	0.257
Control vs. NE	-0.025	-0.133 to 0.083	0.862
Control vs. Mix of 3 drugs	-0.042	-0.341 to 0.257	0.970
Suspected septic ED patients			
Control vs. Ciprofloxacin	0.058	-0.278 to 0.395	0.898
Control vs. Propofol	-0.100	-0.264 to 0.063	0.198
Control vs. NE	0.209	-0.585 to 1.003	0.724
Control vs. Mix of 3 drugs	0.009	-0.171 to 0.189	0.998
Septic ICU patients			
Control vs. Ciprofloxacin	-<0.001	-0.274 to 0.273	>0.999
Control vs. Propofol	-0.313	-0.651 to 0.023	0.065
Control vs. NE	-0.063	-0.222 to 0.094	0.540
Control vs. Mix of 3 drugs	-0.346	-0.699 to 0.006	0.053

Table B31 Pairwise differences in TMRM in monocytes between different drug groups at 24 hours in the absence of LPS.

Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	p-value
Healthy volunteers			
Control vs. Ciprofloxacin	-0.075	-0.199 to 0.047	0.194
Control vs. Propofol	0.205	-0.049 to 0.461	0.096
Control vs. NE	-0.054	-0.200 to 0.091	0.498
Control vs. Mix of 3 drugs	-0.121	-0.295 to 0.052	0.142
Healthy with septic serum			
Control vs. Ciprofloxacin	0.071	-0.012 to 0.167	0.086
Control vs. Propofol	-0.062	-0.232 to 0.107	0.609
Control vs. NE	-0.019	-0.203 to 0.164	0.988
Control vs. Mix of 3 drugs	-0.120	-0.338 to 0.096	0.304
Suspected septic ED patients			
Control vs. Ciprofloxacin	0.014	-0.236 to 0.264	0.997
Control vs. Propofol	-0.121	-0.522 to 0.281	0.643
Control vs. NE	0.367	-0.302 to 1.037	0.253
Control vs. Mix of 3 drugs	0.087	-0.344 to 0.518	0.850
Septic ICU patients			
Control vs. Ciprofloxacin	-0.095	-0.337 to 0.147	0.559
Control vs. Propofol	-0.332	-0.767 to 0.103	0.130
Control vs. NE	0.0223	-0.114 to 0.158	0.949
Control vs. Mix of 3 drugs	-0.287	-0.766 to 0.191	0.254

Table B32 Pairwise differences in TMRM in monocytes between different drug groups at 24 hours in the presence of LPS.

Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	p-value
Healthy volunteers			
Control vs. Ciprofloxacin	0.059	-0.110 to 0.228	0.545
Control vs. Propofol	0.087	-0.263 to 0.437	0.756
Control vs. NE	-0.073	-0.191 to 0.044	0.186
Control vs. Mix of 3 drugs	0.019	-0.304 to 0.343	0.997
Healthy with septic serum			
Control vs. Ciprofloxacin	0.008	-0.158 to 0.174	0.999
Control vs. Propofol	-0.015	-0.109 to 0.079	0.956
Control vs. NE	-0.050	-0.204 to 0.102	0.678
Control vs. Mix of 3 drugs	-0.039	-0.166 to 0.086	0.708
Suspected septic ED patients			
Control vs. Ciprofloxacin	0.079	-0.088 to 0.247	0.343
Control vs. Propofol	-0.060	-0.272 to 0.153	0.683
Control vs. NE	0.263	-0.543 to 1.071	0.591
Control vs. Mix of 3 drugs	0.188	-0.108 to 0.485	0.182
Septic ICU patients			
Control vs. Ciprofloxacin	-0.150	-0.415 to 0.114	0.289
Control vs. Propofol	-0.387	-0.673 to -0.102	0.013
Control vs. NE	-0.033	-0.225 to 0.159	0.939
Control vs. Mix of 3 drugs	-0.341	-0.663 to -0.0184	0.040

Bibliography

1. Al-Hashimi M, Scott SW, Thompson JP, Lambert DG. Opioids and immune modulation: more questions than answers. Br J Anaesth. 2013;111(1):80-8.
2. Chidambaran V, Costandi A, D'Mello A. Propofol: a review of its role in pediatric anesthesia and sedation. CNS Drugs. 2015;29(7):543-63.
3. Jiang S, Liu Y, Huang L, Zhang F, Kang R. Effects of propofol on cancer development and chemotherapy: Potential mechanisms. Eur J Pharmacol. 2018;831:46-51.
4. Moyano J, Aguirre L. Opioids in the immune system: from experimental studies to clinical practice. Rev Assoc Med Bras (1992). 2019;65(2):262-9.
5. Plein LM, Rittner HL. Opioids and the immune system - friend or foe. Br J Pharmacol. 2018;175(14):2717-25.
6. Kallioinen M, Scheinin A, Maksimow M, Långsjö J, Kaisti K, Takala R, et al. The influence of dexmedetomidine and propofol on circulating cytokine levels in healthy subjects. BMC Anesthesiology. 2019;19(1).
7. Khosrow-Khavar F, Kurteva S, Cui Y, Filion KB, Douros A. Opioids and the Risk of Infection: A Critical Appraisal of the Pharmacologic and Clinical Evidence. Expert Opin Drug Metab Toxicol. 2019;15(7):565-75.
8. Zimmermann P, Ziesenitz VC, Curtis N, Ritz N. The Immunomodulatory Effects of Macrolides-A Systematic Review of the Underlying Mechanisms. Front Immunol. 2018;9:302.
9. Bahrambeigi S, Sanajou D, Shafiei-Irannejad V. Major fundamental factors hindering immune system in defense against tumor cells: The link between insufficiency of innate immune responses, metabolism, and neurotransmitters with effector immune cells disability. Immunol Lett. 2019;212:81-7.
10. Ince LM, Weber J, Scheiermann C. Control of Leukocyte Trafficking by Stress-Associated Hormones. Front Immunol. 2018;9:3143.
11. Katsuno G, Takahashi HK, Iwagaki H, Sugita S, Mori S, Saito S, et al. The effect of ciprofloxacin on CD14 and toll-like receptor-4 expression on human monocytes. Shock (Augusta, Ga). 2006;25(3):247-53.
12. Weiss T, Shalit I, Blau H, Werber S, Halperin D, Levitov A, et al. Anti-inflammatory effects of moxifloxacin on activated human monocytic cells: inhibition of NF-kappaB and mitogen-activated protein kinase activation and of synthesis of proinflammatory cytokines. Antimicrob Agents Chemother. 2004;48(6):1974-82.
13. Ziegeler S, Raddatz A, Hoff G, Buchinger H, Bauer I, Stockhausen A, et al. Antibiotics modulate the stimulated cytokine response to endotoxin in a human ex vivo, in vitro model. Acta Anaesthesiol Scand. 2006;50(9):1103-10.
14. Kalghatgi S, Spina CS, Costello JC, Liesa M, Morones-Ramirez JR, Slomovic S, et al. Bactericidal antibiotics induce mitochondrial dysfunction and oxidative damage in Mammalian cells. Sci Transl Med. 2013;5(192):192ra85.

15. Bode C, Muenster S, Diedrich B, Jahnert S, Weisheit C, Steinhagen F, et al. Linezolid, vancomycin and daptomycin modulate cytokine production, Toll-like receptors and phagocytosis in a human in vitro model of sepsis. J Antibiot (Tokyo). 2015;68(8):485-90.
16. Gosain A, Jones SB, Shankar R, Gamelli RL, DiPietro LA. Norepinephrine Modulates the Inflammatory and Proliferative Phases of Wound Healing. The Journal of Trauma: Injury, Infection, and Critical Care. 2006;60(4):736-44.
17. Beis D, von Kanel R, Heimgartner N, Zuccarella-Hackl C, Burkle A, Ehlert U, et al. The Role of Norepinephrine and alpha-Adrenergic Receptors in Acute Stress-Induced Changes in Granulocytes and Monocytes. Psychosom Med. 2018;80(7):649-58.
18. Torres KC, Antonelli LR, Souza AL, Teixeira MM, Dutra WO, Gollob KJ. Norepinephrine, dopamine and dexamethasone modulate discrete leukocyte subpopulations and cytokine profiles from human PBMC. J Neuroimmunol. 2005;166(1-2):144-57.
19. Bone RC, Balk RA, Cerra FB, Dellinger RP, Fein AM, Knaus WA, et al. Definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. The ACCP/SCCM Consensus Conference Committee. American College of Chest Physicians/Society of Critical Care Medicine. Chest. 1992;101(6):1644-55.
20. Singer M, Deutschman CS, Seymour CW, Shankar-Hari M, Annane D, Bauer M, et al. The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3). JAMA. 2016;315(8):801-10.
21. Shankar-Hari M, Harrison DA, Rowan KM. Differences in Impact of Definitional Elements on Mortality Precludes International Comparisons of Sepsis Epidemiology-A Cohort Study Illustrating the Need for Standardized Reporting. Crit Care Med. 2016;44(12):2223-30.
22. Rudd KE, Johnson SC, Agesa KM, Shackelford KA, Tsoi D, Kievlan DR, et al. Global, regional, and national sepsis incidence and mortality, 1990–2017: analysis for the Global Burden of Disease Study. The Lancet. 2020;395(10219):200-11.
23. Bauer M, Gerlach H, Vogelmann T, Preissing F, Stiefel J, Adam D. Mortality in sepsis and septic shock in Europe, North America and Australia between 2009 and 2019- results from a systematic review and meta-analysis. Crit Care. 2020;24(1):239.
24. Angus DC, Linde-Zwirble WT, Lidicker J, Clermont G, Carcillo J, Pinsky MR. Epidemiology of severe sepsis in the United States: analysis of incidence, outcome, and associated costs of care. Crit Care Med. 2001;29:1303–10.
25. Paoli CJ, Reynolds MA, Sinha M, Gitlin M, Crouser E. Epidemiology and Costs of Sepsis in the United States-An Analysis Based on Timing of Diagnosis and Severity Level. Crit Care Med. 2018;46(12):1889-97.
26. Singer M, Inada-Kim M, Shankar-Hari M. Sepsis hysteria: excess hype and unrealistic expectations. The Lancet. 2019;394(10208):1513-4.
27. Martin GS, Mannino DM, Eaton S, Moss M. The epidemiology of sepsis in the United States from 1979 through 2000. N Engl J Med. 2003;348:1546–54.

28. Vincent JL, Sakr Y, Sprung CL, Ranieri VM, Reinhart K, Gerlach H, et al. Sepsis in European intensive care units: results of the SOAP study. Crit Care Med. 2006;34(2):344-53.
29. Vincent JL, Rello J, Marshall J, Silva E, Anzueto A, Martin CD, et al. International study of the prevalence and outcomes of infection in intensive care units. JAMA. 2009;302:2323–9.
30. Luhr R, Cao Y, Soderquist B, Cajander S. Trends in sepsis mortality over time in randomised sepsis trials: a systematic literature review and meta-analysis of mortality in the control arm, 2002-2016. Crit Care. 2019;23(1):241.
31. Stevenson EK, Rubenstein AR, Radin GT, Wiener RS, Walkey AJ. Two decades of mortality trends among patients with severe sepsis: a comparative meta-analysis*. Crit Care Med. 2014;42(3):625-31.
32. Cecconi M, Evans L, Levy M, Rhodes A. Sepsis and septic shock. The Lancet. 2018;392(10141):75-87.
33. Ferrer R, Martin-Loeches I, Phillips G, Osborn TM, Townsend S, Dellinger RP, et al. Empiric antibiotic treatment reduces mortality in severe sepsis and septic shock from the first hour: results from a guideline-based performance improvement program. Crit Care Med. 2014;42(8):1749-55.
34. Kumar A, Ellis P, Arabi Y, Roberts D, Light B, Parrillo JE, et al. Initiation of inappropriate antimicrobial therapy results in a fivefold reduction of survival in human septic shock. Chest. 2009;136(5):1237-48.
35. Hex N, Retzler J, Bartlett C, Arber M. The cost of sepsis care in the UK. University of York, Whitewater Charitable Ttrust; 2017.
36. Arefian H, Heublein S, Scherag A, Brunkhorst FM, Younis MZ, Moerer O, et al. Hospital-related cost of sepsis: A systematic review. J Infect. 2017;74(2):107-17.
37. Medzhitov R. Recognition of microorganisms and activation of the immune response. Nature. 2007;449(7164):819-26.
38. Amarante-Mendes GP, Adjemian S, Branco LM, Zanetti LC, Weinlich R, Bortoluci KR. Pattern Recognition Receptors and the Host Cell Death Molecular Machinery. Front Immunol. 2018;9:2379.
39. Akira S, Takeda K. Toll-like receptor signalling. Nat Rev Immunol. 2004;4(7):499-511.
40. Roh JS, Sohn DH. Damage-Associated Molecular Patterns in Inflammatory Diseases. Immune Netw. 2018;18(4):e27.
41. Kawai T, Akira S. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nat Immunol. 2010;11(5):373-84.
42. Bowie A, O'Neill LA. The interleukin-1 receptor/Toll-like receptor superfamily: signal generators for pro-inflammatory interleukins and microbial products. J Leukoc Biol. 2000;67:508-14.
43. Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. Cell. 2006;124(4):783-801.
44. Mogensen TH. Pathogen recognition and inflammatory signaling in innate immune defenses. Clin Microbiol Rev. 2009;22(2):240-73, Table of Contents.
45. Boekstegers P, Weidenhöfer S, Kapsner T, Werdan K. Skeletal muscle partial pressure of oxygen in patients with sepsis. Crit Care Med. 1994;22(4):640-50.

46. Burgdorff AM, Bucher M, Schumann J. Vasoplegia in patients with sepsis and septic shock: pathways and mechanisms. J Int Med Res. 2018;46(4):1303-10.
47. Rudiger A, Singer M. Mechanisms of sepsis-induced cardiac dysfunction. Crit Care Med. 2007;35(6):1599-608.
48. Benedict CR, Rose JA. Arterial norepinephrine changes in patients with septic shock. Circ Shock. 1992;38(3):165-72.
49. Boyle WA, Parvathaneni LS, Bourlier V, Sauter C, Laubach VE, Cobb JP. iNOS gene expression modulates microvascular responsiveness in endotoxin-challenged mice. Circ Res. 2000;87:E18-24.
50. Levy B, Collin S, Sennoun N, Ducrocq N, Kimmoun A, Asfar P, et al. Vascular hyporesponsiveness to vasopressors in septic shock: from bench to bedside. Intensive Care Med. 2010;36(12):2019-29.
51. Raeburn CD, Calkins CM, Zimmerman MA, Song Y, Ao L, Banerjee A, et al. Vascular cell adhesion molecule--1 expression is obligatory for endotoxin-induced myocardial neutrophil accumulation and contractile dysfunction. Surgery. 2001;130(2):319-25.
52. Kumar A, Thota V, Dee L, Olson J, Uretz E, Parrillo JE. Tumor necrosis factor alpha and interleukin 1beta are responsible for in vitro myocardial cell depression induced by human septic shock serum. . J Exp Med. 1996;183:949-58.
53. Dal-Secco D, DalBo S, Lautherbach NES, Gava FN, Celes MRN, Benedet PO, et al. Cardiac hyporesponsiveness in severe sepsis is associated with nitric oxide-dependent activation of G protein receptor kinase. Am J Physiol Heart Circ Physiol. 2017;313(1):H149-H63.
54. Gogos C, Kotsaki A, Pelekanou A, Giannikopoulos G, Vaki I, Maravitsa P, et al. Early alterations of the innate and adaptive immune statuses in sepsis according to the type of underlying infection. Crit Care. 2010;14(3):R96.
55. Gaudio AR, Rinaldi S, CHelazzi C, Borracci T. Pathophysiology of Sepsis in the Elderly: Clinical Impact and Therapeutic Considerations. Curr Drug Targets. 2009;10(1):60-70.
56. Turnbull IR, Clark AT, Stromberg PE, Dixon DJ, Woolsey CA, Davis CG, et al. Effects of aging on the immunopathologic response to sepsis. Critical Care Medicine. 2009;37(3):1018-23.
57. Rosolem MM, Rabello LSCF, Lisboa T, Caruso P, Costa RT, Leal JVR, et al. Critically ill patients with cancer and sepsis: Clinical course and prognostic factors. Journal of Critical Care. 2012;27(3):301-7.
58. Shankar-Hari M, Ambler M, Mahalingasivam V, Jones A, Rowan K, Rubenfeld GD. Evidence for a causal link between sepsis and long-term mortality: a systematic review of epidemiologic studies. Crit Care. 2016;20:101.
59. Actor JK. Cells and Organs of the Immune System. Elsevier's Integrated Review Immunology and Microbiology2012. p. 7-16.
60. Alves-Filho JC, Spiller F, Cunha FQ. Neutrophil paralysis in sepsis. Shock. 2010;34 Suppl 1:15-21.
61. Urban CF, Ermert D, Schmid M, Abu-Abed U, Goosmann C, Nacken W, et al. Neutrophil extracellular traps contain calprotectin, a cytosolic protein complex involved in host defense against Candida albicans. PLoS Pathog. 2009;5(10):e1000639.

62. Branzk N, Lubojemska A, Hardison SE, Wang Q, Gutierrez MG, Brown GD, et al. Neutrophils sense microbe size and selectively release neutrophil extracellular traps in response to large pathogens. Nat Immunol. 2014;15(11):1017-25.
63. Luan YY, Yao YM, Xiao XZ, Sheng ZY. Insights into the apoptotic death of immune cells in sepsis. J Interferon Cytokine Res. 2015;35(1):17-22.
64. Perianayagam MC, Balakrishnan VS, Pereira BJ, Jaber BL. C5a delays apoptosis of human neutrophils via an extracellular signal-regulated kinase and Bad-mediated signalling pathway. Eur J Clin Invest. 2004;34:50-6.
65. Delano MJ, Thayer T, Gabrilovich S, Kelly-Scumpia KM, Winfield RD, Scumpia PO, et al. Sepsis induces early alterations in innate immunity that impact mortality to secondary infection. J Immunol. 2011;186(1):195-202.
66. Hotchkiss RS, Nicholson DW. Apoptosis and caspases regulate death and inflammation in sepsis. Nat Rev Immunol. 2006;6(11):813-22.
67. Delano MJ, Kelly-Scumpia KM, Thayer TC, Winfield RD, Scumpia PO, Cuenca AG, et al. Neutrophil mobilization from the bone marrow during polymicrobial sepsis is dependent on CXCL12 signaling. J Immunol. 2011;187(2):911-8.
68. Eash KJ, Greenbaum AM, Gopalan PK, Link DC. CXCR2 and CXCR4 antagonistically regulate neutrophil trafficking from murine bone marrow. J Clin Invest. 2010;120(7):2423-31.
69. Cummings CJ, Martin TR, Frevert CW, Quan JM, Wong VA, Mongovin SM, et al. Expression and function of the chemokine receptors CXCR1 and CXCR2 in sepsis. . J Immunol. 1999;162(4):2341-6.
70. Chishti AD, Shenton BK, Kirby JA, Baudouin SV. Neutrophil chemotaxis and receptor expression in clinical septic shock. Intensive Care Med. 2004;30(4):605-11.
71. Stephan F, Yang K, Tankovic J, Soussy CJ, Dhonneur G, Duvaldestin P, et al. Impairment of polymorphonuclear neutrophil functions precedes nosocomial infections in critically ill patients. Crit Care Med. 2002;30:315-22.
72. Riccardi F, Della Porta MG, Rovati B, Casazza A, Radolovich D, De Amici M, et al. Flow cytometric analysis of peripheral blood dendritic cells in patients with severe sepsis. Cytometry B Clin Cytom. 2011;80(1):14-21.
73. Delano MJ, Ward PA. Sepsis-induced immune dysfunction: can immune therapies reduce mortality? J Clin Invest. 2016;126(1):23-31.
74. Hotchkiss RS, Monneret G, Payen D. Sepsis-induced immunosuppression: from cellular dysfunctions to immunotherapy. Nat Rev Immunol. 2013;13(12):862-74.
75. Roquilly A, McWilliam HEG, Jacqueline C, Tian Z, Cinotti R, Rimbert M, et al. Local Modulation of Antigen-Presenting Cell Development after Resolution of Pneumonia Induces Long-Term Susceptibility to Secondary Infections. Immunity. 2017;47(1):135-47 e5.
76. Biswas SK, Lopez-Collazo E. Endotoxin tolerance: new mechanisms, molecules and clinical significance. Trends Immunol. 2009;30(10):475-87.
77. Cavaillon JM, Adib-Conquy M. Bench-to-bedside review: endotoxin tolerance as a model of leukocyte reprogramming in sepsis. Crit Care. 2006;10(5):233.

78. Ertel W, Kremer JP, Kenney J, Steckholzer U, Jarrar D, Trentz O, et al. Downregulation of proinflammatory cytokine release in whole blood from septic patients. Blood. 1995;85(5):1341-7.
79. Döcke WD, Randow F, Syrbe U, Krausch D, Asadullah K, Reinke P, et al. Monocyte deactivation in septic patients: restoration by IFN-gamma treatment. . Nat Med. 1997;3:678-81.
80. Wolk K, Döcke W-D, von Baehr V, Volk H-D, Sabat R. Impaired antigen presentation by human monocytes during endotoxin tolerance. Blood. 2000;96(1):218-23.
81. Landelle C, Lepape A, Voirin N, Tognet E, Venet F, Bohe J, et al. Low monocyte human leukocyte antigen-DR is independently associated with nosocomial infections after septic shock. Intensive Care Med. 2010;36(11):1859-66.
82. Lukaszewicz AC, Grienay M, Resche-Rigon M, Pirracchio R, Faivre V, Boval B, et al. Monocytic HLA-DR expression in intensive care patients: interest for prognosis and secondary infection prediction. Crit Care Med. 2009;37(10):2746-52.
83. Zhang Y, Li J, Lou J, Zhou Y, Bo L, Zhu J, et al. Upregulation of programmed death-1 on T cells and programmed death ligand-1 on monocytes in septic shock patients. . Crit Care. 2011;15:R70.
84. Hotchkiss RS, Monneret G, Payen D. Immunosuppression in sepsis: a novel understanding of the disorder and a new therapeutic approach. The Lancet Infectious Diseases. 2013;13(3):260-8.
85. Guignant C, Lepape A, Huang X, Kherouf H, Denis L, Poitevin F, et al. Programmed death-1 levels correlate with increased mortality, nosocomial infection and immune dysfunctions in septic shock patients. Crit Care. 2011;15(2):R99.
86. Heidecke CD, Hensler T, Weighardt H, Zantl N, Wagner H, Siewert JR, et al. Selective defects of T lymphocyte function in patients with lethal intraabdominal infection. Am J Surg. 1999;178:288-92.
87. Pachot A, Monneret G, Voirin N, Leissner P, Venet F, Bohe J, et al. Longitudinal study of cytokine and immune transcription factor mRNA expression in septic shock. Clin Immunol. 2005;114(1):61-9.
88. Boomer JS, To K, Chang KC, Takasu O, Osborne DF, Walton AH, et al. Immunosuppression in patients who die of sepsis and multiple organ failure. JAMA. 2011;306(23):2594-605.
89. Romani L. Immunity to fungal infections. Nat Rev Immunol. 2011;11(4):275-88.
90. Venet F, Chung CS, Kherouf H, Geeraert A, Malcus C, Poitevin F, et al. Increased circulating regulatory T cells (CD4(+)CD25 (+)CD127 (-)) contribute to lymphocyte anergy in septic shock patients. Intensive Care Med. 2009;35(4):678-86.
91. Moazed D, Noller HF. Interaction of antibiotics with functional sites in 16S ribosomal RNA. Nature. 1987;327(6121):389-94.
92. Gray MW, Burger G, Lang BF. Mitochondrial Evolution. Sci. 1999;283(5407):1476-81.
93. Roger AJ, Munoz-Gomez SA, Kamikawa R. The Origin and Diversification of Mitochondria. Curr Biol. 2017;27(21):R1177-R92.
94. Chinnery PF, Hudson G. Mitochondrial genetics. Br Med Bull. 2013;106:135-59.

95. Andrews RM, Kubacka I, Chinnery PF, al. e. Reanalysis and revision of the Cambridge refer- ence sequence for human mitochondrial DNA. Nat Genet. 1999;23:147.
96. Calvo S, Jain M, Xie X, Sheth SA, Chang B, Goldberger OA, et al. Systematic identification of human mitochondrial disease genes through integrative genomics. Nat Genet. 2006;38(5):576-82.
97. Ma K, Chen G, Li W, Kepp O, Zhu Y, Chen Q. Mitophagy, Mitochondrial Homeostasis, and Cell Fate. Front Cell Dev Biol. 2020;8:467.
98. Youle RJ, van der Bliek AM. Mitochondrial fission, fusion, and stress. Science. 2012;337(6098):1062-5.
99. Pickles S, Vigie P, Youle RJ. Mitophagy and Quality Control Mechanisms in Mitochondrial Maintenance. Curr Biol. 2018;28(4):R170-R85.
100. Kane LA, Lazarou M, Fogel AI, Li Y, Yamano K, Sarraf SA, et al. PINK1 phosphorylates ubiquitin to activate Parkin E3 ubiquitin ligase activity. J Cell Biol. 2014;205(2):143-53.
101. Harper JW, Ordureau A, Heo JM. Building and decoding ubiquitin chains for mitophagy. Nat Rev Mol Cell Biol. 2018;19(2):93-108.
102. Liu YJ, McIntyre RL, Janssens GE, Houtkooper RH. Mitochondrial fission and fusion: A dynamic role in aging and potential target for age-related disease. Mech Ageing Dev. 2020;186:111212.
103. Brealey D, Brand M, Hargreaves I, Heales S, Land J, Smolenski R, et al. Association between mitochondrial dysfunction and severity and outcome of septic shock. The Lancet. 2002;360(9328):219-23.
104. Singer M. The role of mitochondrial dysfunction in sepsis-induced multi-organ failure. Virulence. 2014;5(1):66-72.
105. Hotchkiss RS, Swanson PE, Freeman BD, Tinsley KW, Cobb JP, Matuschak GM, et al. Apoptotic cell death in patients with sepsis, shock, and multiple organ dysfunction. Crit Care Med. 1999;27:1230-51.
106. Rosser DM, Stidwill RP, Jacobson D, Singer M. Oxygen tension in the bladder epithelium rises in both high and low cardiac output endotoxemic sepsis. J Appl Physiol. 1995;79:1878–82.
107. Pool R, Gomez H, Kellum JA. Mechanisms of Organ Dysfunction in Sepsis. Crit Care Clin. 2018;34(1):63-80.
108. Gattinoni L, Vasques F, Camporota L, Meessen J, Romitti F, Pasticci I, et al. Understanding Lactatemia in Human Sepsis. Potential Impact for Early Management. Am J Respir Crit Care Med. 2019;200(5):582-9.
109. Levy B, Gibot S, Franck P, Cravoisy A, Bollaert P-E. Relation between muscle Na+K+ ATPase activity and raised lactate concentrations in septic shock: a prospective study. The Lancet. 2005;365(9462):871-5.
110. Fredriksson K, Tjader I, Keller P, Petrovic N, Ahlman B, Scheele C, et al. Dysregulation of mitochondrial dynamics and the muscle transcriptome in ICU patients suffering from sepsis induced multiple organ failure. PLoS One. 2008;3(11):e3686.
111. Jiroutkova K, Krajcova A, Ziak J, Fric M, Waldauf P, Dzupa V, et al. Mitochondrial function in skeletal muscle of patients with protracted critical illness and ICU-acquired weakness. Crit Care. 2015;19:448.
112. Brealey D, Karyampudi S, Jacques TS, Novelli M, Stidwill R, Taylor V, et al. Mitochondrial dysfunction in a long-term rodent model of sepsis and organ failure. AM J Physiol Regul Integr Comp Physiol. 2003;286:R491-R7.

113. Kreymann G, Grosser S, Buggisch P, Gottschall C, Matthaei S, H. G. Oxygen consumption and resting metabolic rate in sepsis, sepsis syndrome, and septic shock. . Crit Care Med. 1993;21:1012-9.
114. Fredriksson K, Hammarqvist F, Strigard K, Hultenby K, Ljungqvist O, Wernerman J, et al. Derangements in mitochondrial metabolism in intercostal and leg muscle of critically ill patients with sepsis-induced multiple organ failure. Am J Physiol Endocrinol Metab. 2006;291(5):E1044-50.
115. Suliman HB, Carraway MS, Piantadosi CA. Postlipopolysaccharide oxidative damage of mitochondrial DNA. Am J Respir Crit Care Med. 2003;167(4):570-9.
116. Cote HC, Day AG, Heyland DK. Longitudinal increases in mitochondrial DNA levels in blood cells are associated with survival in critically ill patients. Crit Care. 2007;11(4):R88.
117. Pyle A, Burn DJ, Gordon C, Swan C, Chinnery PF, Baudouin SV. Fall in circulating mononuclear cell mitochondrial DNA content in human sepsis. Intensive Care Med. 2010;36(6):956-62.
118. Vanhorebeek I, Gunst J, Derde S, Derese I, Boussemaere M, D'Hoore A, et al. Mitochondrial fusion, fission, and biogenesis in prolonged critically ill patients. J Clin Endocrinol Metab. 2012;97(1):E59-64.
119. Reynolds CM, Suliman HB, Hollingsworth JW, Welty-Wolf KE, Carraway MS, Piantadosi CA. Nitric oxide synthase-2 induction optimizes cardiac mitochondrial biogenesis after endotoxemia. Free Radic Biol Med. 2009;46(5):564-72.
120. Sweeney TE, Suliman HB, Hollingsworth JW, Piantadosi CA. Differential regulation of the PGC family of genes in a mouse model of Staphylococcus aureus sepsis. PLoS One. 2010;5(7):e11606.
121. Hock MB, Kralli A. Transcriptional control of mitochondrial biogenesis and function. Annu Rev Physiol. 2009;71:177-203.
122. de la Cruz Lopez KG, Toledo Guzman ME, Sanchez EO, Garcia Carranca A. mTORC1 as a Regulator of Mitochondrial Functions and a Therapeutic Target in Cancer. Front Oncol. 2019;9:1373.
123. Carre JE, Orban JC, Re L, Felsmann K, Iffert W, Bauer M, et al. Survival in critical illness is associated with early activation of mitochondrial biogenesis. Am J Respir Crit Care Med. 2010;182(6):745-51.
124. Mannam P, Shinn AS, Srivastava A, Neamu RF, Walker WE, Bohanon M, et al. MKK3 regulates mitochondrial biogenesis and mitophagy in sepsis-induced lung injury. Am J Physiol Lung Cell Mol Physiol. 2014;306(7):L604-19.
125. Chang AL, Ulrich A, Suliman HB, Piantadosi CA. Redox regulation of mitophagy in the lung during murine Staphylococcus aureus sepsis. Free Radic Biol Med. 2015;78:179-89.
126. Gunst J, Derese I, Aertgeerts A, Ververs EJ, Wauters A, Van den Berghe G, et al. Insufficient autophagy contributes to mitochondrial dysfunction, organ failure, and adverse outcome in an animal model of critical illness. Crit Care Med. 2013;41(1):182-94.
127. Nakahira K, Haspel JA, Rathinam VA, Lee SJ, Dolinay T, Lam HC, et al. Autophagy proteins regulate innate immune responses by inhibiting the release of mitochondrial DNA mediated by the NALP3 inflammasome. Nat Immunol. 2011;12(3):222-30.

128. Zhao C, Itagaki K, Gupta A, Odom S, Sandler N, Hauser CJ. Mitochondrial damage-associated molecular patterns released by abdominal trauma suppress pulmonary immune responses. J Trauma Acute Care Surg. 2014;76(5):1222-7.
129. Escames G, Lopez LC, Garcia JA, Garcia-Corzo L, Ortiz F, Acuna-Castroviejo D. Mitochondrial DNA and inflammatory diseases. Hum Genet. 2012;131(2):161-73.
130. van der Burgh R, Nijhuis L, Pervolaraki K, Compeer EB, Jongeneel LH, van Gijn M, et al. Defects in mitochondrial clearance predispose human monocytes to interleukin-1beta hypersecretion. J Biol Chem. 2014;289(8):5000-12.
131. Simmons JD, Lee YL, Mulekar S, Kuck JL, Brevard SB, Gonzalez RP, et al. Elevated levels of plasma mitochondrial DNA DAMPs are linked to clinical outcome in severely injured human subjects. Ann Surg. 2013;258(4):591-6; discussion 6-8.
132. Fernandez-Ruiz I, Arnalich F, Cubillos-Zapata C, Hernandez-Jimenez E, Moreno-Gonzalez R, Toledano V, et al. Mitochondrial DAMPs induce endotoxin tolerance in human monocytes: an observation in patients with myocardial infarction. PLoS One. 2014;9(5):e95073.
133. Crimi E, Sica V, Williams-Ignarro S, Zhang H, Slutsky AS, Ignarro LJ, et al. The role of oxidative stress in adult critical care. Free Radic Biol Med. 2006;40(3):398-406.
134. Cherry AD, Suliman HB, Bartz RR, Piantadosi CA. Peroxisome proliferator-activated receptor gamma co-activator 1-alpha as a critical co-activator of the murine hepatic oxidative stress response and mitochondrial biogenesis in Staphylococcus aureus sepsis. J Biol Chem. 2014;289(1):41-52.
135. Belikova I, Lukaszewicz AC, Faivre V, Damoisel C, Singer M, Payen D. Oxygen consumption of human peripheral blood mononuclear cells in severe human sepsis. Crit Care Med. 2007;35:2702–8.
136. Weiss SL, Selak MA, Tuluc F, Perales Villarroel J, Nadkarni VM, Deutschman CS, et al. Mitochondrial dysfunction in peripheral blood mononuclear cells in pediatric septic shock. Pediatr Crit Care Med. 2015;16(1):e4-e12.
137. Clere-Jehl R, Helms J, Kassem M, Le Borgne P, Delabranche X, Charles AL, et al. Septic Shock Alters Mitochondrial Respiration of Lymphoid Cell-Lines and Human Peripheral Blood Mononuclear Cells: The Role of Plasma. Shock. 2019;51(1):97-104.
138. Breda CNS, Davanzo GG, Basso PJ, Saraiva Camara NO, Moraes-Vieira PMM. Mitochondria as central hub of the immune system. Redox Biol. 2019;26:101255.
139. Pearce EL, Pearce EJ. Metabolic pathways in immune cell activation and quiescence. Immunity. 2013;38(4):633-43.
140. Bao Y, Ledderose C, Seier T, Graf AF, Brix B, Chong E, et al. Mitochondria regulate neutrophil activation by generating ATP for autocrine purinergic signaling. J Biol Chem. 2014;289(39):26794-803.
141. Everts B, Amiel E, Huang SC, Smith AM, Chang CH, Lam WY, et al. TLR-driven early glycolytic reprogramming via the kinases TBK1-IKKvarepsilon supports the anabolic demands of dendritic cell activation. Nat Immunol. 2014;15(4):323-32.

142. Lee MKS, Al-Sharea A, Shihata WA, Bertuzzo Veiga C, Cooney OD, Fleetwood AJ, et al. Glycolysis Is Required for LPS-Induced Activation and Adhesion of Human CD14(+)CD16(-) Monocytes. Front Immunol. 2019;10:2054.
143. Raulien N, Friedrich K, Strobel S, Rubner S, Baumann S, von Bergen M, et al. Fatty Acid Oxidation Compensates for Lipopolysaccharide-Induced Warburg Effect in Glucose-Deprived Monocytes. Front Immunol. 2017;8:609.
144. Widdrington JD, Gomez-Duran A, Pyle A, Ruchaud-Sparagano MH, Scott J, Baudouin SV, et al. Exposure of Monocytic Cells to Lipopolysaccharide Induces Coordinated Endotoxin Tolerance, Mitochondrial Biogenesis, Mitophagy, and Antioxidant Defenses. Front Immunol. 2018;9:2217.
145. Tannahill GM, Curtis AM, Adamik J, Palsson-McDermott EM, McGettrick AF, Goel G, et al. Succinate is an inflammatory signal that induces IL-1beta through HIF-1alpha. Nature. 2013;496(7444):238-42.
146. Haschemi A, Kosma P, Gille L, Evans CR, Burant CF, Starkl P, et al. The sedoheptulose kinase CARKL directs macrophage polarization through control of glucose metabolism. Cell Metab. 2012;15(6):813-26.
147. Rodriguez-Prados JC, Traves PG, Cuenca J, Rico D, Aragones J, Martin-Sanz P, et al. Substrate fate in activated macrophages: a comparison between innate, classic, and alternative activation. J Immunol. 2010;185(1):605-14.
148. Caro-Maldonado A, Wang R, Nichols AG, Kuraoka M, Milasta S, Sun LD, et al. Metabolic reprogramming is required for antibody production that is suppressed in anergic but exaggerated in chronically BAFF-exposed B cells. J Immunol. 2014;192(8):3626-36.
149. Jang KJ, Mano H, Aoki K, Hayashi T, Muto A, Nambu Y, et al. Mitochondrial function provides instructive signals for activation-induced B-cell fates. Nat Commun. 2015;6:6750.
150. Tan H, Yang K, Li Y, Shaw TI, Wang Y, Blanco DB, et al. Integrative Proteomics and Phosphoproteomics Profiling Reveals Dynamic Signaling Networks and Bioenergetics Pathways Underlying T Cell Activation. Immunity. 2017;46(3):488-503.
151. Baixauli F, Acin-Perez R, Villarroya-Beltri C, Mazzeo C, Nunez-Andrade N, Gabande-Rodriguez E, et al. Mitochondrial Respiration Controls Lysosomal Function during Inflammatory T Cell Responses. Cell Metab. 2015;22(3):485-98.
152. West AP, Brodsky IE, Rahner C, Woo DK, Erdjument-Bromage H, Tempst P, et al. TLR signalling augments macrophage bactericidal activity through mitochondrial ROS. Nature. 2011;472(7344):476-80.
153. Calvano SE, Xiao W, Richards DR, Felciano RM, Baker HV, Cho RJ, et al. A network-based analysis of systemic inflammation in humans. Nature. 2005;437(7061):1032-7.
154. Garrabou G, Moren C, Lopez S, Tobias E, Cardellach F, Miro O, et al. The effects of sepsis on mitochondria. J Infect Dis. 2012;205(3):392-400.
155. Japiassú AM, Santiago. A.P., d'Avila. J.C., Garcia-Souza. L.F., Galina. A., Castro Faria-Neto. H.C., et al. Bioenergetic failure of human peripheral blood monocytes in patients with septic shock is mediated by reduced F1Fo adenosine-5'-triphosphate synthase activity. Crit Care Med. 2011;39:1056-63.

156. Evans L, Rhodes A, Alhazzani W, Antonelli M, Coopersmith CM, French C, et al. Surviving Sepsis Campaign: International Guidelines for Management of Sepsis and Septic Shock 2021. Crit Care Med. 2021;49(11):e1063-e143.
157. Acheampong A, Vincent JL. A positive fluid balance is an independent prognostic factor in patients with sepsis. Crit Care. 2015;19:251.
158. Investigators P, Rowan KM, Angus DC, Bailey M, Barnato AE, Bellomo R, et al. Early, Goal-Directed Therapy for Septic Shock - A Patient-Level Meta-Analysis. N Engl J Med. 2017;376(23):2223-34.
159. Dunser MW, Takala J, Ulmer H, Mayr VD, Luckner G, Jochberger S, et al. Arterial blood pressure during early sepsis and outcome. Intensive Care Med. 2009;35(7):1225-33.
160. Dellinger RP, Levy MM, Rhodes A, Annane D, Gerlach H, Opal SM, et al. Surviving Sepsis Campaign: international guidelines for management of severe sepsis and septic shock, 2012. Intensive Care Med. 2013;39(2):165-228.
161. Correa TD, Vuda M, Takala J, Djafarzadeh S, Silva E, Jakob SM. Increasing mean arterial blood pressure in sepsis: effects on fluid balance, vasopressor load and renal function. Crit Care. 2013;17(1):R21.
162. Armand-Lefevre L, Angebault C, Barbier F, Hamelet E, Defrance G, Ruppe E, et al. Emergence of imipenem-resistant gram-negative bacilli in intestinal flora of intensive care patients. Antimicrob Agents Chemother. 2013;57(3):1488-95.
163. Rubinstein E, Keynan Y. Short-course therapy for severe infections. Int J Antimicrob Agents. 2013;42 Suppl:S22-4.
164. Nedeva C, Menassa J, Puthalakath H. Sepsis: Inflammation Is a Necessary Evil. Front Cell Dev Biol. 2019;7:108.
165. Venet F, Demaret J, Blaise BJ, Rouget C, Girardot T, Idealisoa E, et al. IL-7 Restores T Lymphocyte Immunometabolic Failure in Septic Shock Patients through mTOR Activation. J Immunol. 2017;199(5):1606-15.
166. Venezio F, Di Vincenzo CA. Effects of Aminoglycoside Antibiotics on Polymorphonuclear Leukocyte Function In Vivo. Antimicrob Agen and Chemo. 1985;27(5):712-4.
167. Colombani T, Haudebourg T, Decossas M, Lambert O, Ada Da Silva G, Altare F, et al. Lipidic Aminoglycoside Derivatives: A New Class of Immunomodulators Inducing a Potent Innate Immune Stimulation. Adv Sci (Weinh). 2019;6(16):1900288.
168. Grassi GG, Fietta A. Antibiotics and their interaction with the host defense system in vivo. J Chemother. 1991;3 Suppl 1:112-5.
169. Guchhait G, Altieri A, Gorityala B, Yang X, Findlay B, Zhanel GG, et al. Amphiphilic tobramycins with immunomodulatory properties. Angew Chem Int Ed Engl. 2015;54(21):6278-82.
170. Kenny MT, Balistreri FJ, Torney HL. Beta-Lactam antibiotic modulation of murine neutrophil cytokinesis. Immunopharmacol Immunotoxicol. 1992;14(4):797-811.
171. Gao Z, Chen Y, Guan MX. Mitochondrial DNA mutations associated with aminoglycoside induced ototoxicity. J Otol. 2017;12(1):1-8.
172. Fourmy D, Recht MI, Blanchard SC, Puglisi JD. Structure of the A-site of Escherichia coli 16S ribosomal RNA complexed with an aminoglycoside antibiotic. Science. 1996;274:1367–71.

173. Mingeot-Leclercq MP, Tulkens PM. Aminoglycosides: Nephrotoxicity. Antimicrobial Agents Chemotherapy. 1999;43:1003 – 12.
174. Henley CM, 3rd, Schacht J. Pharmacokinetics of aminoglycoside antibiotics in blood, inner-ear fluids and tissues and their relationship to ototoxicity. Audiology. 1988;27(3):137-46.
175. Hong S, Harris KA, Fanning KD, Sarachan KL, Frohlich KM, Agris PF. Evidence That Antibiotics Bind to Human Mitochondrial Ribosomal RNA Has Implications for Aminoglycoside Toxicity. J Biol Chem. 2015;290(31):19273-86.
176. Morris JC, Ping-Sheng L, Zhai HX, SHen TS, Mensa-Wilmot K. Phosphatidylinositol Phospholipase C Is Activated Allosterically by the Aminoglycoside G418.pdf>. J Bio Chem. 1995;271(26):15468-77.
177. Ueda N, Guidet B, Shah SV. Gentamicin-induced mobilization of iron from renal cortical mitochondria. Am J Physiol. 1993;265(3 Pt 2):F435-9.
178. Weinberg JM, Harding PG, Humes HD. Mechanisms of gentamicin-induced dysfunction of renal cortical mitochondria. II. Effects on mitochondrial monovalent cation transport. Arch Biochem Biophys. 1980;205(1):232-9.
179. Weinberg JM, Simmons F, Humes HD. Alterations of mitochondrial respiration induced by aminoglycoside antibiotics. Res Commun Chem Pathol Pharmacol. 1980;27(3):521-31.
180. Simmons CF, Bogusky RT, Humes HD. Inhibitory effects of gentamicin on renal mitochondrial oxidative phosphorylation. J Pharm and Exp Ther. 1980;214(3):709-15.
181. O'Reilly M, Young L, Kirkwood NK, Richardson GP, Kros CJ, Moore AL. Gentamicin Affects the Bioenergetics of Isolated Mitochondria and Collapses the Mitochondrial Membrane Potential in Cochlear Sensory Hair Cells. Frontiers in cellular neuroscience. 2019;13:416.
182. Yang CL, Du XH, Han YX. Renal cortical mitochondria are the source of oxygen free radicals enhanced by gentamicin. Ren Fail. 1995;17(1):21-6.
183. Denamur S, Boland L, Beyaert M, Verstraeten SL, Fillet M, Tulkens PM, et al. Subcellular mechanisms involved in apoptosis induced by aminoglycoside antibiotics: Insights on p53, proteasome and endoplasmic reticulum. Toxicol Appl Pharmacol. 2016;309:24-36.
184. Morales AI, Detaille D, Prieto M, Puente A, Briones E, Arevalo M, et al. Metformin prevents experimental gentamicin-induced nephropathy by a mitochondria-dependent pathway. Kidney Int. 2010;77(10):861-9.
185. Desa DE, Nichols MG, Smith HJ. Aminoglycosides rapidly inhibit NAD(P)H metabolism increasing reactive oxygen species and cochlear cell demise. J Biomed Opt. 2018;24(5):1-14.
186. Servais H, Van Der Smissen P, Thirion G, Van der Essen G, Van Bambeke F, Tulkens PM, et al. Gentamicin-induced apoptosis in LLC-PK1 cells: involvement of lysosomes and mitochondria. Toxicol Appl Pharmacol. 2005;206(3):321-33.
187. Abraham OA, Rosa VM, Antonio SSM, Claudia G, María del Rosario MS. Review of Antibiotic and Non-Antibiotic Properties of Beta-lactam Molecules. Anti-Inflamm Anti-Allrg Med Chem. 2016;15:3-14.
188. Mandell LA. Effects of Antimicrobial and Antineoplastic Drugs on the Phagocytic and Microbicidal Function of the Polymorphonuclear Leukocyt. Rev of Infect Dis. 1982;4(3):683-97.

189. Lima CM, Schroeder JT, Galvao CE, Castro FM, Kalil J, Adkinson NF, Jr. Functional changes of dendritic cells in hypersensivity reactions to amoxicillin. Braz J Med Biol Res. 2010;43(10):964-8.
190. Abuaf N, Rostane H, Rajoely B, Gaouar H, Autegarden JE, Leynadier F, et al. Comparison of two basophil activation markers CD63 and CD203c in the diagnosis of amoxicillin allergy. Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology. 2008;38(6):921-8.
191. Rodriguez-Pena R, Lopez S, Mayorga C, Antunez C, Fernandez TD, Torres MJ, et al. Potential involvement of dendritic cells in delayed-type hypersensitivity reactions to beta-lactams. J Allergy Clin Immunol. 2006;118(4):949-56.
192. Smith DM, Kazi A, Smith L, Long TE, Heldreth B, Turos E, et al. A novel beta-lactam antibiotic activates tumor cell apoptotic program by inducing DNA damage. Mol Pharmacol. 2002;61(6):1348-58.
193. Chen D, Falsetti SC, Frezza M, Milacic V, Kazi A, Cui QC, et al. Anti-tumor activity of N-thiolated beta-lactam antibiotics. Cancer Lett. 2008;268(1):63-9.
194. Sugita K, Nishimura T. Effect of antimicrobial agents on chemotaxis of polymorphonuclear leukocytes. J Chemother. 1995;7:118–25.
195. Belsheim JA, Gnarpe GH. Antibiotics and granulocytes. Direct and indirect effects on granulocyte chemotaxis. Acta Pathol Microbiol Scand C. 1981;89(4):217-21.
196. Matera G, Berlinghieri MC, Foci A. Meropenem: effects on human leukocyte functions and interleukin release. Int J of Antimicrobial Agents,. 1995;5:129-33.
197. Fietta A, Sacchi F, Bersani C, Grassi F, Mangiarotti P, Grassi GG. Effect of beta-lactam antibiotics on migration and bactericidal activity of human phagocytes. Antimicrob Agents Chemother. 1983;23(6):930-1.
198. Burgaleta C, Moreno T. Effect of beta-lactams and aminoglycosides on human polymorphonuclear leucocytes. The Journal of antimicrobial chemotherapy. 1987;20(4):529-35.
199. Labro MT, Babin-Chevayeand C, Hakim J. Effects of Cefotaxime and Cefodizime on human granulocyte functions in vitro. The Journal of antimicrobial chemotherapy. 1986;18:233-7.
200. Fietta A, Merlini C, Grassi GG. In vitro activity of two new oral cephalosporins, cefixime and cefdinir on human peripheral mononuclear and polymorphonuclear leukocyte functions. Chemotherapy. 1994;40:317–23.
201. Rodriguez AB, Barriga C, De la Fuente M. In vitro effect of cefoxitin on phagocytic function and antibody-dependent cellular cytotoxicity in human neutrophils. Comp Immunol Microbiol Infect Dis. 1993;16(1):37-50.
202. Periti P. Immunopharmacology of oral betalactams. J Chemother. 1998;10(2):91-6.
203. Scheffer J, Knoller J, Cullmann W, Konig W. Effect of cefaclor, cefemet and Ro40-6890 on inflammatory responses of human granulocytes. The Journal of antimicrobial chemotherapy. 1992;30:57-66.
204. Rodriguez AB, Barriga C, de la Fuente M. Stimulation of phagocytic processes and antibody-dependent cellular cytotoxicity of human neutrophils by cefmetazole. Microbiol Immunol. 1991;35(7):545-56.

205. Nunez RM, Rodriguez AB, Barriga C, De la Fuente M. In vitro and in vivo effects of Imipenem on phagocytic activity of murine peritoneal macrophages. Apmis. 1989;97(10):879-86.
206. Pasqui AL, Di Renzo M, Bruni F, Fanetti G, Campoccia G, Auteri A. Imipenem and immune response: in vitro and in vivo studies. Drugs Exp Clin Res. 1995;21(1):17-22.
207. Bode C, Diedrich B, Muenster S, Hentschel V, Weisheit C, Rommelsheim K, et al. Antibiotics regulate the immune response in both presence and absence of lipopolysaccharide through modulation of Toll-like receptors, cytokine production and phagocytosis in vitro. Int Immunopharmacol. 2014;18(1):27-34.
208. Miyata T, Shinohara M. Effect of antibiotics on rat leukocyte function. J Osaka Dent Univ. 1998;32(1):9-15.
209. Grassi GG, Fietta A, Sacchi F, Derose V. Influence of ceftriaxone on natural defense systems. Am J Med 1984;77:37–41.
210. Pulverer G. Effects of Cefodizime and Cefotaxime on cellular and humoral immune response. Infection. 1992;20:S41-4.
211. Reato G, Cuffinia AM, Tullioa V, Palarchioa AI, Boninoa A, Foab R, et al. Co-amoxiclav affects cytokine production by human polymorphonuclear cells. J Antimicrob Chemo. 1999;43:715-8.
212. Stevens DL, Bryant AE, Hackett SP. Antibiotic Effects on Bacterial Viability, Toxin Production, and Host Response. Clin Infec Dis. 1995;20:S154-7.
213. Neftel KA, Hauser SP, Muller MR. Inhibition of granulopoiesis in vivo and in vitro by beta-lactam antibiotics. The Journal of infectious diseases. 1985;152(1):90-8.
214. Neftel KA, Muller MR, Widmer U, Hugin AW. Beta-lactam antibiotics inhibit human in vitro granulopoiesis and proliferation of some other cell types. Cell Biol Toxicol. 1986;2(4):513-21.
215. Banck G, Forsgren A. Antibiotics and suppression of lymphocyte function in vitro. Antimicrob Agents Chemother. 1979;16(5):554-60.
216. Tune BM, Fravert D. Cephalosporin nephrotoxicity. Transport, cytotoxicity and mitochondrial toxicity of cephaloglycin. JPET. 1980;215:186-90.
217. Tune BM. Mechanisms of nephrotoxicity of beta-lactam antibiotics. Contributions to nephrology. 1990;83:202-7.
218. Tune BM, Hsu CY. Toxicity of Cephaloridine to Carnitine Transport and Fatty Acid Metabolism in Rabbit Renal Cortical Mitochondria: Stanford, California Structure-Activity Relationships. J Pharm and Exp Ther. 1994;270(3):873-80.
219. Oyebode OT, Adebiyi OR, Olorunsogo OO. Toxicity of some broad-spectrum antibacterials in normal rat liver: the role of mitochondrial membrane permeability transition pore. Toxicol Mech Methods. 2019;29(2):128-37.
220. Stogios PJ, Savchenko A. Molecular mechanisms of vancomycin resistance. Protein Sci. 2020;29(3):654-69.
221. Sugimoto Y, Iba Y, Utsugi K, Kamei C. Influences of Everninomicin, Vancomycin and Teicoplanin on Chemical Mediator Release From Rat Peritoneal Mast Cells. Jpn J Pharmacol. 2000;83(4):300-5.

222. Toyoguchi T, Ebihara M, O'hima F, Hosoya J, Shoji T, Nakagawa Y. Histamine Release Induced by Antimicrobial Agents and Effects of Antimicrobial Agents on Vancomycin-induced Histamine Release from Rat Peritoneal Mast Cells. J Pharm Pharmacol. 2000;52:327-31.
223. Hsiao SH, Chang CM, Tsai JC, Lin CY, Liao LH, Lin WL, et al. Glycopeptide-induced neutropenia: cross-reactivity between vancomycin and teicoplanin. Ann Pharmacother. 2007;41(5):891-4.
224. Hsiao SH, Chou CH, Lin WL, Lee EJ, Liao LH, Chang HJ, et al. High risk of cross-reactivity between vancomycin and sequential teicoplanin therapy. J Clin Pharm Ther. 2012;37(3):296-300.
225. Polk RE. Anaphylactoid reactions to glycopeptide antibiotics. J Antimicrob Chemo. 1991;27:17-29.
226. Davenport A. Allergic Cross-Reactivity to Teicoplanin and Vancomycin. Nepron. 1992;63:482.
227. Lewis BB, Buffie CG, Carter RA, Leiner I, Toussaint NC, Miller LC, et al. Loss of Microbiota-Mediated Colonization Resistance to Clostridium difficile Infection With Oral Vancomycin Compared With Metronidazole. The Journal of infectious diseases. 2015;212(10):1656-65.
228. van Opstal E, Kolling GL, Moore JH, 2nd, Coquery CM, Wade NS, Loo WM, et al. Vancomycin Treatment Alters Humoral Immunity and Intestinal Microbiota in an Aged Mouse Model of Clostridium difficile Infection. The Journal of infectious diseases. 2016;214(1):130-9.
229. Lankelma JM, Cranendonk DR, Belzer C, de Vos AF, de Vos WM, van der Poll T, et al. Antibiotic-induced gut microbiota disruption during human endotoxemia: a randomised controlled study. Gut. 2017;66(9):1623-30.
230. Cheng RY, Li M, Li SS, He M, Yu XH, Shi L, et al. Vancomycin and ceftriaxone can damage intestinal microbiota and affect the development of the intestinal tract and immune system to different degrees in neonatal mice. Pathog Dis. 2017;75(8).
231. Brandl K, Plitas G, Mihu CN, Ubeda C, Jia T, Fleisher M, et al. Vancomycin-resistant enterococci exploit antibiotic-induced innate immune deficits. Nature. 2008;455(7214):804-7.
232. Fietta A, Bersani C, De Rose V, Grassi FM, Grassi GG. The effect of teicoplanin on leukocytic activity and intraleukocytic micro-organisms. J Hosp Inf. 1986;7:57-63.
233. Moran FJ, Puente LF, Perez-Giraldo C, Blanco MT, Hurtado C, Gomez-Garcia AC. Activity of vancomycin and teicoplanin against human polymorphonuclear leucocytes: a comparative study. The Journal of antimicrobial chemotherapy. 1991;28(3):415-8.
234. Tawfik AF. Effects of vancomycin, teicoplanin, daptomycin and coumermycin on normal immune capabilities. J Chemother. 1991;3(4):226-31.
235. Barriga C, Pedrera I, Rodriguez AB. Comparative study of the effect of teicoplanin and vancomycin upon the phagocytic process of peritoneal macrophages. Rev Esp Fisiol. 1996;52(4):215-22.
236. Capodicasa E, Scaringi L, Rosati E, De Bellis F, Sbaraglia G, Marconi P, et al. In-vitro effects of teicoplanin, teicoplanin derivative MDL 62211 and vancomycin on human polymorphonuclear cell function. The Journal of antimicrobial chemotherapy. 1991;27(5):619-26.

237. Foca A, Matera G, Berlinghieri MC. Inhibilion of Endotoxin-lnduced Interleukin 8 Release by Teicoplanin in Human Whole Blood. European journal of clinical microbiology & infectious diseases : official publication of the European Society of Clinical Microbiology. 1993;12:940-4.
238. Arimura Y, Yano T, Hirano M, Sakamoto Y, Egashira N, Oishi R. Mitochondrial superoxide production contributes to vancomycin-induced renal tubular cell apoptosis. Free radical biology & medicine. 2012;52(9):1865-73.
239. Sakamoto Y, Yano T, Hanada Y, Takeshita A, Inagaki F, Masuda S, et al. Vancomycin induces reactive oxygen species-dependent apoptosis via mitochondrial cardiolipin peroxidation in renal tubular epithelial cells. Eur J Pharmacol. 2017;800:48-56.
240. Qu S, Dai C, Guo H, Wang C, Hao Z, Tang Q, et al. Rutin attenuates vancomycin-induced renal tubular cell apoptosis via suppression of apoptosis, mitochondrial dysfunction, and oxidative stress. Phytother Res. 2019;33(8):2056-63.
241. Zuckerman JM, Qamar F, Bono BR. Review of macrolides (azithromycin, clarithromycin), ketolids (telithromycin) and glycylcyclines (tigecycline). Med Clin North Am. 2011;95(4):761-91, viii.
242. Alvarez-Elcoro S, Enzler MJ. The Macrolides: Erythromycin, Clarithromycin and Azithromycin. Mayo Clin Proc. 1999;74:613-34.
243. Mazzei T, Mini E, Novelli A, Perti P. Chemistry and mode of action of macrolides. J Antimicrob Chemo. 1993;31:1-9.
244. Oda H, Kadota J, Kohno S, Hara K. Erythromycin inhibits neutrophil chemotaxis in bronchoalveoli of diffuse panbronchiolitis. Chest. 1994;106(4):1116-23.
245. Oda H, Kadota J, Kohno S, Hara K. Leukotriene B4 in bronchoalveolar lavage fluid of patients with diffuse panbronchiolitis. Chest 108116–122. Chest. 1995;108:116-22.
246. Park SJ, Lee YC, Rhee YK, Lee HB. The effect of long-term treatment with erythromycin on Th1 and Th2 cytokines in diffuse panbronchiolitis. Biochem Biophys Res Commun. 2004;324(1):114-7.
247. Kadota J, Sakito O, Kohno S, Sawa H, Mukae H, Oda H, et al. A mechanism of erythromycin treatment in patients with diffuse panbronchiolitis. The American review of respiratory disease. 1993;147(1):153-9.
248. Sakito O, Kadota J, Kohno S, Abe K, Shirai R, Hara K. Interleukin 1b, tumor necrosis factor alpha, and interleukin 8 in bronchoalveolar lavage fluid of patients with diffuse panbronchiolitis: a potential mechanism of macrolide therapy. Respiration; international review of thoracic diseases. 1996;63:42-8.
249. Suzuki H, Shimomura A, Ikeda K, Oshima T, Takasaka T. Effects of long-term low-dose macrolide administration on neutrophil recruitment and IL-8 in the nasal discharge of chronic sinusitis patients. Tohoku J Exp Med. 1997;182(2):115-24.
250. Cervin A, Wallwork B, Mackay-Sim A, Coman WB, Greiff L. Effects of long-term clarithromycin treatment on lavage-fluid markers of inflammation in chronic rhinosinusitis. Clin Physiol Funct Imaging. 2009;29(2):136-42.
251. Wallwork B, Coman W, Mackay-Sim A, Greiff L, Cervin A. A double-blind, randomized, placebo-controlled trial of macrolide in the treatment of chronic rhinosinusitis. Laryngoscope. 2006;116(2):189-93.

252. Yamada T, Fujieda S, Mori S, Yamamoto H, Saito H. Macrolide treatment decreased the size of nasal polyps and IL-8 levels in nasal lavage. Am J Rhinol. 2000;14(3):143-8.
253. Kohyama T, H. Takizawa, S. Kawasaki, N. Akiyama, M. Sato, and K. Ito. Fourteen-member macrolides inhibit interleukin-8 release by human eosinophils from atopic donors. Antimicrob Agen and Chemo. 1999;43:907-11.
254. Piacentini GL, Peroni DG, Bodini A, Pigozzi R, Costella S, Loiacono A, et al. Azithromycin reduces bronchial hyperresponsiveness and neutrophilic airway inflammation in asthmatic children: a preliminary report. Allerg Asthma Proc. 2007;28(2):194-8.
255. Fonseca-Aten M, Okada PJ, Bowlware KL, Chavez-Bueno S, Mejias A, Rios AM, et al. Effect of clarithromycin on cytokines and chemokines in children with an acute exacerbation of recurrent wheezing: a double-blind, randomized, placebo-controlled trial. Ann Allerg Asthma Immunol. 2006;97(4):457-63.
256. Amayasu H, Yoshida S, Ebana S, Yamamoto Y, Nishikawa T, Shoji T, et al. Clarithromycin suppresses bronchial hyperresponsiveness associated with eosinophilic inflammation in patients with asthma. Ann Allerg Asthma Immunol. 2000;84(6):594-608.
257. Kraft M, Cassell GH, Pak J, Martin RJ. Mycoplasma pneumoniae and Chlamydia pneumoniae in asthma: effect of clarithromycin. Chest. 2002;121(6):1782-8.
258. Simpson JL, Powell H, Boyle MJ, Scott RJ, Gibson PG. Clarithromycin targets neutrophilic airway inflammation in refractory asthma. Am J Respir Crit Care Med. 2008;177(2):148-55.
259. Shoji T, Yoshida S, Sakamoto H, Hasegawa H, Nakagawa H, Amayasu H. Anti- inflammatory effect of roxithromycin in patients with aspirin-intolerant asthma. Clin Exp Allergy. 1999;29:7.
260. Kamoi H, Kurihara N, Fujiwara H, Hirata K, Takeda T. The macrolide antibacterial roxithromycin reduces bronchial hyperresponsiveness and superoxide anion production by polymorphonuclear leukocytes in patients with asthma. J Asthma. 1995;32(3):191-7.
261. Schultz MJ, Speelman P, Hack CE, Buurman WA, van De-venter SJ, Van Der Poll T. Intravenous infusion of erythromycin inhibits CXC chemokine production, but augments neutrophil degranulation in whole blood stimulated with Streptococcus pneumoniae. J Antimicrob Chemo. 2000;46:235-40.
262. Schultz MJ, Speelman P, Zaat S, van Deventer SHJ, Van Der Poll T. Erythromycin inhibits tumor necrosis factor alpha and interleukin 6 production induced by heat-killed Streptococcus pneumoniae in whole blood. Antimicrob Agen and Chemo. 1998;42:1605-9.
263. Ianaro A, Lalenti A, Maffia P, Sautebin L, Rombola L, Carnuccio R, et al. Anti-inflammatory activity of macrolide antibiotics. J Pharmacol Exp Ther. 2000;292:156-63.
264. Ohshima A, Tokura Y, Wakita H, Furukawa F, Takigawa M. Roxithromycin down-modulates antigen-presenting and interleukin-1 beta-producing abilities of murine Langerhans cells. J Dermatol Sci. 1998;17(3):214-22.

265. Takahashi E, Indalao IL, Sawabuchi T, Mizuno K, Sakai S, Kimoto T, et al. Clarithromycin suppresses induction of monocyte chemoattractant protein-1 and matrix metalloproteinase-9 and improves pathological changes in the lungs and heart of mice infected with influenza A virus. Comp Immunol Microbiol Infect Dis. 2018;56:6-13.
266. Banerjee D, Honeybourne D, Khair OA. The effect of oral clarithromycin on bronchial airway inflammation in moderate-to-severe stable COPD: a randomized controlled trial. Treat Respir Med. 2004;3(1):59-65.
267. Cameron EJ, Chaudhuri R, Mair F, McSharry C, Greenlaw N, Weir CJ, et al. Randomised controlled trial of azithromycin in smokers with asthma. . The European respiratory journal. 2013;42(5):1412-5.
268. Iino Y, Sasaki Y, Kojima C, Miyazawa T. Effect of macrolides on the expression of HLA-DR and costimulatory molecules on antigen-presenting cells in nasal polyps. Ann Otol Rhinol Laryngol. 2001;110(5 Pt 1):457-63.
269. Karrow NA, McCay JA, Brown RD, Musgrove DL, Germolec DR, White KL, Jr. Evaluation of the immunomodulatory effects of the macrolide antibiotic, clarithromycin, in female B6C3F1 mice: a 28-day oral gavage study. Drug Chem Toxicol. 2001;24(1):19-37.
270. Konno S, Adachi M, Asano K, Kawazoe T, Okamoto K, Takahashi T. Influences of roxithromycin on cell-mediated immune responses. Life Sci. 1992;51(10):Pl107-12.
271. Anderson R. Erythromycin and roxithromycin potentiate human neutrophil locomotion in vitro by inhibition of leukoattractant activated superoxide generation and autooxidation. J Infect Dis. 1989;5:966-72.
272. Yamaryo T, Oishi K, Yoshimine H, Tsuchihashi Y, Matsushima K, Nagatake T. Fourteen-member macrolides promote the phosphatidylserine receptor-dependent phagocytosis of apoptotic neutrophils by alveolar macrophages. Antimicrob Agents Chemother. 2003;47(1):48-53.
273. Hodge S, Hodge G, Brozyna S, Jersmann H, Holmes M, Reynolds PN. Azithromycin increases phagocytosis of apoptotic bronchial epithelial cells by alveolar macrophages. The European respiratory journal. 2006;28(3):486-95.
274. Herrera-Insúa I, Jacques-Palaz K, Murray BE, Rakita RM. The effect of antibiotic exposure on adherence to neutrophils of Enterococcus faecium resistant to phagocytosis. The Journal of antimicrobial chemotherapy. 1997;39 Suppl A:109-13.
275. Noma T, Hayashi M, Yoshizawa I, Aoki K, Shikishima Y, Kawano Y. A comparative investigation of the restorative effects of roxithromycin on neutrophil activities. International journal of immunopharmacology. 1998;20(11):615-24.
276. Scaglione F, Ferrara F, Dugnani S, Demartini G, Triscari F, Fraschini F. Immunostimulation by clarithromycin in healthy volunteers and chronic bronchitis patients. J Chemother. 1993;5(4):228-32.
277. Wenisch C, Parschalk B, Zedtwitz-Liebenstein K, Weihs A, el Menyawi I, Graninger W. Effect of single oral dose of azithromycin, clarithromycin, and roxithromycin on polymorphonuclear leukocyte function assessed ex vivo by flow cytometry. Antimicrob Agents Chemother. 1996;40(9):2039-42.

278. Ortega E, Escobar MA, Gaforio JJ, Algarra I, Alvarez De Cienfuegos G. Modification of phagocytosis and cytokine production in peritoneal and splenic murine cells by erythromycin A, azithromycin and josamycin. The Journal of antimicrobial chemotherapy. 2004;53(2):367-70.
279. Braga PC, Maci S, Dal Sasso M, Fonti E, Ghessi A. Effects of rokitamycin on phagocytosis and release of oxidant radicals of human polymorphonuclear leukocytes. Chemotherapy. 1997;43(3):190-7.
280. Mitsuyama T, Tanaka T, Hidaka K, Abe M, Hara N. Inhibition by erythromycin of superoxide anion production by human polymorphonuclear leukocytes through the action of cyclic AMP-dependent protein kinase. Respiration; international review of thoracic diseases. 1995;62(5):269-73.
281. Cui CH, Honda K, Saito N, Yamada Y, Sannohe S, Ueki S, et al. Effect of roxithromycin on eotaxin-primed reactive oxygen species from eosinophils. . International archives of allergy and immunology. 2001;125:38-41.
282. Eswarappa SM, Basu N, Joy O, Chakravortty D. Folimycin (concanamycin A) inhibits LPS-induced nitric oxide production and reduces surface localization of TLR4 in murine macrophages. Innate immunity. 2008;14(1):13-24.
283. Goormaghtigh E, Pollakis G, Ruysschaert JM. Mitochondrial membrane modification by adriamycin-mediated electron transport. Biochemical Pharm. 1983;32(5):889-93.
284. Bravo-Sagua R, Lopez-Crisosto C, Parra V, Rodriguez-Pena M, Rothermel BA, Quest AF, et al. mTORC1 inhibitor rapamycin and ER stressor tunicamycin induce differential patterns of ER-mitochondria coupling. Sci Rep. 2016;6:36394.
285. Hooper DC. Mode of action of fluoroquinolones. 1999;58:6-10.
286. Wohlkonig A, Chan PF, Fosberry AP, Homes P, Huang J, Kranz M, et al. Structural basis of quinolone inhibition of type IIA topoisomerases and target-mediated resistance. Nat Struct Mol Biol. 2010;17(9):1152-3.
287. Neuman MG, Cohen LB, Nanau RM. Quinolones-induced hypersensitivity reactions. Clin Biochem. 2015;48(10-11):716-39.
288. Schmid DA, Campi P, Pichler WJ. Hypersensitivity reactions to quinolones. Curr Pharm Des. 2006;12(26):3313-26.
289. Schmid DA, Depta JP, Pichler WJ. T cell-mediated hypersensitivity to quinolones: mechanisms and cross-reactivity. Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology. 2006;36(1):59-69.
290. Scherer K, Bircher AJ. Hypersensitivity reactions to fluoroquinolones. Curr Allergy Asthma Rep. 2005;5(1):15-21.
291. Strzępa A, Majewska-Szczepanik M, Kowalczyk P, Woźniak D, Motyl S, Szczepanik M. Oral treatment with enrofloxacin early in life promotes Th2-mediated immune response in mice. Pharmacol Rep. 2016;68(1):44-50.
292. Juanola O, Gómez-Hurtado I, Zapater P, Moratalla A, Caparrós E, Piñero P, et al. Selective intestinal decontamination with norfloxacin enhances a regulatory T cell-mediated inflammatory control mechanism in cirrhosis. Liver international : official journal of the International Association for the Study of the Liver. 2016;36(12):1811-20.

293. Bailly S, Fay M, Gougerot-Pocidalo JJ. Effects of quinolones on tumor necrosis factor production by human monocytes. Int J Immunopharmacol. International journal of immunopharmacology. 1990;12:31-6.
294. Ogino H, Fujii M, Ono M, Maezawa K, Hori S, Kizu J. In vivo and in vitro effects of fluoroquinolones on lipopolysaccharide-induced pro-inflammatory cytokine production. Journal of infection and chemotherapy : official journal of the Japan Society of Chemotherapy. 2009;15(3):168-73.
295. Riesbeck K, Forsgren A. Selective enhancement of synthesis of interleukin-2 in lymphocytes in the presence of ciprofloxacin. European journal of clinical microbiology & infectious diseases : official publication of the European Society of Clinical Microbiology. 1990;9:409-13.
296. Roche Y, Fay M, Gougerot-Pocidalo MA. Effects of quinolones on interleukin 1 production in vitro by human monocytes. Immunopharmacol. 1987;13:99-109.
297. Roche Y, Gougerot-Pocidalo MA, Fay M, Etienne D, Forest N, Pocidalo JJ. Comparative effects of quinolones on human mononuclear leucocyte functions. The Journal of antimicrobial chemotherapy. 1987;19(6):781-90.
298. Bailly S, Fay M, Roche Y, Gougerot-Pocidalo MA. Effects of quinolones on tumor necrosis factor production by human monocytes. International journal of immunopharmacology. 1990;12:31-6.
299. Bailly S, Mahe Y, Ferrua B, Tursz T, Wakasugi H, Gougerot-Pocidalo MA. Quinolone-induced differential modification of IL-1 alpha and IL-1 beta production by LPS-stimulated human monocytes. Cell Immunol. 1990;128:277-88.
300. Yoshimura T, Kurita C, Usami E, Nakao T, Watanabe S, Kobayashi J, et al. Immunomodulatory action of levofloxacin on cytokine production by human peripheral blood mononuclear cells. . Chemotherapy. 1996;1996:459-64.
301. Khan AA, Slifer TR, Remington JS. Effect of trovafloxacin on production of cytokines by human monocytes. Antimicrob Agen and Chemo. 1998;42:1713-7.
302. Petit JC, Daguet GL, Richard G, Burghoffer B. Influence of ciprofloxacin and piperacillin on interleukin-1 production by murine macrophages. J Antimicrob Chemo. 1987;20:615-7.
303. Mori S, Takahashi HK, Liu K, Wake H, Zhang J, Liu R, et al. Ciprofloxacin inhibits advanced glycation end products-induced adhesion molecule expression on human monocytes. Br J Pharmacol. 2010;161(1):229-40.
304. Vickers IE, Smikle MF. The immunomodulatory effect of antibiotics on the secretion of tumour necrosis factor alpha by peripheral blood mononuclear cells in response to Stenotrophomonas maltophilia stimulation. West Indian Med J. 2006;55(3):138-41.
305. Yao M, Gao W, Tao H, Yang J, Huang T. The regulation effects of danofloxacin on pig immune stress induced by LPS. Res Vet Sci. 2017;110:65-71.

306. Ives TJ, Schwab UE, Ward ES, Hall IH. In-vitro anti-inflammatory and immunomodulatory effects of grepafloxacin in zymogen A- or Staphylococcus aureus-stimulated human THP-1 monocytes. Journal of infection and chemotherapy : official journal of the Japan Society of Chemotherapy. 2003;9(2):134-43.
307. Ouo Y, Ohmoto Y, Ouo K, Sakata Y, Murata K. Effect of grapafloxacin on cytokine production in vitro. J Antimicrob Chemo. 2000;46:91-4.
308. Riesbeck K. Immunomodulating activity of quinolones: review. J Chemother. 2002;14(1):3-12.
309. Mato R, Corrales I, Prieto J. Influence of lomefloxacin on phagocytosis and killing activity of macrophages and neutrophils. The Journal of antimicrobial chemotherapy. 1992;30(4):558-9.
310. Forsgren A, Bergkvist PI. Effect of ciprofloxacin on phagocytosis. Eur J Clin Microbiol. 1985;4(6):575-8.
311. Gruger T, Morler C, Schnitzler N, Brandenburg K, Nidermajer S, Horre R, et al. Influence of fluoroquinolones on phagocytosis and killing of Candida albicans by human polymorphonuclear neutrophils. Med Mycol. 2008;46(7):675-84.
312. Nielsen SL, Obel N, Storgaard M, Andersen PL. The effect of quinolones on the intracellular killing of Staphylococcus aureus in neutrophil granulocytes. The Journal of antimicrobial chemotherapy. 1997;39(5):617-22.
313. Lianou PE, Votta EG, Papavassiliou JT, Bassaris HP. In vivo potentiation of polymorphonuclear leukocyte function by ciprofloxacin. J Chemother. 1993;5(4):223-7.
314. Chide OE, Orisakwe OE. Structural development, haematological immunological and pharmacological effects of quinolones. Recent Pat Antiinfect Drug Discov. 2007;2(2):157-68.
315. Plekhova NG, Kondrashova NM, Somova LM, Drobot EI, Lyapun IN. Effects of immunomodulators on functional activity of innate immunity cells infected with Streptococcus pneumoniae. Bull Exp Biol Med. 2015;158(4):461-4.
316. Hangas A, Aasumets K, Kekalainen NJ, Paloheina M, Pohjoismaki JL, Gerhold JM, et al. Ciprofloxacin impairs mitochondrial DNA replication initiation through inhibition of Topoisomerase 2. Nucleic Acids Res. 2018;46(18):9625-36.
317. Lawrence JW, Claire DC, Weissig V, Rowe TC. Delayed cytotoxicity and cleavage of mitochondrial DNA in ciprofloxacin-treated mammalian cells. Mol Pharmacol. 1996;50(5):1178-88.
318. Kaminski MM, Sauer SW, Klemke CD, Suss D, Okun JG, Krammer PH, et al. Mitochondrial reactive oxygen species control T cell activation by regulating IL-2 and IL-4 expression: mechanism of ciprofloxacin-mediated immunosuppression. J Immunol. 2010;184(9):4827-41.
319. Koziel R, Zablocki K, Duszynski J. Calcium signals are affected by ciprofloxacin as a consequence of reduction of mitochondrial DNA content in Jurkat cells. Antimicrob Agents Chemother. 2006;50(5):1664-71.
320. Herold C, Ocker M, Ganslmayer M, Gerauer H, Hahn EG, Schuppan D. Ciprofloxacin induces apoptosis and inhibits proliferation of human colorectal carcinoma cells. Br J Cancer. 2002;86(3):443-8.

321. Aranha O, Zhu L, Alhasan S, Wood DP, Jr., Kuo TH, Sarkar FH. Role of mitochondria in ciprofloxacin induced apoptosis in bladder cancer cells. J Urol. 2002;167(3):1288-94.
322. Yu M, Li R, Zhang J. Repositioning of antibiotic levofloxacin as a mitochondrial biogenesis inhibitor to target breast cancer. Biochem Biophys Res Commun. 2016;471(4):639-45.
323. Song M, Wu H, Wu S, Ge T, Wang G, Zhou Y, et al. Antibiotic drug levofloxacin inhibits proliferation and induces apoptosis of lung cancer cells through inducing mitochondrial dysfunction and oxidative damage. Biomed Pharmacother. 2016;84:1137-43.
324. Leach KL, Swaney SM, Colca JR, McDonald WG, Blinn JR, Thomasco LM, et al. The site of action of oxazolidinone antibiotics in living bacteria and in human mitochondria. Mol Cell. 2007;26(3):393-402.
325. Milosevic TV, Payen VL, Sonveaux P, Muccioli GG, Tulkens PM, Van Bambeke F. Mitochondrial Alterations (Inhibition of Mitochondrial Protein Expression, Oxidative Metabolism, and Ultrastructure) Induced by Linezolid and Tedizolid at Clinically Relevant Concentrations in Cultured Human HL-60 Promyelocytes and THP-1 Monocytes. Antimicrob Agents Chemother. 2018;62(3).
326. Nagiec EE, Wu L, Swaney SM, Chosay JG, Ross DE, Brieland JK, et al. Oxazolidinones inhibit cellular proliferation via inhibition of mitochondrial protein synthesis. Antimicrob Agents Chemother. 2005;49(9):3896-902.
327. McKee EE, Ferguson M, Bentley AT, Marks TA. Inhibition of mammalian mitochondrial protein synthesis by oxazolidinones. Antimicrob Agents Chemother. 2006;50(6):2042-9.
328. De Vriese AS, Coster RV, Smet J, Seneca S, Lovering A, Van Haute LL, et al. Linezolid-induced inhibition of mitochondrial protein synthesis. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. 2006;42(8):1111-7.
329. Santini A, Ronchi D, Garbellini M, Piga D, Protti A. Linezolid-induced lactic acidosis: the thin line between bacterial and mitochondrial ribosomes. Expert Opin Drug Saf. 2017;16(7):833-43.
330. Garrabou G, Soriano À, Pinós T, Casanova-Mollà J, Pacheu-Grau D, Morén C, et al. Influence of Mitochondrial Genetics on the Mitochondrial Toxicity of Linezolid in Blood Cells and Skin Nerve Fibers. Antimicrob Agents Chemother. 2017;61(9).
331. Protti A, Ronchi D, Bassi G, Fortunato F, Bordoni A, Rizzuti T, et al. Changes in Whole-Body Oxygen Consumption and Skeletal Muscle Mitochondria During Linezolid-Induced Lactic Acidosis. Crit Care Med. 2016;44(7):e579-82.
332. Garcia-Roca P, Mancilla-Ramirez J, Santos-Segura A, Fernandez-Aviles M, Calderon-Jaimes E. Linezolid diminishes inflammatory cytokine production from human peripheral blood mononuclear cells. Arch Med Res. 2006;37(1):31-5.
333. Pichereau S, Moran JJ, Hayney MS, Shukla SK, Sakoulas G, Rose WE. Concentration-dependent effects of antimicrobials on Staphylococcus aureus toxin-mediated cytokine production from peripheral blood mononuclear cells. The Journal of antimicrobial chemotherapy. 2012;67(1):123-9.

334. Franks Z, Campbell RA, Vieira de Abreu A, Holloway JT, Marvin JE, Kraemer BF, et al. Methicillin-resistant Staphylococcus aureus-induced thrombo-inflammatory response is reduced with timely antibiotic administration. Thromb Haemost. 2013;109(4):684-95.
335. Yanagihara K, Kihara R, Araki N, Morinaga Y, Seki M, Izumikawa K, et al. Efficacy of linezolid against Panton-Valentine leukocidin (PVL)-positive meticillin-resistant Staphylococcus aureus (MRSA) in a mouse model of haematogenous pulmonary infection. Int J Antimicrob Agents. 2009;34(5):477-81.
336. Luna CM, Bruno DA, Garcia-Morato J, Mann KC, Risso Patron J, Sagardia J, et al. Effect of linezolid compared with glycopeptides in methicillin-resistant Staphylococcus aureus severe pneumonia in piglets. Chest. 2009;135(6):1564-71.
337. Breslow-Deckman JM, Mattingly CM, Birket SE, Hoskins SN, Ho TN, Garvy BA, et al. Linezolid decreases susceptibility to secondary bacterial pneumonia postinfluenza infection in mice through its effects on IFN-gamma. J Immunol. 2013;191(4):1792-9.
338. Jacqueline C, Broquet A, Roquilly A, Davieau M, Caillon J, Altare F, et al. Linezolid dampens neutrophil-mediated inflammation in methicillin-resistant Staphylococcus aureus-induced pneumonia and protects the lung of associated damages. The Journal of infectious diseases. 2014;210(5):814-23.
339. Kaku N, Morinaga Y, Takeda K, Kosai K, Uno N, Hasegawa H, et al. Antimicrobial and immunomodulatory effects of tedizolid against methicillin-resistant Staphylococcus aureus in a murine model of hematogenous pulmonary infection. Int J Med Microbiol. 2016;306(6):421-8.
340. Verma AK, Bauer C, Yajjala VK, Bansal S, Sun K. Linezolid attenuates lethal lung damage during post-influenza methicillin-resistant staphylococcus aureus pneumonia. Infection and immunity. 2019;87(10):e00538–19.
341. Naess A, Stenhaug Kilhus K, Nystad TW, Sornes S. Linezolid and human polymorphonuclear leukocyte function. Chemotherapy. 2006;52(3):122-4.
342. Ballesta S, Pascual A, García I, Perea EJ. Effect of linezolid on the phagocytic functions of human polymorphonuclear leukocytes. Chemotherapy. 2003;49(4):163-6.
343. Kushiya K, Nakagawa S, Taneike I, Iwakura N, Imanishi K, Uchiyama T, et al. Inhibitory effect of antimicrobial agents and anisodamine on the staphylococcal superantigenic toxin-induced overproduction of proinflammatory cytokines by human peripheral blood mononuclear cells. Journal of infection and chemotherapy : official journal of the Japan Society of Chemotherapy. 2005;11(4):192-5.
344. Molinoff PB, Axelrod J. Biochemistry of catecholamines. Annu Rev Biochem. 1971;40:465-500.
345. Shore PA. Transport and Storage of Biogenic Amines. Ann Rev Pharm. 1972;12:209-26.
346. Brodde OE, Engel G, Hoyer D, Block K.D., Weber F. The beta-adrenergic receptor in human lymphocytes-sub- classification by the use of a new radio- ligand (±)[125 Iodo]cyanopindolol]. Life Sci. 1981;29(21):89-98.

347. Fuchs BA, Campbell KS, Munson AE. Norepinephrine and serotonin content of the murine spleen: its relationship to lymphocyte beta-adrenergic receptor density and the humoral immune response in vivo and in vitro. Cell Immunol. 1988;117(2):339-51.
348. Landmann R, Burgisser E, West M, F.R. B. Beta adrenergic receptors are different in subpopulations of human circulating lymphocytes. J Recept Res. 1985;4:37-50.
349. Titinchi S, Clark B. Alpha 2-adrenoceptors in human lymphocytes: direct characterisation by [3H]yohimbine binding. Biochem Biophys Res Commun. 1984;121:1-7.
350. Anstead MI, Hunt TA, Carlson SL, Burki NK. Variability of peripheral blood lymphocyte beta-2-adrenergic receptor density in humans. . Am J Respir Crit Care Med. 1998;157:990-2.
351. Williams JM, Felten DL. Sympathetic innervation of murine thymus and spleen: a comparative histofluorescence study. Anat Rec. 1981;199:531-42.
352. Weihe E, Nohr D, Michel S, Müller S, Zentel HJ, Fink T, et al. Molecular anatomy of the neuro-immune connection. Int J Neurosci. Weihe E, Nohr D, Michel S, Müller S, Zentel HJ, Fink T, Krekel J. Molecular anatomy of the neuro-immune connection. Int J Neurosci. 1991 Jul;59(1-3):1-23.;59:1-23.
353. Bergquist J, Tarkowski A, Ekman R, Ewing A. Discovery of endogenous catecholamines in lymphocytes and evidence for catecholamine regulation of lymphocyte function via an autocrine loop. Proc Natl Acad Sci U S A. 1994;91:12912–6.
354. Josefsson E, Bergquist J, Ekman R, Tarkowski A. Catecholamines are synthesized by mouse lymphocytes and regulate function of these cells by induction of apoptosis. Immunol. 1996;88:140-6.
355. Cosentino M, Bombelli R, Ferrari M, Marino F, Rasini E, Maestroni GJ, et al. HPLC-ED measurement of endogenous catecholamines in human immune cells and hematopoietic cell lines. Life Sci. 2000;68(3):283-95.
356. Musso NR, Brenci S, Setti M, Indiveri F, Lotti G. Catecholamine content and in vitro catecholamine synthesis in peripheral human lymphocytes. J Clin Endocrinol Metab. 1996;81(10):3553-7.
357. Balsa MD, Gómez N, Unzeta M. Characterization of monoamine oxidase activity present in human granulocytes and lymphocytes. Biochem Biophys Acta. 1989;992(2):140-4.
358. Bidart JM, Motte P, Assicot M, Bohuon C, Bellet D. Catechol-O-methyltransferase activity and aminergic binding sites distribution in human peripheral blood lymphocyte subpopulations. . Clin Immunol Immunopathol. 1983;26:1-9.
359. van der Poll T, Coyle SM, Barbosa K, Braxton CC, Lowry SF. Epinephrine inhibits tumor necrosis factor-alpha and potentiates interleukin 10 production during human endotoxemia. J Clin Invest. 1996;97:713-9.
360. Norris JG, Benveniste EN. nterleukin-6 production by astrocytes: induction by the neurotransmitter norepinephrine. J Neuroimmunol. 1993;45:137-45.
361. Kavelaars A, van de Pol M, Zijlstra J, Heijnen CJ. Beta 2-adrenergic activation enhances interleukin-8 production by human monocytes. . J Neuroimmunol. 1997;77:211-6.

362. Hasko G, Shanley TP, Egnaczyk G, Németh ZH, Salzman AL, Vizi ES, et al. Exogenous and endogenous catecholamines inhibit the production of macrophage inflammatory protein (MIP) 1a via a b adrenoceptor mediated mechanism. Br J Pharmacol. 1998;125:1297-303.
363. Hetier E, Ayala J, Bousseau A, Prochiantz A. Modulation of interleukin-1 and tumor necrosis factor expression by beta-adrenergic agonists in mouse ameboid microglial cells. Exp Brain Res. 1991;86:407-13.
364. Severn A, Rapson NT, Hunter CA, Liew FY. Regulation of tumor necrosis factor production by adrenaline and beta-adrenergic agonists. . J Immunol 1992;48:3441-5.
365. Spengler RN, Chensue SW, Giacherio DA, Blenk N, Kunkel SL. Endogenous norepinephrine regulates tumor necrosis factor-alpha production from macrophages in vitro. J Immunol. 1994;152(6):3024-31.
366. Koff WC, Dunegan MA. Neuroendocrine hormones suppress macrophage-mediated lysis of herpes simplex virus-infected cells. . J Immunol. 1986;136:705-9.
367. Bermudez LE, Wu M, Young LS. Effect of stress-related hormones on macrophage receptors and response to tumor necrosis factor. Lymphokine Res. 1990;9:137-45.
368. Wieduwild E, Girard-Madoux MJ, Quatrini L, Laprie C, Chasson L, Rossignol R, et al. beta2-adrenergic signals downregulate the innate immune response and reduce host resistance to viral infection. J Exp Med. 2020;217(4).
369. Wenisch C, Parschalk B, Weiss A, Zedwitz-Liebenstein K, Hahsler B, Wenisch H, et al. High-dose catecholamine treatment decreases polymorphonuclear leukocyte phagocytic capacity and reactive oxygen production. Clin Diagn Lab Immunol. 1996;3:423-8.
370. Elliott L, Brooks W, Roszman T. Inhibition of anti-CD3 monoclonal antibody-induced T-cell proliferation by dexamethasone, isoproterenol, or prostaglandin E2 either alone or in combination. Cell Mol Neurobiol. 1992;12:411-27.
371. Elenkov IJ, Papanicolaou DA, Wilder RL, Chrousos GP. Modulatory effects of glucocorticoids and catecholamines on human interleukin-12 and interleukin-10 production: clinical implications. . Proc Assoc Am Physicians. 1996;108:374-81.
372. Huang HW, Tang JL, Han XH, Peng YP, Qiu YH. Lymphocyte-derived catecholamines induce a shift of Th1/Th2 balance toward Th2 polarization. Neuroimmunomodulation. 2013;20(1):1-8.
373. Swanson MA, Lee WT, Sanders VM. IFN-gamma production by Th1 cells generated from naive CD4+ T cells exposed to norepinephrine. J Immunol. 2001;166(1):232-40.
374. Sanders VM, Baker RA, Ramer-Quinn DS, Kasprowicz DJ, Fuchs BA, Street NE. Differential expression of the b2-adrenergic receptor by Th1 and Th2 clones: implications for cytokine production and B cell help. J Immunol. 1997;158:4200-10.
375. Broug-Holub E, Persoons JH, Schornagel K, Mastbergen SC, Kraal G. Effects of stress on alveolar macrophages: a role for the sympathetic nervous system. Am J Respir Crit Care Med. 1998;19:842-8.

376. Miles BA, Lafuse WP, Zwilling BS. Binding of alpha-adrenergic receptors stimulates the anti-mycobacterial activity of murine peritoneal macrophages. . J Neuroimmunol. 1996;71:19-24.
377. Spengler RN, Allen RM, Remick DG, Strieter RM, Kunkel SL. Stimulation of a-adrenergic receptor augments the production of macrophage-derived tumor necrosis factor. J Immunol. 1990;145:1430-4.
378. Cao CJ, Eldefrawi AT, Eldefrawi ME. ATP-regulated neuronal catecholamine uptake: a new mechanism. Life Sci. 1990;47(7):655-67.
379. Taylor SC, Shaw SM, Peers C. Mitochondrial inhibitors evoke catecholamine release from pheochromocytoma cells. Biochem Biophys Res Commun. 2000;273(1):17-21.
380. Lim DY, Park HG, Miwa S. CCCP enhances catecholamine release from the perfused rat adrenal medulla. Auton Neurosci. 2006;128(1-2):37-47.
381. Genova ML, Abd-Elsalam NM, Mahdy el SM, Bernacchia A, Lucarini M, Pedulli GF, et al. Redox cycling of adrenaline and adrenochrome catalysed by mitochondrial Complex I. Arch Biochem Biophys. 2006;447(2):167-73.
382. Manoli I, Alesci S, Blackman MR, Su YA, Rennert OM, Chrousos GP. Mitochondria as key components of the stress response. Trends Endocrinol Metab. 2007;18(5):190-8.
383. Stolk RF, van der Pasch E, Naumann F, Schouwstra J, Bressers S, van Herwaarden AE, et al. Norepinephrine Dysregulates the Immune Response and Compromises Host Defense during Sepsis. Am J Respir Crit Care Med. 2020;202(6):830-42.
384. Napolitano G, Barone D, Di Meo S, Venditti P. Adrenaline induces mitochondrial biogenesis in rat liver. J Bioenerg Biomembr. 2018;50(1):11-9.
385. Barke RA, Brady PS, Brady LJ. The regulation of mitochondrial fatty acid oxidation and hepatic gene expression by catecholamine. J Surg Res. 1993;54(2):95-101.
386. Porta F, Takala J, Weikert C, Kaufmann P, Krahenbuhl S, Jakob SM. Effect of endotoxin, dobutamine and dopamine on muscle mitochondrial respiration in vitro. J Endotoxin Res. 2006;12(6):358-66.
387. Regueira T, Banziger B, Djafarzadeh S, Brandt S, Gorrasi J, Takala J, et al. Norepinephrine to increase blood pressure in endotoxaemic pigs is associated with improved hepatic mitochondrial respiration. Crit Care. 2008;12(4):R88.
388. Vuda M, Brander L, Schröder R, Jakob SM, Takala J, Djafarzadeh S. Effects of catecholamines on hepatic and skeletal muscle mitochondrial respiration after prolonged exposure to faecal peritonitis in pigs. Innate Immun. 2012;18(2):217-30.
389. Youn YC, Kwon OS, Han ES, Song JH, Shin YK, Lee CS. Protective effect of boldine on dopamine-induced membrane permeability transition in brain mitochondria and viability loss in PC12 cells. Biochem Pharmacol. 2002;63:495-505.
390. Lee CS, Han JH, Jang YY, Song JH, Han ES. Differential effect of catecholamines and MPP(+) on membrane permeability in brain mitochondria and cell viability in PC12 cells. . Neurochem Int. 2002;40:361-9.
391. Kim KJ, Jang YY, Han ES, Lee CS. Modulation of brain mitochondrial membrane permeability and synaptosomal Ca2+ transport by dopamine oxidation. Mol Cell Biochem. 1999;201:89-98.

392. Lunemann JD, Buttgereit F, Tripmacher R, Baerwald CG, Burmester GR, Krause A. Norepinephrine inhibits energy metabolism of human peripheral blood mononuclear cells via adrenergic receptors. Biosci Rep. 2001;21:627–35.
393. Smith MA, Hibino M, Falcione BA, Eichinger KM, Patel R, Empey KM. Immunosuppressive aspects of analgesics and sedatives used in mechanically ventilated patients: an underappreciated risk factor for the development of ventilator-associated pneumonia in critically ill patients. Ann Pharmacother. 2014;48(1):77-85.
394. Darandale SS, Shevalkar GB, Vavia PR. Effect of Lipid Composition in Propofol Formulations: Decisive Component in Reducing the Free Propofol Content and Improving Pharmacodynamic Profiles. AAPS PharmSciTech. 2017;18(2):441-50.
395. Alam S, Laughton DL, Walding A, Wolstenholme AJ. Human peripheral blood mononuclear cells express GABAA receptor subunits. Mol Immunol. 2006;43(9):1432-42.
396. Dionisio L, Jose De Rosa M, Bouzat C, Esandi Mdel C. An intrinsic GABAergic system in human lymphocytes. Neuropharmacology. 2011;60(2-3):513-9.
397. Prud'homme GJ, Glinka Y, Wang Q. Immunological GABAergic interactions and therapeutic applications in autoimmune diseases. Autoimmun Rev. 2015;14(11):1048-56.
398. Bhat R, Axtell R, Mitra A, Miranda M, Lock C, Tsien RW, et al. Inhibitory role for GABA in autoimmune inflammation. Proc Natl Acad Sci U S A. 2010;107(6):2580-5.
399. Jun IJ, Jo JY, Kim JI, Chin JH, Kim WJ, Kim HR, et al. Impact of anesthetic agents on overall and recurrence-free survival in patients undergoing esophageal cancer surgery: A retrospective observational study. Sci Rep. 2017;7(1):14020.
400. Zheng X, Wang Y, Dong L, Zhao S, Wang L, Chen H, et al. Effects of propofol-based total intravenous anesthesia on gastric cancer: a retrospective study. Onco Targets Ther. 2018;11:1141-8.
401. Devlin EG, Clarke RS, Mirakhur RK, McNeill TA. Effect of four i.v. induction agents on T-lymphocyte proliferations to PHA in vitro. Br J Anaesth. 1994;73(3):315-7.
402. Schneemilch CE, Hachenberg T, Ansorge S, Ittenson A, Bank U. Effects of different anaesthetic agents on immune cell function in vitro. Eur J Anaesthesiol. 2005;22:616-23.
403. Yuki K, Soriano SG, Shimaoka M. Sedative drug modulates T-cell and lymphocyte function-associated antigen-1 function. Anesth Analg. 2011;112(4):830-8.
404. Pirttikangas CO, Perttila J, Salo M, Vainio O, Liukko-Sipi S. Propofol infusion anaesthesia and immune response in minor surgery. Anaesthesia. 1994;49:13-6.
405. Inada T, Yamanouchi Y, Jomura S, Sakamoto S, Takahashi M, Kambara T, et al. Effect of propofol and isoflurane anaesthesia on the immune response to surgery. Anaesthesia. 2004;59:954-9.
406. Visvabharathy L, Freitag NE. Propofol Sedation Exacerbates Kidney Pathology and Dissemination of Bacteria during Staphylococcus aureus Bloodstream Infections. Infect Immun. 2017;85(7).

407. Visvabharathy L, Xayarath B, Weinberg G, Shilling RA, Freitag NE. Propofol Increases Host Susceptibility to Microbial Infection by Reducing Subpopulations of Mature Immune Effector Cells at Sites of Infection. PLoS One. 2015;10(9):e0138043.
408. Park HJ, Piao L, Seo EH, Lee SH, Kim SH. The effect of repetitive exposure to intravenous anesthetic agents on the immunity in mice. Int J Med Sci. 2020;17(4):428-36.
409. Takaono M, Yogosawa T, Okawa-Takatsuji M, Aotsuka S. Effects of intravenous anesthetics on interleukin (IL)-6 and IL-10 production by lipopolysaccharide-stimulated mononuclear cells from healthy volunteers. . Acta Anaesthesiol Scand. 2002;46:176-9.
410. Colluci DG, Puig NR, Hernandez-Pando R. Influence of anaesthetic drugs on immune response: from inflammation to immunosuppression. OA Anaesthetics. 2013;1:2-7.
411. Gonzalez-Correa JA, Cruz-Andreotti E, Arrebola MM, Lopez-Villodres JA, Jodar M, De La Cruz JP. Effects of propofol on the leukocyte nitric oxide pathway: in vitro and ex vivo studies in surgical patients. Naunyn Schmiedebergs Arch Pharmacol. 2008;376(5):331-9.
412. Lisowska B, Szymanska M, Nowacka E, Olszewska M. Anesthesiology and the cytokine network. . Postepy Hig Med Dosw (Online). 2013;67:761-9.
413. Kochiyama T, Li X, Nakayama H, Kage M, Yamane Y, Takamori K, et al. Effect of Propofol on the Production of Inflammatory Cytokines by Human Polarized Macrophages. Mediators Inflamm. 2019;2019:1919538.
414. Galley HF, Dubbels AM, Webster NR. The effect of midazolam and propofol on IL-8 from human PBMCs. Anaesth Analg. 1998;86:1289-93.
415. Tang J, Chen X, Tu W, Guo Y, Zhao Z, Xue Q, et al. Propofol inhibits the activation of p38 through up-regulating the expression of annexin A1 to exert its anti-inflammation effect. PLoS One. 2011;6(12):e27890.
416. Tanaka T, Kai S, Matsuyama T, Adachi T, Fukuda K, Hirota K. General anesthetics inhibit LPS-induced IL-1beta expression in glial cells. PLoS One. 2013;8(12):e82930.
417. Li W, Hao X, Liu Y, Tong T, Xu H, Jia L. Effects of anesthetic agents on inflammation in Caco-2, HK-2 and HepG2 cells. Exp Ther Med. 2021;21(5):487.
418. Zha H, Matsunami E, Blazon-Brown N, Koutsogiannaki S, Hou L, Bu W, et al. Volatile anesthetics affect macrophage phagocytosis. PLoS One. 2019;14(5):e0216163.
419. Ploppa A, Kiefer RT, Nohe B, Haeberle HA, Dieterich HJ, Unertl KE, et al. Monocyte phagocytosis of viable Staphylococcus aureus is impaired by barbiturates, but not by propofol. Infection. 2008;36(3):220-5.
420. Ploppa A, Kiefer RT, Nohe B, Haeberle HA, Dieterich HJ, Unertl KE, et al. Dose-dependent influence of barbiturates but not of propofol on human leukocyte phagocytosis of viable Staphylococcus aureus. Crit Care Med. 2006;34(2):478-83.
421. Davidson JA, Boom SJ, Pearsall FJ, Zhang P, Ramsay G. Comparison of the effects of four i.v. anaesthetic agents on polymorphonuclear leucocyte function. Br J Anaesth. 1995;74(3):315-8.

422. Wheeler DW, Thompson AJ, Corletto F, Reckless J, Loke JC, Lapaque N, et al. Anaesthetic impairment of immune function is mediated via GABA(A) receptors. PLoS One. 2011;6(2):e17152.
423. Hsing CH, Chen MS, Chen CL, Huang WC, Lin MC, Tseng PC, et al. Anesthetic Propofol Causes Glycogen Synthase Kinase-3b-regulated Lysosomal/Mitochondrial Apoptosis in Macrophages. CCM. 2012;116(4):868-81.
424. Chen RM, Wu CH, Chang HC, Wu GJ, Lin YL, Sheu JR, et al. Propofol Suppresses Macrophage Functions and Modulates Mitochondrial Membrane Potential and Cellular Adenosine Triphosphate Synthesis. Anesthesiology. 2003;98:1178-85.
425. Chen L, Wan Y, Liu Y, Li T. Propofol inhibits biological functions of leukaemia stem and differentiated cells through suppressing Wnt/beta-catenin and Akt/mTOR. Clin Exp Pharmacol Physiol. 2020;47(1):127-34.
426. Chen MS, Lin WC, Yeh HT, Hu CL, Sheu SM. Propofol specifically suppresses IL-1beta secretion but increases bacterial survival in Staphylococcus aureus-infected RAW264.7 cells. Mol Cell Biochem. 2018;449(1-2):117-25.
427. Shiratsuchi H, Kouatli Y, Yu GX, Marsh HM, Basson MD. Propofol inhibits pressure-stimulated macrophage phagocytosis via the GABAA receptor and dysregulation of p130cas phosphorylation. Am J Physiol Cell Physiol. 2009;296(6):C1400-10.
428. Hulse D, Kusel JR, O''Donnell NG, Wilkinson PC. Effects of anaesthetics on membrane mobility and locomotor responses of human neutrophils. FEMS Immunol and Med Micro. 1994;8:241-8.
429. Heller A, Heller S, Blecken S, Urbaschek R, Koch T. Effects of intravenous anesthetics on bacterial elimination in human blood in vitro. Acta Anaes Scan. 2008;42(5):518-26.
430. Zhao J, Juettner B, Scheinichen D, Jaeger K, Heine J, Piepenbrock S. Respiratory burst activity of polymorphonuclear cells is dependent on the cell preparation technique. Acta Anaesthesiol Scand. 2003;47(6):702-6.
431. Yang SC, Chung PJ, Ho CM, Kuo CY, Hung MF, Huang YT, et al. Propofol inhibits superoxide production, elastase release, and chemotaxis in formyl peptide-activated human neutrophils by blocking formyl peptide receptor 1. J Immunol. 2013;190(12):6511-9.
432. Cai X, Li Y, Zheng X, Hu R, Li Y, Xiao L, et al. Propofol suppresses microglial phagocytosis through the downregulation of MFG-E8. J Neuroinflammation. 2021;18(1):18.
433. Kotani N, Hashimoto H, Sessler DI, Kikuchi A, Suzuki A, Takahashi S, et al. Intraoperative Modulation of Alveolar Macrophage Function during Isoflurane and Propofol Anesthesia. Anaesthesiology. 1998;89:1125-32.
434. Heine J, Jaeger K, Osthaus A, Weingaertner N, Munte S, Piepenbrock S, et al. Anaesthesia with propofol decreases FMLP-induced neutrophil respiratory burst but not phagocytosis compared with isoflurane. Br J Anaesth. 2000;85(3):424-30.
435. Erol A, Reisli R, Reisli I, Kara R, Otelcioglu S. Effects of desflurane, sevoflurane and propofol on phagocytosis and respiratory burst activity of human polymorphonuclear leucocytes in bronchoalveolar lavage. Eur J Anaesthesiol. 2009;26(2):150-4.

436. Sun L, Ma W, Gao W, Xing Y, Chen L, Xia Z, et al. Propofol directly induces caspase-1-dependent macrophage pyroptosis through the NLRP3-ASC inflammasome. Cell Death Dis. 2019;10(8):542.
437. Wu GJ, Tai YT, Chen TL, Lin LL, Ueng YF, Chen RM. Propofol specifically inhibits mitochondrial membrane potential but not complex I NADH dehydrogenase activity, thus reducing cellular ATP biosynthesis and migration of macrophages. Ann N Y Acad Sci. 2005;1042:168-76.
438. Delogu G, Antonucci A, Moretti S, Marandola M, Tellan G, Signore M, et al. Oxidative stress and mitochondrial glutathione in human lymphocytes exposed to clinically relevant anesthetic drug concentrations. J Clin Anesth. 2004;16(3):189-94.
439. Kokita N, Hara A. Propofol Attenuates Hydrogen Peroxide-induced Mechanical and Metabolic Derangements in the Isolated Rat Heart. Anaesthesiology. 1996;84:117-27.
440. Shao H, Li J, Zhou Y, Ge Z, Fan J, Shao Z, et al. Dose-dependent protective effect of propofol against mitochondrial dysfunction in ischaemic/reperfused rat heart: role of cardiolipin. Br J Pharmacol. 2008;153(8):1641-9.
441. Yu DJ, Gao HY. Effect of propofol on mitochondrial ATP content and ATPase activity in hippocampus of rats with cerebral ischemia-reperfusion injury. Saudi J Biol Sci. 2017;24(2):246-50.
442. Musacchio E, Rizzoli V, Bianchi M, Bindoli A, Galzigna L. Antioxidant Action of Propofol on Liver Microsomes, Mitochondria and Brain Synaptosomesin the Rat. Pharm & Toxicol. 1991;69:75-7.
443. Ranjbar A, Sharifzadeh M, Karimi J, Tavilani H, Baeeri M, Shayesteh TH, et al. Propofol Attenuates Toxic Oxidative Stress by CCl4 in Liver Mitochondria and Blood in Rat. Iran J Pharm Res. 2014;13(1):253-62.
444. Bellanti F, Mirabella L, Mitarotonda D, Blonda M, Tamborra R, Cinnella G, et al. Propofol but not sevoflurane prevents mitochondrial dysfunction and oxidative stress by limiting HIF-1alpha activation in hepatic ischemia/reperfusion injury. Free Radic Biol Med. 2016;96:323-33.
445. Zhong H, Song R, Pang Q, Liu Y, Zhuang J, Chen Y, et al. Propofol inhibits parthanatos via ROS-ER-calcium-mitochondria signal pathway in vivo and vitro. Cell Death Dis. 2018;9(10):932.
446. Urabe T, Yanase Y, Motoike S, Harada K, Hide I, Tanaka S, et al. Propofol induces the elevation of intracellular calcium via morphological changes in intracellular organelles, including the endoplasmic reticulum and mitochondria. Eur J Pharmacol. 2020;884:173303.
447. Liu XR, Cao L, Li T, Chen LL, Yu YY, Huang WJ, et al. Propofol attenuates H2O2-induced oxidative stress and apoptosis via the mitochondria- and ER-medicated pathways in neonatal rat cardiomyocytes. Apoptosis. 2017;22(5):639-46.
448. Zhang Q, Cai S, Guo L, Zhao G. Propofol induces mitochondrial-associated protein LRPPRC and protects mitochondria against hypoxia in cardiac cells. PLoS One. 2020;15(9):e0238857.
449. Zhao L, Zhuang J, Wang Y, Zhou D, Zhao D, Zhu S, et al. Propofol Ameliorates H9c2 Cells Apoptosis Induced by Oxygen Glucose Deprivation and Reperfusion Injury via Inhibiting High Levels of Mitochondrial Fusion and Fission. Front Pharmacol. 2019;10:61.

450. Sumi C, Okamoto A, Tanaka H, Nishi K, Kusunoki M, Shoji T, et al. Propofol induces a metabolic switch to glycolysis and cell death in a mitochondrial electron transport chain-dependent manner. PLoS One. 2018;13(2):e0192796.
451. Qin J, Li Y, Wang K. Propofol induces impairment of mitochondrial biogenesis through inhibiting the expression of peroxisome proliferator-activated receptor-gamma coactivator-1alpha. J Cell Biochem. 2019;120(10):18288-97.
452. Liang C, Sun M, Zhong J, Miao C, Han X. The Role of Pink1-Mediated Mitochondrial Pathway in Propofol-Induced Developmental Neurotoxicity. Neurochem Res. 2021;46(9):2226-37.
453. Kido K, Ito H, Yamamoto Y, Makita K, Uchida T. Cytotoxicity of propofol in human induced pluripotent stem cell-derived cardiomyocytes. J Anesth. 2018;32(1):120-31.
454. Urban T, Waldauf P, Krajcova A, Jiroutkova K, Halacova M, Dzupa V, et al. Kinetic characteristics of propofol-induced inhibition of electron-transfer chain and fatty acid oxidation in human and rodent skeletal and cardiac muscles. PLoS One. 2019;14(10):e0217254.
455. Bergamini C, Moruzzi N, Volta F, Faccioli L, Gerdes J, Mondardini MC, et al. Role of mitochondrial complex I and protective effect of CoQ10 supplementation in propofol induced cytotoxicity. J Bioenerg Biomembr. 2016;48(4):413-23.
456. Kam PC, Cardone D. Propofol infusion syndrome. Anaesthesia. 2007;62(7):690-701.
457. Finsterer J, Frank M. Propofol Is Mitochondrion-Toxic and May Unmask a Mitochondrial Disorder. J Child Neurol. 2016;31(13):1489-94.
458. Bains R, Moe MC, Vinje ML, Berg-Johnsen J. Sevoflurane and propofol depolarize mitochondria in rat and human cerebrocortical synaptosomes by different mechanisms. Acta Anaesthesiol Scand. 2009;53(10):1354-60.
459. Yue ZY, Dong H, Wang YF, Liu Y, Song CY, Yang WC, et al. Propofol prevents neuronal mtDNA deletion and cerebral damage due to ischemia/reperfusion injury in rats. Brain Res. 2015;1594:108-14.
460. Shao H, Zhang Y, Dong Y, Yu B, Xia W, Xie Z. Chronic treatment with anesthetic propofol improves cognitive function and attenuates caspase activation in both aged and Alzheimer's disease transgenic mice. J Alzheimers Dis. 2014;41(2):499-513.
461. Lotz C, Stumpner J, Smul TM. Sevoflurane as opposed to propofol anesthesia preserves mitochondrial function and alleviates myocardial ischemia/reperfusion injury. Biomed Pharmacother. 2020;129:110417.
462. Grundmanova M, Jarkovska D, Suss A, Tuma Z, Markova M, Grundman Z, et al. Propofol-induced mitochondrial and contractile dysfunction of the rat ventricular myocardium. Physiol Res. 2016;65(Suppl 5):S601-S9.
463. Osonwa UE, Ugochukwu JI, Ajaegbu EE, Chukwu KI, Azevedo RB, Esimone CO. Enhancement of antibacterial activity of ciprofloxacin hydrochloride by complexation with sodium cholate. Bulletin of Faculty of Pharmacy, Cairo University. 2017;55(2):233-7.

464. Gai XY, Bo SN, Shen N, Zhou QT, Yin AY, Lu W. Pharmacokinetic-pharmacodynamic analysis of ciprofloxacin in elderly Chinese patients with lower respiratory tract infections caused by Gram-negative bacteria. Chin Med J (Engl). 2019;132(6):638-46.
465. Sanchez Navarro MD, Sayalero Marinero ML, Sanchez Navarro A. Pharmacokinetic/pharmacodynamic modelling of ciprofloxacin 250 mg/12 h versus 500 mg/24 h for urinary infections. J Antimicrob Chemother. 2002;50(1):67-72.
466. Al-Omar MA. Ciprofloxacin: Drug Metabolism and Pharmacokinetic Profile. Profiles of Drug Substances, Excipients and Related Methodology2005. p. 209-14.
467. Peck TE, Hill SA, Williams M. Pharmacology fo Anaesthesia and Intensive Care (Third Ed). Third ed: Cambridge University Press; 2009. 360 p.
468. Sahinovic MM, Struys M, Absalom AR. Clinical Pharmacokinetics and Pharmacodynamics of Propofol. Clin Pharmacokinet. 2018;57(12):1539-58.
469. Eleveld DJ, Colin P, Absalom AR, Struys M. Pharmacokinetic-pharmacodynamic model for propofol for broad application in anaesthesia and sedation. Br J Anaesth. 2018;120(5):942-59.
470. Dinis-Oliveira RJ. Metabolic Profiles of Propofol and Fospropofol: Clinical and Forensic Interpretative Aspects. Biomed Res Int. 2018;2018:6852857.
471. Dominguez-Andres J, Arts RJW, Bekkering S, Bahrar H, Blok BA, de Bree LCJ, et al. In vitro induction of trained immunity in adherent human monocytes. STAR Protoc. 2021;2(1):100365.
472. Manohar SM, Shah P, Nair A. Flow cytometry: principles, applications and recent advances. Bioanalysis. 2021;13:181-98.
473. Perry SW, Norman JP, Barbieri J, Brown EB, Gelbard HA. Mitochondrial membrane potential probes and the proton gradient: a practical usage guide. Biotechniques. 2011;50(2):98-115.
474. Vangapandu HV, Gandhi V. Chronic Lymphocytic Leukemia. Methods in Molecular Biology. New York, NY: Humana Press; 2019.
475. Geiss GK, Bumgarner RE, Birditt B, Dahl T, Dowidar N, Dunaway DL, et al. Direct multiplexed measurement of gene expression with color-coded probe pairs. Nat Biotechnol. 2008;26(3):317-25.
476. Lekkou A, Karakantza M, Mouzaki A, Kalfarentzos F, Gogos CA. Cytokine production and monocyte HLA-DR expression as predictors of outcome for patients with community-acquired severe infections. Clin Diagn Lab Immunol. 2004;11(1):161-7.
477. Johnson SB, Lissauer M, Bochicchio GV, Moore R, Cross AS, Scalea TM. Gene expression profiles differentiate between sterile SIRS and early sepsis. Ann Surg. 2007;245(4):611-21.
478. Ramilo O, Allman W, Chung W, Mejias A, Ardura M, Glaser C, et al. Gene expression patterns in blood leukocytes discriminate patients with acute infections. Blood. 2007;109(5):2066-77.
479. Tang BM, McLean AS, Dawes IW, Huang SJ, Cowley MJ, Lin RC. Gene-expression profiling of gram-positive and gram-negative sepsis in critically ill patients. Crit Care Med. 2008;36(4):1125-8.
480. Lu H, Wen D, Wang X, Gan L, Du J, Sun J, et al. Host genetic variants in sepsis risk: a field synopsis and meta-analysis. Crit Care. 2019;23(1):26.

481. Engoren M, Jewell ES, Douville N, Moser S, Maile MD, Bauer ME. Genetic variants associated with sepsis. PLoS One. 2022;17(3):e0265052.
482. Kwok AJ, Mentzer A, Knight JC. Host genetics and infectious disease: new tools, insights and translational opportunities. Nat Rev Genet. 2021;22(3):137-53.
483. Davenport EE, Burnham KL, Radhakrishnan J, Humburg P, Hutton P, Mills TC, et al. Genomic landscape of the individual host response and outcomes in sepsis: a prospective cohort study. The Lancet Respiratory Medicine. 2016;4(4):259-71.
484. Tang BM, Huang SJ, McLean A, S. Genome-wide transcription profiling of human sepsis: a systematic review. Crit Care. 2010;14(6):R237.
485. Cajander S, Backman A, Tina E, Stralin K, Soderquist B, Kallman J. Preliminary results in quantitation of HLA-DRA by real-time PCR: a promising approach to identify immunosuppression in sepsis. Crit Care Medicine. 2013;17:R223.
486. Boomer JS, To K, Chang KC, Takasu O, Osborne DF, Walton AH, et al. Immunosuppression in Patients Who Die of Sepsis and Multiple Organ Failure. JAMA. 2011;306(23):2594- 605.
487. Merz TM, Pereira AJ, Schurch R, Schefold JC, Jakob SM, Takala J, et al. Mitochondrial function of immune cells in septic shock: A prospective observational cohort study. PLoS One. 2017;12(6):e0178946.
488. McBride MA, Owen AM, Stothers CL, Hernandez A, Luan L, Burelbach KR, et al. The Metabolic Basis of Immune Dysfunction Following Sepsis and Trauma. Front Immunol. 2020;11:1043.
489. Brealey D, Karympudi S, Jacques TS, Novelli M, Stidwil R, Taylor V, et al. Mitochondrial dysfunction in a long-term rodent model of sepsis and organ failure. Am J Physiol Regul Integr Comp Physiol. 2003;286:R491–R7.
490. Schildberger A, Rossmanith E, Eichhorn T, Strassl K, Weber V. Monocytes, peripheral blood mononuclear cells, and THP-1 cells exhibit different cytokine expression patterns following stimulation with lipopolysaccharide. Mediators Inflamm. 2013;2013:697972.
491. Brooks D, Barr LC, Wiscombe S, McAuley DF, Simpson AJ, Rostron AJ. Human lipopolysaccharide models provide mechanistic and therapeutic insights into systemic and pulmonary inflammation. Eur Respir J. 2020;56(1).
492. Vivas MC, Villamarin Guerrero HF, Tascon AJ, Valderrama-Aguirre A. Plasma interleukin-6 levels correlate with survival in patients with bacterial sepsis and septic shock. Interventional Medicine and Applied Science. 2021;11(4):224-30.
493. Damas P, Ledoux D, Nys M, Vrindts Y, De Groote D, Franchimont P, et al. Cytokine serum level during severe sepsis in human IL-6 as a marker of severity. Ann Surg. 1992;215(4):356-62.
494. Chaudhry H, Zhou J, Zhong Y, Ali MM, McGuire F, Nagarkatti PS, et al. Role of cytokines as a double-edged sword in sepsis. . In Vivo. 2013;27(6):669-84.
495. Yang N, Sin DD, Drscheild DR. Various factors affect lipopolysaccharide sensitization in cell cultures. Biotechniques. 2020;69:127-33.
496. Tateda K, Matsumoto T, Miyazaki S, Yamaguchi K. Lipopolysaccharide-induced lethality and cytokine production in aged mice. Infect Immun. 1996;64:769-74.

497. Vollmar B, Pradarutti S, Nickels RM, Menger MD. Age-associated loss of immunomodulatory protection by granulocyte-colony stimulating factor in endotoxic rats. Shock. 2002;18:348-54.
498. Gardner ID. The effect of aging on susceptibility to infection. Rev Infect Dis. 1980;2:801-10.
499. Sarker P, Mily A, Mamun AA, Jalal S, Bergman P, Raqib R, et al. Ciprofloxacin Affects Host Cells by Suppressing Expression of the Endogenous Antimicrobial Peptides Cathelicidins and Beta-Defensin-3 in Colon Epithelia. Antibiotics (Basel). 2014;3(3):353-74.
500. van der Poll T, Jansen J, Endert E, Sauerwein HP, van Deventer SJ. Noradrenaline inhibits lipopolysaccharide-induced tumor necrosis factor and interleukin 6 production in human whole blood. . Infect Immun. 1994;62:2046-50.
501. Sharma D, Farrar JD. Adrenergic regulation of immune cell function and inflammation. Semin Immunopathol. 2020;42(6):709-17.
502. Saraiva M, O'Garra A. The regulation of IL-10 production by immune cells. Nat Rev Immunol. 2010;10(3):170-81.
503. Agac D, Estrada LD, Maples R, Hooper LV, Farrar JD. The beta2-adrenergic receptor controls inflammation by driving rapid IL-10 secretion. Brain Behav Immun. 2018;74:176-85.
504. Fabri A, Kandara K, Coudereau R, Gossez M, Abraham P, Monard C, et al. Characterization of Circulating IL-10-Producing Cells in Septic Shock Patients: A Proof of Concept Study. Front Immunol. 2020;11:615009.
505. Tamulyte S, Kopplin J, Brenner T, Weigand MA, Uhle F. Monocyte HLA-DR Assessment by a Novel Point-of-Care Device Is Feasible for Early Identification of ICU Patients With Complicated Courses-A Proof-of-Principle Study. Front Immunol. 2019;10:432.
506. Monneret G, Venet F. Monocyte HLA-DR in sepsis: shall we stop following the flow? Crit Care. 2014;18(1):102.
507. Haas LEM, van Dillen LS, de Lange DW, van Dijk D, Hamaker ME. Outcome of very old patients admitted to the ICU for sepsis: A systematic review. European Geriatric Medicine. 2017;8(5-6):446-53.
508. McAleer JP, Vella AT. Understanding how lipopolysaccharide impacts CD4 T cell immunity. Crit Rev Immunol. 2008;28(4):281-99.
509. Ai W, Li H, Song N, Li L, Chen H. Optimal method to stimulate cytokine production and its use in immunotoxicity assessment. Int J Environ Res Public Health. 2013;10(9):3834-42.
510. Bertani B, Ruiz N. Function and Biogenesis of Lipopolysaccharides. EcoSal Plus. 2018;8(1).
511. Morgan MP, Szakmany T, Power SG, Olaniyi P, Hall JE, Rowan K, et al. Sepsis Patients with First and Second-Hit Infections Show Different Outcomes Depending on the Causative Organism. Front Microbiol. 2016;7:207.
512. Morgan A, Babu D, Reiz B, Whittal R, Suh LYK, Siraki AG. Caution for the routine use of phenol red - It is more than just a pH indicator. Chem Biol Interact. 2019;310:108739.
513. Kerbel RS, Blakeslee D. Rapid adsorption of a foetal calf serum component by mammalian cells in culture. Immunology. 1976;31:881-91.

514. Morgan JF, Morton HJ, Parker RC. Nutrition of Animal Cells in Tissue Culture. I. Initial Studies on a Synthetic Medium. Exp Bio Med. 1950;73(1):1-8.
515. Cossarizza A, Chang HD, Radbruch A, Acs A, Adam D, Adam-Klages S, et al. Guidelines for the use of flow cytometry and cell sorting in immunological studies (second edition). Eur J Immunol. 2019;49(10):1457-973.
516. Feng C, Wang H, Lu N, Chen T, He H, Lu Y, et al. Log-transformation and its implications for data analysis. Shanghai Arch Psychiatry. 2014;26(2):105-9.
517. Turrens JF, Freeman BA, Levitt JG, Crapo JD. The effect of hyperoxia on superoxide production by lung submitochondrial particles. Arch Biochem Biophys. 1982;217:401-10.
518. Boveris A, Oshino N, Chance B. The cellular production of hydrogen peroxide. Biochem J. 1972;128:617–30.
519. Lebiedzinska M, Karkucinska-Wieckowska A, Giorgi C, Karczmarewicz E, Pronicka E, Pinton P, et al. Oxidative stress-dependent p66Shc phosphorylation in skin fibroblasts of children with mitochondrial disorders. Biochim Biophys Acta. 2010;1797(6-7):952-60.
520. Li X, Fang P, Yang WY, Chan K, Lavallee M, Xu K, et al. Mitochondrial ROS, uncoupled from ATP synthesis, determine endothelial activation for both physiological recruitment of patrolling cells and pathological recruitment of inflammatory cells. Can J Physiol Pharmacol. 2017;95(3):247-52.
521. Boulos M, Astiz ME, Barua RS, Osman M. Impaired mitochondrial function induced by serum from septic shock patients is attenuated by inhibition of nitric oxide synthase and poly(ADP-ribose) synthase. Crit Care Med. 2003;31(2):353-8.
522. Trentadue R, Fiore F, Massaro F, Papa F, Iuso A, Scacco S, et al. Induction of mitochondrial dysfunction and oxidative stress in human fibroblast cultures exposed to serum from septic patients. Life Sci. 2012;91(7-8):237-43.
523. Dikalov SI, Harrison DG. Methods for detection of mitochondrial and cellular reactive oxygen species. Antioxid Redox Signal. 2014;20(2):372-82.
524. Korshunov SS, Skulachev VP, Starkov AA. High protonic potential actuates a mechanism of production of reactive oxygen species in mitochondria. FEBS Lett. 1997;416:15-8.
525. Polster BM, Nicholls DG, Ge SX, Roelofs BA. Use of potentiometric fluorophores in the measurement of mitochondrial reactive oxygen species. Methods Enzymol. 2014;547:225-50.
526. Xiao B, Deng X, Zhou W, Tan EK. Flow Cytometry-Based Assessment of Mitophagy Using MitoTracker. Front Cell Neurosci. 2016;10:76.
527. Robinson KM, Janes MS, Pehar M, Monette JS, Ross MF, Hagen TM, et al. Selective fluorescent imaging of superoxide in vivo using ethidium-based probes. Proc Natl Acad Sci U S A. 2006;103(41):15038-43.
528. Kalyanaraman B, Dranka BP, Hardy M, Michalski R, Zielonka J. HPLC-based monitoring of products formed from hydroethidine-based fluorogenic probes--the ultimate approach for intra- and extracellular superoxide detection. Biochim Biophys Acta. 2014;1840(2):739-44.

529. Keij JF, Bell-Prince C, Steinkamp JA. Staining of mitochondrial membranes with 10-nonyl acridine orange, MitoFluor Green, and MitoTracker Green is affected by mitochondrial membrane potential altering drugs. Cytometry. 2000;39(3):203-10.
530. Brand MD, Nicholls DG. Assessing mitochondrial dysfunction in cells. Biochem J. 2011;435(2):297-312.