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Antibiotics exacerbate features of sepsis-induced immunosuppression

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A thesis submitted to University College London in candidature for the degree of Doctor of Philosophy

"This letter is written by excellent investigators without an in-depth understanding of the subject matter that regress by selecting random articles to support their views and without reading the actual content."

Reviewer 1

“No ‘p’, no value”

Dr Tobias Zimmermann

Declaration of Originality

I, Timothy Snow confirm that the work presented in this thesis is my own. Where information has been derived from other Sources, I confirm this has been referenced in the thesis.

Signed:

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Abstract

Critical illness is associated with an immunocompromised state, characterised by functional impairments in monocytes and lymphocytes, predisposing to subsequent infections. Critically ill patients represent the highest *per capita* users of antibiotics. Understanding the effect of antibiotics on immune (dys)function in critically ill patients is therefore imperative, particularly as antibiotic levels are extremely variable due to impaired pharmacokinetics.

I hypothesised that antibiotics exacerbate features of critical illness-induced immunosuppression in patients with sepsis, surgery and COVID-19.

I conducted a cohort study of patients admitted to Critical Care. Using cell culture, spectral flow cytometry, and ELISA, I characterised patient immunophenotype followed by *ex vivo* experiments to evaluate the effect of antibiotics on monocyte and lymphocyte function.

Compared to mild infection, septic patients had lower monocyte HLA-DR expression and lymphopenia which were associated with mortality. Multiple other functional pathways were also impaired. Beta-lactam antibiotics (amoxicillin, cefuroxime, meropenem, and piperacillin) exacerbated these features, particular monocyte antigen presentation. Many of the effects were dose-dependent.

I demonstrate commonality between the immunophenotype of patients undergoing elective major surgery with sepsis. Additional effects were identified in monocytes on chemokine receptor expression and T-cell suppression following a secondary stimulus *in vitro*. Contrary to my hypothesis, cefuroxime (but not amoxicillin or metronidazole) ameliorated the effect of surgery on lymphocyte function.

Finally, I characterised serum levels of multiple biomarkers that were targets for immunomodulators in clinical trials for COVID-19. Only seven biomarkers, including IL-6 and neutralising antibodies differentiated between mild and severe disease. Many of the immunomodulatory drugs trialled during COVID targeted biomarkers which did not differentiate between disease severity, which may explain their lack of benefit in clinical trials. Clarithromycin then demonstrated an immunomodulatory effect on spike-protein stimulated cytokine release from volunteer lymphocytes.

My work supports ongoing antimicrobial stewardship attempts to reduce inappropriate use of antibiotics.

Impact Statement

My thesis highlights the need for antimicrobial stewardship in critically ill patients.

I demonstrate that beta-lactam antibiotics exacerbate some of the immunosuppressive features associated with sepsis. Effects on immune cells were more prominent with broad-spectrum antibiotics (compared to narrow-spectrum antibiotics) and at higher (but clinically relevant) doses. This supports the judicious use of narrow- over broad-spectrum beta-lactams, supporting the recently updated National Institute of Clinical Excellence sepsis guideline, which recommends antimicrobial prescribing guided by severity of illness. In addition, the dose-dependent effects of antibiotics support the need to investigate the potential benefit of therapeutic drug monitoring in the ICU to achieve therapeutic serum concentrations above the required minimum inhibitory concentration (MIC) but below the level associated with immune toxicity.

In patients undergoing major elective surgery, I demonstrate that cefuroxime, but not other commonly used antibiotics for antimicrobial prophylaxis, can ameliorate the detrimental effects of surgery on the immune system. This supports the appropriate selection and duration of antimicrobial prophylaxis in clean-contaminated surgery. Additionally, specific antibiotics may have beneficial immunomodulatory properties over and above their antimicrobial function. Further work is required however to identify whether the beneficial effects of cefuroxime are clinically relevant.

Randomised trials of azithromycin in viral pneumonia did not demonstrate benefit. I demonstrated, in a retrospective observational study, that the use of clarithromycin in patients with COVID-19 was associated with a mortality benefit. I*n vitro* stimulation of healthy volunteer peripheral blood mononuclear cells with viral spike protein showed immunomodulatory effects with clarithromycin (but not azithromycin). The specific immunomodulatory properties of different macrolides required further investigation.

My data have been presented at international meetings and published in peer reviewed journals. The research techniques developed are being adapted to investigate the immunopharmacological effects of other commonly used drugs in critical care including steroids, plasmapheresis, and liposomal antibiotic preparations. Pilot data generated using these techniques has been awarded a £10,000 grant from the British Infection Association and a €20,000 Fundamental Research award from the European Society of Intensive Care Medicine.

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1 Introduction

1.1 Antibiotic use in critical care

A major barrier to improved antimicrobial stewardship is the widespread belief that, for the individual patient, the benefits of prompt initiation and continued use of antimicrobials outweigh any potential harm. Antimicrobial stewardship may be perceived by some clinicians as the utilitarian rationing of a vital healthcare resource, potentially sacrificing individual patient wellbeing for a collective good. This view is highly pertinent to patients admitted to the Intensive Care Unit (ICU), the highest *per-capita* consumers of antibiotics within the hospital population,[1] and who are at greatest risk of harm from both untreated or undertreated infection.[2,3] However, unnecessary or unduly prolonged antimicrobial use is also associated with adverse effects to the individual patient.[4] Indeed, over-use of antimicrobials is associated with adverse patient outcomes, including increased mortality.[4-6] If more emphasis is placed on this potential iatrogenic harm, clinicians may be more judicious about prescribing antimicrobials. Antimicrobial stewardship aims to improve appropriate antimicrobial commencement, duration, and minimise co-administration of multiple agents.

A key driver for the early initiation of antibiotic therapy were the Surviving Sepsis Campaign guidelines which emphasised the importance of empirical broad-spectrum antimicrobial therapy ideally within one hour of diagnosis.[7] This recommendation was based on retrospective analyses of observational data from patients with septic shock showing an association between early appropriate antibiotic administration and survival benefit.[3,8] This association was extrapolated to all patients with sepsis, regardless of the presence of shock, and had the unintended effect of indiscriminate empirical broad-spectrum antimicrobial prescribing in patients with uncomplicated infection or no bacterial infection at all.[9,10] This aggressive approach may be causing harm compared to a more judicious use.[11] Systematic reviews indicate that delayed antimicrobial administration strategies in less sick patients can be safe and are associated with non-inferior clinical outcomes.[12] The most recent National Institute for Health and Care Excellence (NICE) sepsis guideline now advocates an individualised and more thoughtful antimicrobial prescribing approach guided by patient illness severity.[13]

In addition to reducing inappropriate commencement of antimicrobials, stewardship efforts in the ICU also target the duration of antimicrobial therapy. Randomised clinical trials utilising biomarkers to guide antimicrobial duration demonstrated a reduction in antimicrobial exposure; of note, these were associated with reduced mortality and do imply an unrecognized iatrogenic harm.[4,14] Even in the absence of biomarkers, shorter course therapies were non-inferior compared to longer courses in critically ill patients.[15-18] In mild infection, higher rates of readmission were associated with a longer

duration of antimicrobial treatment.[19] This has led to calls to abandon the concept of a defined 'antibiotic course' and move to a more individualised approach.

Co-administration of multiple antimicrobial agents is also associated with adverse events. A prospective observational study evaluating the impact of American Thoracic Society guideline compliance in the management of pneumonia caused by potentially resistant organisms demonstrated an excess mortality associated with guideline compliance.[5] The main reasons for non-compliance were non-use of dual treatment for Gram negative pathogens and non-coverage for methicillin-resistant *Staphylococcus aureus.* Empirical concomitant use of several antibiotics may be associated with an excess mortality risk compared to monotherapy, although causality cannot be determined by observational data alone.

The most commonly prescribed antimicrobials in critical care patients include broad-spectrum beta-lactams (particularly piperacillin-tazobactam and meropenem), narrow-spectrum beta-lactams, cephalosporins (particularly newer generations e.g. ceftazidime), and macrolides.[20,21] Beyond antimicrobial stewardship programs, several approaches are being used to minimise overuse. This includes use of biomarkers (e.g. procalcitonin) to either guide commencement or cessation of antimicrobials, and molecular pathogen diagnostics to enable earlier initiation of narrow-spectrum antibiotics.[22] Additionally, given the wide variations in pharmacokinetics and pharmacodynamics seen in a critically ill population, there is growing use of extended duration infusions and therapeutic drug monitoring in an attempt to optimise antibiotic dosing.[23]

1.2 Antibiotic-induced harm in critical care patients

Antibiotics are associated with many side-effects, both overt including hepatotoxicity, nephrotoxicity and bone marrow dyscrasias, and covert such as their impact on the microbiome and immune cell function. Mechanisms underlying these toxicities are not completely understood.[24]

1.2.1 Antibiotic-induced idiosyncratic drug reactions

Antimicrobials are associated with numerous adverse drug reactions (ADRs), most often gastrointestinal disturbances but also rashes, anaphylaxis, nephrotoxicity (e.g. from aminoglycosides), hepatotoxicity (e.g. from rifampicin), fluoroquinolone-induced QT prolongation and tendon rupture, β-lactam-induced seizures, and interactions with other medications (e.g. macrolide-induced cytochrome p450 enzyme inhibition).[25]

Allergic and hypersensitivity reactions can range from mild eosinophilic drug rashes to potentially life-threatening anaphylaxis or severe cutaneous adverse reactions such as toxic epidermal necrolysis and

Stevens-Johnson syndrome. While many other drugs and allergens can provoke such reactions, antimicrobials (particularly beta-lactams and sulphonamides) are a predominant cause.[26] These reactions may be particularly difficult to recognize in the critically ill patient.

Conversely, inaccurate documentation of antibiotic allergy is also associated with harm. Rates of reported penicillin allergy far outstrip the rates of actual allergy on skin prick testing,[27] resulting in unnecessarily broad-spectrum antibiotic choices that increase the risk of antimicrobial-induced harm.

1.2.2 Antimicrobial resistance

The most widely recognised consequence of antibiotic-associated harm is the emergence of antimicrobial resistance (AMR), a major threat to global health. Infections caused by multi-drug resistant (MDR) pathogens are associated with increased mortality and length of stay.[28] As these pathogens are more likely to be resistant to commonly prescribed empirical antimicrobials, this may delay initiation of appropriate, effective therapies and thereby compromise patient outcomes. MDR infections may also necessitate treatment with antimicrobials with inferior bactericidal activity and/or undesirable pharmacological properties/toxicities.[29,30]

Globally there is a general increase in the rate of MDR pathogens being reported in ICU patients. One European estimate suggests that more than two-thirds of cases of ICU-acquired bacteraemia are caused by MDR bacteria,[31] with rates of specific MDR Gram negative infections (e.g. carbapenem-resistant *Pseudomonas aeruginosa* and *Acinetobacter baumanii* and carbapenem-resistant/extended spectrum beta-lactamase (ESBL) producing *Enterobacterales*) rapidly increasing.[32-34]

Patients in critical care are particularly susceptible to acquiring multi-drug resistant (MDR) organisms, either as pathogens or colonisers, in part related to the large antimicrobial burden they are exposed to prior and during their ICU stay.[35,36] Studies in patients with ventilator-associated pneumonia report a significant increase in subsequent MDR-related superinfection and no mortality benefit in patients receiving longer duration antimicrobial therapy.[16,37] This includes susceptibility to MDRs of different classes,[38] and is likely to be mediated by complex selection pressures driven by the antimicrobials.[39]

1.2.3 Disruption of the microbiome

Critical illness itself alters the human microbiome and causes dysbiosis.[40,41] A decrease in diversity and numbers of commensal organisms, and an increase in potential pathogens, have been described for skin, oral, respiratory and gastrointestinal microbiota.[40,42,43] While multifactorial in origin,[44] frequent use of antibiotics, especially broad-spectrum agents, certainly impacts upon microbiome diversity.[41,45] The impact of this antibiotic-exacerbated dysbiosis is unclear. Beyond being a primary cause for

Clostridium difficile-associated diarrhoea, it may contribute to worsening spinal cord injury,[46] the risk of pseudomonal lung infection,[84] and an impaired immune response to influenza A.[47,48]

1.2.4 Antibiotic-induced mitochondrial dysfunction

Mitochondrial dysfunction is implicated in the pathophysiology of organ dysfunction in sepsis,[49] although precise mechanisms require clarification. The common phylogenetic origin between mitochondria and bacteria suggest that antibiotics can directly affect human mitochondrial function and may contribute to the mitochondrial dysfunction and associated organ failure seen in sepsis.[50]

Mitochondrial reactive oxygen species (mROS) production, antioxidant depletion and associated oxidative damage has been described in several clinical studies.[51-53] The mitochondrial electron transport chain (ETC) is the major source of ROS production in non-immune cells.[54] A number of antibiotics including ciprofloxacin and ampicillin inhibit ETC complexes I and III,[55] the major sites of mitochondrial ROS production.[56] Excessive production of reactive oxygen and nitrogen species such as superoxide, nitric oxide and peroxynitrite can lead to irreversible protein oxidation and nitration, lipid peroxidation and membrane damage, DNA damage and perpetuation of mitochondrial dysfunction leading to cellular and organ dysfunction.[55,57]

Antibiotics including beta-lactams and cephalosporins cause both reversible and irreversible changes to carriers of mitochondrial substrates due to acylation of the transporters.[58] At high doses, several antibiotics can inhibit mitochondrial oxidative phosphorylation.[59] The resulting impairment in substrate availability, ETC complex activity and decreased ATP production may further contribute to mitochondrial-associated organ dysfunction in sepsis.[51]

Recovery from sepsis is heralded by increased mitochondrial biogenesis,[52] which requires mitochondrial topoisomerase II-is for mitochondrial DNA (mtDNA) replication and transcription. This enzyme is susceptible to direct inhibition by antibiotics including ciprofloxacin, resulting in site-specific double-stranded breaks in mtDNA.[60] Prolonged use of antibiotics may therefore perpetuate organ dysfunction by impairing mitogenesis.

Given the ability of antibiotics to adversely affect mitochondrial function in multiple cell types, it is plausible they could also affect immune cell function through similar mechanisms.

1.3 The immune response to bacterial infections

Most infections lead to a localised inflammatory response mediated by the immune system, with resolution achieved either with or without the assistance of antibiotics. Both innate and adaptive components of the immune system are involved in clearance of pathogens, as discussed below.

1.3.1 Innate immune system

Innate immunity is a rapid, antigen-independent, and evolutionary conserved system comprised of non-cellular and cellular components. Physical barriers (e.g. mucosa, epithelial cells, etc) separate the external environment from the host. If pathogens compromise these defences, non-cellular (e.g. defensins, complement, etc) and cellular (e.g. neutrophils, monocytes, etc.) components aim to limit the spread of infection.

1.3.1.1 Non-cellular immunity

Non-cellular components of the innate immune system include various proteins that have both direct anti-pathogen effects as well as facilitating rapid pathogen clearance by the cellular components. Antimicrobial peptides are secreted by cells of the innate immune system and barrier cells and have broad activity against fungi, bacteria and viruses.[61] They are constitutively expressed although expression is further enhanced during infection. They can cause direct pathogen death through interruption of biological pathways. Cathelicidins, for example, bind to and disrupt the negatively charged bacterial cell wall, as well as inhibiting bacterial RNA and DNA synthesis.[62] Defensins also disrupt bacterial cell membranes and neutralise secreted toxins but also possess direct antiviral effects, inhibiting viral replication.[63]

The complement system consists of several inactive circulating proteins that are activated upon contact with a pathogen. Binding of these proteins to the bacterial cell stimulates the complement cascade, leading to cleavage and activation of central complement proteins and formation of the membrane-attack complex which causes bacteria cell death through both membrane disruption and opsonisation to facilitate phagocytosis.[64]

1.3.1.2 Cell-based immunity

Cells of the innate immune system include neutrophils, monocytes (including macrophages and dendritic cells), basophils, eosinophils, mast cells and natural killer cells. They have two primary functions: phagocytosis of invading pathogens and signalling to the adaptive immune system to facilitate clearance of the infection. Contrary to conventional understanding, these cells may possess a degree of immunological memory.[65] To recognise pathogens, innate cells express numerous germline-encoded pattern recognition receptors (PRRs) e.g. c-type lectin-like, nucleotide-binding oligomerisation domain-like, retinoic acid inducible gene 1-like, and toll-like receptors. These receptors recognise evolutionary conserved components of pathogens, broadly known as pathogen-associated molecular patterns (PAMPs). These may be cell wall constituents, e.g. peptidoglycans or β-glucans, or intracellular components such as nucleic acids.[66] Additionally, host tissue and cellular damage leads to release of cellular contents including DNA, actin and mitochondrial components. These are defined immunologically as damage-associated molecular pattern (DAMPs) and also recognised by PRRs.[67]

Activation of PRRs leads to induction of multiple cell-signalling pathways that mediate the inflammatory response. Binding to and activation of toll-like receptors causes receptor dimerization and recruitment of adaptor molecules. Adapter protein activation acts via two main pathways; MyD88-dependant induction of inflammatory cytokines through transcription factors such as NF-κB (nuclear factor-κB) and PI3K, or TRIF-dependant interferon induction through interferon regulatory protein-3 and -7.[68]

Neutrophils are the most abundant human leukocytes, making up to 70% of myeloid cells in humans. They differentiate within the bone marrow before being released into the circulation where they survive for up to 24 hours. In the presence of infection, neutrophils become tethered to the endothelium by a process regulated by selectin molecules (e.g. P-selectin on the endothelium, L-selectin on neutrophils) in response to local release of chemokines. The neutrophils then migrate along the chemokine gradient, extravasate and cross the epithelial layer to the site of infection.[69] Here they clear pathogens and cell debris by phagocytosis, reactive oxygen species production, release of granules containing microbicidal molecules and extracellular traps (NETs). They also signal to the rest of the immune system, recruiting and activating other immune cells through release of cytokines and chemokines including macrophage inflammatory protein-1α, as well as by direct signalling.[70]

Antigen-presenting cells include circulating monocytes and tissue macrophages and dendritic cells. These cells can phagocytose pathogens, and present processed pathogen components as antigens on the major histocompatibility complex (MHC) class 2 receptor, HLA-DR to activate the adaptive immune system.[71]

Monocytes represent approximately 10% of circulating leukocytes and are a heterogenous population of myeloid cells differentiated by their relative expression of CD14 (involved in the LPS receptor complex) and CD16 (an IgG immunoglobulin receptor). The so-called 'classical' population ($CD14^{++}CD16^{-}$) represents the majority; these cells are primarily proinflammatory (secreting TNF-α, IL-6 and IL-10 cytokines) and phagocytotic with high peroxidase activity. The intermediate population ($CD14^{++}CD16^{+}$) are specialised at antigen presentation, strong stimulators of T-cells and also secrete IL-6, IL-8 and TNF-α. The 'non-classical' ($CD14^{-}CD16^{++}$) population is the smallest; these are phagocytic with T-cell stimulatory roles, and secrete IL-1β, IL-6 and TNF-α.[72] Circulating monocytes survive for up to three days and thereafter migrate to tissues. Increased trafficking during infection is mediated via a chemokine gradient. Once in the tissues, monocytes differentiate into macrophages or dendritic cells. This is dependent on local growth factors and the presence of proinflammatory cytokines and pathogens.[73] Macrophages have either a M1 pro-inflammatory phenotype or M2 anti-inflammatory phenotype.[74] Dendritic cells primarily have surveillance and antigen presentation roles.[75]

In addition to antigen presentation via HLA-DR, antigen-presenting cells also signal through other mechanisms including programmed-death ligand-1 (PD-L1) and cytokine release. They are therefore

key intermediaries between the innate and adaptive immune systems and can have either pro- or anti-inflammatory effects.[76]

1.3.2 Adaptive immune system

Adaptive immunity differs from the innate system by being antigen-dependent and highly specific, but are slower in response (days rather than hours). This system is prominently cellular with the ability to retain immunological memory enabling an enhanced response following secondary exposure to the same pathogen. The two main cell types are T- and B-cells, each having multiple subtypes.

T-cells are derived from the bone marrow and mature in the thymus. They express the T-cell receptor (CD3), an antigen-binding receptor on their membrane which binds to antigens presented to HLA-DR by antigen-presenting cells. Activation of this receptor, in the presence of co-stimulatory receptor CD28 and lack of inhibitory receptor CTLA-4, leads to proliferation and differentiation of the cell into separate populations. Differentiation is altered by the relative presence of cytokines and the type of antigen-presenting cell being activated.[77]

T-helper ($CD4^+$) cells marshal the immune response, activating other immune cells including the innate immune cells and B-cells. T_{h1}-cells are involved in elimination of intracellular pathogens and develop in response to the presence of IL-10 and IFN-γ. T_{h2}-cells help to clear extracellular pathogens, and these develop in the presence of IL-2 and IL-4. T_{h17}-cells act against extracellular bacteria and fungi and develop in the presence of IL-21, IL-6, IL-23 and TGF-ß. T_{reg}-cells are involved in the development of tolerance and negatively regulate the immune response.[77]

Cytotoxic T-cells ($CD8^+$) help clear infection by killing infected cells and releasing cytokines at peripheral sites of infection. These cells are activated by antigen-presenting cells or via antigens presented by other cells to MHC class I molecules, causing proliferation and differentiation into effector cells.[78,79]

B-cells are generated by, and mature from, haematopoietic stem cells in the bone marrow. They have a specific receptor (B-cell receptor, CD19) that recognizes soluble and particulate antigens in addition to multiple other receptors that recognize other unique antigens. These receptors can be activated by direct antigen binding or via T_h-cells, leading to proliferation and differentiation into antibody-secreting plasma cells, or memory cells. Secreted antibodies bind to the pathogen, aiding phagocytosis by opsonization, neutralizing toxins, and enhancing the complement system. Memory B-cells are long-lived; in response to subsequent repeat infection, they quickly proliferate and produce antibodies.

1.3.3 Summary

Pathogens are targeted by the non-cellular innate immune system which can directly cause pathogen death or facilitate phagocytosis via opsonisation. Antigens are also presented to the adaptive immune system which facilitates antibody-mediated pathogen clearance as well as proliferation and differentiation of lymphocytes. Finally, a subset of lymphocytes differentiate into memory cells, facilitating enhanced clearance upon repeated infection with the same pathogen.

1.4 Sepsis

1.4.1 Definition

Sepsis is a clinical syndrome that has various descriptions throughout history. Initially described by Avicenna in 1000 BCE as 'putrification of blood and tissues with fever',[80] and Hippocrates in 400 BCE as 'biological decay leading to auto-intoxication',[81] it is now defined as 'life-threatening organ dysfunction caused by a dysregulated host response to infection'.[82] Organ dysfunction is defined by an acute increase of 2 points or more in the Sequential (or sepsis-related) Organ Failure Assessment (SOFA) score. This score comprises several clinical and laboratory value-based assessments of organ function.[83] Defining the presence of infection however, is left to the discretion of the treating clinician.

Sepsis has a spectrum of severities, with the most severe, septic shock, defined as profound circulatory, cellular, and metabolic abnormalities that increase the risk of mortality over sepsis alone.[82] It is clinically characterised by a requirement for vasopressors to maintain mean arterial pressure ≥65 mmHg and a persistent hyperlactatemia (>2 mmol/l) despite adequate volume resuscitation.

1.4.2 Epidemiology

The precise incidence of sepsis remains unclear due to the heterogeneity of sepsis, inaccuracies in diagnosis (especially of underlying infection), and differences in definitions between databases.[10,84,85] Sepsis is estimated to result in 40,000 ICU admissions in the United Kingdom annually, with a hospital mortality of 27%.[86] Worldwide it is estimated that sepsis claims almost 11 million lives annually, accounting for nearly 20% of all global deaths.[87]

Certain subgroups are more likely to die with sepsis, either as a consequence of the acute infection or further compromise of their chronic health status. This includes those at both extremes of age, of lower socio-economic status, with co-morbidities and immunosuppression, and in those whose infection is caused by resistant organisms.[10,81,86,88]

In the UK, sepsis is estimated to cost the NHS €21,000 per admission,[89] totalling almost 0.2% of GDP. These costs however exclude the long-term physical, cognitive and psychological disabilities of survivors, which has been estimated at £10 billion annually.[90,91]

1.4.3 Treatments

Despite the definition of sepsis highlighting infection and dysregulated host response, existing management is largely limited to the former while supporting the consequences of the latter.

Treatment of sepsis requires appropriate source control of the invading pathogen(s) with appropriate antimicrobial therapy. Source control can include surgery, interventional radiology, or removal of an infective locus, e.g. indwelling lines and catheters. Initial appropriately targeted antimicrobial therapy is often limited by the lack of identity of the causative pathogen(s). Broad-spectrum antibiotics are often initiated in septic patients with choice based on the likely causative organisms adjudged by site of infection, risk of immunosuppression, previous hospital exposure, and local resistance patterns. Blood and other specimens should be taken prior to commencing antimicrobial therapy, as identification of the pathogen and its antimicrobial susceptibility pattern would enable narrowing of the antimicrobial spectrum. Early versions of management guidelines mandated rapid administration of IV antibiotics upon suspicion of sepsis.[7] However with lack of evidence to support this dogma and a progressive increase in antimicrobial resistance, most recent guidelines advocate a more individualised approach dependent on patient severity.[13]

1.4.4 Inflammation in sepsis

The infecting pathogen triggers an inflammatory response by the innate immune system and endothelium, leading to alterations in cardiovascular, coagulation, hormonal, metabolic, bioenergetic and neurological systems resulting in end-organ dysfunction.[92] Simultaneous activation of pattern recognition receptors by both PAMPs and DAMPs induces multiple intracellular signalling systems through activation of janus kinases (JAKs), mitogen-activated protein kinases (MAPKs), signal transducers and activators of transcription (STATs) and NF-κB leading to release of inflammatory cytokines such as TNF-α, IL-6, IL- 1β and interferons. These, in turn, initiate cascades of other pro-inflammatory cytokines and chemokines.[93,94]

Activation of the complement system, specifically C3a and C5a, act as both chemoattractants and stimulants for innate immune cells. The complement system promotes neutrophil synthesis and release of pro-inflammatory cytokines and chemokines, and initiates neutrophil degranulation with release of enzymes and reactive oxygen species. While useful for killing pathogens, this may also result in host tissue damage and additional release of DAMPs.[95]

Neutrophil and monocyte infiltration at the site of infection is facilitated by disruption of the vascular endothelium, shedding of the glycocalyx and upregulation of adhesion molecules enabling immune cell tethering and engagement. The resulting increase in leak of capillary fluid via gap junctions into tissues may contributes to tissue oedema and impaired perfusion. Sepsis is also associated with a hypercoagulable state. This is driven by endothelial disruption with exposure of tissue factor to von Willebrand factor, by cytokine-activated platelets, and by consumption of anticoagulants. This leads to thrombin activation, which fuels the inflammatory process, and fibrin deposition which may lead to clot formation.[96] However, micro-thrombi, and occasionally, macro-thrombi are only occasionally seen. Depletion of clotting factors can subsequently lead to spontaneous bleeding.

1.4.5 Sepsis-induced immunosuppression

Sepsis is associated with significant short and long-term mortality and morbidity.[82,97] Many patients with sepsis survive their initial insult but die several days following initial presentation.[98] Persistent and secondary infections are commonplace among patients with longer durations of stay within the intensive care unit (ICU). This is often associated with impaired immune function, and is described as sepsis-induced immunosuppression.[1,99] Key cardinal features include persistent downregulation of monocyte HLA-DR and lymphopenia. Multiple other immunosuppressive changes are also described.[100-103] (Figure 1.1)

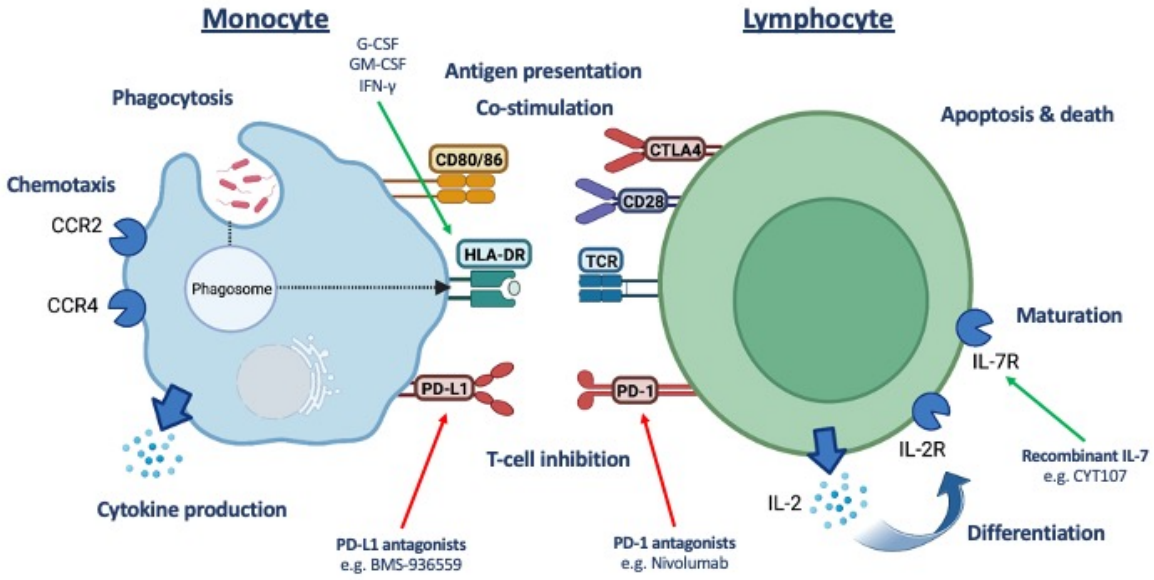

Figure 1.1: Summary of pathways implicated and therapeutic agents under investigation in sepsis-induced immunosuppression
Abbreviations: CCR2: C-C motif chemokine receptor 2; CXCR4: C-X-C motif chemokine receptor 4; CD: cluster of differentiation; IL: Interleukin.

1.4.5.1 Monocyte HLA-DR

Monocytes are a key interface between innate and adaptive immune systems. A key signalling pathway is via antigen presentation through HLA-DR. In critical illness, there is marked reduction in monocyte surface HLA-DR expression. Persistent downregulation of monocyte HLA-DR is associated with an increased risk of secondary infections and mortality.[104] Reduced HLA-DR expression is associated with impaired release of pro-inflammatory TNF-α, and IL- 1β,[105] enhanced release of anti-inflammatory IL-10, and inhibition of T-cell proliferation.[106]

Ex vivo exposure of healthy volunteer monocytes to bacterial products, and active bacterial infection, are associated with early upregulation of monocyte HLA-DR.[107] HLA-DR expression is regulated transcriptionally by CIITA (class II, major histocompatibility complex, transactivator).[108,109] Expression of monocyte HLA-DR is regulated by other factors including IFN-γ and IL-10 which increase and decrease expression, respectively.[110] Newly synthesised MHC class II heterodimers are stabilised within the endoplasmic reticulum by binding the dedicated invariant chain chaperone protein (Ii or CD74) to prevent premature loading of antigens. This complex is subsequently degraded to CLIP (class II-associated Ii peptide) before HLA-DR is finally released in the late compartments via the action of HLA-DM.[111]

1.4.5.2 Lymphopenia

Following resolution of infection, there is a natural process of lymphocyte death, leaving memory cells to reactivate upon subsequent restimulation. During sepsis, however, there is marked apoptosis of $CD4^+$ lymphocytes, $CD8^+$ lymphocytes and B-cells. This is mediated through upregulation of the programmed death-1 (PD-1) receptor and mitochondrial death pathways,[104,112,113] and downregulation of proliferation receptors IL-2R and IL-7R.[104,114] PD-1 mediated cell death may be mediated by monocytes which demonstrate increased upregulation of the receptor ligand, PD-L1.[115,116] Surviving lymphocytes demonstrate impaired functionality; this is characterised by anergy and increased percentages of regulatory phenotypes.[117,118]

$CD4^+$ lymphocytes show a loss of population diversity with a reduction in the percentage of T_{h1} and T_{h2} helper cells, mediated by reduced transcription factor expression of T-bet and GATA3 respectively, and a reduction in T_{h17} cells through reduced expression of RORγt. The anti-inflammatory T_{reg} population remains stable or even increases.[119-121] This could be due to their increased resistance to apoptosis as these cells have increased expression of the anti-apoptotic BCL-2 protein.[122] T_{h2} cells demonstrate impaired polarisation.[123] $CD8^+$ lymphocytes demonstrate reduced proliferation with loss of population diversity and impaired cytotoxic function.[124]

Both T-cell classes exhibit features of anergy, identified by a functional phenotype of inability to produce cytokines (IFN-γ and TNF-α) in response to a secondary infective stimulus, as well as increased expression of PD-1 and decreased levels of IL-2 and IL-7 receptors.[104,114] Concurrent lack of lymphocyte co-stimulatory pathway activation (via monocyte CD86), and increased expression of inhibitory pathways (PD-1, PD-L1, CTLA-4), may explain these findings. Simultaneous engagement of the monocyte co-stimulatory molecules CD80 and CD86 with the T-cell CD28 receptor is required for T-cell activation. Engagement of monocyte CD80 and CD86 with cytotoxic T-cell lymphocyte-associated protein 4 (CTLA-4) results in T-cell inhibition. Features associated with lymphocyte exhaustion, including increased PD-1 expression, decreased IL-2R and IL-7R expression, and increased CTLA-4 expression are evident in sepsis.

B-cells also show features of exhaustion, with decreased expression of HLA-DR, reduced CD69 expression but increased IL-10 production after exogenous stimulation.[125]

1.4.5.3 Other cell types

While the above features are the most frequently described, other cell types are also affected. For example, neutrophil apoptosis is delayed, leading to ageing of the neutrophil population.[126-128] This phenotype is associated with impaired chemotaxis,[129] suppressed reactive oxygen species burst,[130] and reduced recruitment to infected tissues and is associated with an increased risk of developing secondary infections.[131]

In contrast, dendritic cell apoptosis is enhanced, with reductions in both circulating and tissue populations.[132] Viable dendritic cells have impaired capacity for antigen presentation, and increased release of IL-10. As a result, they induce T-cell anergy and increase proliferation of the T_{reg} population.[133,134]

Circulating natural killer cell count is reduced in sepsis.[135] Cells demonstrate a reduced ability to secrete pro-inflammatory cytokines in response to secondary infective stimuli.[136]

1.4.5.4 Immunomodulatory treatments

Given sepsis incorporates both pro- and anti-inflammatory components, both immunosuppressive and immunostimulatory therapies have been trialled. Patient selection for these trials has however been broad, including different underlying infection aetiologies, timing of administration, and dosing. Few studies have measured circulating levels of the immunomodulatory targets, either as an enrolment criterion or to show an effect.[137]

Several immunosuppressive therapies have targeted the pro-inflammatory effects of cytokines and include blockade of TNF-α, and IL-1β receptors to inhibit downstream effects. None have demonstrated a benefit in clinical trials.[137]

Immunostimulatory treatments have targeted both monocytes and lymphocytes to reverse sepsis-induced immunosuppression and reduce the risk of secondary or persistent infection. Both GM-CSF and IFN-γ enhance monocyte HLA-DR expression and modulate cytokine production away from an anti-inflammatory phenotype *ex vivo*.[138] In clinical trials, however, while use of GM-CSF increased monocyte HLA-DR expression, the only apparent clinical benefit was a reduction in days requiring mechanical ventilation.[139] A trial assessing IFN-γ therapy, where HLA-DR levels were not measured, was stopped early due to an increased incidence of adverse events.[140]

To reverse lymphopenia, both recombinant IL-7 (to stimulate proliferation) and anti-PD-1 therapies (to prevent apoptosis) have shown theoretical benefits *ex vivo* and in small safety trials. Results from larger clinical trials are awaited.[141,142] (Figure 1.1)

1.5 Antibiotics and immunity

Antibiotics remain the mainstay treatment for sepsis. However, given the lack of suitable rapid diagnostic tests, most patients with sepsis are commenced on broad-spectrum antibiotics and transitioned to narrow-spectrum if cultures identify a causative organism. However, given the poor sensitivity of traditional cultures many patients are not de-escalated to narrow-spectrum antibiotics. Additionally, the length of antibiotic course is highly variable; often between 3 and 14 days.[143]

Many patients with sepsis develop immunosuppression, increasing their risk of secondary infection.[100] Mechanisms underpinning sepsis-induced immunosuppression are multifactorial, but likely to include off-target effects of medications including antibiotics. Although adverse effects of antibiotics on immune cell function are well described,[144,145] their specific effects on immunosuppression after sepsis and critical illness are unknown. Prolonged use of antibiotics may exacerbate this immunosuppression, leaving septic patients more vulnerable to subsequent infection.[146]

Data on antibiotic modulation of immunity have been mainly characterised in cell lines and animal models;[24,145] clinical data are limited.[147] Most antibiotic classes suppress both innate and adaptive immune responses.[24] It is therefore imperative to understand potential off-target immune effects of the specific antibiotic classes and to determine underlying mechanisms.

Antibiotics target (prokaryotic) bacterial cellular processes, although the antibiotic-related side-effects experienced by patients clearly indicate off-target effects. It is unclear if the mechanism(s) by which

antibiotics impact on human immune cells are directly related to their antibacterial effects on DNA transcription (ciprofloxacin) or protein translation (clarithromycin, gentamicin). The effect of beta-lactams on human immune cells is clearly unrelated to their mechanism of action on bacteria. Several pathways have been implicated in antibiotic-induced immunosuppression (Table 9.1 and Figure 1.2).

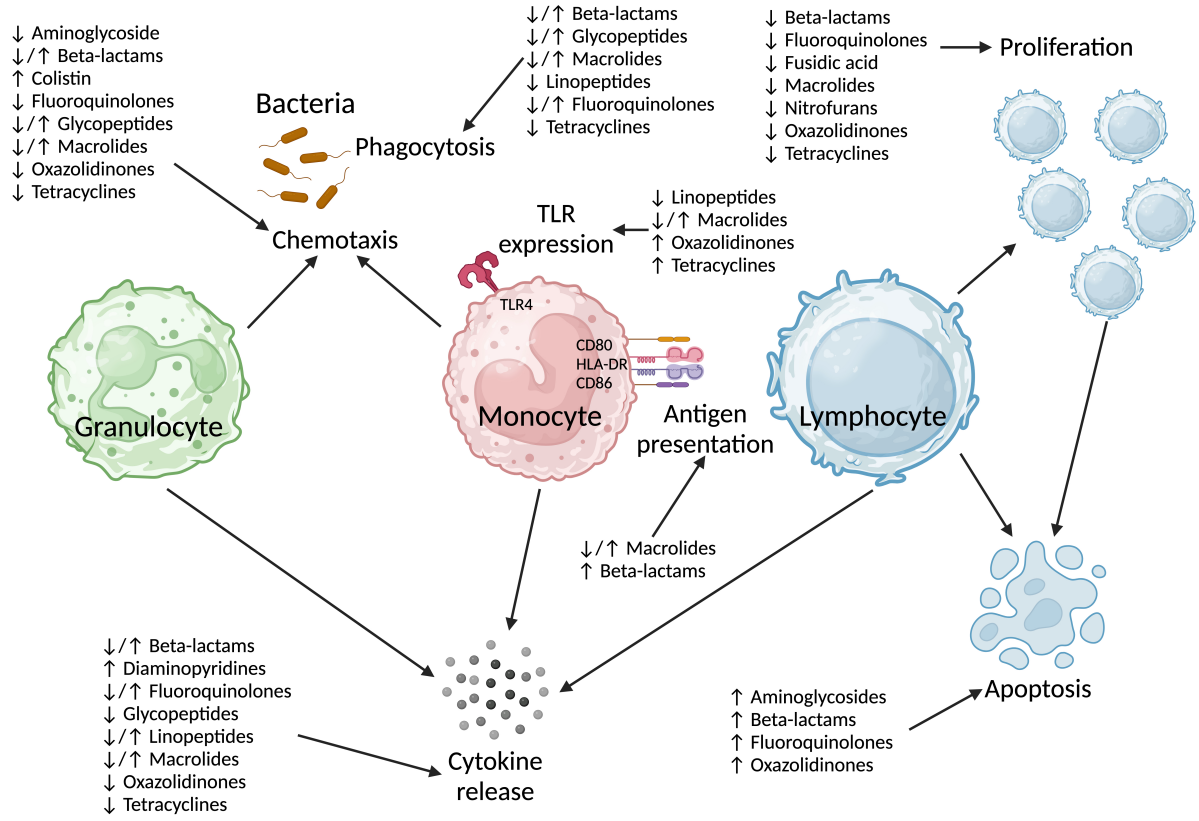

Figure 1.2: Summary of immunosuppressive effects of antibiotics by class on immune cell function
TLR: Toll-like receptor, HLA-DR: Human Leukocyte Antigen—DR isotype, CD: cluster of differentiation. Up arrow indicates antibiotics increase or enhance the effect, down arrow a decrease or impairment.

1.5.1 Mitochondrial dysfunction

Mitochondria are integral to regulating immune function; defects in leukocyte energy metabolism in septic patients are associated with immunosuppression.[148] The direct roles of mitochondria in innate and adaptive immune cells are wide-ranging, suggesting that mitochondrial dysfunction may play a significant causative role.[149] Given the current understanding of the prokaryotic origins of mitochondria, it is plausible that antibiotics targeting bacteria have detrimental effects on mitochondrial functionality.

For example, ETC adaptations serve as an early immunological-metabolic checkpoint for innate immune responses to bacterial infection.[150] Synthesis of mitochondrial DNA induced after engagement of Toll-like receptors mediates NLRP3 inflammasome signalling in macrophages.[151] Antibiotics including lincosamides, macrolides and fluoroquinolones accumulate in phagocytes and may interfere

with the above processes.[152] The highly energy-dependent respiratory burst required for bacterial killing by macrophages is impaired by a dose-dependent inhibition of mitochondrial respiratory activity by ciprofloxacin.[153]

The effects of antibiotics on immune system function are complex; observations from *ex vivo* experiments may not necessarily translate to the *in vivo* situation. For instance, although ciprofloxacin decreases release of IL-1ß from human monocytes in response to LPS *ex-vivo*,[154] *in vivo* production of pro-inflammatory cytokines in healthy volunteers is enhanced by ciprofloxacin.[155] However, previous work by our group has shown that fluroquinolones impair immune cell function but not via mitochondrial pathways.[146]

Aminoglycoside antibiotics are a family of amino-modified sugars containing hydrophilic portions and cationic amine moieties that preferentially bind nucleic acids due to their negative charge. They can cause translational errors and assembly of incorrect amino acid products, or premature termination of protein synthesis.[156,157] While their effects on immune cell mitochondria are yet to be delineated, they do impact upon renal tubular epithelial mitochondria in animal models.[158]

Aminoglycosides bind to human mitochondrial ribosomes.[159] In isolated mitochondria from rat renal cells, aminoglycosides induced electron transport chain uncoupling, increased mitochondrial membrane cation permeability,[158] and collapse of the mitochondrial membrane potential.[160] This reduced oxidative phosphorylation,[161] and also production of mitochondrial reactive oxygen species.[162] However, there may be differing effects on different aspects of mitochondrial respiration,[163] as why some studies demonstrated an increase in ROS.[164] Oxazolidinone antibiotics bind to mitochondrial ribosomes, reducing mitochondrial protein in non-immune cells[165] and the THP-1 monocyte cell line.[166]

In a rat model of gentamicin-induced toxicity, respiratory components (including cytochrome C and NADH were depleted. This was associated with opening of the mitochondrial transition pore and an increase in ROS production.[167] The potency of the aminoglycosides in producing these effects correlates with the number of ionizable amino groups present on the aminoglycoside molecule. suggesting that cationic charge is an important molecular determinant of toxic effect.[163] Similar effects have been demonstrated in other cell types including mouse cochlear cells,[168] but not liver cells,[161] suggesting certain cell types are at increased risk.

1.5.2 Chemotaxis and migration

Mouse macrophage chemotaxis was increased by carbapenems,[169] teicoplanin and vancomycin,[170] but decreased by beta-lactams, clindamycin and tetracycline.[171] Mouse neutrophil migration was decreased by linezolid,[172] and rat neutrophil migration increased by colistin.[173]

In volunteer immune cells and PBMCs, erythromycin and roxithromycin increased migration or chemotaxis,[174] while aminoglycosides and tetracyclines were inhibitory.[175,176] Penicillins,[176,177] carbapenems,[177,178] and linezolid had no effect.[179,180] Cephalosporins,[175,177,181-185] teicoplanin,[186,187] and vancomycin had differing model-dependent effects.[187,188] In *in vivo* healthy volunteer models erythromycin impaired neutrophil migration via reduced IL-8,[189] and ceftriaxone impaired chemotaxis.[190]

In patients, macrolides inhibited neutrophil chemotaxis and migration predominantly through reduced IL-8 in patients with COPD,[191-195] bronchial hyperreactivity,[196] chronic sinusitis,[197-200] and allergy.[201] This effect was however not seen consistently with clarithromycin.[202]

1.5.3 Toll-like receptor expression

In a mouse model, folimycin decreased surface expression of TLR mediated by inhibition of V-ATPases.[203] In THP-1 cell lines, linezolid increased TLR expression (-1, -2 and -6), while daptomycin decreased it.[204] Erythromycin, moxifloxacin and doxycycline increased TLR expression (-1, -2, -4, and -6) in both the THP-1 cell line and in patients following cardiac bypass.[205]

1.5.4 Cytokine release

Most antibiotics inhibit cytokine production and release. In *ex vivo* mouse models on antigen-presenting cells, roxifloxacin,[206] erythromycin,[207] azithromycin,[207] and doxycycline inhibited release of multiple pro-inflammatory cytokines.[208] One postulated mechanism was through inhibition of mitochondrial protein translation and NLRP3 inflammasome assembly in bone marrow-derived macrophages.[208]

Using *in vivo* and *in vitro* animal models, fluroquinolones inhibited some pro-inflammatory cytokines, although there were in-class differences related to antibiotic structure.[209,210] Macrolides were anti-inflammatory,[211] while roxifloxacin had time-dependent effects, increasing pro-inflammatory release initially but caused inhibition after ≥2 weeks' treatment.[212,213] Linezolid and vancomycin also reduced cytokine release in pneumonia models.[172,214-217] In large animal pneumonia models, azithromycin inhibited IL-6 release,[218] linezolid had no effect,[219] and danofloxacin was predominately anti-inflammatory, reducing pro-inflammatory cytokine release yet increasing IL-10.[220]

In J774 macrophage cell lines, macrolides inhibited pro-inflammatory cytokine release through reduced COX-2 and nitric oxide synthase expression.[211] In THP-1 monocyte cell lines, linezolid and vancomycin increased both pro- and anti-inflammatory cytokine release,[204] erythromycin, doxycycline and moxifloxacin increased pro-inflammatory cytokine release,[205] grepafloxacin inhibited pro-inflammatory release,[221] while daptomycin had mixed effects on pro-inflammatory cytokine release.[204]

In volunteer whole blood and PBMC models, cytokine release was reduced by linezolid,[222,223] clindamycin,[224] teicoplanin,[225] erythromycin,[223,226] ceftazidime,[227] and tigecycline.[228] Meropenem had mixed effects, reducing release of some pro-inflammatory cytokines.[178] Amoxicillin and trimethoprim however were pro-inflammatory,[228,229] while penicillin and metronidazole had no effect.[224,226,230] Several studies yielded conflicting results. Vancomycin either decreased release or had no effect,[222,230] while fluroquinolones either reduced,[154,178,227,231-237] had no effect,[238] or increased release.[239]

In patient studies, clarithromycin given to COPD and asthmatic patients had either no effect,[195,240] or reduced both pro- and anti-inflammatory cytokine release.[241,242] Erythromycin given to wheezy children reduced cytokine levels,[243] while amoxicillin and penicillin given to allergy patients increased pro-inflammatory cytokine levels.[244] Norfloxacin in cirrhotic patients induced an immunosuppressive phenotype with an increased proportion of T_{regs} and IL-10 release.[245] Clarithromycin in septic patients with ventilator-associated pneumonia increased IL-10 and caused a reduction in TNF-α,[246] yet in patients with community-acquired pneumonia there was no effect on stimulated PBMC release of IL-10 and IL-17, but an increase in TNF-α release.[247] Suggested mechanisms include a direct fluroquinolone effect on protein synthesis,[154,231,236] mitochondrial ETC inhibition,[237] inhibition of COX-2,[235] and upregulation of the *rag1* gene (responsible for T-cell receptor formation).[245]

1.5.5 Phagocytosis

In mouse and rat macrophages, carbapenems increased phagocytosis,[169] while beta-lactams, clindamycin, azithromycin and erythromycin impaired it.[171,207] Vancomycin and teicoplanin had differing effects with both enhancement and impairment.[170,248] Daptomycin and lomefloxacin had no effect.[248,249] In the THP-1 cell line, the antifungal agents liposomal amphotericin B and itraconazole suppressed phagocytosis.[250]

In volunteer immune cells and PBMCs, meropenem and macrolides reduced neutrophil phagocytosis.[178,251] Cephalosporins, co-amoxiclav and imipenem increased neutrophil phagocytosis,[182-184,251-253] while macrolides increased monocyte phagocytosis.[254] Rokitamycin and linezolid had no effects.[179,180,255] Teicoplanin and vancomycin had differing dose-dependent effects.[186,187] Fluroquinolones including ciprofloxacin also had differing effects with low doses enhancing phagocytosis,[256,257] or having no effect,[249,258] while inhibition could occur at supra-pharmacological doses.[259]

In volunteer *in vivo* models, carbapenems increased phagocytosis,[253] while ceftriaxone had no effects.[190] In patients, piperacillin, doxycycline, and moxifloxacin inhibited monocyte phagocytosis after cardiac surgery.[205] Azithromycin increased macrophage phagocytosis,[260] and clarithromycin increased neutrophil phagocytosis in COPD patients.[202] Roxifloxacin also increased neutrophil phagocytosis.[261]

1.5.6 Antigen presentation

In mice, roxithromycin impairs antigen-presenting cell MHCII presentation,[206] and CD80 and CD86 on B-cells,[262,263] although this effect was only seen with longer courses.[264]

In volunteer PBMCs, pefloxacin and ciprofloxacin had no effect on antigen presentation,[265] in PBMCs isolated from patients with allergies, there was an upregulation of HLA-DR, CD80 and CD86 with amoxicillin,[266] while in PBMCs from cirrhotic patients, norfloxacin impaired CD80/86 expression.[245] Macrolides increased CD80 but not HLA-DR in patients with chronic sinusitis,[267] clarithromycin increased HLA-DR in patients with pneumonia and sepsis,[268] and increased CD86 in patients with ventilator-associated pneumonia and sepsis.[246]

1.5.7 Lymphocyte proliferation

The fluroquinolone levofloxacin inhibited proliferation in breast and lung cancer cell lines by damaging mitochondria and deactivating PI3K/Akt/mTOR and MAPK/ERK pathways.[269,270] However, in T-cells of patients with allergies, quinolone antibiotics directly cross-reacted with the T-cell receptor stimulating proliferation.[271] Amoxicillin may have similar effects.[266] However, in volunteer PBMCs, fluroquinolones impaired proliferation,[232,239] although this effect was not consistent.[235]

Erythromycin, clindamycin, rifampicin, fusidic acid, nitrofurantoin, and doxycycline all inhibited proliferation of healthy volunteer lymphocytes, whereas penicillin, cephalosporins, aminoglycosides, chloramphenicol, sulfamethoxazole, trimethoprim,[272] and macrolides did not.[273,274] However, in a mouse model, cefotaxime did inhibit lymphocyte proliferation.[275] Other cephalosporins and penicillins (including piperacillin) impaired proliferation but with differing effects on chick embryos, lymphocyte cell lines, and mouse lymphocytes.[276]

1.5.8 Lymphocyte apoptosis

Cell apoptosis is mediated by two main pathways, mitochondrial (which includes caspases-3 and -8, Bcl-2 proteins) and non- mitochondrial pathways.[277]

Linezolid induced lymphocyte apoptosis through mitochondrial pathways by inhibiting mitochondrial protein synthesis and complex IV activity in volunteer peripheral blood monocytes (PBMCs) and skin nerve fibres,[278] and in patient and rat skeletal muscle and liver.[279] Protein levels were reduced while mitochondrial DNA levels remained similar suggesting a direct action on the mitochondrial ribosome; certain polymorphisms appear to be at increased risk. Moxifloxacin increased murine macrophage cell death, although this could be ameliorated by the use of immunomodulatory compounds tinrostim and licopid.[280]

The experimental beta-lactam, lactam 1, induced t-cell apoptosis in a Jurkat cell line,[281] and a mouse breast cancer model through direct damage to, and inhibition of, DNA replication.[282] This led to p38 mitogen-activated protein kinase activation, S phase arrest, and apoptotic cell death mediated by caspase-3, -8, and -9 activation, cleavage of the pro-apoptotic Bcl-2 family protein Bid, and release of mitochondrial cytochrome c.

The fluroquinolone ciprofloxacin also induced Jurkat cell apoptosis through mitochondrial pathways by causing direct damage to mitochondrial DNA, inhibiting the respiratory chain and decreasing membrane potential.[283] Similar effects of mitochondrial-induced apoptosis have been demonstrated by ciprofloxacin on other cell lines including colon and bladder tumour cells,[284,285] and by levofloxacin in breast and lung cancer cell lines.[269,270]

Gentamicin-induced electron transport chain inhibition activated caspases -3 and -9 leading to mitochondrial-induced cellular apoptosis in renal cell lines.[286,287]

1.6 Summary

Antibiotics are associated with multiple deleterious effects beyond their immediate side-effect profile. These include patient-centred effects of idiosyncratic drug reactions, disruption of microbiome and mitochondrial toxicity, and population-level effects including antimicrobial resistance. A growing body of evidence shows they directly impact immune cell function, although the extent and mechanisms by which these occur remains relatively unexplored.

Critical illness is associated with multiple immune defects which lead to the development of an immunosuppressed state associated with an increased risk of subsequent infection. While reduced HLA-DR expression and lymphopenia are well described, it is unclear whether these are isolated defects or symptomatic of wider immune cell dysfunction. The lack of benefit demonstrated by immunomodulatory treatments targeting these pathways suggests the latter, however further research is required to explore this further.

Given the significant use of antibiotics in the critically ill, it is plausible that antibiotics may directly affect immune cell function exacerbating the immunosuppressive state seen in critical illness. Confirmation of this would add support to the ongoing antimicrobial stewardship goals aiming to reduce antimicrobial use, especially if the deleterious effects are related to duration of course, use of broad-spectrum agents, or there is evidence of a dose-dependent effect.

Given beta-lactam antibiotics are the most widely used class of antibiotics in the critically ill, they represent the best target for identification of immunosuppressive effects. The growing use of

therapeutic drug monitoring for them also presents an opportunity to incorporate dosing regimens which ensure appropriate serum concentrations for bacterial killing whilst preventing supra-clinical concentrations which could have deleterious effects on immune cell function.

1.7 Hypothesis and aims

1.7.1 Hypothesis

I hypothesise that antibiotics amplify the immunosuppressive effects of critical illness on monocyte and lymphocyte function.

1.7.2 Aims

a. To characterise the immunophenotype of peripheral blood mononuclear cells (PBMCs) from patients with mild and critical illness.
b. To develop an *ex vivo* model using a stimulus to induce features of immunosuppression in healthy volunteer PBMCs.
c. Use the *ex vivo* model to mimic a secondary infection in immunosuppressed PBMCs taken from critically ill patients.
d. To utilise the *ex vivo* model to see whether commonly prescribed antibiotic used in critically ill patients exacerbate features of immunosuppression.

2 Materials and Methods

2.1 Chapter context

Prior to commencing my PhD, I was awarded a National Institute of Health Research Academic Clinical Fellowship. This gave me dedicated research time as part of my clinical training to generate pilot data for my PhD. I developed techniques including cell culture and flow cytometry.

2.2 Ethical approvals

2.2.1 Healthy volunteers - Immunosuppressive effect of antibiotics study

2.2.1.1 Approval

I was granted ethical approval by the University College London Research Ethics Committee (UCL REC) on 25th February 2021 lasting for 3 years with the approval number 19181/001. A one-year extension was granted on 16th February 2024.

2.2.1.2 Participants

At least 12 volunteers will be recruited. This number of volunteers should be enough to collect sufficient samples of blood for the experiments without individual volunteers being required too many times. With 12 volunteers, I will not need to bleed volunteers more than once each 6 to 8 weeks.

The age range will be from 21 to 60 years old. Both male and female volunteers will be approached

2.2.1.3 Recruitment

Volunteers from other members of the laboratory or Intensive Care Unit. I acknowledge that it is important that volunteers do not feel pressured to give consent. I will not approach individuals but will approach staff as a group. Therefore, individuals will not feel pressured to provide a response that they are not comfortable with. This will allow people to either actively volunteer themselves, or to decline (either direct refusal by stating their refusal, or implied refusal by not responding). Members of staff will be verbally approached as a group, with no individual being singled out.

If a volunteer decides that they no longer wish their blood to be used in an experiment/ no longer wish to give blood they will be able to withdraw their consent at any point without penalty. This information will be conveyed to them prior to the venepuncture procedure.

2.2.1.4 Protocol summary

Whole human blood (10-30 ml) will be obtained from willing volunteers in the laboratory and intensive care unit. Venepuncture will be performed from an arm vein by a qualified clinician or nurse. Repeat samples may be requested but on different days with a minimum of 6 weeks apart.

2.2.2 Patients - An observational study to evaluate the diagnostic and predictive accuracy of Calprotectin in patients with severe infections and sepsis study

2.2.2.1 Approval

My secondary supervisor was approached by an industry partner, Gentian AS (Moss, Norway), who wished to study calprotectin, a novel biomarker for the diagnosis of sepsis. The study protocol was similar to ethical approval I had been drafting for my own research and I therefore approached the funder to enquire whether they would object to additional samples being collected for my project. They did not object, thus the study protocol and ethics application were submitted including sample for my research. The study was granted ethical approval by the London – Queen Square Research Ethics Committee on 20th October 2020 with the approval number 20/LO/2024 and IRAS number 266594. The final patient was recruited on 26th January 2023.

2.2.2.2 Funding

The study was sponsored by Gentian who funded research nurse time for patient recruitment and sample collection. The funder had no involvement in my study.

2.2.2.3 Study design

This was an observational cohort study of patients with potential severe infection. At no point did the execution of the study impact on the clinical management of the patient. The calprotectin results and associated research assays will not be provided to treating clinicians or used in any manner to affect patient care.

The study was a prospective, single centre, observational, cohort study of patients to determine whether calprotectin can identify patients with infection from those without, including those with other reasons for inflammation (e.g. post-operative). It also assessed whether it has the potential to judge the severity of illness, prognosticate outcome and guide antibiotic therapy.

The aim was to recruit patients who are "representative" of patients with suspected sepsis, uncomplicated infection, or non-infection related critical illness that require critical care intervention and assessment.

The study aimed to take place over approximately an 12-18-month period and expected approximately 400 patients would be collectively enrolled. Due to COVID-related delays, recruitment occurred over 27 months to achieve its recruitment target.

2.2.2.4 Patient population:

The target population was those patients being investigated for potentially serious infection. A control group of patients with 'sterile' inflammation following major elective surgery was also included to assess the ability of calprotectin to distinguish between inflammation caused by infection and non-infective causes (e.g. surgery). These patients were identified in 2 environments:

1. The Emergency Department. Patients presenting with a potential infection, significant enough to warrant blood cultures (250 patients). Patients were identified through the Electronic Patient Management System.
2. The Critical Care Unit. Patients being managed on the CCU for potential sepsis and COVID-19 (100 patients) and following elective major surgery e.g. oesophagectomy (50 patients). These patients were identified through the Electronic Health Record System (Epic) and the Operating Theatre Schedules.

2.2.2.5 Eligibility criteria

Patients meeting the inclusion criteria were screened by the study team using the electronic health records and medical notes. Potential participants (or their consultee) were approached by a delegated member of the study team to obtain consent/agreement in order to enrol into the study. (Table 2.1).

Suspected infection cohort		**Surgical cohort**	
Inclusion criteria	**Exclusion criteria**	**Inclusion criteria**	**Exclusion criteria**
• ≥ 18 years old • Investigated for potential infection (the clinical need for a blood culture)	• <18 years old • Severe anaemia (<60g/dl) and contra-indication to transfusion • Unable to gain consent or agreement • Treated with palliative intent • Blood culture indicated for screening or monitoring	• ≥ 18 years old • Had major elective surgery • Admitted to high dependency area post-operatively as part of elective care pathway • Infection not suspected	• <18 years old • Severe anaemia (<60g/dl) and contra-indication to transfusion • Unable to gain consent or agreement • Treated with palliative intent • No vascular access by which to obtain blood samples • Infection suspected or known to be present • COVID-19 diagnosis

Table 2.1: Inclusion and exclusion criteria for the clinical study

Potential participants were given a Participant Information Sheet and following a period of time to allow for questions and discussion the participant asked for their consent. After agreement, a consent form was signed by the participant and the member of the study team. A copy of the consent was given to the patient, a copy filed in the notes and the original filed in the site file. However, due to alterations in conscious level caused by illness and therapeutic sedation, it was expected that a large proportion of patients would be unable to give consent. Agreement was therefore sought from next of kin (Personal Consultee) in line with the Mental Capacity Act 2005. The Personal Consultee advised on the presumed wishes of the patient; authorized staff described the study to the Consultee, supplementing the oral information with the Consultee Patient Information Sheet (CPIS). Following a period of time to allow for questions and discussion the Consultee was asked for their will to sign the form and the research team would countersign it.

If there was no Personal Consultee present, agreement was obtained via the telephone. If agreement was obtained, the research investigators completed the Consultee Telephone Agreement Form and written agreement obtained once the consultee was available.

If there was no Personal Consultee available, then the patient was provided with a Nominated Consultee appointed by the Trust. Agreement was be addressed in the same manner as for the Personal Consultee. Copies of the signed Consultee Agreement Form or Consultee Telephone Agreement Form and CPIS was placed the hospital notes.

If the patient regained their capacity to give consent, an informed retrospective consent was sought with the aid of the Retrospective Patient Information Sheet.

If any patient refused retrospective consent or if any patient or their consultee withdrew their consent or agreement at any time during the study, then the patient's data was destroyed.

2.2.2.6 Study Procedures

An initial 20ml blood sample was taken at the time of the clinically indicated blood culture or admission to the CCU following surgery (control cohort). Blood samples for my study were processed within 30 minutes, whilst those for the calprotectin biomarker were centrifuged within 4 hours, aliquoted and frozen for later analysis. This index point was referred to as Day 0. Providing the patient is enrolled in the study further, daily samples were taken in the Critical Care cohorts at between 12-24 hours and between day 5-7.

Study data was collected in a standard way onto a study specific case report form by the clinical research team transcribing from the patient's notes (paper or electronic). (Table 2.2) During the trial, paper data was held in a locked cupboard in the secure research office, whilst electronic data was held

on UCLH Trust computers, where the data is password protected and can only be accessed by the research team. Stored patient data was de-identified and given a unique patient identifier.

Baseline Data (at the time of Enrolment/Entry)	**Daily Data:**	**Specimen Collection Information**	**Discharge Data:**
• Patient demographics (e.g.: age, gender) • Date and time of hospital admission • Date and time of admission to location at entry to study • Location of patient (e.g. emergency department, ward, ICU) • Major comorbid conditions (e.g. diabetes, cardiac, renal) • Immune status (immunosuppressed/immunocompetent) • Physiologic site of any suspected or confirmed infection • Antimicrobial course prior to study enrolment • Surgery/procedures prior to enrolment • Sequential Organ Failure Assessment (SOFA) Score (assessment of organ failures) • Laboratory data	• Antimicrobial/Antibiotic therapy: • Includes duration of therapy (date therapy was initiated and stopped) • Component parts of SOFA (and NEWS and qSOFA) scores. • Physiology • Surgical and other procedures for diagnosis/treatment of infection. • Radiological testing for diagnosis/evaluation of potential infection. • Microbiology results and antimicrobial sensitivities • Laboratory data	• Date & time of draw or collection	• Date of discharge (hospital) • Date of discharge (ICU) – if applicable • Discharge destination (ward, skilled nursing facility, other hospital, home, etc.) • Vital status at discharge from hospital/ICU (survival/death)

Table 2.2: List of data collected for individual participants
Abbreviations: ICU: Intensive Care Unit, SOFA: Sequential organ failure assessment score, qSOFA: quick SOFA score.

2.2.3 Patients – Immunophenotyping patients with COVID-19 pneumonia

2.2.3.1 Approval

Ethical approval was granted on 26th March 2020 by the London Westminster Ethics Research Committee, Health Research Authority and Healthcare Research Wales with the 20/HRA/2505 and IRAS ID 284088. The final patient was recruited on 30th June 2020.

2.2.3.2 Study design

This was a retrospective cohort study of patients admitted to UCLH with confirmed COVID-19 designed to characterize immunophenotype of patients through measurement of serum biomarkers associated with severity of illness. Approval was granted to utilise remaining serum samples left over following routine biochemistry evaluation. Informed patient consent was deemed not to be required.

2.2.3.3 Eligibility criteria

Blood samples taken from adult (age greater than 17) patients within 5 days of admission through the emergency department at University College London Hospitals with a pneumonic illness with a

positive real-time reverse transcription-polymerase chain reaction (PCR) test for SARS-CoV2 RNA were included. Patients receiving immunomodulatory agents were excluded.

2.3 Cell culture

2.3.1 Sample collection

Healthy volunteer blood (10mL) was obtained by venepuncture and collected in PST lithium heparin™ (used during the initial COVID-19 pandemic due to limited availability of vacutainers) or K2 EDTA™ (Ethylenediaminetetraacetic acid) vacutainers (both Becton Dickinson (BD), Wokingham, UK) and processed immediately.

Patient whole blood was collected after recruitment by the Critical Care Unit research nurses in CPT™ (8ml), K2 EDTA (4ml), and SST Advance™ vacutainers (all BD) and processed within 30 minutes.

2.3.2 PBMC isolation

2.3.2.1 Ficoll gradient separation

Healthy volunteer blood was transferred to a 50ml Falcon™ (Thermo Fischer (TF) Scientific, Oxford, UK) and diluted 1:2 with phosphate buffered saline (PBS, TF). In a separate 50ml Falcon, Ficoll-Paque™ (GE Healthcare, Hatfield, UK) was added in a 2:1 ratio and the diluted blood was then layered on top using a Scipippette Leto™ (SciQuip Ltd, Newton, UK) with Corning Stripette™ (TF). The layered blood was then centrifuged at 400rcf for 30minutes at room temperature without brake. The resulting PBMC layer was then removed using a 1000ul pipette, transferred to a new 50ml Falcon, mixed with 20mls PBS (TF) and then centrifuged at 400rcf for 5minutes at room temperature with brake. The supernatant was discarded and the PBMC pellet resuspended in 20mls PBS (TF) and re-centrifuged at 400rcf for 5minutes at room temperature with brake. The supernatant was again discarded and the PBMC pellet resuspended in either:

a. 1ml of cell medium (Dulbeccos' modified eagle medium (DMEM, TF) or Roswell Park Memorial Institute medium (RPMI, TF)), counted using a Countess 3TM Automated cell counter (TF) and diluted to a working concentration for immediate experimentation.
b. 1ml freezing media (foetal bovine serum (FBS) (Gibco, TF) with 10% Dimethylsulfoxide (DMSO) (Sigma-Aldrich, Gillingham, UK)) and transferred to a CryotubeTM (Greiner, Stonehouse, UK). PBMCs were then cooled to -80°C using isopropyl alcohol gradient cooling (Mr Frosty™, TF). After 24-48 hours, the PBMCs were transferred for storage in liquid nitrogen for subsequent defrosting and experimentation.

2.3.2.2 CPT vacutainer separation

CPT vacutainers (BD) were centrifuged at 1500rcf for 15minutes at room temperature. The PBMC layer was transferred using a 1000ul pipette into two Eppendorf™ microtubes (TF) and then centrifuged at 400rcf for 5minutes at room temperature with break. The supernatant was discarded and the PBMC pellet resuspended in PBS (TF) and then re-centrifuged at 400g for 5minutes at room temperature with brake. The supernatant was again discarded and the PBMC pellet resuspended in 1ml freezing media and processed as described above.

Frozen PBMCs were defrosted in batches for subsequent analysis or stimulation. Samples were removed from the liquid nitrogen and kept on dry ice. 5mls of PBS (if samples were to be immediately analysed) or RPMI (for samples undergoing further stimulation) was added to a 15ml Falcon (TF) and using a 1000ul pipette, PBS or medium was added to each frozen sample, agitated briefly and transferred to the Falcon. This process was continued until the whole sample had defrosted. The defrosted PBMCs were then centrifuged at 400g for 5minutes at room temperature with break, the supernatant discarded and the PBC pellet resuspended in 2mls PBS or medium. The defrosted PBMCs were then re-centrifuged at 400g for 5minutes at room temperature with break, the supernatant discarded and the PBC pellet resuspended in PBS or medium for subsequent staining or stimulation.

2.3.3 Whole blood stimulation

To assess cytokine release in response to an additional stimulus, 1000ul of EDTA blood to an Eppendorf and incubating for 1hr at 37°C, 5% CO_2 with 100ng/ml of lipopolysaccharide (LPS, E. *coli* 0111:B4, Merck Millipore (MM), Livingstone, UK). The sample was then centrifuged at 1500g for 5mins at room temperature and the resulting plasma transferred to a cryovial (Greiner) and stored at -80°C until analysis.

2.3.4 Serum and plasma isolation

After removal of blood for whole blood stimulation as per Section 2.3.3 above, the K2 EDTA, and SST Advance™ vacutainers (BD) were centrifuged at 1500g for 15minutes at room temperature. The resulting serum and plasma were aspirated, transferred to cryovials (Greiner) and stored at -80°C until analysis.

2.3.5 Cell stimulation

2.3.5.1 Heat-killed bacteria stimulation

To model bacterial infection *ex vivo*, I purchased commercially available heat-killed bacteria as this would enable me to assess immune response to an infection whilst excluding potential confounding from different bacterial replication rates in my samples. I chose to compare both Gram positive (S.

aureus) and negative (E. *coli*) bacteria given there are immunological differences in response to different organisms. Doses were compared using preliminary dose titrations and E. *coli* subsequently used in the model as it gave the greatest stimulus (Section 3.2.1).

200µl of PBMCs resuspended in medium (1-2x10^6 PBMCs/ml) per well were plated on to a 96-well plate and stimulated with heat-killed E. *coli* (InvivoGen, TF) at a concentration of 1x10^8 per ml for 24 hours. Unstimulated samples were used as negative controls. Plates were centrifuged 400g for 5mins, the supernatant aspirated and stored at -80°C for future ELISA. The cells were then ready for flow cytometry staining.

2.3.5.2 LPS stimulation

To assess monocyte response using spectral flow cytometry, PBMCs (1x10^6/ml) were plated into 96-well plates and incubated at 37°C, 5% CO_2 for 24 hours with LPS (E. *coli* 0111:B4, MM) at a concentration of 100ng/ml. Choice of stimulus was identified following preliminary experiments. (Section 3.2.2)

2.3.5.3 CD3/CD28 bead stimulation

For *in vitro* stimulation, PBMCs (1x10^6/ml) were plated into 96-well plates and incubated at 37°C, 5% CO_2 with CD3-28 beads (Miltenyi Biotec (MB), Woking, UK) at a concentration of 4:1 for 48 hours (for lymphocyte analysis). Following incubation, plates were centrifuged at 400g for 5minutes at room temperature in in preparation for cell staining. Dose was identified through preliminary experiments. (Section 3.2.3)

2.3.5.4 SARS-CoV-2 spike protein stimulation

To model COVID-19 infection *ex vivo*, I purchased what was at the time, the only commercially available recombinant SARS-CoV-2 S protein S1 + S2 (Biolegend (BL), London, UK). There was limited data regarding its use given its novelty, therefore I performed a series of exploratory experiments to identify a suitable model. (Section 3.2.4)

250ul of PBMCs resuspended in medium (1x10^6 PBMCs/ml) were plated on to a 96-well plate with recombinant SARS-CoV-2 S protein S1 + S2 (BL) at a concentration of 15µg/ml for 6 hours at 37°C. Cell stimulation cocktail (TF) at a 1x concentration for 6 hours was used as a positive control and Cytochalasin-D at 1µM (MM) was used as a negative control.

2.3.6 Influence of antibiotics on the immune response in critical illness

Antibiotics were initially dissolved in sterile water as per manufacturer recommendations before being diluted in PBS to working stock concentrations.

2.3.6.1 Sepsis model

I assessed of the effects of beta-lactams, which are commonly used in UK clinical practice,[288] including amoxicillin (Wockhardt, Wrexham, UK), cefuroxime (Flynn Pharma, Stevenage, UK), meropenem (Milpharm, South Ruislip, UK), and piperacillin (Fresenius Kabi, Runcorn, UK). Several previously identified *ex vivo* antibiotic-induced immunosuppressive effects have been identified only in supra-clinical concentrations. I used 2 doses of each antibiotic based on the range of measured mean concentrations from the published pharmacokinetic literature.[289-292] Amoxicillin and cefuroxime were used at doses of 5 and 25μg/ml, meropenem at 20 and 60μg/ml, and piperacillin at 50 and 250μg/ml,

2.3.6.2 Surgical model

I identified the mostly commonly prescribed antibiotics for perioperative antimicrobial prophylaxis using a meta-analysis of worldwide practise, which included penicillins, cephalosporins, nitroimidazoles, and combination cephalosporin and nitroimidazole.[293] I chose to assess one drug from each class, based on UK practice including amoxicillin (Wockhardt, Wrexham, UK), cefuroxime (Flynn Pharma, Stevenage, UK), and metronidazole (B Braun Medical, Sheffield, UK). For patient samples, I used 2 doses based on the range of measured mean concentrations from the published pharmacokinetic literature (5 and 25μg/ml).[290,294-296]

2.3.6.3 COVID-19 model

During the COVID-19 pandemic, in the search for potential treatments, the known immunomodulatory effect of macrolide antibiotics made them attractive candidates.[297,298] Whilst these immunomodulatory properties have theoretical benefits in the management of inflammatory diseases including viral severe acute respiratory illness (SARI),[299] the theoretical benefits have not been borne out in randomised control trials (RCTs), either in the management of non-viral[300] nor viral SARI,[301] including COVID-19.[302-305] Furthermore, immunomodulatory properties may vary between azithromycin and other macrolides including clarithromycin.[306]

I chose to model the effect of two macrolide antibiotics, azithromycin (Aspire Pharma Ltd, Petersfield, UK) and clarithromycin (Mylan Products Ltd, Potters Bar, UK) compared to a beta-lactam (amoxicillin, Ibigen SRL, Aprilia, Italy) as this was commonly co-prescribed. The three antibiotics were used at a concentration of 10μg/ml.

2.4 Enzyme-linked immunosorbent assays

Released cytokines (IL-1β, IL-6, IL-10, TNF-α, PD-1, and PD-L1) were measured using Duoset Enzyme-Linked Immunosorbent Assay (ELISA) kits (R&D Systems, Abingdon, UK), based on the sandwich ELISA technique as per manufacturer protocol.

Reagents were made up as per manufacturer instructions. High-bind plates were incubated with 100µl/well of relevant capture antibody overnight before being washed 3 times with PBS-Tween (PBS with 0.05% Tween) and blocked with 300µl/well reagent diluent (1% bovine serum albumin (BSA) solution) for 1 hour. Plates were washed as described before 100µl/well of relevant standard or samples were added in duplicates and incubated at room temperature for 2 hours. Samples were diluted either 1:2 for unstimulated or 1:10 for stimulated samples in reagent dilutant (based on previous dose titrations). Plates were washed and 100µl/well of the relevant detection antibody incubated for a further 2 hours. Plates were again washed and 100µl/well of Streptavidin-Peroxidase added and incubated for 20 minutes. Plates were washed, and 100µl/well substrate solution added and incubated for 20 minutes. Finally, 50µl/well stop solution was added.

Optical densities were acquired on a SPECTROstar Nano™ microplate reader (BMG Labtech, Aylesbury, UK) running MARS™ (version 3.42, BMG) software. Standard curves were generated, and sample concentrations extrapolated using GraphPad prism (version 9, GraphPad, San Diego, CA). (Section 3.3)

2.5 Electrochemiluminescent immunoassay

Electrochemiluminescent immunoassays using two U-PLEX inflammatory marker panels including IFN-γ, IL-4, IL-6, IL-8, IL-12p70, IL-5, GM-CSF, G-CSF, IFN-α2a, IFN-β, IL-1RA, IL-7, IL-19, IP-10, MCP-1, MIP-1a, and VEGF-a were performed according to the manufacturer's protocol (Meso Scale Discovery (MSD), Rockville, MD). This technique is similar to a sandwich ELISA approach but uses electrical current to generate emission of light from $[Ru(bpy)_3]^{2+}$. Reagents were made up as per manufacturer instructions. A U-PLEX™ linker-coupled solution was made by incubating each biotinylated antibody to its assigned linker for 30 minutes before adding stop solution and incubating for an additional 30 minutes. 10 U-PLEX™ linker-coupled solutions were combined and 50µl/well added to the supplied 96-well plate which was incubated for 1 hour. The plate was then washed 3 times with wash buffer before 50µl/well sample or calibrator standard was added and incubated for 1hour. The plate was washed, and 50µl/well detection antibody solution added and incubated for 1hour. The plate was washed and 150µl/well Read buffer added before electrochemiluminescence was acquired and analysed using a Meso QuickPlex SQ120™ microplate reader (MSD) running Discovery Workbench™ (version 4.0, MSD). (Section 3.4)

2.6 Flow cytometry

Flow cytometry panels were designed to assess immune cell functions. The following markers were used, (Table 2.3) concentrations were determined using dose titrations based on manufacturers recommendations (Section 3.5).

Phagocytosis was assessed using pHRodo red *E. coli* bioparticles (TF). Bioparticles were added to the wells at a final concentration of 100µg/ml 1 hour prior to completion of stimulation. Cells were then resuspended in PBS with cell surface markers: CD14, CD16 and HLA-DR and viability stain (Blue UV Live/Dead) added and incubated for 30 minutes. The cells were then washed once and resuspended in ice cold PBS and placed on ice for acquisition.

Viability was assessed using a fixable amine-based fluorescent dye. The dye reacts with free amines both inside and outside of cells with compromised membranes leading to intense fluorescent staining. Dyes used included aqua, blue and zombie near-infrared (all TF).

Monocyte markers of chemotaxis, antigen presentation, co-stimulation and T-cell suppression were assessed by resuspending PBMCs in PBS with the following antibodies: CD14, CD16, HLA-DR, CD80, CD86, CD192 (CCR-2), CD184 (CXCR-4), and CD274 (PD-L1), and viability stain (Aqua UV Live/Dead) and incubated for 30 minutes. The cells were then washed once and resuspended in PBS for acquisition.

Monocyte intracellular cytokine levels was assessed by initially resuspending PBMCs in PBS with the following cell surface markers: CD14, CD16 and HLA-DR and viability stain (Blue UV Live/Dead) added and incubated for 30 minutes. The cells were then washed and resuspended in fixation/permeabilization buffer (BD) and incubated for 20 minutes at 4°C. The cells were then washed and resuspended in wash/permeabilization buffer (BD) with the following antibodies to intracellular cytokines: IL-1β, IL-6, IL-10, IFN-γ, and TNF-α, and incubated for 30 minutes at 4°C. The cells were then washed and resuspended in PBS for acquisition on flow cytometry.

Lymphocyte markers of viability, death pathways, differentiation, proliferation and activation were assessed by resuspending PBMCs in annexin binding buffer with the following antibodies: CD3, CD4, CD8, CD19, CD25 (IL-2R), CD28, CD127 (IL-7R), CD152 (CTLA-4), CD274 (PD-L1), and CD279 (PD-1) with viability stain (Aqua UV Live/Dead and Annexin V).

Marker type	Cell Marker	Cat no	Fluorochrome	Species	Isotype	Dilution
Cell surface	CD3	BD 564001	BUV395	Mouse	IgG1, κ	1:250
	CD4	BL 317442	BV785	Mouse	IgG2b, κ	1:250
	CD8	BD 563677	BV711	Mouse	IgG1, κ	1:250
	CD14	BL 301840	BV785	Mouse	IgG2a, κ	1:250
	CD16	BD 563785	BUV395	Mouse	IgG1, κ	1:250
	HLA-DR	BL 307618	APC-Cy7	Mouse	IgG2a, κ	1:250
	CD19	BD 557791	APC-Cy7	Mouse	IgG1, κ	1:250
	CD25 (IL-2R)	BD 562442	BV421	Mouse	IgG1, κ	1:250
	CD28	BD 748475	BUV737	Mouse	IgG1, κ	1:250
	CD80	BL 305208	PE	Mouse	IgG1, κ	1:250
	CD86	BL 374218	PE-Dazzle	Mouse	IgG1, κ	1:250
	CD127 (IL-7R)	BL 351304	PE	Mouse	IgG1, κ	1:250
	CD152 (CTLA-4)	BL 349922	PE-Dazzle	Mouse	IgG1, κ	1:250
	CD184 (CXCR4)	BL 306518	BV421	Mouse	IgG2a, κ	1:250
	CD192 (CCR2)	BL 357232	BV711	Mouse	IgG2a, κ	1:250
	CD274 (PD-L1)	BD 563741	APC	Mouse	IgG1, κ	1:250
	CD279 (PD-1)	BD 561272	PE-Cy7	Mouse	IgG1, κ	1:250
Phagocytosis	pHRodo EC	TF P35361	PE	-	-	1:20
	pHRodo SA	TF A10010	PE	-	-	1:20
Viability	Annexin	TF A23202	Alexa Fluro 350	-	-	1:250
	L/D Aqua	TF L34957	Aqua UV	-	-	1:1000
	L/D Blue	TF L34962	Blue UV	-	-	1:1000
Intracellular cytokine	IL-1β	TF 11701842	FITC	Mouse	IgG1, κ	1:100
	IL-2	BL 500304	FITC	Rat	IgG2a, κ	1:100
	IL-6	BD 561441	APC	Rat	IgG1, κ	1:100
	IL-10	BL 501422	BV421	Rat	IgG1, κ	1:100
	IFN-γ	BL 502544	BV510	Mouse	IgG1, κ	1:100
	TNF-α	BL 502930	PE- Cy7	Mouse	IgG1, κ	1:100

Table 2.3: Flow cytometry fluorochromes
Abbreviations: APC: Allophycocyanin, BD: Beckton Dickinson, BL: Biolegend, BUV: Brilliant ultraviolet, BV: Brilliant violet, CCR2: C-C motif chemokine receptor 2, CXCR4: C-X-C motif chemokine receptor 4, CD: cluster of differentiation, CTLA-4: Cytotoxic T-lymphocyte associated protein-4, Cy: Cyanine, FITC: Fluorescein isothiocyanate, HLA-DR: Human leukocyte antigen – DR isotope, IFN: Interferon, IL: Interleukin, L/D: Live/Dead, PD-1: Programmed death receptor 1 PD-L1: Programmed death-ligand 1, PE: Phycoerythrin, TF: Thermofisher, TNF: Tissue necrosis factor, UV: Ultraviolet.

Cells were acquired on an LSRII™ or Fortessa X20™ flow cytometer (BD) running FACSDiva™ (version 9, BD).

Calibration beads (BD) were used prior to commencing acquisition to ensure consistency in baseline MFI (median fluorescence intensity) between experimental days. Compensation controls were applied to all samples prior to acquisition. Single-stained unstimulated healthy donor cells were used as compensation controls for cell surface markers. Healthy donor cells were heat-treated at 60°C for 10 minutes as a positive control for cell death. Single-stains for pHRodo used healthy donor cells stimulated with eBioscience cell stimulation cocktail (PMA 81nM with ionomycin 1.34μM). Compensation beads (BD) were used as positive controls for intracellular cytokines.

Gates were drawn with the use of single stains and FMOs (fluorescence minus one) for all markers. The stopping gate was set on $CD14^+$ monocytes or $CD4^+$ lymphocytes and set at 10,000 events.

2.7 Spectral flow cytometry

Spectral flow cytometry panels were designed to assess the mechanisms responsible for immune cell functions. The following markers were used, (Table 2.4) concentrations were determined using dose titrations based on manufacturers recommendations. (Section 3.6.3)

After stimulation, fluorochromes against cell surface antigens were assessed by resuspending PBMCs in cell staining buffer (BL) with relevant antibodies and viability stain added. After 30 minutes, the PBMCs were washed in cell staining buffer before being fixed and permeabilised using the True-Nuclear kit (BL). (Section 3.6.2) PBMCs were resuspended in the fixation-permeabilisation buffer and incubated at 4°C for 40 minutes. The plates were then centrifuged and resuspended in the permeabilisation-wash buffer with fluorochromes to intracellular cytokines, intracellular proteins and transcription factors added and incubated at 4°C for 40 minutes. The PBMCS were then washed in the permeabilization-wash buffer before being resuspended in cell staining buffer.

Cells were acquired on an ID7000 spectral cell analyser (Sony Biotechnology Inc, Weybridge, UK) and analysed using ID7000 software (version 1.2, Sony). Alignment check beads (Sony) were used prior to running each experiment and spectral references for each fluorochrome were added using either single stain labelled heat-killed cells (60°C for 10minutes, live/dead stain) or compensation beads (BD, all other markers) with appropriate negative controls. FMO (fluorescence minus one) samples were used to identify cell populations. The stopping gate was set at 10,000 events for either $CD14^+$ monocytes or $CD4^+$ lymphocytes.

Function	Marker type	Cell Marker	Fluorochrome	Cat no	Species	Isotope	Dilution
Antigen presenting cells							
Gating	Cell surface	CD11b	SBUV445	BR MCA711	Rat	IgG2b	1:250
Gating	Cell surface	CD14	SBB580	BR MCA1568	Mouse	IgG2a	1:250
Gating	Cell surface	CD16	SB702	TF 67-0168-42	Mouse	IgG1, κ	1:250
Gating/ Antigen presentation	Cell surface	HLA-DR	BUV805	BD 748338	Mouse	IgG2a, κ	1:250
Phagocytosis	Cell surface	CD64	BUV737	BD 612776	Mouse	IgG1, κ	1:250
Cell activation	Cell surface	CD66a	BUV563	BD 741410	Mouse	IgG2a, κ	1:250
Gating	Cell surface	CD66b	BB515	BD 564679	Mouse	IgM, κ	1:250
Antigen presentation	Cell surface	CD74	BV650	BD 743734	Mouse	IgG1, κ	1:250
Antigen presentation	Cell surface	CD80	BV480	BD 751735	Mouse	IgG1, κ	1:250
Antigen presentation	Cell surface	CD86	BUV496	BD 749895	Mouse	IgG1, κ	1:250
Chemokine receptor	Cell surface	CD192	BUV395	BD 747854	Mouse	IgG2a, κ	1:250
T-cell suppression	Cell surface	CD274	RB545	BD 756359	Mouse	IgG1, κ	1:250
Toll-like receptor	Cell surface	CD284	SB600	TF 63-9917-42	Mouse	IgG2a, κ	1:250
Antigen presentation	Cell surface	HLA-DM	APC	MB 130-124-252	Human	IgG1	1:250
Antigen presentation	Cell surface	HLA-DP	RB780	BD 755757	Mouse	IgG1, κ	1:250
Viability	Viability	Live/Dead	Zombie NIR	BL 423106	-	-	1:1000
Cytokine	Intracellular cytokine	IL-1ß	AF750	BT FAB10349S-100UG	Human	IgG1	1:100
Cytokine	Intracellular cytokine	IL-10	BB700	BD 566567	Rat	IgG2a, κ	1:100
Cytokine	Intracellular cytokine	IFN-γ	BV750	BD 566357	Mouse	IgG1, κ	1:100

Cytokine	Intracellular cytokine	TNF-α	BV785	BL 502948	Mouse	IgG1, κ	1:100
Antigen presentation	Transcription factor	CIITA	DY680	BT NBP2-59072FR	Rabbit	IgG	1:100
Antigen presentation	Intracellular protein	NF-κB p65	PE-CF594	BD 565447	Mouse	IgG2b, κ	1:100
Inflammasome	Intracellular protein	NLRP3	AF405	BT IC7578V-100UG	Rat	IgG2a	1:100
Phagocytosis	Intracellular protein	Nox-2	PE-Cy7	BT NBP1-41012PECY7	Mouse	IgG1	1:100
Lymphocytes							
Proliferation	Proliferation	CellTrace	FarRed	TF C34564	-	-	1:1000
Gating	Cell surface	CD3	SBUV445	BR MCA463	Mouse	IgG1	1:250
Gating	Cell surface	CD4	BUV805	TF 368-0047-42	Mouse	IgG1, κ	1:250
Gating	Cell surface	CD8	APC-Fire 750	BL 301066	Mouse	IgG1, κ	1:250
Gating	Cell surface	CD19	BUV395	TF 363-0198-42	Mouse	IgG1, κ	1:250
Gating/Differentiation	Cell surface	CD25	SBV570	BR MCA2127	Mouse	IgG1	1:250
Activation	Cell surface	CD28	BUV496	BD 741168	Mouse	IgG1, κ	1:250
	Cell surface	CD95	BUV615	BD 752346	Mouse	IgG1, κ	1:250
Differentiation	Cell surface	CD127	PerCP-eFluor 710	TF 46-1278-42	Mouse	IgG1, κ	1:250
Activation	Cell surface	CD152	AF532	BT NBP2-50286	Mouse	IgG1, κ	1:250
Gating	Cell surface	CD194	BUV563	BD 752566	Mouse	IgG1, κ	1:250
Gating	Cell surface	CD196	BV786	BD 563704	Mouse	IgG1, κ	1:250
T-cell suppression/ Activation	Cell surface	CD274	FITC	BL 393606	Mouse	IgG1, κ	1:250
Cell death	Cell surface	CD279	BV480	BD 566112	Mouse	IgG1, κ	1:250
Activation/ Antigen presentation	Cell surface	HLA-DR	BV711	BD 563696	Mouse	IgG2a, κ	1:250
Viability	Viability	Live-Dead	Zombie NIR	BL 423106	-	-	1:1000
Cytokine	Intracellular cytokine	IL-2	BV650	BL 500334	Rat	IgG2a, κ	1:100
Gating	Intracellular cytokine	IL-4	PE-Cy7	BD 560672	Mouse	IgG1, κ	1:100
Cytokine	Intracellular cytokine	IL-10	BUV737	TF 367-7108-42	Rat	IgG1, κ	1:100
Gating	Intracellular cytokine	IL-17A	APC-R700	BD 565163	Mouse	IgG1, κ	1:100
Cytokine	Intracellular cytokine	IFN-γ	BV750	BD 566357	Mouse	IgG1, κ	1:100
Gating	Transcription factor	Fox-p3	PE-Cy5	TF 15-4776-42	Rat	IgG2a, κ	1:100
Activation	Intracellular protein	NF-κB	PE-CF594	BD 565447	Mouse	IgG2b, κ	1:100
Gating	Intracellular protein	STAT5	RB780	BD 568759	Mouse	IgG1, κ	1:100
Gating	Intracellular protein	T-bet	BV605	BL 644817	Mouse	IgG1, κ	1:100

Table 2.4: Spectral flow cytometry fluorochromes

Abbreviations: APC: Allophycocyanin, AF: Alexa Fluor, BD: Beckton Dickinson, BL: Biolegend, BR: Biorad, BT: Biotechne, BUV: Brilliant ultraviolet, BV: Brilliant violet, CCR2: C-C motif chemokine receptor 2, CD: cluster of differentiation, CF: Cyanine-based fluorescent dye, CIITA: Class II major histocompatibility complex transactivator, CTLA-4: Cytotoxic T-lymphocyte associated protein-4, Cy: Cyanine, FITC: Fluorescein isothiocyanate, Fox-P3: Forkhead box P3, HLA-DR/M/P: Human leukocyte antigen – DR/M/P isotype, IFN: Interferon, IL: Interleukin, L/D: Live/Dead, MB: Miltenyi Biotec, NLRP3: NOD-, LRR-, and pyrin domain-containing protein 3, NF-κB: Nuclear Factor Kappa B, NIR: Near-infrared, Nox-2: Nicotinamide adenine dinucleotide phosphate oxidase 2, PD-1: Programmed death receptor 1 PD-L1: Programmed death-ligand 1, PE: Phycoerythrin, PerCP: Peridinin-chlorophyll-protein, RB: RealBlue, SBV: StarBright violet, SBUV: StarBright ultraviolet, STAT5: Signal transducer and activator of transcription 5, T-bet: T-box transcription factor TBX21, TF: Thermofischer, TNF: Tissue necrosis factor, UV: Ultraviolet.

2.8 HPLC

High-performance liquid chromatography (HPLC; Agilent 1260 II HPLC (Agilent Technologies UK Ltd Cheshire, UK)) was used to determine the concentration of meropenem and amoxicillin over 5 days. The integrated temperature-controlled column compartment was set at 35°C and the autosampler was set at 4°C. An Agilent Porshell 120 EC-C18 4.6 x 150mm, 4µm analytical column was used. Data signals were processed and presented using Open LAB CDS LC ChemStation (Agilent, Cheshire, UK).

The mobile phase for the HPLC study for meropenem and amoxicillin was prepared by using monobasic sodium phosphate ($NaH_2PO_4 \cdot 2H_2O$), dibasic sodium phosphate ($Na_2HPO_4 \cdot 12H_2O$) and acetonitrile (all from Sigma); and pH corrected to 7.4. A final solution of 10mM phosphate (buffer: acetonitrile) 90:10 (v/v) was used. Meropenem and amoxicillin stock solutions were prepared to a concentration of 1000µg/ml by reconstitution of 1 mg relevant antibiotic with 1ml HPLC water. The mobile phase flow rate was set at 1.3ml/min with a retention time set at 4 minutes. The peak absorbance of meropenem was read at 290nm and absorbance of amoxicillin read at 200nm.

A standard curve was prepared by serial dilutions of the antibiotic stock solution with a concentration range from 800 µg/mL to 0.97 µg/ml, and HPLC gradient water was used as a negative control. 50µl of each sample was injected by the autosampler. (Section 3.7)

2.9 Statistics

Flow cytometry data were analysed using FlowJo (version 10.7.1, BD) or ID7000 software (version 1.2, Sony). Samples with cell counts fewer than 10 in the population of interest were excluded. Data are presented as either median fluorescence intensity (MFI; arbitrary units) or percentage positive cells. Multiplex data were analysed using MSD Discovery Workbench (version 4.0, MSD). ELISA data were analysed using MARS (version 3.42, BMG).

Clinical and demographic data are reported as either median (interquartile range) or number (%). Categorical data were compared using the chi-square test. Continuous data were compared for two groups using either non-parametric Wilcoxon test for unpaired data or t-test for paired data, and between three or more groups non-parametric Kruskal-Wallis test for unpaired data or Friedmans test for paired data, both without Dunnett's correction. Graphs were constructed, and statistical analysis performed using Prism (version 10, GraphPad, San Diego, CA) or SPSS version 26.0 (IBM Corp, Armonk, NY, USA).

Detailed statistical testing for each chapter is described below.

2.9.1 Immune responses to infection and sepsis

Simple linear regression was used to investigate correlations between variables. Principal component analysis (PCA) was performed to identify whether patients with different illness severities demonstrate unique immune signatures. To assess if patients with sepsis demonstrate an immune signature, I undertook a principal component analysis (PCA) of immune markers and age, for all patients for whom full datasets were available. Heat maps were generated using percentage change. Graphs were constructed, and statistical analysis performed using Prism (version 10, GraphPad, San Diego, CA)

2.9.2 Immune responses to sterile inflammation

Mixed-effects two-way ANOVA was used to assess the difference in continuous data over time (before and 24 hours after surgery) between patients with and without subsequent infections. Data are presented as differences over time, between groups, and the difference in the change over time between the two groups (interaction term).

To assess if patients undergoing surgery who develop a post-operative infection demonstrate a characteristic immunophenotype compared to those who do not develop an infection, I undertook a principal component analysis (PCA) of 62 immune markers, age, and body mass index (BMI) for all patients for whom full datasets were available. Immune markers consisted of nine serological markers, nine monocyte markers, six CD4 lymphocyte markers, and six CD8 lymphocyte markers. Each immunological marker was assessed prior to and 24 hours following surgery.

To identify statistically significant discriminators between patients with and without subsequent infections, I conducted multiple comparisons using a Mann–Whitney test and calculated a corrected p-value (−log10) with a false discovery rate (FDR) of 1% using a two-stage step-up method of Benjamini, Krieger, and Yekutieli, and area under the receiver operating characteristic curve (AUROC); and data are presented using a volcano plot. I conducted a regression analysis to assess independent risk factors associated with post-operative infection.

2.9.3 Immune responses to COVID-19

Analysis was performed using anonymized data. Clinical data were collated with viral loads, levels of SARS-CoV-2-specific antibodies, and plasma cytokines and chemokines. Continuous and categorical variables are reported as median (interquartile range) and *n* (%), respectively. Mann-Whitney *U* tests without post hoc correction for comparison between subgroups were performed for comparison of continuous variables between groups. Categorical data were compared using the chi-square test. The association between biomarkers and clinical severity was assessed using AUROC. Pearson's

correlation coefficient was used to assess correlation between various clinical and therapeutic biomarkers.

Changes in continuous variables over time between groups was assessed using two-way ANOVA. Unadjusted survival differences were assessed using log-rank test and displayed using a Kaplan-Meier curve. Adjusted hazards ratios of factors associated with mortality was analysed using Cox regression.

3 Validation of laboratory experimental techniques

3.1 Sample preparation and storage

3.1.1 Peripheral blood mononuclear cell isolation method comparison

CPT™ tubes were chosen for isolating peripheral blood mononuclear cells (PBMC) from patient samples as they enabled rapid processing (30 minutes vs. 60 minutes) which was deemed beneficial, given patients could be recruited at any time. To ensure validity compared to standard ficoll gradient separation, PBMC count and viability was compared between 8mls of volunteer blood using ficoll gradient separation and from 8mls blood collected in a CPT vacutainer. After isolation, cells were resuspended in 1ml media and cell count and viability assessed using Trypan blue™ (TF) and a Countess 3™ Automated cell counter (TF). Cell counts and viability were similar between the two methods. (Table 3.1)

Volunteer	**Ficoll gradient**		**CPT vacutainer**	
	Count (x10^6/ml)	Viability (%)	Count (x10^6/ml)	Viability (%)
1	5.4	97	7.4	97
2	2.6	98	2.8	97
3	4.2	96	3.7	97

Table 3.1: Comparison of PBMC count and viability between ficoll gradient and CPT vacutainer separation

3.1.2 Liquid nitrogen viability

PBMCs were stored in a liquid nitrogen dewar which was regularly topped up. To confirm long term storage viability and no difference in viability between samples stored in the bottom versus top racks, two samples stored from each rack level were defrosted, resuspended in 1ml media and cell count and viability assessed using Trypan blue™ (TF) and a Countess 3™ Automated cell counter (TF). Rack position did not alter viability nor did duration of storage. (Table 3.2)

Rack	**Storage duration (days)**			**Viability (%)**		
	Sample 1	Sample 2	Mean	Sample 1	Sample 2	Mean
A	41	41	41	99	99	99
B	31	75	53	98	87	92.5
C	32	32	32	98	99	98.5
D	98	98	98	98	97	97.5

Table 3.2: Comparison of depth and duration of liquid nitrogen storage on sample viability

3.2 Cell culture

3.2.1 Dose titration of heat-killed bacteria

To assess monocyte function, PBMCs were incubated for 24 hours with three different concentrations (10^{7-9}/ml)) of heat-killed E. *coli* and S. *aureus* and their effect on classical monocyte cell surface marker assessed using flow cytometry. A concentration of 10^8 bacteria per ml provided the best stimulus. (Figure 3.1)

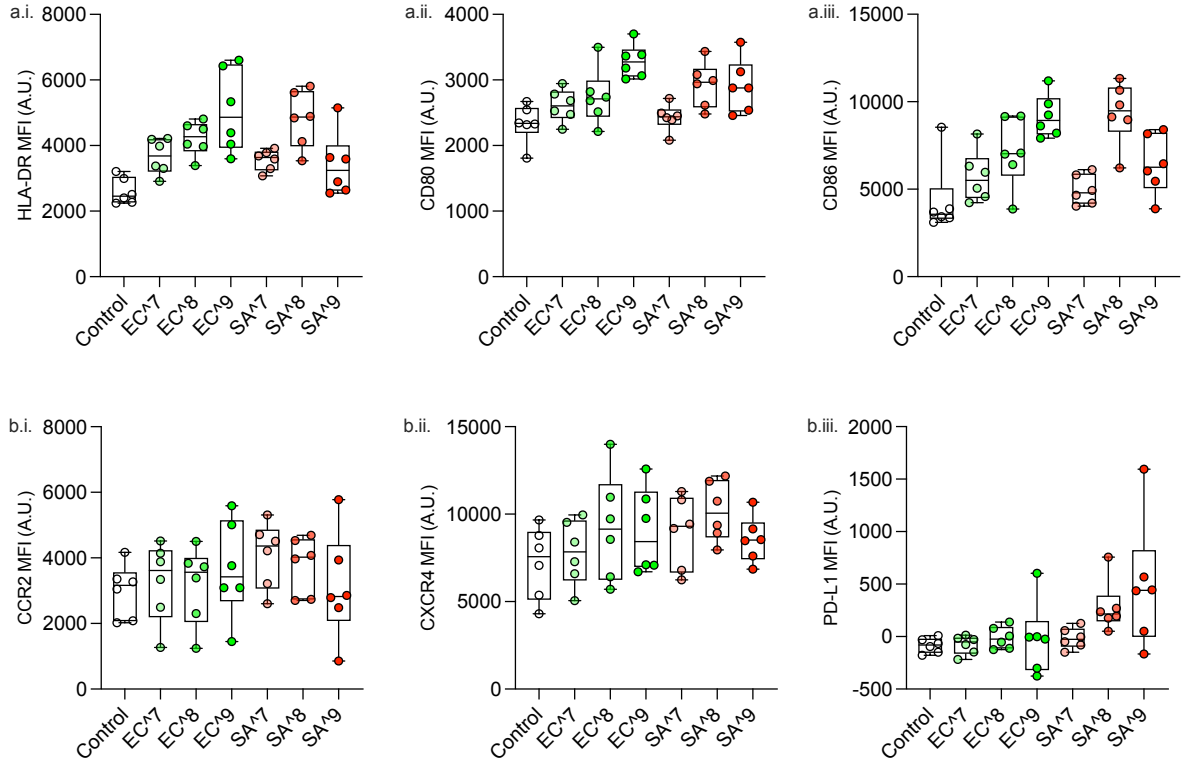

Figure 3.1: 24 hour heat-killed bacteria dose titration on classical monocytes
Healthy volunteer PBMCs (n=6) were stimulated with heat-killed E. *coli* (EC, green) or S aureus (SA, red) at three concentrations (10^{7-9}/ml) for 24 hours and changes in classical monocyte surface marker expression measured including markers associated with antigen presentation (HLA-DR, a.i. CD80, a.ii. and CD86, a.iii.), chemokine receptors (CCR2, b.i. and CXCR4, b.ii.) and PD-L1 (b.iii.) compared to unstimulated cells (white). Data displayed as median fluorescence intensity (MFI). Dots represent individual volunteers, horizontal line the median, box the interquartile range, and whisker the range.

3.2.2 LPS stimulation

To identify the best *in vitro* model that caused an increase in classical monocyte surface HLA-DR expression, I compared LPS with three concentrations of three different heat-killed bacteria at 2 timepoints. LPS consistently outperformed the heat-killed bacteria and was used in the model. (Figure 3.2)

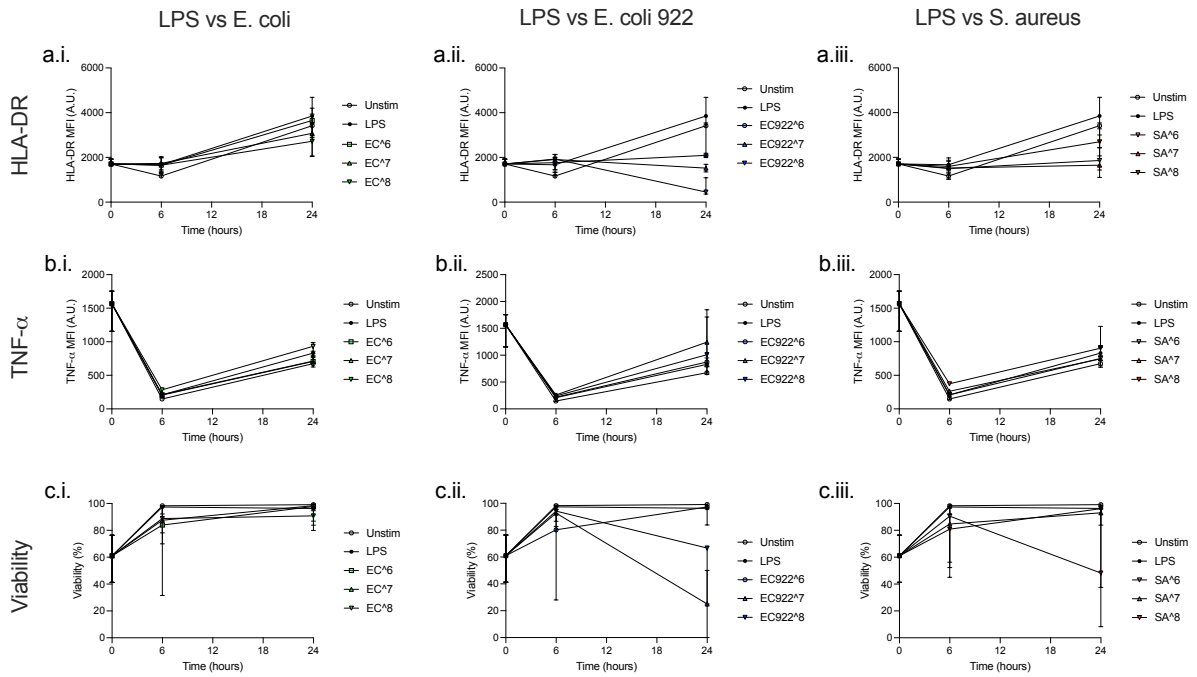

Figure 3.2: Comparison of LPS with heat-killed bacteria on classical monocytes
Volunteer PBMCs (n=4) were stimulated for 6 and 24 hours were stimulated with LPS (100ng/ml, black) or two different E. *coli* (EC, green, column i. and EC922, blue, column ii.)) or S. *aureus* (SA, red, column iii.) heat-killed bacteria and effect on classical monocyte HLA-DR expression (row a.), intracellular TNF-α concentration (row b.) or viability (row c.) compared to unstimulated cells (white). Data displayed as median fluorescence intensity (MFI) or % of cell population. Dots represent median value of the 4 volunteers, and whisker the range.

3.2.3 CD3/CD28 bead stimulation

To identify the optimal dose of beads for subsequent antibiotic stimulation experiments, healthy volunteer PBMCs were stimulated with 3 different concentrations of CD3/CD28 stimulatory beads for 24 and 48hours and the effect on lymphocyte proliferation (measured using carboxyfluorescein succinimidyl ester (CFSE)) and IFN-γ concentration compared. A concentration ratio of 1:4 (cells:beads) offered the best stimulation (IFN-γ) and use of proliferation index to measure proliferation would not be suitable given the low cell counts I would encounter in septic patients. (Figure 3.3)

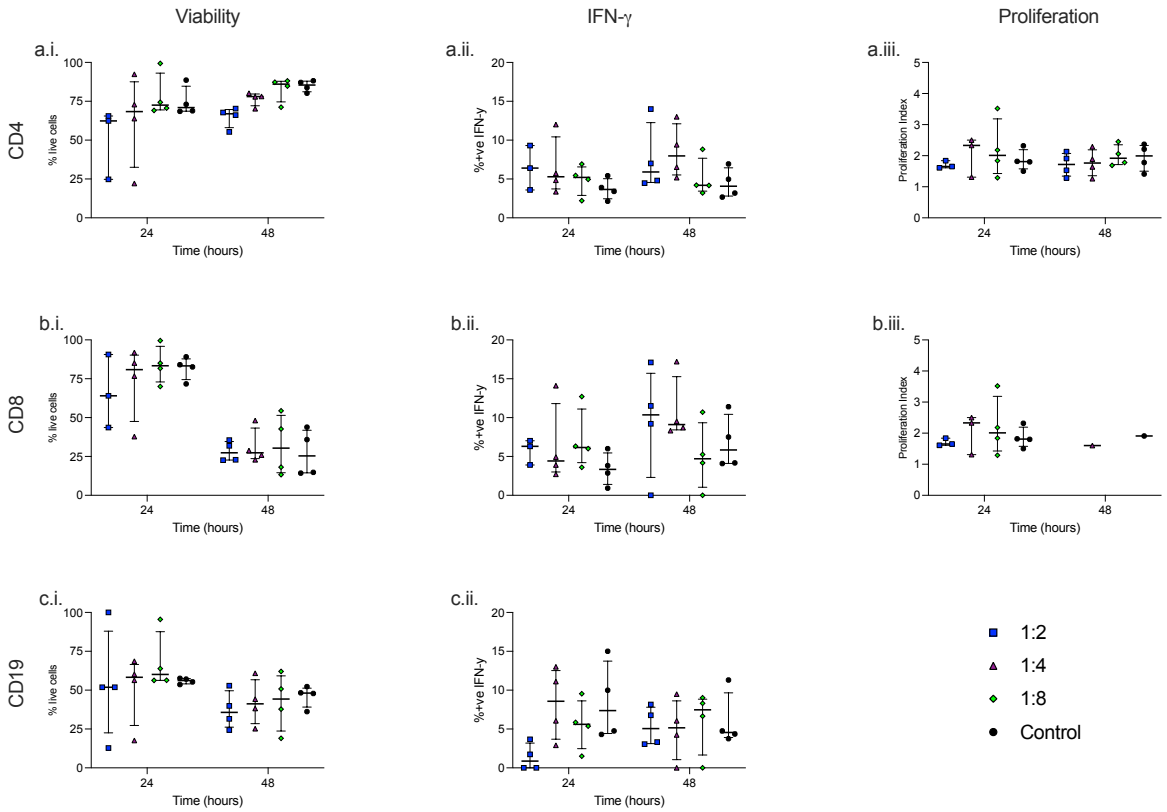

Figure 3.3: Dose titration of CD3/CD28 beads for lymphocyte stimulation
Volunteer PBMCs (n=4) were stimulated with CD3/CD28 beads for 24 or 48 hours at a ratio (cells:beads) of 1:2 (blue), 1:4 (purple) or 1:8 (green) and compared to control (black) and effect on $CD4^+$ (a.), $CD8^+$ (b.) and $CD19^+$ lymphocyte (c.) viability (i.), IFN-γ production (ii.) and proliferation (iii., Measured using carboxyfluorescein succinimidyl ester (CFSE)). Data displayed as % of cell population or proliferation index. Dots represent individual volunteers, horizontal line the median, and whisker the range.

3.2.4 SAR-CoV-2 model development

3.2.4.1 24 hour model

I initially trialled a PBMC model. Healthy volunteer PBMCs were incubated with SARS-CoV-2 S1 and S2 spike protein at three concentrations (2.5, 5, and 15μg/ml) for 18 hours at 37◦C, 5% CO_2. LPS (100ng/ml) was used as a positive control, and unstimulated for a negative control. Golgistop (BD) was then added to all samples to inhibit cytokine release before the cells were incubated for a further 6 hours before undergoing flow cytometry staining and analysis.

Whilst the addition of LPS increased monocyte HLA-DR expression and IL-10 intracellular concentration, no changes were seen with SARS-CoV-2. (Figure 3.4)

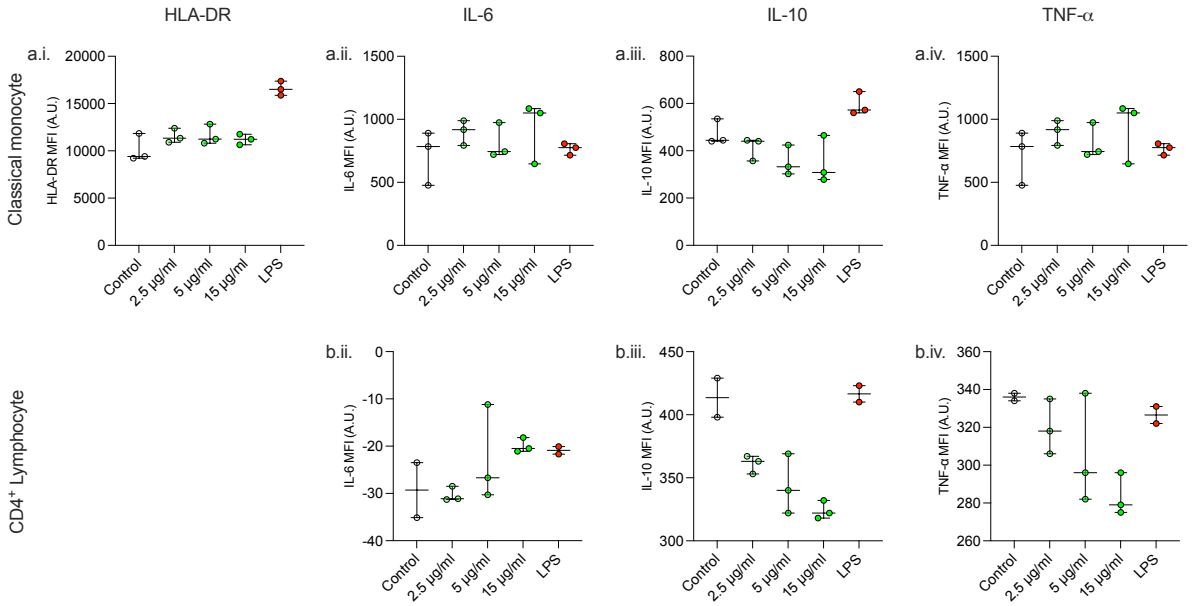

Figure 3.4: Dose titration effect of SARS-CoV-2 protein on classical monocytes and CD4⁺ lymphocytes

Volunteer (n=3) PBMCs were incubated for 24 hours with three concentrations of SARS-CoV-2 spike protein (2.5, 5 and 15µg/ml, green) and LPS (red) as a positive control and effect on classical monocytes (a.) and CD4⁺ lymphocyte (b.) HLA-DR expression (i.), IL-6 (ii.), IL-10 (iii.) and TNF-α (iv.) concentration compared to control (white). Data displayed as median fluorescence intensity (MFI). Dots represent individual volunteers, horizontal line the median, and whisker the range.

3.2.4.2 6 hour model

Given the lack of effect seen at 24 hours, in case this was due to a time dependant effect, I repeated the experiment using the same concentrations of SARS-CoV-2 protein over 6 hours in both a PBMC and whole blood model.

This demonstrated a potential dose dependant effect at 6 hours although there was no apparent difference between the whole blood or PBMC model. (Figure 3.5)

3.2.4.3 Effect of Golgistop

To identify an optimal dose at the 6 hour incubation, I repeated the experiment with higher dose SARS-CoV-2 (5 and 15µg/ml) and I saved the post-stimulation supernatant and used ELISA to assess whether released cytokines could be measured despite the addition of Golgistop. This acts by inhibiting protein transport, therefore would prevent the release of cytokines.

The higher dose (15µg/ml) gave a greater response and consistency was better between samples in the PBMC model thus these were taken forwards. (Figure 3.6) Golgistop did appear to impair release of cytokines in the PBMC media SARS-CoV-2 stimulated samples, therefore I did not use Golgistop in the subsequent model. (Figure 3.7)

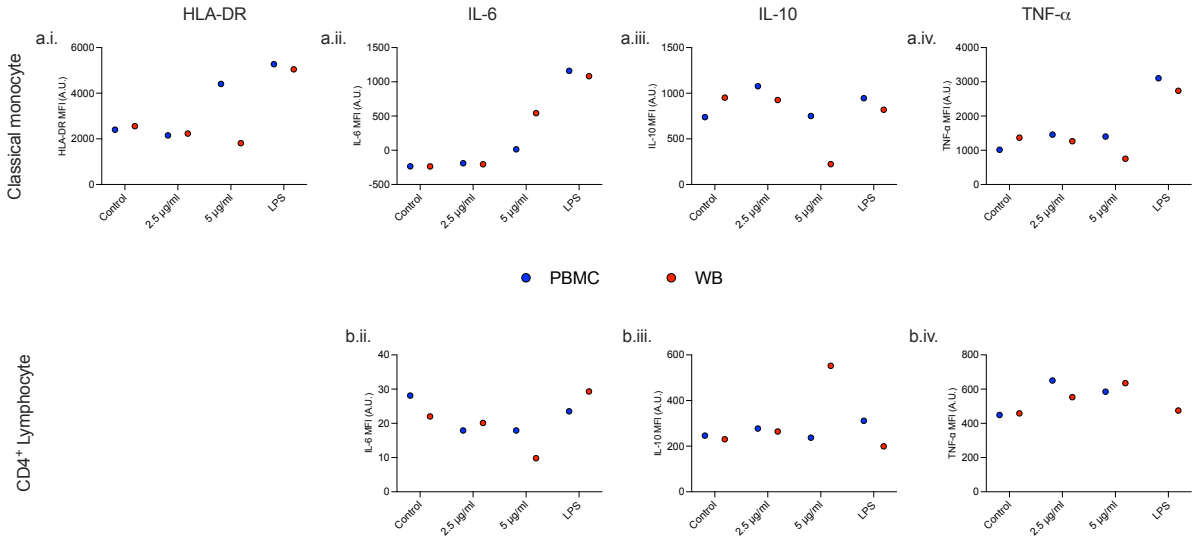

Figure 3.5: Dose titration effect of SARS-CoV-2 protein on classical monocyte and CD4+ lymphocytes at 6 hours

Volunteer (n=3) PBMCs or whole blood was incubated with 2 concentrations of SARS-CoV-2 spike protein (2.5 and 5µg/ml)) and LPS as a positive control and effect on classical monocytes (a.) and $CD4^+$ lymphocyte (b.) HLA-DR expression (i.), IL-6 (ii.), IL-10 (iii.) and TNF-α (iv.) concentration. Data displayed as median fluorescence intensity (MFI). Dots represent median of the three volunteers.

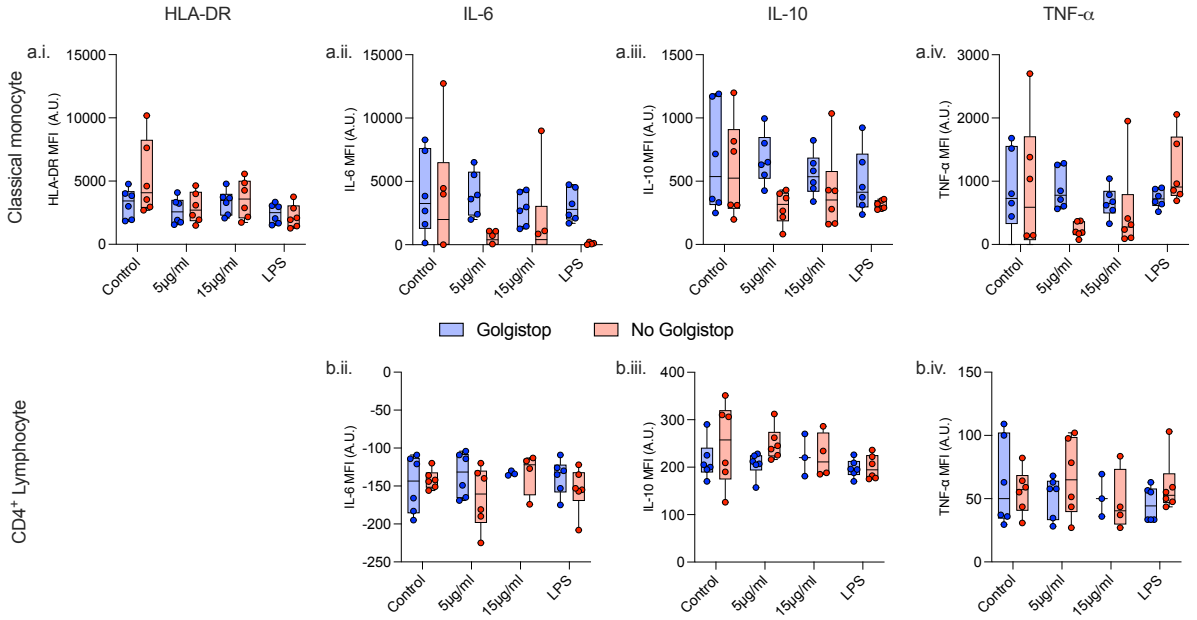

Figure 3.6: Dose titration effect of SARS-CoV-2 protein on monocytes and lymphocytes at 6 hours with and without Golgistop

Volunteer (n=6) PBMCs were incubated with SARS-CoV-2 spike protein (5 and 15µg/ml) and LPS as a positive control in the presence (blue) or absence (red) of Golgistop and the effect on classical monocytes (a.) and $CD4^+$ lymphocyte (b.) HLA-DR expression (i.), IL-6 (ii.), IL-10 (iii.) and TNF-α (iv.) concentration assessed. Data displayed as median fluorescence intensity (MFI). Dots represent individual volunteers, horizontal line the median, box the interquartile range, and whisker the range.

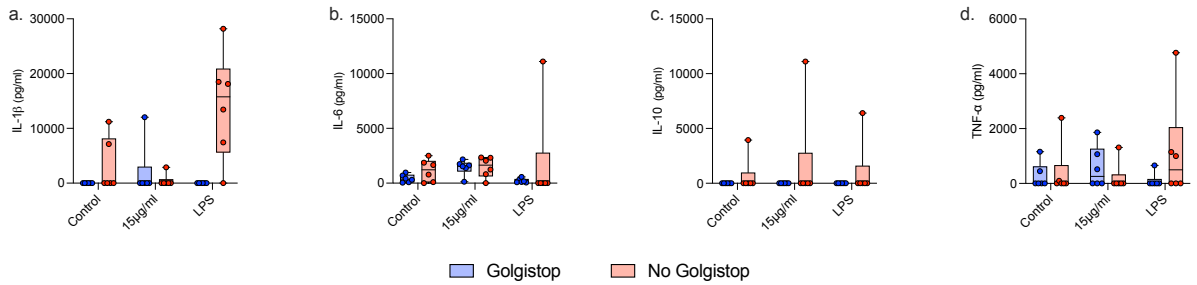

Figure 3.7: Cytokine release was impaired by the co-incubation of Golgistop
Volunteer (n=6) PBMCs were incubated with SARS-CoV-2 spike protein (15μg/ml) and LPS as a positive control in the presence (blue) or absence (red) of Golgistop and the released cytokines including IL-1ß (a.), IL-6 (b.), IL-10 (c.) and TNF-α (d.) in the released supernatant. Data displayed as concentration calculated from a standard curve. Dots represent individual volunteers, horizontal line the median, and whisker the range.

3.3 Enzyme-linked immunosorbent assay validity

Standard curves were generated using the included standard, diluted as per manufacturer's instructions and interpolated using GraphPad Prism. (Figure 3.8) Standards and samples were analysed in duplicates. Co-efficient of variation between each sample in the duplicate was <10%.

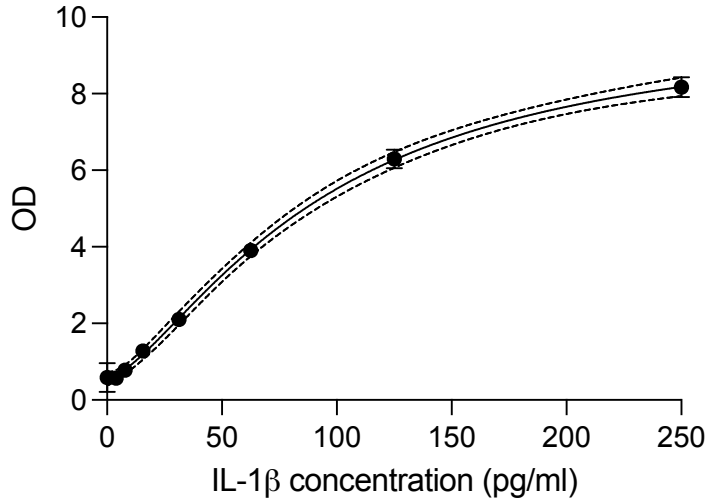

Figure 3.8: Example standard curve of IL-1β concentration
An 8-point standard curve was generated from the supplied standard as per manufacturer instructions. Samples were run in duplicate. Data presented as optical density. Dots represent the median value, and whisker the range.

3.4 Electrochemiluminescent immunoassay

Standard curves were generated using the included standard, diluted as per manufacturer's instructions and interpolated using the MSD Discovery Software. (Figure 3.9) Standards and samples were analysed in duplicates. Co-efficient of variation between each sample in the duplicate was <10%.

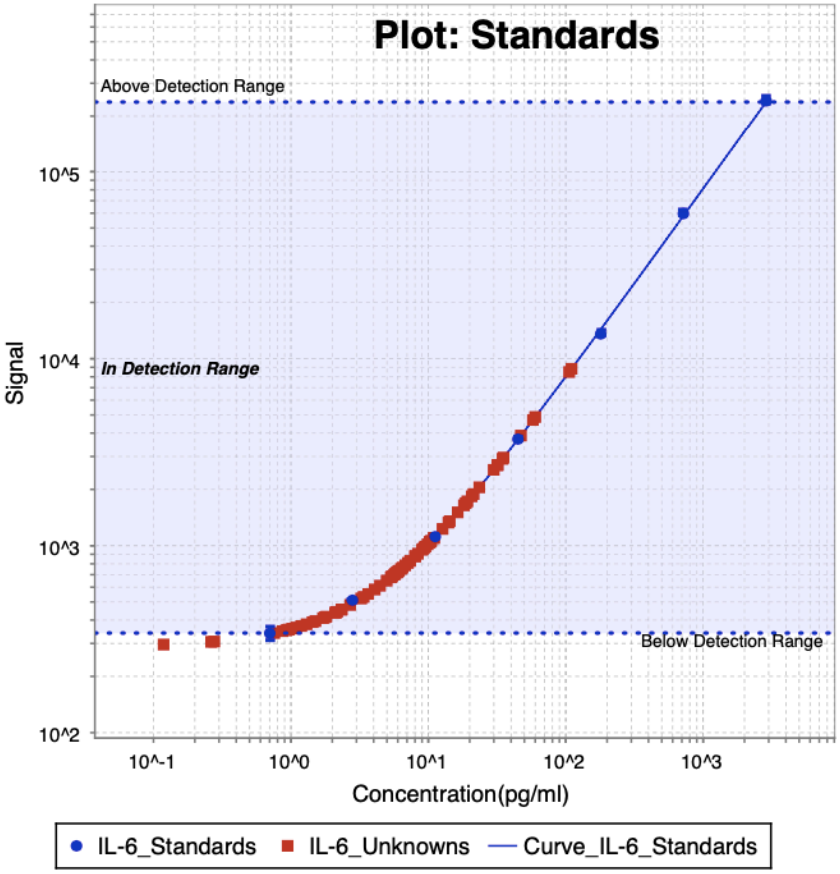

Figure 3.9: Example standard curve of IL-6 concentration
An 8-point standard curve was generated from the supplied calibrator standard(s) (blue) as per manufacturer instructions and used to interrogate the samples (red). Samples were run in duplicate. Data presented as measured luminescence signal. Blue dots represent the median value, and whisker the range of each standard, red dots represent each sample.

3.5 Flow cytometry

3.5.1 Dose titration of cell surface marker antibodies

To ascertain the optimal concentration of fluorochrome labelled cell surface marker antibodies, healthy volunteer PBMCs (n=3) were incubated for 30 minutes with 5 different dilutions of fluorochrome (250µl of PBS:antibody solution was used per sample with the antibody solution diluted volume/volume in PBS either 1:20, 100, 250, 500 or 1000) and analysed by flow cytometry. A dilution of 1:250 was adequate for both classical monocytes and $CD4^+$ lymphocytes. (Figure 3.10)

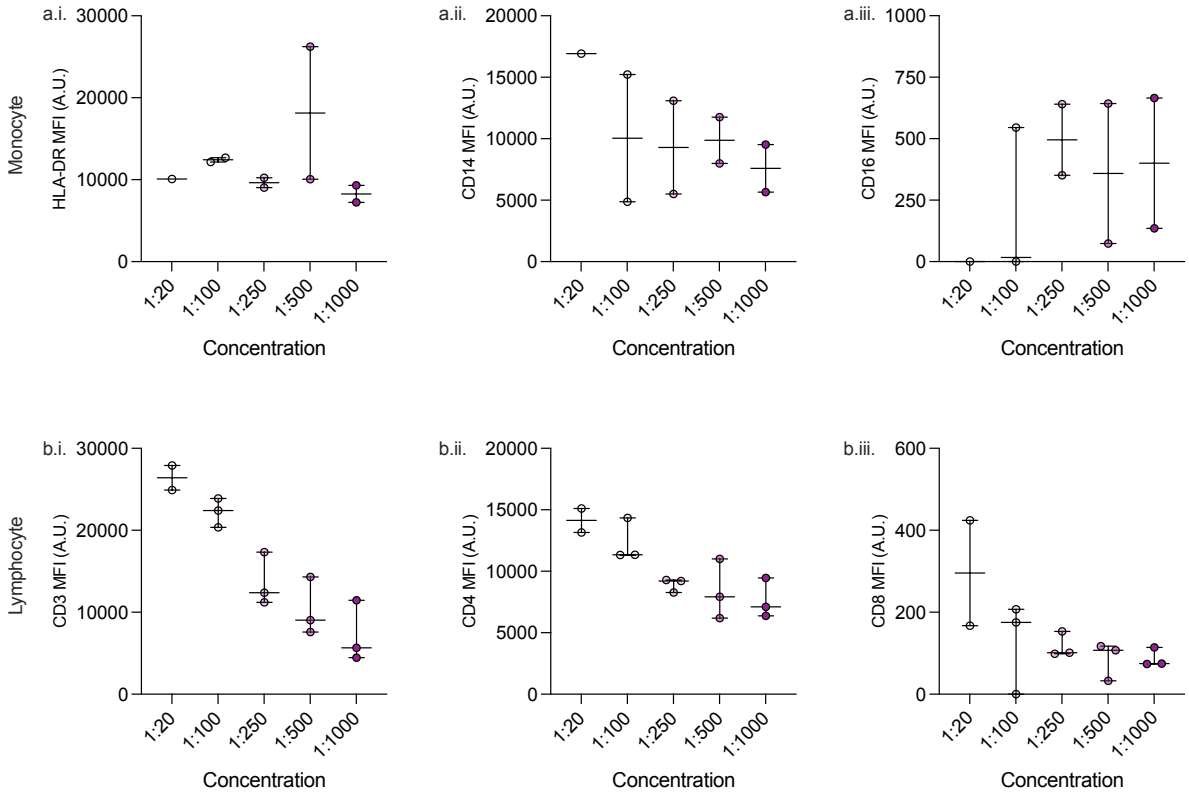

Figure 3.10: Dose titration to identify suitable concentration of cell surface marker antibody labelled fluorochromes

Healthy volunteer PBMCS (n=3) were incubated with monocyte (HLA-DR, a.i. CD14, a.ii. and CD16, a.iii.) and lymphocyte (CD3, b.i. CD4, b.ii. and CD8, b.iii.) cell surface markers resuspended in PBS for 30 minutes at different dilutions (1:20-1000). Data displayed as median fluorescence intensity (MFI). Dots represent individual volunteers, horizontal line the median, and whisker the range.

3.5.2 Dose titration of pHRodo phagocytosis bioparticles

To identify the optimal concentration of pHrodo labelled bioparticles used to measure phagocytosis, healthy volunteer PBMCs (n=2) were incubated for 1 hour with seven different concentrations of bioparticles. In addition, to investigate the effect of prolonged co-culture I incubated the PBMCs with the highest concentration for 6 hours. A concentration of 100 μg/ml for 1 hour gave best phagocytosis by classical monocytes. (Figure 3.11)

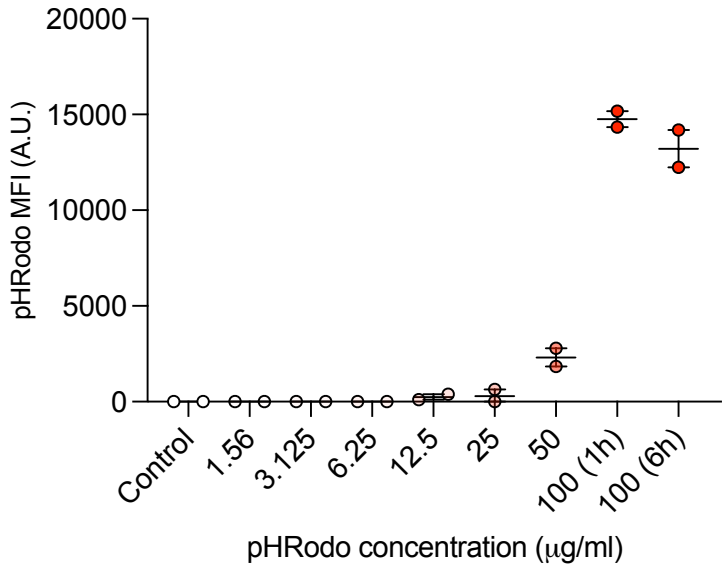

Figure 3.11: Dose titration to identify optimal concentration of pHRodo phagocytosis bioparticles

Healthy volunteer PBMCs (n=2) were incubated with pHRodo at increasing concentrations (1.56-100 μg/ml) for 1 hour and at 100 μg/ml for 6 hours and effective phagocytosis by classical monocytes measured. Data displayed as median fluorescence intensity (MFI). Dots represent individual volunteers, horizontal line the median, and whisker the range.

3.5.3 Intra-person variability

To assess whether flow cytometry experiments could be performed in singlets or duplicates, healthy volunteer PBMCs (n=2) were incubated with and without heat-killed E. *coli* for 6 hours in triplicate. Cells were labelled with cell surface markers, fixed and permeabilised and then incubated with intracellular cytokines. Mean CV values for the 6 measured variables for control samples was 10% and for HKB- stimulated samples 14%. (Figure 3.12) Samples were therefore run as singlets for future experiments.

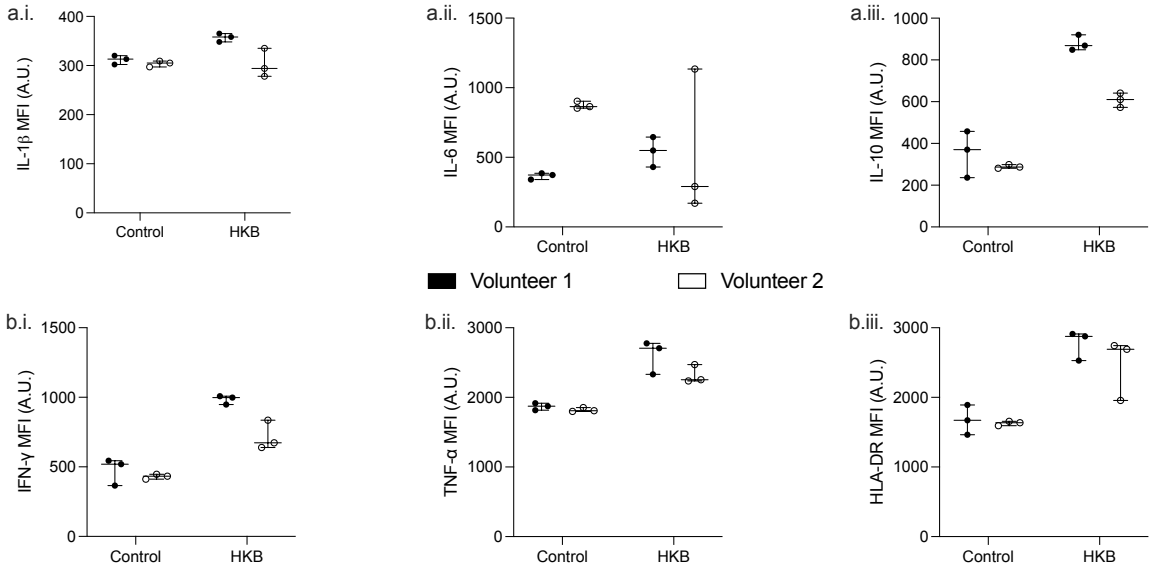

Figure 3.12: Assessment of intra-person variability on flow cytometry measured variables
Healthy volunteer PBMCs (n=2, black and white, in triplicate) were incubated with or without heat-killed E. *coli* (HKB) for 6 hours and intracellular cytokine concentration measured using flow cytometry. Data displayed as median fluorescence intensity (MFI). Dots represent individual replicate, horizontal line the median, and whisker the range.

3.5.4 Flow cytometry gating strategy

The following gating strategy was used to identify cell populations of interest. (Figure 3.13) Populations were guided using frequency-minus ones. (Figure 3.14)

- Monocytes: Cells, single cells, live-HLA-DR$^+$ cells, CD14/CD16 subset differentiation
- Lymphocytes: Cells, single cells, live cells, CD3$^+$ or CD19$^+$, CD4$^+$ or CD8$^+$ T-cell differentiation

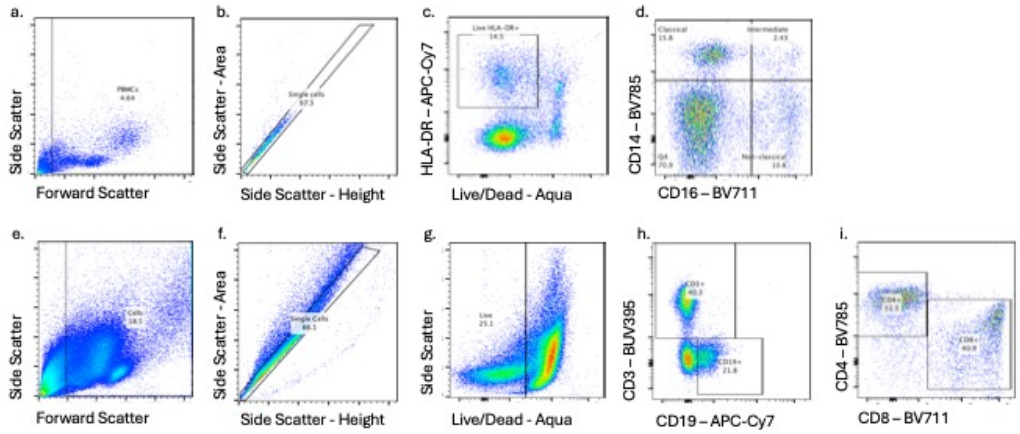

Figure 3.13: Monocyte and lymphocyte gating strategy
Monocytes: a. Peripheral blood mononuclear cells; b. Single cells; c. Live HLA-DR +ve cells; d. Monocyte subset differentiation (CD14 and CD16)
Lymphocytes: e. Lymphocytes; f. Single cells; g. Live cells; h. CD3$^+$ or CD19$^+$; i. T-cell differentiation (CD4$^+$ or CD8$^+$)

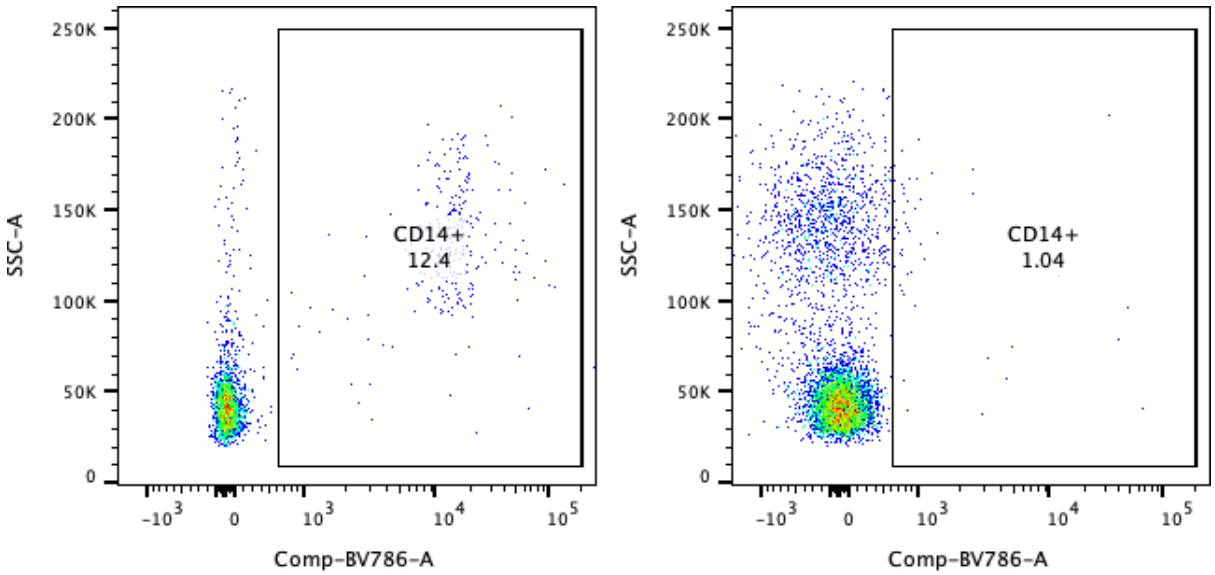

Figure 3.14: Example plot of use of a fluorescence minus-one gating strategy to guide placement of gates for % positive cells

In these dot plots, each dot represents an event (or cell) with changes in colour representing the number of events measured at the given wavelength (blue indicating single events and red many events). The CD14 gate was drawn based on comparing the fully stained sample (left) with the fluorescence minus-one (stained with all markers except CD14, right).

3.6 Spectral Flow cytometry

3.6.1 Panel design

Relevant cell surface marker, intracellular cytokines, intracellular proteins, and transcription factors were identified through literature review. Once candidate markers were identified, the panel was initially designed with the use of online panel design tools including, EasyPanel 2 (Paris, France), Fluorofinder (Broomfield, Colorado) and Flow Panel Builder (Biocompare, San Francisco, California). Additional optimisations were then further performed by our facility manager, Jamie Evans and Sony field application specialist Karim Boustani.

3.6.2 Fixation-permeabilisation buffer comparison

Because the chosen panel included a mixture of intracellular cytokines, proteins, and transcription factors, all of which have different recommended fixation-permeabilisation buffers. To ascertain which was the most optimal to use, the median fluorescent intensity of intracellular proteins and transcription factors were compared with 3 different buffers: CytoFix/Perm (BD), Phosphoflow (BD) and True-Nuclear (BL) buffers. The True-Nuclear buffer was chosen for use subsequently. (Figure 3.15)

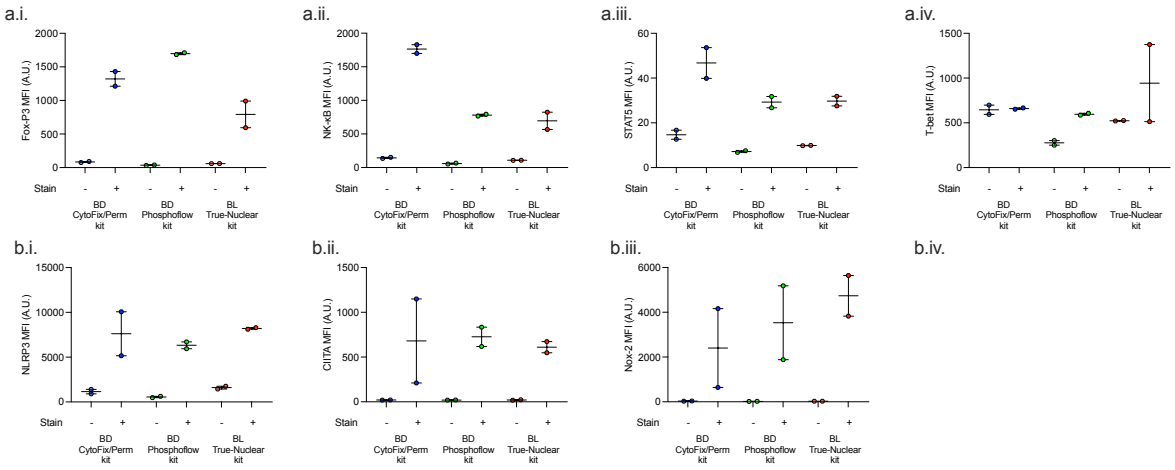

Figure 3.15: The True-Nuclear kit had better overall performance for measurement of intracellular proteins and transcription factors

The median florescent intensity of Fox-P3 (a.i.), NF-κB (a.ii.), STAT5 (a.iii.), T-bet (a.iv.), NLRP3 (b.i.), CIITA (b.ii.), and Nox-2 (b.iii.) (all at 1:100 concentration) was measured after preparation of healthy volunteer PBMCs (n=2) using either the Cytofix/perm kit (BD), Phosphoflow kit (BD), and True-nuclear kit (BL). The true-nuclear kit had better overall staining and was used. Dots represent individual volunteers, horizontal line the median, and whisker the range.

3.6.3 Panel marker dose titration

To identify the best dilution of individual markers to use in the panel, a comparison of 4 different dilution was performed and differences in MFI used. A dilution of 1:250 was acceptable for cell surface markers and 1:1000 for live-dead stain for a 30 minute staining period, and 1:100 for intracellular cytokines, proteins and transcription for the 40 minute staining period. (Figure 3.16)

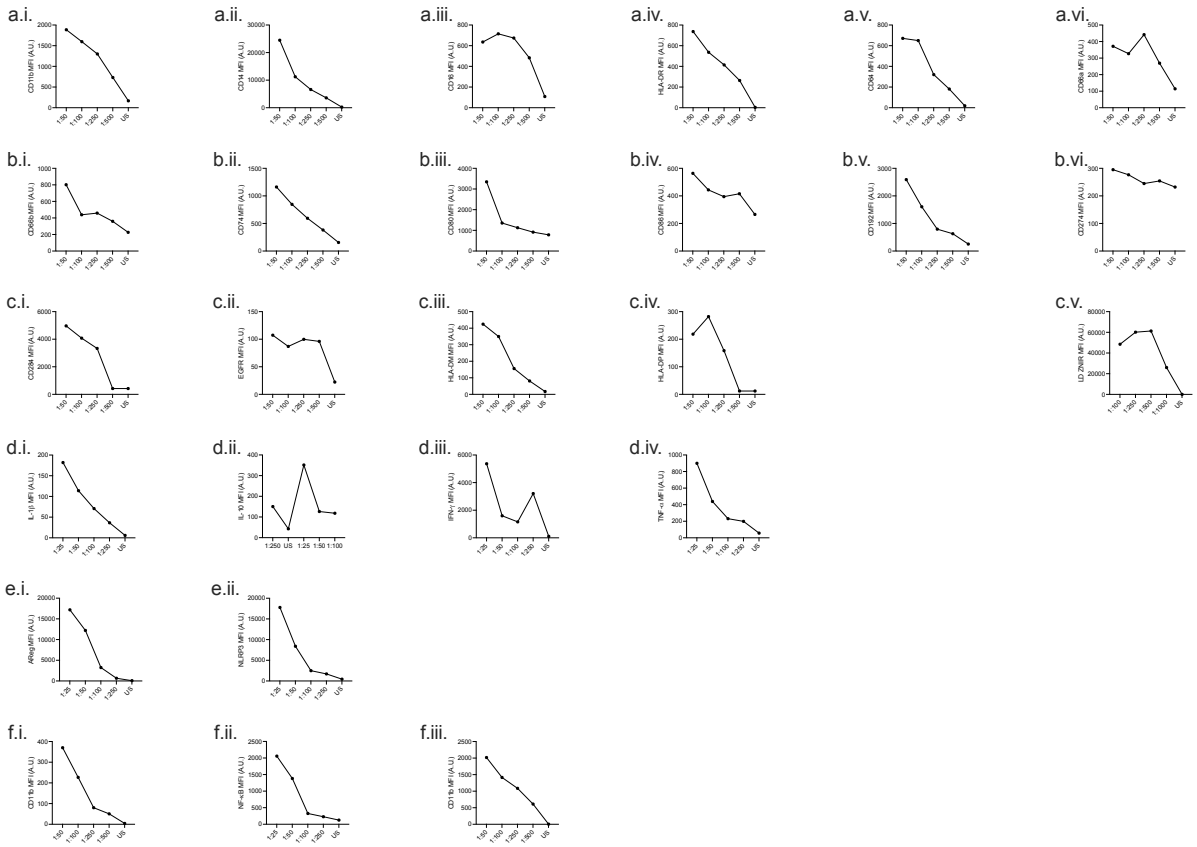

Figure 3.16: Dose titrations identified optimal dilutions to use for cell staining
Healthy volunteer PBMCs (n=1) were incubated with 4 different dilutions of fluorochromes, for cell surface markers (a-c.iv.) 1:50, 1:100, 1:250 and 1:500 was used, for live-dead (c.v.)1:100, 1:250, 1:500 and 1:1000, and for intracellular cytokines (d.), proteins (e.) or transcription factors (f.), 1:25, 1:50, 1:100 and 1:200.

3.6.4 Panel marker combination assessment

Given the number of fluorochromes used in the panel, bound to a multitude of different antibodies from different animal species and the proximity on the cell surface of some co-expressed markers (e.g. CD80 and HLA-DR), there was a risk that one or more of my fluorochromes would not be compatible in the panel. To test this, I divided my fluorochromes into the following three panels:

- Panel 1 – CD11b, HLA-DR, CD66b, CD86, CD284, HLA-DP, IL-10, NLRP3
- Panel 2 – CD14, CD64, CD74, CD192, Live-dead, IFN-γ, CIITA, Nox-2
- Panel 3 – CD16, CD66a, CD80, CD274, HLA-DM, IL-1β, TNF-α, NF-κB

I then stained volunteer PBMCs with each fluorochrome as a single stain and compared the MFI to PBMCs stained with the panels stained separately and in combination with each other. Splitting the panel in this way would allow me to identify the cause of any possible interactions faster. (Figure 3.17) As an example, the MFI of CD14 was approximately 7000-10000 in the single stain, panel 2 (in which it was included), combined panels 1+2 and 2+3, and fully combined panels 1+2+3. The MFI was 0 in

the panels in which it was not included, panel 1, panel 3, and combined panels 1+3. This confirmed that CD14 was compatible with the panel.

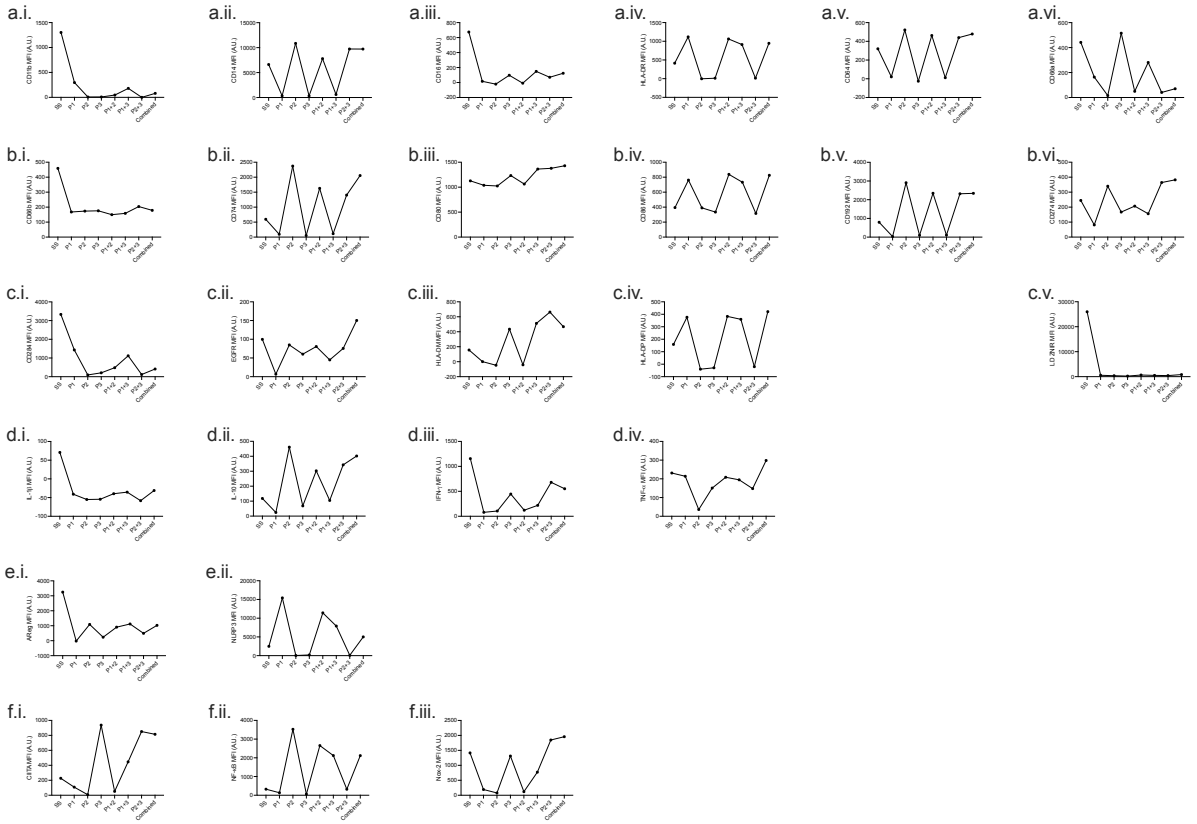

Figure 3.17: Different combinations of fluorochromes were compared to confirm compatibility of the spectral flow cytometry panel
Healthy volunteer PBMCs (n=1) were incubated with 3 different combinations of fluorochromes (panel 1; P1, panel 2; P2 and panel 3; P3), alone and in combination and the MFI in each panel was compared to the single stain value (SS).

3.7 High-performance liquid chromatography

The concentration of antibiotics over 5 days was characterised using high-performance liquid chromatography (HPLC). Due to the drop in measured meropenem concentrations over the course of the experiment, levels were topped up after 48 hours. (Figure 3.18)

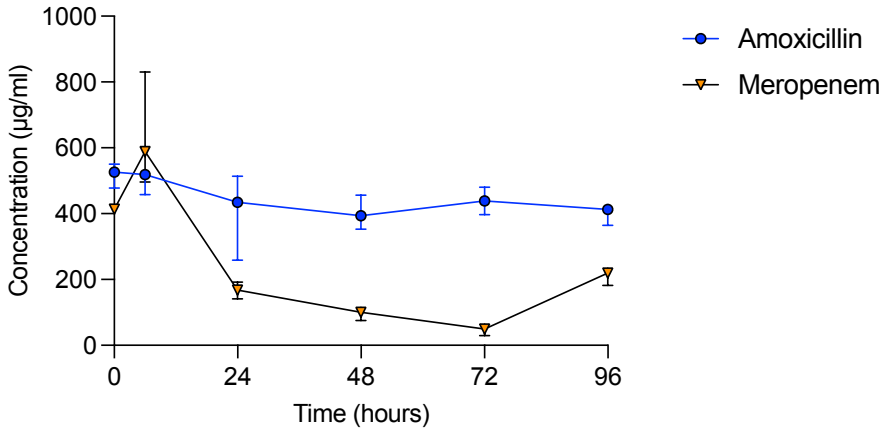

Figure 3.18: Stability of antibiotics over 96 hours in PBS

Amoxicillin (blue) and meropenem (orange) were diluted in phosphate buffered saline (PBS) (n=3) and the concentration measured by high-performance liquid chromatography at 6, 24, 48, 72, and 96 hour timepoints. Dots represent the mean value of the 3 samples and whisker the range.

4 Immune responses to infection and sepsis

4.1 Chapter context

The cause of sepsis-induced immunosuppression is multifactorial and includes inadvertent effects of routinely administered medications. The ability of antibiotics to modulate the immune system is well-recognised, although poorly characterised in sepsis. Paradoxically, antibiotics may weaken the patient's immune system, increasing the risk of recurrent or secondary infections. Additionally, patients administered antibiotics unnecessarily for non-bacterial infections or non-infectious conditions may suffer from unintended harm. This may be mediated via direct effect on immune cells or indirectly via alterations of the microbiome.

Sepsis research places significant emphasis on the interaction between the host immune system and pathogenic bacteria, and the interaction between antibiotics and pathogenic bacteria. However, the interaction between antibiotics and the host immune system has been largely neglected.

4.2 Introduction

Overuse of antibiotics is associated with numerous overt consequences to the individual patient, including side-effects such as hepatotoxicity, nephrotoxicity and bone marrow dyscrasias, and many covert effects that pass unrecognised in clinical practice including reductions in microbiome diversity, and impairment of immune and bioenergetic function. At a societal level, the rise of antimicrobial resistance is a particular concern. These consequences are especially pertinent to critically ill patients admitted to the Intensive Care Unit (ICU) in whom antibiotic use is high.

Patients in ICU are at increased risk of infection due to a multitude of factors, ranging from proximity of harmful pathogens, breaches of normal protective skin or mucosal barriers, and critical illness-associated immunosuppression.[24] Immunosuppression associated with sepsis may be related to the underlying disease (e.g. sepsis, trauma, or surgery) and may be exacerbated by other factors including medications. There is a growing body of evidence demonstrating the immunomodulating effects of antibiotics. However, data on the immunomodulatory effects of beta-lactam antibiotics in septic patients are limited, despite these being the most commonly used antibiotics given to one of the highest risk hospital populations.

Two well-characterised features of sepsis-induced immunosuppression include monocyte HLA-DR expression and lymphopenia.[1] I first characterised the immunosuppressive phenotype of PBMCs isolated from infected patients with increasing illness severity ranging from uncomplicated infection

through to septic shock. Having identified the immunosuppressive phenotypes, I then conducted an in-depth *ex vivo* analysis of the effect of two narrow-spectrum and two broad-spectrum beta-lactam antibiotics on features of sepsis-induced immunosuppression in PBMCs from patients presenting to the emergency department with acute infection.

4.3 Methods Summary

I performed a prospective cohort study of adult patients (>17 years of age) presenting with suspected infection either to the Emergency Department or ICU. Suspected infection was defined as clinical need for a blood culture. Patients were excluded if they had severe anaemia (Hb<60/dl) with a contraindication to transfusion, unable to gain consent or agreement, treated with a palliative intent or the blood culture was performed for screening or monitoring. Presence of infection was adjudicated by two independent examiners with disagreements resolved by a third.

At time of culture, and in the ICU cohort on days one and five, peripheral blood mononuclear cells (PBMCs) were isolated, stored and analysed in batches using flow cytometry. Serum and serum isolated following whole blood in vitro LPS-stimulation was stored for batch analysis by ELISA and multiplex to identify cytokines and other biomarkers. Relevant laboratory, clinical and outcome data was also stored and combined with experimental results at study completion.

PBMCs were defrosted and stained for immunophenotyping, or underwent LPS-stimulation for 24 hours or with CD3/CD28 beads for 72 hours with and without beta-lactam antibiotics (amoxicillin, cefuroxime, meropenem, and piperacillin) prior to staining.

4.4 Results

4.4.1 Immune function in mild infection and sepsis

4.4.1.1 Clinical demographics

One hundred and eleven patients and 20 healthy volunteers were enrolled to provide blood samples. Patients included 57 adjudicated to have infection who attended the Emergency Department (ED), of whom 29 (32%) were discharged home and 28 (30%) were admitted to hospital for general ward level care. A further 35 patients (38%) had been admitted to the ICU, of whom 15 (43%) subsequently died in hospital. Control groups consisted of 19 ED non-infected patients (age matched controls) and the healthy volunteers.

Patients discharged home from the ED were younger and had lower CRP values ($p<0.001$) than those ED patients admitted to the hospital or in the ICU. Monocyte, lymphocyte, and neutrophil count were similar between patients. (Table 4.1)

4.4.1.2 Monocyte antigen presentation and T lymphocyte activation

Increasing illness severity was significantly associated with lower monocyte HLA-DR. ICU patients had a lower monocyte HLA-DR MFI compared to ED patients admitted to hospital ($p<0.05$), discharged ED patients, age matched ED controls, and healthy volunteers (all $p<0.001$) who had similar levels of HLA-DR MFI. Similarly, $CD86^+$ MFI on monocytes was lower in ICU patients compared to hospitalised ED patients, discharged ED patients (both $p<0.001$), age-matched non-infected ED controls ($p<0.01$), and healthy volunteers ($p<0.05$). CD86 MFI was similar between the other groups. CD80 MFI was higher in ICU patients compared to all groups ($p<0.05$) except age-matched controls. (Figure 4.1)

$CD4^+$ and $CD8^+$ lymphocyte CTLA-4 MFI was similar between groups. ICU patients had higher $CD4^+$ lymphocyte CD28 MFI compared to hospitalised patients and those discharged home (both $p<0.05$). (Figure 4.2)

A principal component analysis (PCA) was conducted in 59 individuals for whom full datasets were available including age, CRP and the 12 monocyte variables (HLA-DR, CD80, CD86, CCR2, CXCR4, PD-L1, pHRodo, IL-1β, IL-10, and TNF-α). PCA provided separation between ICU patients and volunteers. ED patients discharged home had an overlapping phenotype with volunteers, while admitted ED patients had an overlapping phenotype with ED patients discharged home and ICU patients. Monocyte HLA-DR (loading vector coefficient of 0.77), followed by co-stimulatory molecule CD86 had the greatest discrimination between patients and were responsible for 39% of the cumulative proportion of variance. Monocyte HLA-DR expression correlated positively with CD86 ($r^2=0.48$, $p<0.0001$) and phagocytic capacity (pHrodo) ($r^2=0.13$, $p<0.01$). (Figure 4.3)

	Volunteers (n=20)	Age Matched Controls (n=19)	Discharged from ED (n=29)	Hospitalised (n=28)	ICU (n=35)
Age (years)	37 (31-42)	53 (27-69)	31 (23-46)	58 (50-79)	55 (45-74)
Sex (male) n(%)	15 (88%)	9 (53%)	10 (48%)	21 (57%)	19 (66%)
Ethnicity n(%)					
Asian	6 (35%)	3 (18%)	3 (14%)	1 (3%)	5 (17%)
Black	1 (6%)	1 (6%)	1 (5%)	3 (8%)	4 (14%)
Other/Not Stated	0	3 (18%)	6 (29%)	7 (19%)	0
White	10 (59%)	10 (59%)	11 (52%)	26 (70%)	20 (69%)
Co-morbidities n(%)					
Diabetes	-	0	2 (10%)	8 (22%)	5 (17%)
COPD	-	3 (18%)	0	4 (11%)	4 (14%)
Heart failure	-	3 (18%)	1 (5%)	5 (14%)	2 (7%)
Ischaemic heart disease	-	0	0	3 (8%)	2 (7%)
Source of infection n(%)					
Pulmonary	-	-	8 (38%)	17 (46%)	11 (38%)
GU	-	-	4 (19%)	8 (22%)	2 (7%)
GI	-	-	0	1 (3%)	12 (41%)
ENT	-	-	8 (38%)	5 (14%)	0
Soft tissue	-	-	1 (5%)	4 (11%)	0
Bacteraemia	-	-	0	2 (5%)	3 (10%)
Other	-	-	0	0	1 (3%)
Clinical parameters					
Temperature	-	37.0 (36.4-37.9)	38.0 (36.8-39.0)	37.5 (36.7-38.5)	38.4 (38.0-38.7)
GCS	-	15 (15-15)	15 (15-15)	15 (15-15)	14 (11-15)
Respiratory rate	-	19 (18-23)	18 (16-20)	20 (18-24)	27 (23-36)
Systolic blood pressure	-	135 (123-153)	131 (116-136)	134 (118-157)	93 (85-106)
qSOFA score	-	0 (0-1)	0 (0-0)	0 (0-1)	2 (2-3)
Laboratory values					
WBC (x10^6/ml)	-	8.5 (6.8-11.4)	11.0 (8.1-15.7)	12.0 (8.0-15.9)	10.6 (6.9-16.8)
Neutrophils (x10^6/ml)	-	6.7 (4.6-9.2)	6.5 (3.9-8.9)	10.2 (6.7-12.8)	6.8 (4.0-9.5)
Lymphocytes (x10^6/ml)	-	1.0 (0.6-1.8)	0.9 (0.6-2.3)	1.0 (0.4-1.5)	0.8 (0.4-1.4)
Monocytes (x10^6/ml)	-	0.8 (0.5-1.0)	0.8 (0.5-1.2)	0.8 (0.6-1.2)	0.5 (0.2-1.1)
CRP (mg/l)	1 (1-1)	19 (8-40)	21 (8-45)	89 (39-138)	188 (54-282)
Lactate (mmol/l)	-	1.3 (0.8-2.1)	1.0 (0.7-1.3)	1.2 (0.9-1.6)	1.2 (0.8-2.0)

Table 4.1 Clinical parameters of included patients

Demographic and laboratory values for included volunteers and patients. Data presented as mean (interquartile range). Abbreviations: COPD: Chronic obstructive pulmonary disease, CRP: C-reactive protein, ED: Emergency department, ENT: Ear, nose and throat, GCS: Glasgow coma scale, GI: Gastrointestinal, GU: Genitourinary, n: number, ICU: Intensive care unit, qSOFA: quick sequential organ failure assessment score, WBC: white blood cell.

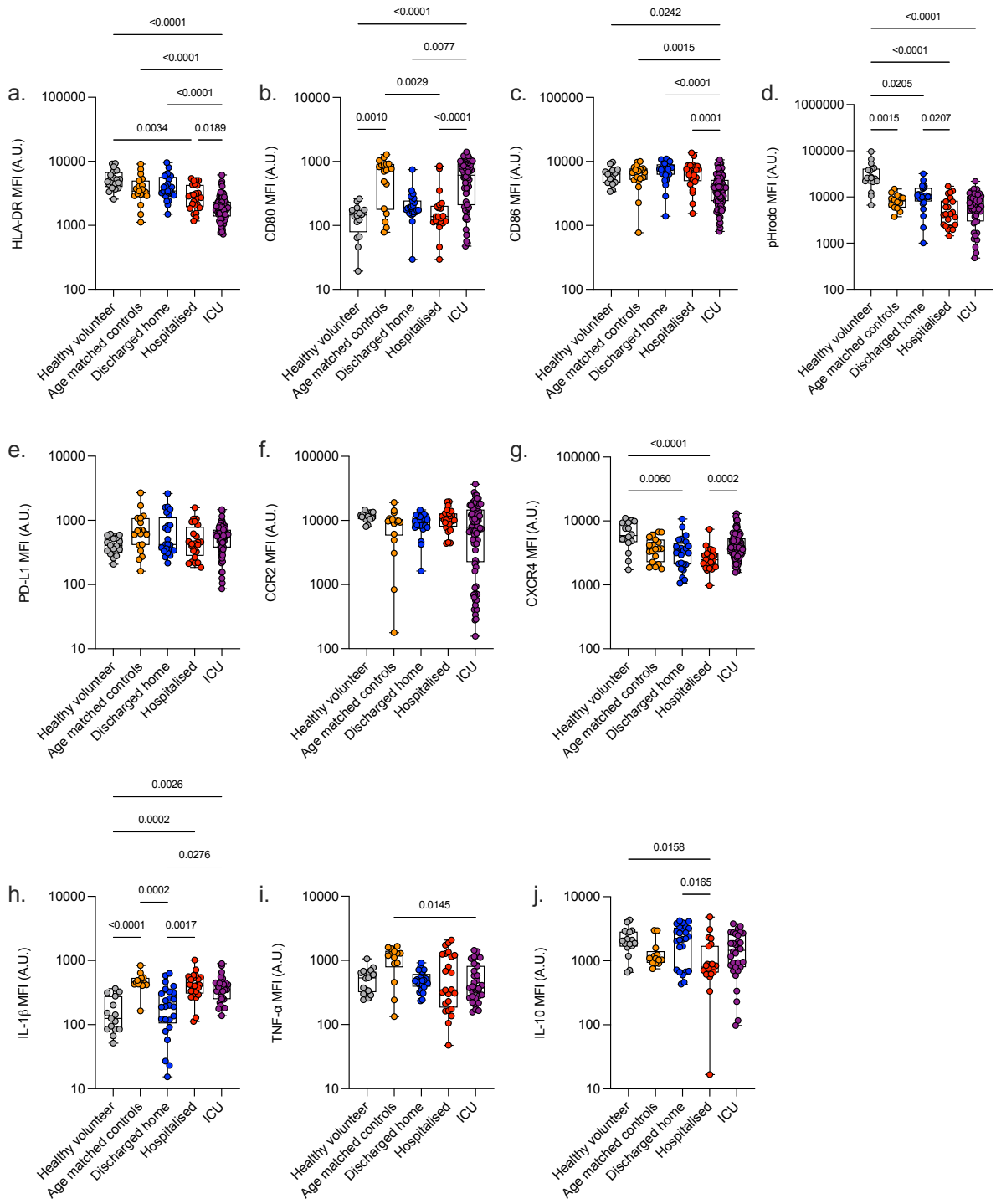

Figure 4.1: Sepsis alters classical monocyte immunophenotype

Classical monocyte immunophenotype was characterised in healthy volunteers, patients admitted to the Emergency Department either without infection (age matched controls), discharged home or hospitalised with infection, and patients admitted to the Intensive Care Unit (ICU) with sepsis. The following markers were assessed: HLA-DR (a.), CD80 (b.), CD86 (c.), pHRodo (d.), PD-L1 (e.), CCR2 (f.), CXCR4 (g.), IL-1β (h.), TNF-α (i.), and IL-10 (j.). Data expressed as median fluorescent intensity (MFI) measured in arbitrary units (A.U.). Individual patients represented as dots, horizontal line represents median, box interquartile range and whisker range. Differences between all groups were compared using a non-parametric Kruskal-Wallis test without Dunnett's correction. Only p-values <0.05 shown.

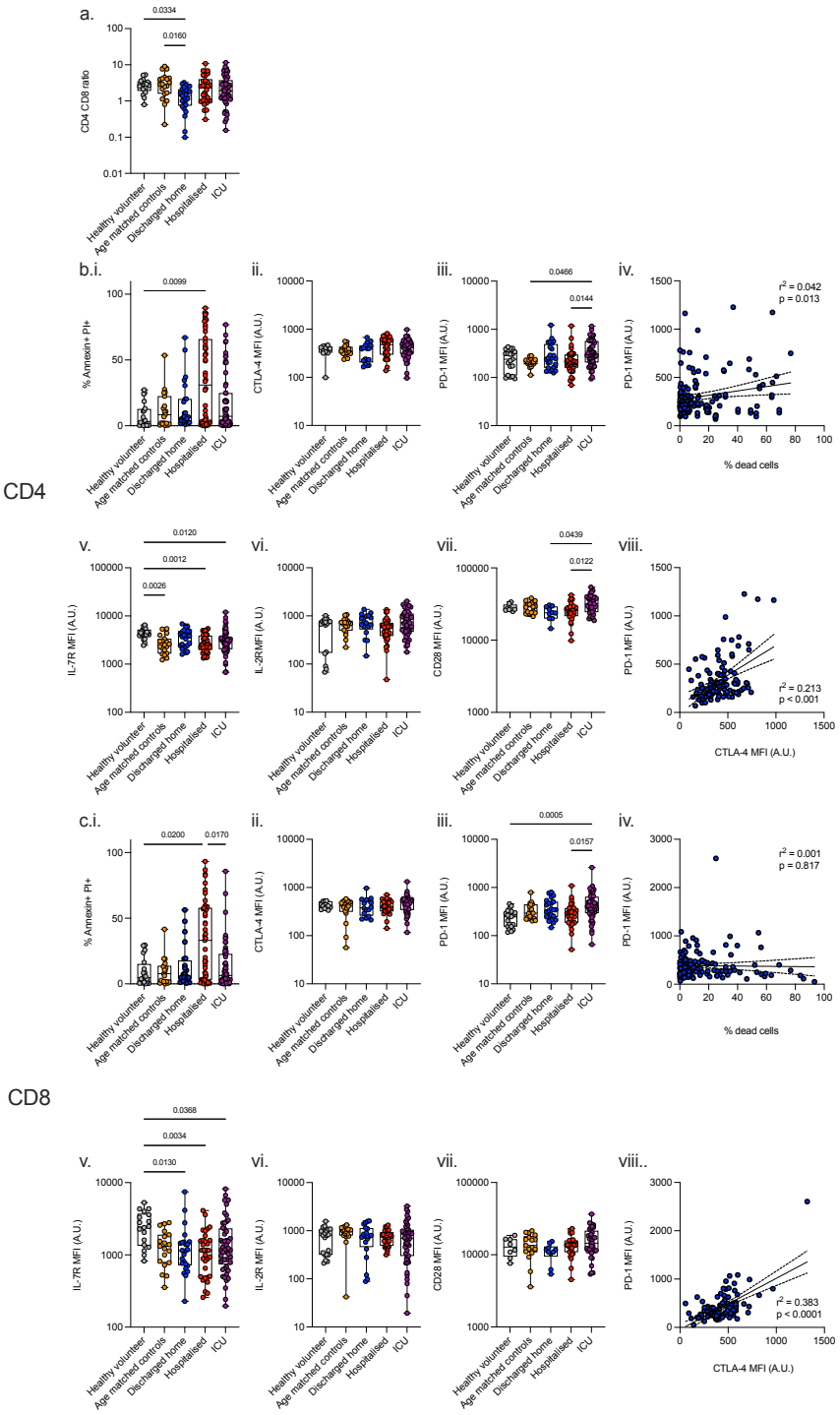

Figure 4.2: Sepsis alters the immunophenotype of CD4⁺ and CD8⁺ lymphocytes

T-cell immunophenotype was characterised in healthy volunteers, patients admitted to the Emergency Department (ED) either without infection (age matched controls), discharged home of hospitalised with infection, and patients admitted to the Intensive Care Unit (ICU) with sepsis. The following markers were assessed: CD4/CD8 ratio (a.), $CD4^+$ (b.) and $CD8^+$ (c.) Apoptosis (i.), CTLA-4 (ii.), PD-1 (iii.), correlation between PD-1 and apoptosis (iv.), IL-7R (v.), IL-2R (vi.), CD28 (vii.), correlation between PD-1 and CTLA-4 (viii.). Data expressed as median fluorescent intensity (MFI) measured in arbitrary units (A.U.), ratio, or percentage positive cells. Individual patients represented as dots, horizontal line represents median, box interquartile range and whisker range. Differences between all groups were compared using a non-parametric Kruskal-Wallis test without Dunnett's correction. Only p-values <0.05 shown. Correlation plots show all patients represented as a dot with line of best fit and confidence interval, r^2 and p-values.

4.4.1.3 Monocyte phagocytosis

Compared to healthy volunteers, monocyte phagocytosis was reduced in all other groups (all $p<0.05$). Phagocytosis was also reduced in hospitalised ED patients compared to ED discharged patients ($p<0.05$). (Figure 4.1)

4.4.1.4 Monocyte chemokine receptors

No between-group differences were seen with the chemokine receptor CCR2 MFI. Compared to healthy volunteers CXCR4 MFI was reduced in discharged ED patients ($p<0.01$) and those hospitalised ($p<0.0001$). ICU patient monocytes had higher CXCR4 MFI compared to hospitalised ED patients ($p=0.001$). (Figure 4.1) No correlation was seen between CXCR4 and CCR2. (Figure 4.3)

4.4.1.5 Programmed death receptor-1 pathway and T lymphocyte death.

There were no differences in monocyte PD-L1 MFI between groups. (Figure 4.1) $CD4^+$ lymphocyte PD-1 MFI was increased in ICU patients compared to both healthy volunteers ($p<0.05$) and ED hospitalised patients ($p<0.05$). However, only hospitalised ED patients showed increased lymphocyte cell death compared to volunteers ($p<0.05$). Near-identical effects were seen in $CD8^+$ lymphocytes although, in addition, ICU patients had reduced cell death ($p<0.01$). (Figure 4.2)

4.4.1.6 T-cell differentiation and proliferation

Compared to healthy volunteers, $CD4^+$ lymphocyte IL-7R MFI was reduced in ICU patients ($p<0.02$), hospitalised ED patients ($p<0.05$) and age-matched controls ($p<0.01$). Likewise, for $CD8^+$ lymphocytes, IL-7R MFI was reduced compared to discharged ED patients ($p<0.05$), hospitalised ED patients ($p<0.01$) and ICU patients ($p<0.05$). IL-2R MFI was unchanged in both cell types. (Figure 4.2)

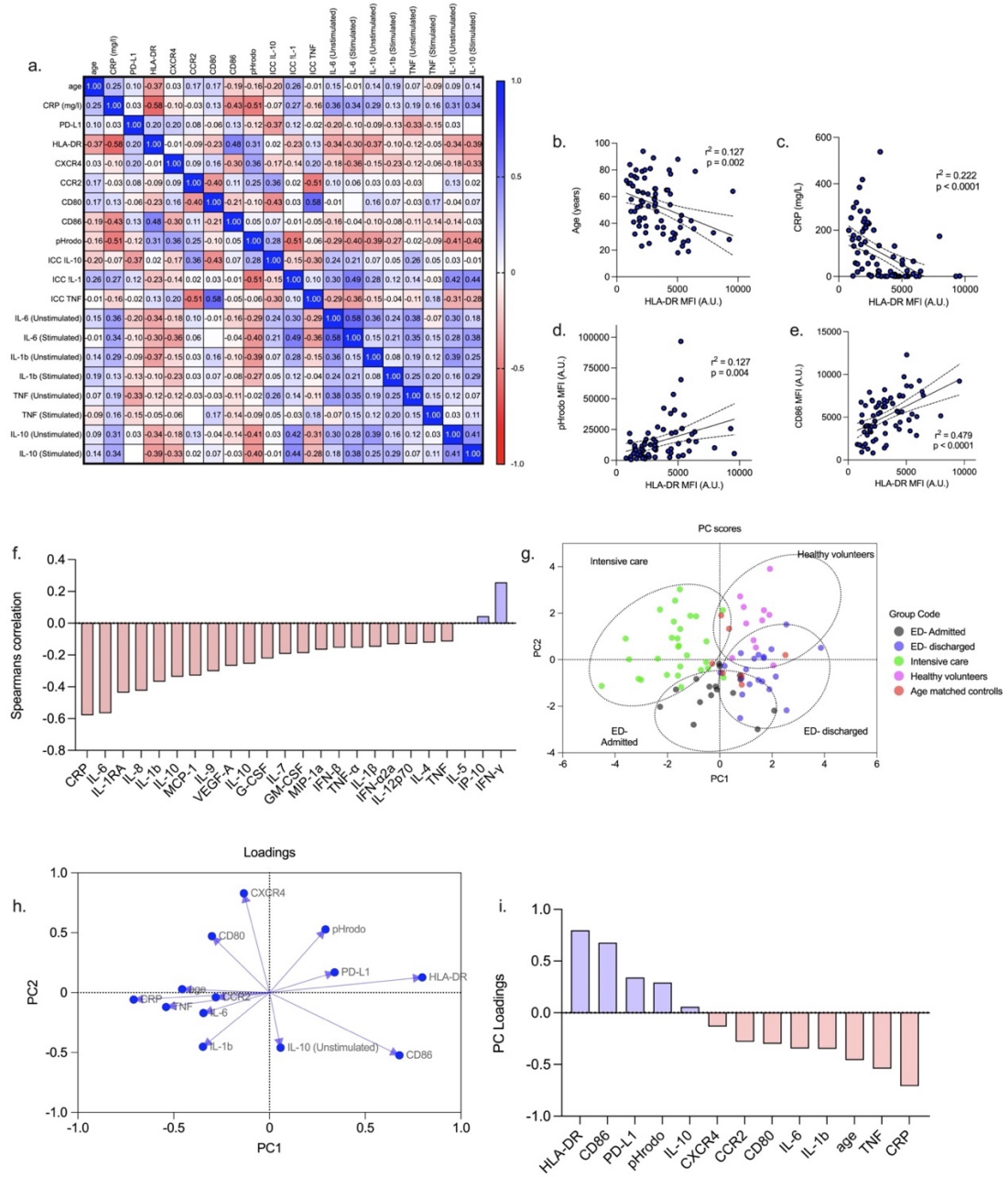

Figure 4.3: Reduced classical monocyte HLA-DR in sepsis correlates with multiple other immunophenotype markers suggestive of broad immunosuppressive defects

To further explore the effects of sepsis on immunophenotype, a **correlation** matrix (a.) was made incorporating age, CRP, 12 monocyte immunophenotype markers, serum cytokines **and** LPS-stimulated cytokines (IL-6, IL-1β, TNF-α, and IL-10). To explore factors which may be responsible for the sepsis-induced reduction in classical monocyte HLA-DR MFI, correlation plots were formed comparing HLA-DR MFI with age (b.), CRP (c.), pHRodo (d.) and CD86 (d.). Data expressed as individual patients represented as dots with line of best fit and confidence interval, r^2 and p-values. To identify possible surrogate biomarkers for monocyte HLA-DR, monocyte HLA-DR MFI was compared with other serum biomarkers (CRP, IL-6, IL-1RA, IL-8. IL-1β, IL-10, MCP-1, IL-9, VEGF-A, IL-10, G-CSF, IL-7, CM-CSF, MIP-1α, IFN-β, TNF-α, IFN-α2a, IL-12p70, IL-4, IL-5, IP-10, IFN-γ) using Spearman's correlation. Data expressed as r-value, red represents negative and blue positive correlation. Principal component analysis (12 monocyte markers, age and CRP) was performed to identify immunophenotypes. This showed separation of patients into immunophenotype clusters (g.), with loadings shown in (h.) and (i.) identifying HLA-DR and CD86 as the largest determinates.

4.4.1.7 Cytokines

Several cytokines and chemokines demonstrated an association with increasing illness severity. Inflammatory markers including IL-1RA, IL-7, G-CSF, and IP-10 were significantly higher in all patient groups but did not discriminate between patient groups, albeit some did demonstrate trends. Levels of IL-12p70, IL-8, IL-9, MIP-1α, VEGF-a, IL-6, soluble PD-L1 and CRP demonstrated an association with increasing illness severity, with ICU patients having significantly higher levels that all (or most other) patient groups. Inflammatory markers that provided the strongest association with monocyte HLA-DR were IL-8 (r^2=0.60; p<0.001) and CRP (r^2=-0.38; p<0.001). (Figure 4.4)

Cytokine (TNF-α, IL-1β and IL-10) release following whole blood LPS stimulation was assessed. Increased TNF-α and IL-10 release was seen in all groups (all p<0.05) except in ICU patients. IL-1β was only released in ED patients both discharged (p=0.01) and hospitalised (p<0.05). Following LPS stimulation, monocyte intracellular TNF-α was increased only in healthy volunteers (p<0.01), IL-1β was increased in discharged ED patients (p<0.001) and ICU patients (p<0.05), but no change was seen in IL-10 levels. There was poor correlation between serum cytokine levels and percentage positive monocytes expressing the cytokines TNF-α, IL-1β and IL-10. (Figure 4.5)

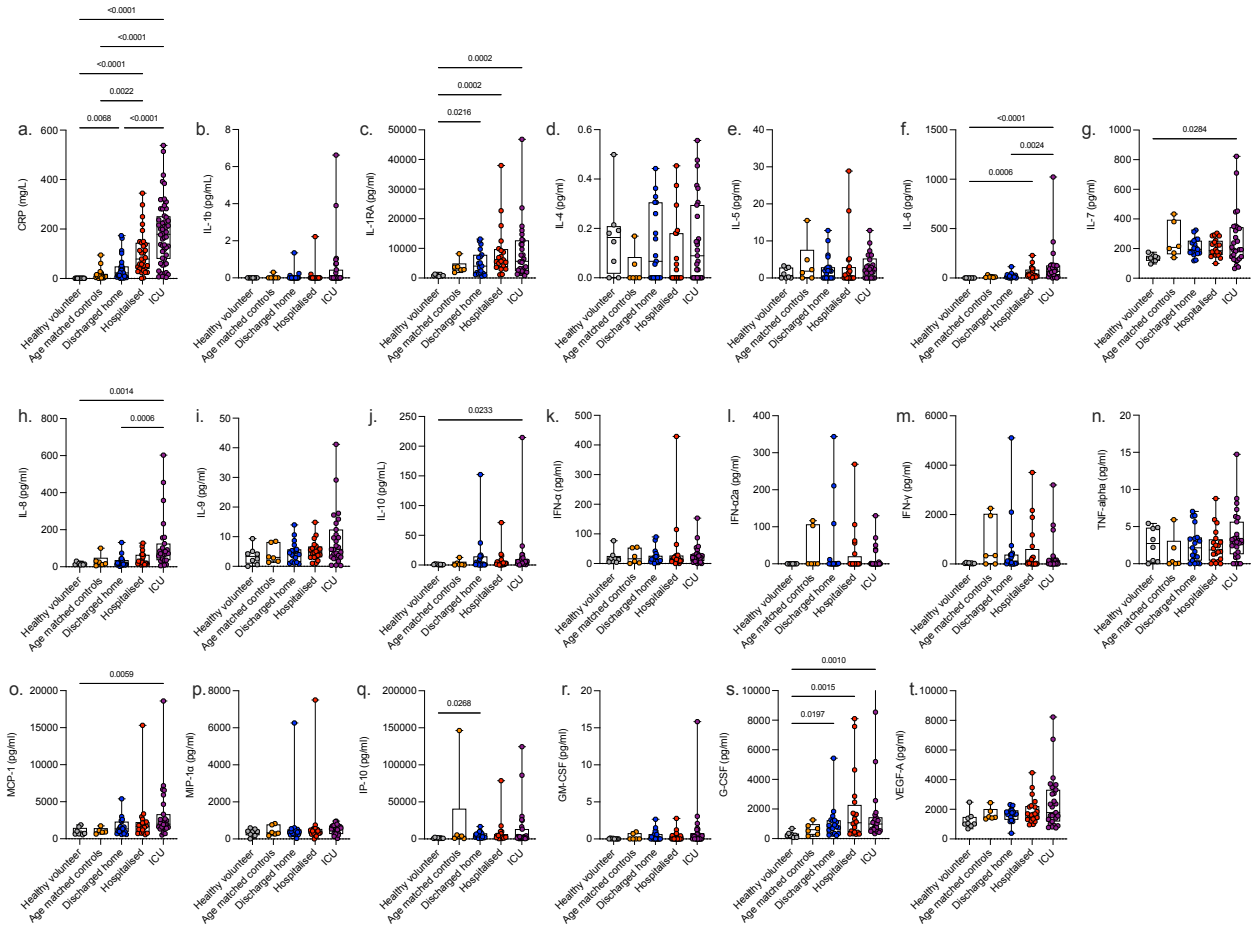

Figure 4.4: Sepsis alters the immunophenotype of serum biomarker concentrations

The immunophenotype of serum biomarkers including chemokines and cytokines was characterised in healthy volunteers, patients admitted to the Emergency Department (ED) either without infection (age matched controls), discharged home of hospitalised with infection, and patients admitted to the Intensive Care Unit (ICU) with sepsis. The following markers were assessed: were measured a. CRP, b. IL-1β, c. IL-1RA, d. IL-4, e. IL-5, f. IL-6, g. IL-7, h. IL-8, i. IL-9, j. IL-10, k. IFN-α, l. IFN-α2a, m. IFN-γ, n. TNF-α, o. MCP-1, p. MIP-1α, q. IP-10, r. GM-CSF, s. G-CSF, t. VEGF-A. Data expressed as measured values. Individual patients represented as dots, horizontal line represents median, box interquartile range and whisker range. Differences between all groups were compared using a non-parametric Kruskal-Wallis test without Dunnett's correction. Only p-values <0.05 are shown.

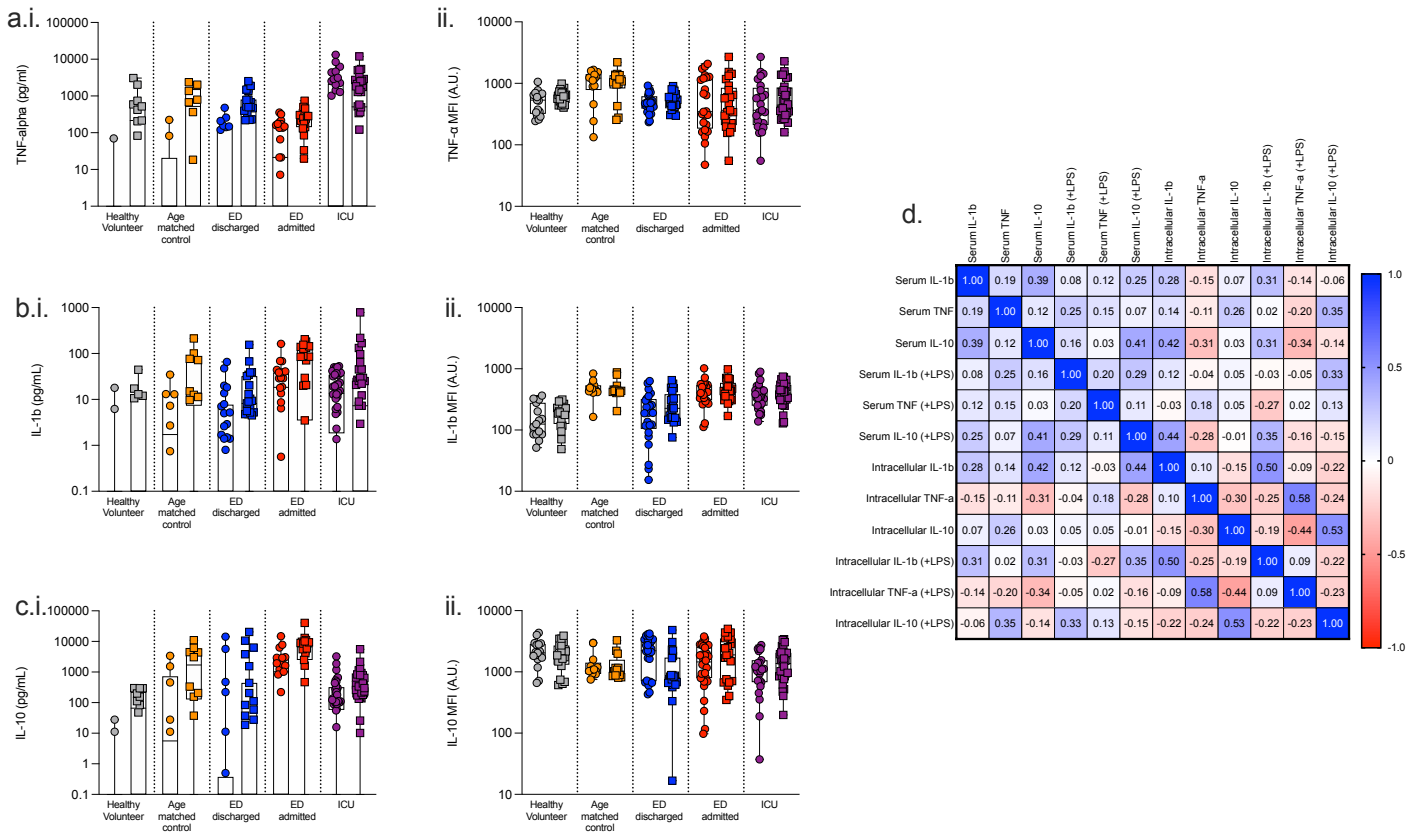

Figure 4.5: Cytokine release in response to LPS-stimulation is altered by sepsis

To assess how sepsis would alter the ability of immune cells to release cytokines in response to an additional stimuli, whole blood (i.) or PBMCs (ii.) isolated from healthy volunteers, patients admitted to the Emergency Department (ED) either without infection (age matched controls), discharged home of hospitalised with infection, and patients admitted to the Intensive Care Unit (ICU) with sepsis were incubated with 100ng/ml of LPS for 1 hour. The intracellular concentration of TNF-α (a.), IL-1β (b.), and IL-10 (c.) in classical monocytes (PBMCs) or released (whole blood) were then compared to unstimulated levels. Data expressed as measured value or median fluorescent intensity (MFI) measured in arbitrary units (A.U.). Individual patients represented as dots, horizontal line represents median, box interquartile range and whisker range. Within each patient group, the left column is the unstimulated data, the right column the LPS-stimulated data. Differences between the unstimulated and LPS-stimulated concentrations were compared using Wilcoxon test. Only p-values <0.05 are shown. The correlation between the unstimulated and stimulated cytokines are shown in the correlation matrix (d.).

4.4.2 Effect of antibiotics on sepsis-induced immunosuppression

I investigated whether beta-lactam antibiotics were associated with an exacerbation of features of sepsis-induced immunosuppression (*ex vivo*) in PBMCs isolated from patients presenting to the emergency department with infection. I assessed the effect of two narrow and two broad-spectrum beta-lactam antibiotics. PBMCs from patients on ICU were not used as all patients admitted with sepsis were already receiving antibiotics prior to blood sampling.

4.4.2.1 Effect of antibiotics on unstimulated PBMCs

All beta-lactams caused a reduction in markers associated with monocyte activation (CD14) (all $p<0.05$). (Figure 4.6)

Amoxicillin reduced monocyte NF-κB ($p<0.05$) and PD-L1 ($p<0.05$). (Figure 4.6 and Figure 9.1) In $CD4^+$ lymphocytes, amoxicillin increased markers associated with differentiation (IL-2R and T_{reg} population; both $p<0.05$) and decreased viability ($p<0.05$). (Figure 4.7 and Figure 9.2) In $CD8^+$ lymphocytes, amoxicillin increased markers associated with activation (CD8 ($p<0.01$) and differentiation (IL-2R and T_{c1} population; both $p<0.05$). (Figure 4.7 and Figure 9.3)

Cefuroxime increased monocyte viability ($p<0.01$). In $CD4^+$ lymphocytes, it increased markers associated with activation (TCR and NF-κB, both $p<0.05$), suppression (CTLA-4, $p<0.01$), differentiation (IL-2R, $p<0.05$), T_{h1}, T_{h2} and T_{reg} populations (all $p<0.05$), transcription factor expression (T-bet, $p<0.05$), and cytokine concentrations (IL-10 and IL-17A, both $p<0.02$). In $CD8^+$ lymphocytes, cefuroxime increased NF-κB ($p<0.05$), markers associated with suppression (CTLA-4, $p<0.01$ and PD-L1, $p<0.05$), proliferation (IL-7R, $p<0.01$), differentiation (IL-2R, $p<0.05$), transcription factor expression (STAT5, $p<0.05$), chemokine receptor expression (CCR4, $p<0.05$) and increased PD-1 expression ($p<0.01$).

Meropenem decreased markers associated with monocyte antigen presentation (CLIP, $p<0.05$), and cytokines (IL-1β, $p<0.05$). In $CD4^+$ lymphocytes, meropenem increased markers associated with activation (CD28 and NF-κB, both $p<0.05$) and transcription factor expression (T-bet, $p<0.001$). In $CD8^+$ lymphocytes, meropenem caused an increase in markers associated with activation (NF-κB, $p<0.05$) and transcription factor expression (STAT5, $p<0.05$).

Piperacillin decreased markers associated with monocyte activation (NF-κB), cytokine concentration (IL-10) and inflammasome (NLRP3) (all $p<0.05$), with mixed effects on markers associated with antigen presentation with a decrease in HLA-DR but an increase in HLA-DM (both $p<0.05$). In $CD4^+$ lymphocytes, meropenem increased markers associated with differentiation (T_{h2}, $p<0.05$ and T_{reg},

$p<0.001$ populations) chemokine receptor expression (CCR4, $p<0.005$) and viability ($p<0.05$). In $CD8^+$ lymphocytes, meropenem increased markers associated with activation (CD8, $p<0.05$), and chemokine receptor expression (CCR4, $p<0.005$ and CCR6 $p<0.05$).

4.4.2.2 Effect of stimulation

LPS increased markers associated with monocyte antigen presentation (HLA-DP), inflammasome (NLRP3) and T-cell suppression (PD-L1) (all $p<0.05$), and a non-significant increase in NF-κB and IL-1β (both $p<0.1$). (Figure 4.6 and Figure 9.4)

CD3/CD28 bead stimulation decreased $CD4^+$ lymphocyte markers associated with activation (TCR), proliferation (IL-7R) and viability (all $p<0.05$). This was associated with an increase in PD-1 ($p<0.01$), an increase in differentiation (T_{reg} and T_{h2} populations (both $p<0.01$)), and intracellular cytokine concentration (IFN-γ, $p<0.05$). (Figure 4.7 and Figure 9.5) In $CD8^+$ lymphocytes, CD3/CD28 bead stimulation increased markers associated with suppression (CTLA-4), differentiation (IL-2R and T_{c1}) (all $p<0.05$), and transcription factor expression (T-bet, $p<0.01$). It also decreased viability ($p<0.01$) which was associated with an increase in PD-1 ($p<0.01$) and intracellular cytokine concentration (IL-4, $p<0.05$ and IFN-γ $p<0.01$). (Figure 4.7 and Figure 9.6)

4.4.2.3 Effect of antibiotics on stimulated PBMCs

Beta-lactams reduced (all $p<0.05$) monocyte markers associated with antigen presentation (decreased HLA-DR and CLIP) and activation (reduced CD14); although CD80 rose. Beta-lactams also increased markers associated with suppression (CTLA-4) in $CD4^+$ lymphocytes, and increased viability in both $CD4^+$ and $CD8^+$ lymphocytes (with no effect on PD-1 or Fas). (Figure 4.6 and Figure 4.7)

Amoxicillin reduced monocyte NOX-2 at high dose (HD), intracellular cytokine concentration (IL-1β at HD, and IL-10 at low dose (LD), and inflammasome (NLRP3) at HD; all $p<0.05$. (Figure 9.7) Amoxicillin increased $CD4^+$ lymphocyte markers associated with activation (NF-κB, HD $p<0.05$), proliferation (IL-7R, HD $p<0.05$), and anti-inflammatory intracellular cytokine concentrations (IL-4 (HD $p<0.01$) and IL-10 (LD $p<0.01$, HD $p<0.05$)). (Figure 9.8) Amoxicillin also increased $CD8^+$ lymphocyte chemokine receptor expression (CCR6, $p<0.05$). (Figure 9.9)

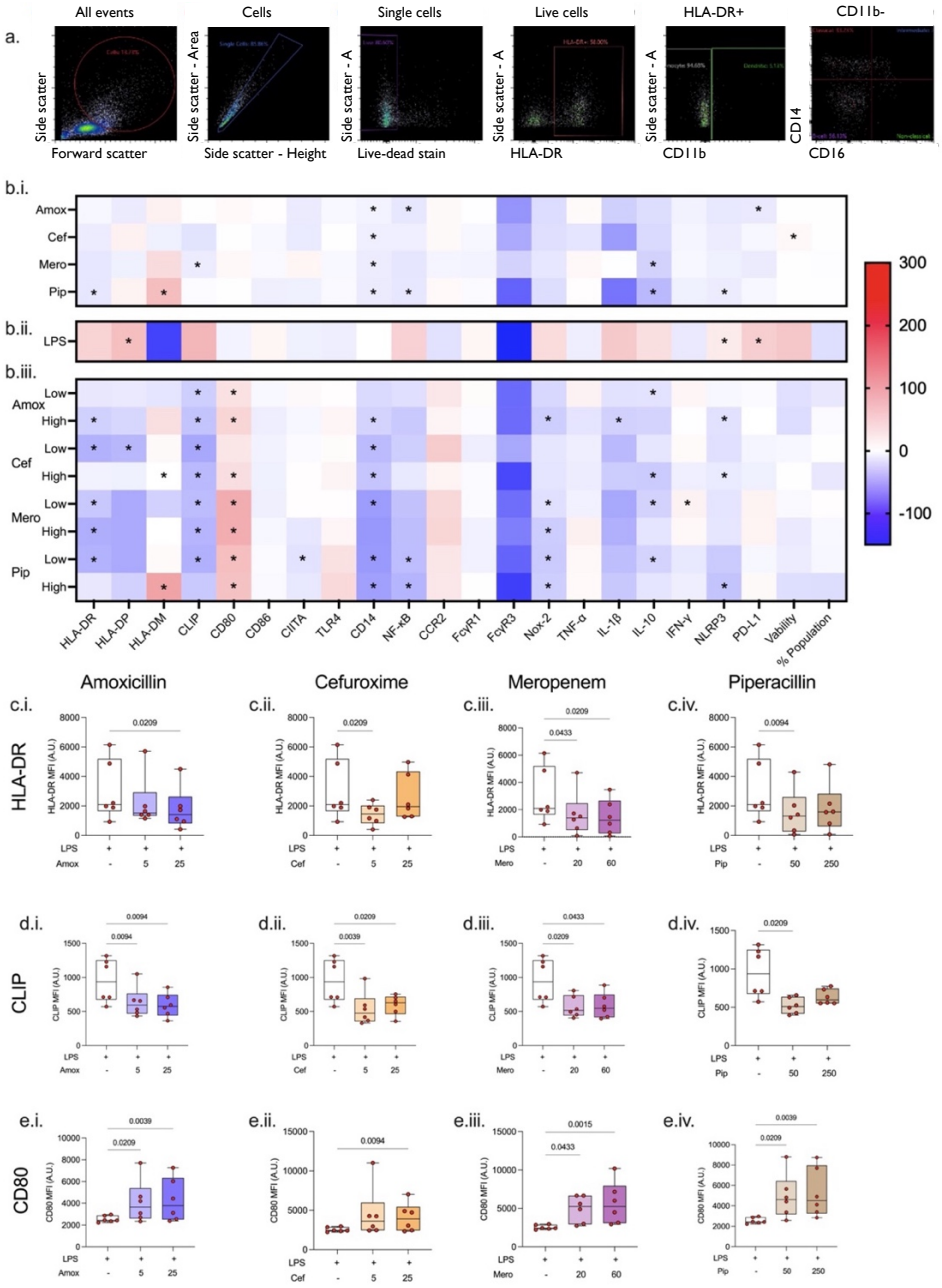

Figure 4.6: Beta-lactam antibiotics have an immunomodulatory effect on classical monocyte phenotype in sepsis.

PBMCs from ED patients diagnosed with bacterial infection (n=12) were incubated for 24 hours with amoxicillin (5 and 25μg/ml, blue), cefuroxime (5 and 25μg/ml, orange), meropenem (20 and 60μg/ml, purple) and piperacillin (50 and 250μg/ml, brown) at low and high clinically relevant doses, both with and without LPS (100ng/ml). (a.) Example gating strategy to identify classical monocytes (PBMCs-Singlets-Live-$CD11b^-$-$HLA\text{-}DR^+$-$CD14^{hi}CD16^{lo}$), (b.) Heat-map of median percentage change of (i.) effect of antibiotics compared to unstimulated monocytes, (ii.) effect of LPS stimulation compared to unstimulated, and (iii.) antibiotic effect on stimulated monocytes. Raw median fluorescent intensity (MFI) of each antibiotic compared to control or LPS using Friedman multiple comparisons test without Dunnett's correction, only results with $p<0.05$ indicated by * reported. Effect on markers associated with antigen presentation pathways including HLA-DR (c.), CLIP (d.) and CD80 (e.) expressed as MFI measured in arbitrary units (A.U.) with individual patients represented as dots, horizontal line median, box interquartile range and whisker range. Only values with $p<0.05$ are shown. Six patients were excluded due to cell counts <10.

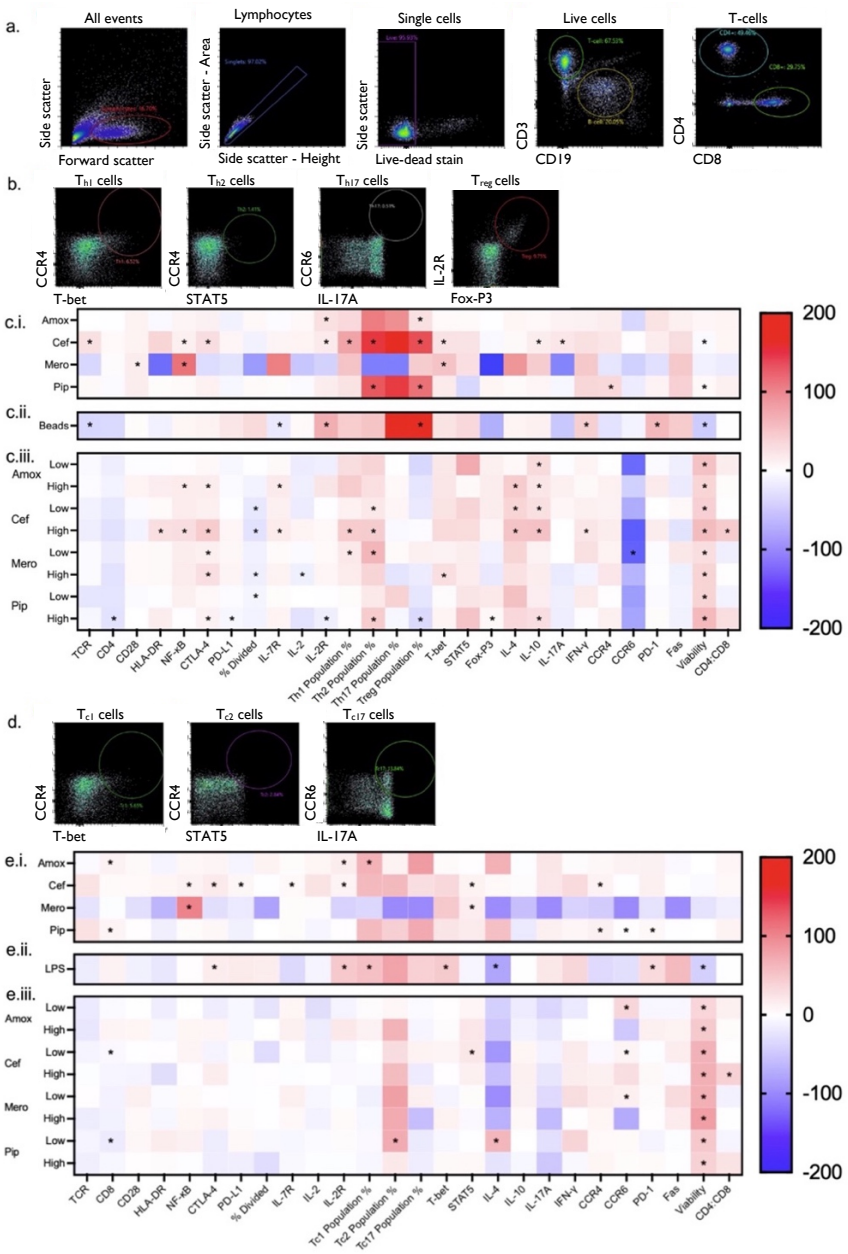

Figure 4.7: Beta-lactam antibiotics have immunomodulatory effects on CD4⁺ and CD8⁺ lymphocyte function in sepsis

PBMCs from ED patients (n=12) were incubated with amoxicillin (5 and 25μg/ml, blue), cefuroxime (5 and 25μg/ml, orange), meropenem (20 and 60μg/ml, purple) and piperacillin (50 and 250μg/ml, brown) at low and high clinically relevant doses, both with and without CD3/CD28 beads. (a.) Example gating strategy for lymphocytes (Lymphocytes-singlets-live-CD3/CD19-CD4/CD8, (b.) Example gating strategy for $CD4^+$ lymphocyte subpopulations (T_{h1} ($CCR4^-Tbet^+$), T_{h2} ($CCR4^+STAT5^+$), T_{h17} ($CCR6^+IL\text{-}17A^+$) and T_{reg} ($CD25^+FoxP3^+$)), (c.) heat-map of median percentage change of (i.) effect of antibiotics on unstimulated $CD4^+$ lymphocytes, (ii.) effect of LPS stimulation on unstimulated $CD4^+$ lymphocytes, and (iii.) effect of antibiotics on stimulated $CD4^+$ lymphocytes. Raw median fluorescent intensity (MFI) of each antibiotic compared to control or beads using Friedman multiple comparisons test without Dunnett's correction, only results with $p<0.05$ indicated by * reported. (d.) example gating strategy for $CD8^+$ lymphocyte subpopulations (T_{c1} ($CCR4^-Tbet^+$), T_{c2} ($CCR4^+STAT5^+$), and T_{c17} ($CCR6^+IL\text{-}17A^+$)), (e.) heat-map of median percentage change of effect of antibiotics on unstimulated $CD8^+$ lymphocytes, (ii.) effect of LPS stimulation on unstimulated $CD8^+$ lymphocytes, and (iii.) effect of antibiotics on stimulated $CD8^+$ lymphocytes. Four patients were excluded due to cell counts <10.

Meropenem in monocytes altered markers associated with phagocytosis (reduced NOX-2 at LD and HD) and intracellular cytokine concentrations (decreased IL-10 at LD, increased IFN-γ at LD, all $p<0.05$). (Figure 9.13) In $CD4^+$ lymphocytes, it increased transcription factor expression (T-bet at HD), decreased proliferation (percentage divided, at HD) and chemokine receptor expression (CCR6 at LD), all $p<0.05$). It had mixed effects on differentiation (decreased IL-2 at HD, $p<0.01$) but increased T_{h1} and T_{h2} populations (at LD, both $p<0.05$). (Figure 9.14) In $CD8^+$ lymphocytes, it only increased chemokine receptor expression (CCR6, $p<0.05$). (Figure 9.15)

Piperacillin in monocytes altered markers associated with antigen presentation (increased HLA-DM and CIITA at HD, both $p<0.005$), and reduced markers associated with activation (NF-κB at both LD, $p<0.05$ and HD, $p<0.01$), phagocytosis (reduced NOX-2 at LD and HD (both $p<0.05$), intracellular cytokine concentration (IL-10 at LD, $p<0.05$), and inflammasome assembly (NLRP at HD, $p<0.01$). (Figure 9.16) In $CD4^+$ lymphocytes, it increased intracellular cytokine concentration (IL-10, at HD $p<0.01$) but decreased markers associated with activation (CD4 at HD $p<0.05$) and transcription factor expression (Fox-P3 at HD $p<0.05$). It had mixed effects on markers associated with suppression (increased CTLA-4 at HD but decreased PD-L1 at HD, both $p<0.05$) and differentiation (decreased IL-2R and T_{reg} population at HD but increased the T_{h2} population, all $p<0.05$). (Figure 9.17) In $CD8^+$ lymphocytes, piperacillin increased differentiation (T_{c2} population) and intracellular cytokine concentration (IL-4), and decreased markers associated with activation (CD8), all $p<0.05$. (Figure 9.18)

4.5 Discussion

4.5.1 Effect of infection and sepsis on immune function

In this study I was able to confirm the significant association between lower monocyte HLA-DR expression and greater sepsis illness severity. *Ex vivo* exposure of healthy volunteer monocytes to bacterial products, and mild bacterial infection in patients with inflammatory bowel disease, are associated with upregulation of monocyte HLA-DR.[107] Yet, in critically ill patients with persistent or secondary infections, or those who subsequently die, monocyte HLA-DR downregulation is often evident as early as ICU admission.[100-102] Low monocyte HLA-DR is also associated with the development of sepsis in patients presenting to the ED with suspected acute infection, although none of my ED patients developed sepsis or required ICU admission. It is also associated with secondary infections following major trauma or surgery suggesting a continuum of illness severity.[307-310] I have previously shown an early (<24 hours) downregulation of monocyte HLA-DR in response to surgery (non-infectious inflammation).[311]

The reason for monocyte HLA-DR downregulation is unclear, although it may represent an exaggerated response to the normal resolution of infection. Low monocyte HLA-DR expression may be associated with an impaired ability to present antigen, resulting in impaired activation of the adaptive immune system and thereby contributing to the persistence or recurrence of bacterial infection.

Given the pivotal role of monocyte HLA-DR in antigen presentation and T-cell activation, I assessed an associated panel of monocyte and lymphocyte markers. My findings highlight that downregulation of monocyte HLA-DR in sepsis is associated with other changes to monocyte and effector cell function. Changes included decreased phagocytosis with simultaneous lower expression of co-stimulatory receptor CD86 but increased CD80. This particular pattern of co-stimulatory receptor expression was specific to ICU patients, and has been previously associated with increased illness severity.[312,313]

To identify whether reduced monocyte HLA-DR expression was a consequence of impaired upstream antigen presentation pathways, I assessed whether impaired phagocytosis led to reduced antigen processing and subsequently reduced HLA-DR expression.[314] However, HLA-DR only weakly correlated with phagocytosis, suggesting impaired phagocytosis is unlikely to be the primary cause for impaired antigen presentation. The weak correlation between HLA-DR and CD80 or CD86 also suggests that their regulation is independent of one another.

Monocyte HLA-DR is typically regulated at the transcriptional level via Class II transactivator (CIITA).[315] During receptor synthesis, HLA-DR is bound to the class II associated invariant chain peptide, CLIP (CD74) to prevent binding of peptides located in the endoplasmic reticulum and to facilitate transit through the Golgi apparatus to late endosomic compartments. Here CLIP is removed from HLA-DR by HLA-DM to allow peptide binding before surface expression with co-stimulatory receptors CD80 and CD86 to facilitate signalling to lymphocytes.[316] The exact roles of CD80 and CD86 have not been characterised in sepsis in any detail, but they do appear to have opposing patterns of expression with increased CD80 and decreased CD86 being associated with mortality.[312] While both co-stimulatory receptors stimulate naïve T-cells, in differentiated lymphocytes they have differing effects on the individual populations and this may partly explain their opposing effects.[317] Both co-stimulatory receptors bind to CD28 and CTLA-4 on lymphocytes, although CD80 binds more avidly. Binding to CD28 causes T-cell activation while binding to CTLA-4 leads to receptor transendocytosis preventing activation. Following transendocytosis, CD86 (but not CD80) remains bound to CTLA-4 preventing receptor recycling and this can lead to unopposed T-cell activation eventually leading to cell exhaustion and anergy.[318] An increase in monocyte CD80 expression and decrease in CD86 expression would favour a transient/reversible effect on CTLA-4 induced T-cell inhibition in sepsis.

Sepsis causes multiple impairments in the regulation of monocyte HLA-DR both at transcriptional and post-transcriptional levels. Expression of both HLA-DR and CD74/CLIP genes are reduced in

sepsis,[319,320] due to upregulation of CCCTC-Binding Factor (CTCF), an architectural protein and superordinate regulator of transcription which inhibits transcription.[321] Glucocorticoids decrease monocyte HLA-DR expression via suppression of CIITA transcription.[322] mRNA levels of CD74 and IL-10 both correlate (positively and negatively, respectively) with decreased HLA-DR expression.[323] CD74 prevents antigen binding and surface transit while IL-10 enhances endocytosis, resulting in intracellular sequestration.[324,325] My data are consistent with this finding, showing a significant negative correlation between serum IL-10 levels and monocyte HLA-DR expression.

Age is associated with immune senescence and a reduction in monocyte HLA-DR expression.[326] While the younger healthy volunteer and discharged ED patient cohorts had higher HLA-DR expression than ICU patients, expression in the ICU patient cohort was lower than both age-matched control and admitted ED patient groups who were of similar age, suggesting these changes were primarily related to illness severity. The significant inverse association between CRP and monocyte HLA-DR supports this notion.

Patients either discharged from ED or admitted to hospital demonstrated an increase in TNF-α, IL-1β and IL-10 release on whole blood stimulation with lipopolysaccharide. ICU patients however did not demonstrate any increase in TNF-α or IL-1β on LPS stimulation, although IL-10 was significantly increased. Data on LPS-induced cytokine production in septic patients are conflicting.[314,327-329] The preserved IL-10 response to LPS among septic patients is however consistent with an anti-inflammatory phenotype, with IL-10 having inhibitory effects on both monocyte HLA-DR expression and TNF-α release.[325] I found no correlation between monocyte HLA-DR and baseline serum TNF-α, LPS-stimulated TNF-α, or monocyte intracellular TNF-α. Prior research has suggested monocyte HLA-DR and LPS-induced TNF-α release are positively correlated, although this is more apparent in the first few days of ICU admission and may be lost after a week of illness.[328,329] Some of my patients had been admitted to hospital prior to developing sepsis prompting their ICU admission which may explain this difference.

Among my panel of 22 serum biomarkers, eight demonstrated a clear association with increasing illness severity, with ICU patients having significantly higher levels than all or most other patient groups. In the absence of bedside flow cytometry to assess monocyte HLA-DR expression, I assessed whether serum biomarkers could be used as a surrogate for low monocyte HLA-DR. CRP, which is routinely measured in many ICUs, and serum IL-8 provided the strongest association with monocyte HLA-DR. Further work on their role in predicting and monitoring sepsis-induced immunosuppression is required.[330,331]

I assessed the phenotype of relevant effector cells in addition to the assessment of monocyte phenotype. While $CD4^+$ lymphocytes demonstrated increased activation (as measured by CD28)

compared to other infected patient groups, both $CD4^+$ and $CD8^+$ lymphocytes expressed markers associated with anergy, including increased PD-1 expression and decreased IL-7R expression, which are evident in sepsis.[114] $CD4^+$ lymphocyte apoptosis was positively correlated with both CTLA-4 and PD-1 expression, demonstrating early activation of cell death mechanisms. However, I did not find any significant alterations in cell viability; this may relate to samples being taken early in the disease course, or as a consequence of performing the assay on stored rather than fresh PBMCs.[332] Direct cell-cell contact between antigen-presenting cell and lymphocyte may underpin several phenotypic changes seen in lymphocytes.[333,334] Therefore further work is required to ascertain whether impaired lymphocyte function in sepsis is a consequence of impaired activation via antigen presenting cells or a direct immunosuppressive effect on the lymphocyte itself.

The use of immunomodulatory therapies to reverse immunosuppressive phenotypes may offer therapeutic value. Clinical trials have focused on modulation of monocyte HLA-DR (with GM-CSF or IFN-γ)[335-337] and lymphopenia (recombinant IL-7 and anti-PD-1 or -PD-L1).[141,338] However, many of these therapies have either not demonstrated a clinical benefit, or in the case of IFN-γ, have demonstrated harm.[140] Given the multitude of impairments seen in each cell type, it is unlikely that targeted treatments to one impaired immune cell pathway would improve the wider immunosuppressive phenotype.

There are a number of limitations to report. Despite the breadth of data presented in this study, I did not report differences between ICU survivors and non-survivors due to the relatively small sample size. I have not performed sequential measurements to assess the trajectory of immune cell function over time. I did not quantify monocyte HLA-DR (receptors per cell) as this requires the use of flow cytometry performed within hours of blood sampling.[329] Data have only been provided on classical monocytes and not intermediate or non- classical monocytes due to limitations of sample volume (and hence cell number).

I investigated the phenotypes of monocytes and their effector cells given the robust association between monocyte HLA-DR / lymphopenia and mortality in critically ill patients. However, I did not examine neutrophil or B-cell function which are also impaired in sepsis.[113,339] All stimulatory experiments were performed using a single dose of LPS or phagocytotic particles. Interpretation of intracellular cytokines following a stimulus reflects changes to pre-formed, newly synthesised and released cytokines. Concentration is often assessed in the presence of an inhibitor of intracellular protein transport e.g. brefeldin A. However, use of this agent would prevent reliable measurement of released cytokines into the supernatant.[147] The number of assays I could perform were also limited by cell numbers.

In summary, surface monocyte HLA-DR expression was lower among patients with greater illness severity. Reduction in monocyte HLA-DR was also seen among patients with relatively mild and early infection compared to healthy individuals. Reduced monocyte HLA-DR was associated with impairments in associated proteins regulating antigen presentation and other features of monocyte function. Therapeutic agents modulating HLA-DR expression alone may therefore fail to improve overall monocyte function. Concurrent assessment of a wider monocyte functional phenotype is thus required. I also found $CD4^+$ lymphocyte dysfunction with evidence of activated apoptotic cell pathways and reduced proliferation receptor expression. The direct impact of monocyte signalling on lymphocyte dysfunction and scope for therapeutic modulation on cellular immunity requires further evaluation.

4.5.2 Effect of antibiotics on sepsis-induced immunosuppression

Several routinely administered drugs in sepsis, including sedatives, catecholamines and antibiotics, impair immune function.[145,340,341] While antibiotic treatment forms the cornerstone of sepsis management, they may exacerbate sepsis-associated immunosuppression, paradoxically leaving patients at greater risk of secondary infection or endogenous clearance of the primary infection. High serum levels of antibiotics resulting from impaired drug metabolism and elimination in critically ill patients may further exacerbate this issue. A better understanding of the interaction between antibiotics and the immune system is thus imperative. Having identified key features of sepsis-induced immunosuppression in my patient cohort, I chose to assess the effect of beta-lactam antibiotics in the presence of a secondary stimulus using an in-depth analysis of classical monocyte HLA-DR regulatory pathways and lymphocyte proliferation, differentiation, and death pathways.

Beta-lactam antibiotics reduced monocyte HLA-DR and increased co-stimulatory receptor CD80 expression; both features are seen in monocytes from septic patients.[312,313] Amoxicillin has previously been shown to cause upregulation of HLA-DR, CD80 and CD86.[266] However, this study was performed in PBMCs isolated from patients with an amoxicillin allergy suggesting direct activation of monocytes, rather than an effect in already stimulated cells.

I investigated key transcriptional and post translational proteins associated with the regulation of monocyte HLA-DR. HLA-DR expression is regulated at the transcriptional level by CIITA while CLIP prevents antigen binding. In sepsis, CLIP mRNA is decreased in blood whilst increased CLIP expression inhibits HLA-DR expression.[323,342] HLA-DM is responsible for removing CLIP for HLA-DR binding.[316] I did not find any changes in either CLIP, CIITA or HLA-DM, suggesting alternative post-transcriptional pathways are responsible.[321]

IL-10 reduces HLA-DR expression through enhanced endocytosis of the surface receptor,[324] however I found that the beta-lactam agents reduced monocyte intracellular IL-10. While this suggests the lack of an autocrine effect of monocyte IL-10, it should be recognised that IL-10 is released by many cell types during sepsis, including lymphocytes. Beta-lactams are reported to have mixed effects on serum IL-10 concentrations, although effects appear to depend on the model used. For example, IL-10 was decreased by penicillin and cephalosporins in a 4 day non-infected IL-10 gene depleted colitis mouse model,[344] but increased by carbapenems and amoxicillin in a 5 day rat model of ear infection,[345] and after 7 days in patients with S. *aureus* infection.[343] No study looked at intracellular concentrations.

Findings from my prospective observational cohort study demonstrate impaired monocyte phagocytosis in sepsis. The *ex vivo* data demonstrate that broad-spectrum beta-lactam antibiotics suppress NOX-2 expression and a trend toward a reduction in both FcyR1 and FcyR3. Phagocytosis assays were not conducted in this experiment due to limitations in cell numbers. This raises the possibility that beta-lactams may contribute to impaired phagocytosis in septic patients. Previous research has demonstrated mixed effects of beta-lactams on phagocytosis *in vitro*.[169,171,219] However, none of the studies were performed in patients with sepsis. *Ex vivo*, carbapenems are reported to both increase or decrease phagocytosis,[169,219] While cephalosporins decreased phagocytosis at supra-clinical doses and penicillin had no effect.[171] *In vivo*, carbapenems increased healthy volunteer phagocytosis while cephalosporins had no effect.[253] Piperacillin, however, was associated with a reduction in phagocytosis in postoperative patients.[205]

In $CD4^+$ lymphocytes, beta-lactam antibiotics improved cell viability. There has been little previous research on the role of beta-lactams on lymphocyte apoptosis and death, although a beta-lactam based treatment induced apoptosis through direct DNA damage.[281,282] The mechanism responsible for this enhanced viability is unclear given the expression of death pathways receptors including PD-1 and Fas, and proliferation including IL-7R, were unchanged. However, independent of changes in IL-2 or IL-2R expression,[344] beta-lactams did enhance $CD4^+$ lymphocyte differentiation, with increases in T_{h2} cell populations and non-significant reductions in the T_{reg} population, findings that are consistent with published data.[345]

Beta-lactams also increased expression of CTLA-4. This, to my knowledge, is the first study to provide evidence of a direct effect of these agents on CTLA-4 expression. Beta-lactam antibiotic use with immune checkpoint inhibition (including antibodies to CTLA-4) for cancer is associated with worse survival;[346] this was attributed to alterations in gut microbiota, although it remains unknown whether mortality was related to cancer progression or secondary infection. Beta-lactams have previously been demonstrated to cause lymphocyte suppression, reducing transformation and blastogenesis, although

there may be within-class differences between penicillin (no effect) and cephalosporins (enhanced suppression).[347]

Few effects were seen on $CD8^+$ lymphocytes, although the improvement in $CD4^+$ lymphocyte viability was mirrored in this population. Previous evidence exists for an immunosuppressive effect of cephalosporins, but penicillin can also modulate $CD8^+$ lymphocyte functions including proliferation and differentiation albeit in a viral model of infection.[348]

While several class effects were demonstrated, there were a few additional within-class effects on immune function. In monocytes, piperacillin had additional effects on markers associated with antigen presentation (increased HLA-DM expression in both stimulated and unstimulated cells) and activation (suppressed NF-κB at both low and high doses). Amoxicillin, cephalosporin and piperacillin at high dose suppressed NLRP3. In $CD4^+$ lymphocytes, amoxicillin and cefuroxime increased some intracellular cytokine concentrations (IL-4 and IL-10) but not IFN-γ. Lymphocyte cytokine expression is regulated by multiple factors including the presence of other cytokines.[349] Both IL-10 and IL-4 are known inhibitors of IFN-γ expression which may explain the differential effects on cytokine production.[350,351] Previous research has also demonstrated within-class differences between the immunomodulatory effects of beta-lactams, including on cytokine production,[352] and inhibition of the PI3K-Akt-mTOR pathway.[353] The reason for these differences remains to be elucidated, however it may be related to the binding of, and intracellular sequestration of albumin,[354] which alters expression of GATA3, responsible for regulation of cytokine production.[345] This binding is however dependent on the structure of the beta-lactam ring which partly explains the within class differences.[355] There were no particular differences between narrow-spectrum and broad-spectrum antibiotics.

I chose to assess beta-lactam effects at two doses, representing low and high clinically relevant doses. While effects on antigen presentation and lymphocyte viability occurred at both doses, several effects were predominantly demonstrated only at high dose, including monocyte NLRP3 and $CD4^+$ lymphocyte activation (NF-κB and HLA-DR) and suppression (CTLA-4). The pharmacokinetics and pharmacodynamics of many drugs are altered in critical illness. Due to concerns regarding underdosing of antibiotics, there is growing interest in administering beta-lactams as extended or continuous infusions, often without therapeutic drug monitoring, to hopefully ensure serum levels remain above the target minimum inhibitory concentration (MIC).[356,357] Given there is a dose-dependent effect of the beta-lactams on immune dysfunction, therapeutic drug monitoring should have additional benefits by ensuring serum concentrations are maintained above target MIC but below the concentration which causes immunotoxicity. Further *in vivo* research is required to see whether this would be feasible and whether this leads to clinical benefit. Some effects were only seen at low doses. Additional effects

may have been seen if higher patient numbers were included as several effects appeared dose dependent, however only low or high dose reached a $p<0.05$.

In terms of limitations, I conducted all *in vitro* work using PBMCs isolated from patients in the emergency department with infection but without new organ dysfunction, as ICU patients were already in receipt of antibiotics. While this enabled me to model the effect of an additional stimulus on the cells, other effects seen may be different in septic patients, and those recovering from sepsis. I chose a single dose of LPS and CD3/CD28 beads; effects may differ with other doses or stimuli. I assessed relative expression of markers associated with common immune cell functions, but due to limitations in cell numbers could not assess function. Observations from *in vitro* experiments may not translate to *in vivo* functionality. Additional work is required to demonstrate whether a similar effect occurs *in vivo,* including interactions with other medications and co-morbidities. Other mechanisms by which antibiotics may modulate immunity, including effects on other cell types, the innate immune system, and on the microbiome,[24,113,339,358] have not been explored in my work. While the panel of antibiotics I chose to investigate is not exhaustive, the four antibiotics include the most commonly used beta-lactams in UK clinical practice.[288] Other antibiotic classes may have other clinically relevant effects.[359] The immunomodulatory effects are presumed to be related to the antibiotic, however other compounds with potentially immunomodulatory effects may be included in the vial as part of the powdered form.

At clinically relevant doses, beta-lactam antibiotics exacerbate features of sepsis-induced immunosuppression. The mechanisms, duration and reversibility of this phenomenon warrant investigation. A better understanding of toxicity of antibiotics on immune cells and functional impact has significant clinical implications and will impact the type and duration of antibiotics administered. In light of the potential harm, judicious limitation of antibiotic courses may reduce the generation of antibiotic-resistant organisms.

4.6 Chapter summary

Low expression of monocyte HLA-DR is associated with greater illness severity. Concurrent changes to pathways regulating antigen presentation and global monocyte and $CD4^+$ lymphocyte cell function occurs. Further work is required to identify the underlying mechanisms, to what degree lymphocyte phenotype is a direct consequence of monocyte phenotype, and whether current therapeutic agents which target the HLA-DR receptor can improve overall monocyte and immune function.

Beta-lactam antibiotics can exacerbate the immunosuppressive phenotype in sepsis, particularly related to the antigen presentation pathway. This appears to occur by a mechanism independent of the key regulatory transcription factor CIITA and the proteins responsible for intracellular trafficking (CLIP

and HLA-DM). Further work is required to identify this mechanism, and whether the effects demonstrated are clinically relevant *in vivo*.

5 Immune responses to sterile inflammation

5.1 Chapter context

The immune response to sterile inflammation following surgery or trauma shares many features with inflammation following infection. Changes following surgery consistent with an immunosuppressive phenotype also share several features with sepsis-induced immunosuppression. Patients often receive antibiotics as prophylaxis against post-operative bacterial infections. Antibiotics are known to modulate immune function, and may therefore have an independent effect on the risk of developing postoperative infection independent of its direct antimicrobial effect. I characterised changes to the immune system following major surgery, and investigated the effect of antibiotics on these changes *ex vivo*.

5.2 Introduction

Post-operative infections are a significant cause of morbidity, affecting up to 40% of patients undergoing major surgery.[360,361] Surgery activates the immune system in response to physical damage to tissues ('sterile inflammation'), with many similarities with infections.[362] This response is influenced by a myriad of factors including patient age, medical conditions (e.g. cancer), and medications. Several immune responses following surgery are associated with subsequent infections.[363,364] Two well-characterised changes associated with post-operative infections are a reduction in monocyte HLA-DR and persistent lymphopenia.[310,365] Similar changes are seen in patients who die from sepsis.[104]

Despite these known associations, the duration, intensity and characteristics of the immune response to surgery, and its impact on the response to infections, remain poorly characterised. Better understanding may facilitate identification of high-risk patients, the risk period, and preventative therapies.

Whilst the traditional approach to preventing these infections postoperatively has been the liberal usage of perioperative antimicrobial prophylaxis, there has been a concerted effort over the last decade to reduce their usage due to the risk of antimicrobial resistance, and an increasing awareness of their side-effects, including immunosuppression.[366]

The most commonly used antimicrobials include the beta-lactams (penicillins and cephalosporins) and nitroimidazoles (metronidazole).[293] Cefuroxime and metronidazole may be better in preventing postoperative infections than amoxicillin.[367] This may relate to their effects on different microbial cover including on the microbiome, but could also be caused by their immunomodulatory

effects.[147,345,368] Different beta-lactams have different effects on the immune system,[276,345] as evidenced by the different risks of allergies, which is likely due to the presence of only some cross-reactivity between amoxicillin and cephalosporins.[369]

I hypothesised that immune pathways which are reproducibly perturbed early after major surgery may guide approaches to modulate immune responses to mitigate the risk of subsequent infections. I therefore evaluated if there were differences in changes to immune cell phenotype before and 24hours following surgery between patients who did and did not develop a post-operative infection. I also investigated the *in vitro* immune response to an infectious challenge before and after surgery, to determine if surgery altered the immune response to a subsequent infectious challenge, and if this was different between patients who did and did not develop a post-operative infection.

I also hypothesised that antimicrobial prophylaxis would modulate the immune changes seen early after major surgery which may modify the risk of developing subsequent postoperative infections. I evaluated the effect of amoxicillin, cefuroxime, metronidazole and combined cefuroxime-metronidazole on immune cell phenotype in patients who had undergone surgery.

I chose to focus on the same immune cells and functional pathways impaired in sepsis due to the commonality in the immune response between the two conditions.[104,114,362]

5.3 Methods summary

I performed a prospective cohort study of adult patients (>17 years of age) undergoing major elective surgery requiring post-anaesthetic care unit admission. Patients were excluded if they had severe anaemia (Hb<60/dl) with a contraindication to transfusion, unable to gain consent or agreement, treated with a palliative intent, no vascular access to obtain blood or were suspected of having a current infection.

Samples were taken at induction of anaesthesia, immediately post-operatively, and on days one and five. Peripheral blood mononuclear cells were isolated, stored and analysed in batches using flow cytometry. Serum and serum isolated following whole blood in vitro LPS-stimulation was stored for batch analysis by ELISA and multiplex to identify cytokines and other biomarkers. Relevant laboratory, clinical and outcome data was also stored and combined with experimental results at study completion.

PBMCs were defrosted and stained for immunophenotyping, or underwent heat-killed bacterial stimulation for 24 hours or with CD3/CD28 beads for 72 hours with and without antibiotics (amoxicillin, cefuroxime, metronidazole and combined cefuroxime-metronidazole) prior to staining.

5.4 Results

5.4.1 Effect of surgery on immune function

5.4.1.1 Study participants

48 patients and 16 healthy volunteers were recruited. (Figure 5.1). There was no difference in age (68 vs 66), sex (64% vs 79% male), BMI (24.73 vs 25.23), ASA grade (2 vs 3), co-morbidities, surgical site, use of dexamethasone for postoperative nausea and vomiting prophylaxis, or use of or duration of peri-operative antibiotics between patients who did or did not develop a post-operative infection. All patients received their first dose of prophylaxis within 30 minutes prior to skin incision. (Table 5.1) Compared to healthy volunteers, patients were older (67 vs 36; $p<0.001$), although sex (72% vs 66%) and BMI were similar (25.0 vs 23.54).

Twenty-six (54%) patients developed a post-operative infection as defined by the standardized endpoints in perioperative medicine – core outcome measures for perioperative and anaesthetic care (StEP-COMPAC) criteria.[370] Site of infection included pneumonia (n=17), wound (n=4), anastomotic leak (n=2), urinary tract (n=2), and unknown (n=1, this patient had clinical features consistent with an infection including fever, however the source of the infection was unknown). Infections were diagnosed a median of 3 (2-4) days following surgery, with 10 patients having positive microbial cultures. Patients who developed an infection were more likely to have cancer ($p<0.1$), with distant spread ($p<0.05$) and receiving neoadjuvant chemotherapy ($p<0.1$). Their perioperative risk of mortality, as measured by SORT (Surgical Outcome Risk Tool) score was higher ($p<0.05$) and operative times were longer ($p<0.05$). Patients who developed an infection had a longer hospital length of stay ($p<0.001$) and higher Clavien-Dindo score ($p<0.001$), severity of postoperative complications scored by the type of intervention required for correction) although there were no differences in unplanned ICU readmission or mortality. (Table 5.1) Antibiotic duration was a median of 5 (3-12) days for treatment of post-operative infections.

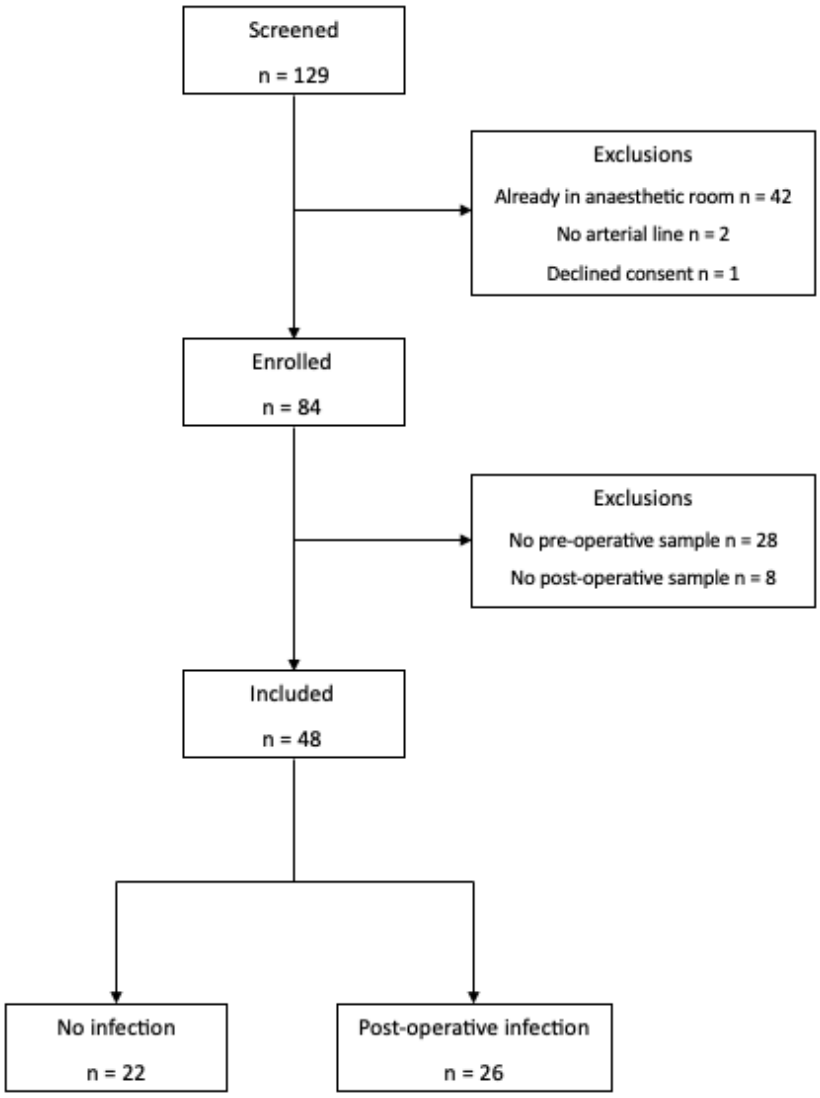

Figure 5.1: Flow diagram of patients screened and included in the study

5.4.1.2 Changes to immune cell phenotype 24 hours following major surgery

Twenty-four hours following surgery, there was a significant fall in lymphocyte count, and rise in neutrophil count, neutrophil: lymphocyte ratio, CRP (all $p<0.0001$), serum IL-7, and serum IL-6 (both $p<0.01$) among patients with and without post-operative infections. There was a significant increase in monocyte CCR2 expression, and decreased CXCR4, PD-L1, HLA-DR and CD86 (all $p<0.001$) expression. $CD4^+$ lymphocytes demonstrated a decrease in CD28 expression and an increase in IL-7R (both $p<0.001$) expression, whilst $CD8^+$ lymphocyte expression of CD28 was decreased ($p<0.05$). (Figure 5.2**,** Figure 5.3**,** and Figure 5.4)

Variable		Healthy volunteer (n=16)	No post-op infection (n=22)	Post-op infection (n=26)	p-value (patient groups only)
Age (years)		36 (35-38)	68 (56-72)	66 (56-72)	0.9556
Gender (% male)		66%	64%	79%	0.7749
BMI		23.54	24.73	25.23	0.5426
Co-morbidities					
	Hypertension (%)	-	41%	27%	0.3057
	Cardiovascular disease (%)	-	23%	19%	0.7663
	Respiratory disease (%)	-	27%	38%	0.4126
	Type 2 diabetes (%)	-	9%	19%	0.3213
	ASA Grade (%)	-	2 (2-3)	3 (2-3)	0.3561
	Active cancer (%)	-	64%	96%	0.0822
	Cancer staging	-	1 (2-3)	2 (2-3)	0.0261
	Neoadjuvant chemotherapy (%)	-	36%	67%	0.0822
	Other immunosuppressive medication (%)	-	9%	8%	0.8613
	Long term steroids	-	9%	4%	0.4545
SORT Score (%)		-	0.47 (0.25-0.87)	1.48 (0.37-3.17)	0.0169
Type of surgery					
	Upper GI (%)	-	50%	75%	0.3218
	Lower GI (%)	-	27%	21%	
	Maxillofacial (%)	-	9%	13%	
	Gynaecological (%)	-	9%	0%	
	Other (%)	-	5%	0%	
Peri-operative antibiotics					
	Prophylaxis administered (%)	-	95%	100%	0.2931
	Duration of prophylaxis (days)	-	1 (0-1)	1 (0-1)	0.7640
Intra-operative dexamethasone use (%)		-	95%	84%	0.2206
Operation duration (mins)		-	174 (112-280)	287 (204-350)	0.0138
Blood loss (mls)		-	500 (500-500)	500 (100-500)	0.2019
Peri-operative blood transfusion (%)		-	5%	4%	0.9038
Unplanned ICU readmission (%)		-	5%	19%	0.1253
Clavien-Dindo classification		-	1 (0-1)	2 (2-3a)	<0.0001
Hospital length of stay (days)		-	8 (7-13)	15 (11-27)	0.0001
Death (%)		-	9%	4%	0.4545

Table 5.1: Baseline clinical data and outcomes

Continuous data was analysed using Mann Whitney-U for comparison of two groups, whilst chi-squared test was used for analysis of categorical data. BMI: Body Mass Index, ASA: American Society of Anesthesiologists, GI: Gastrointestinal, GU: Genitourinary, ICU: Intensive Care Unit. Blood loss was categorised as <100, 100-500, 500-1000, 1000-2000 or >2000mls. For analysis the higher value of the range was used.

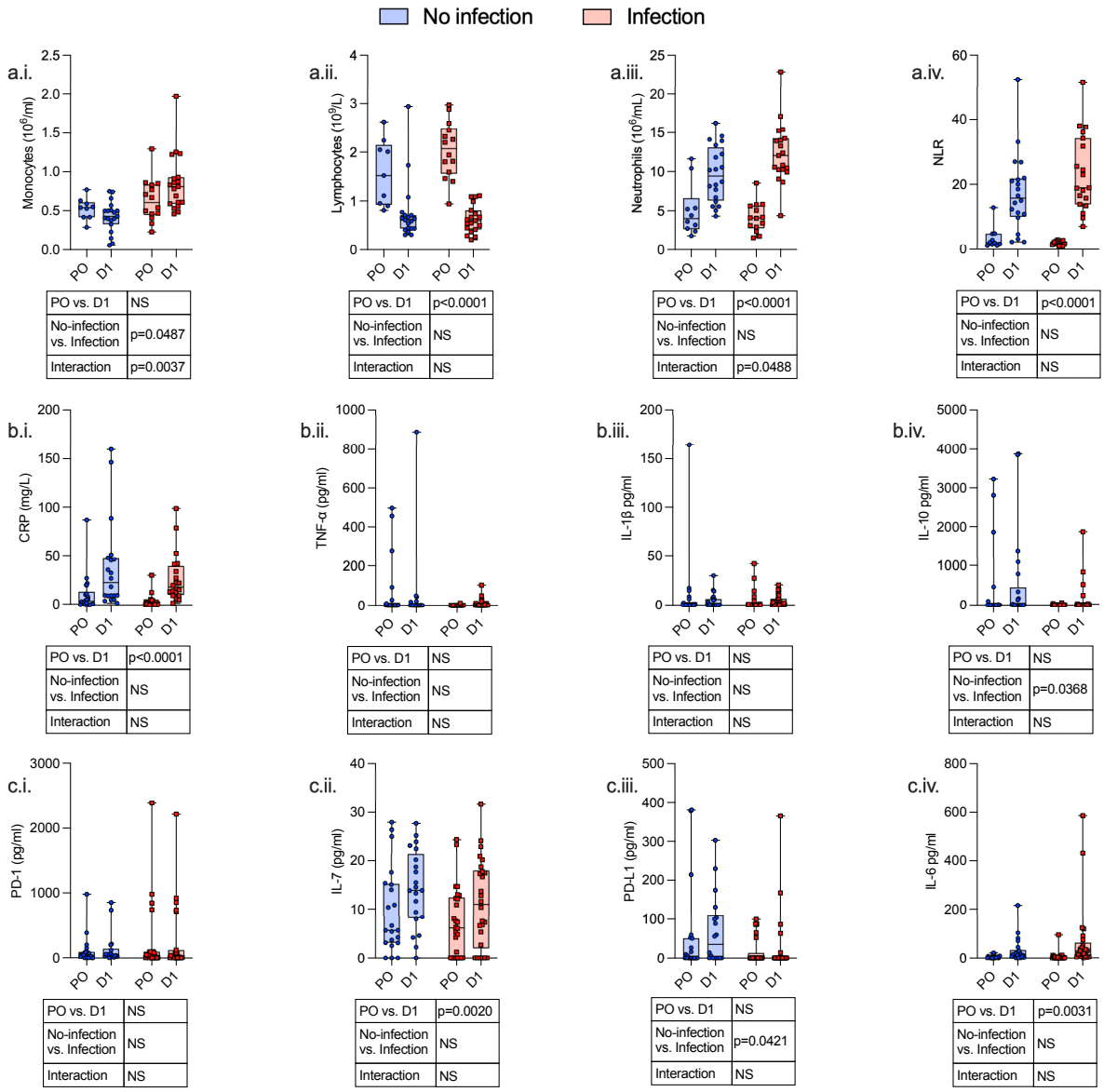

Figure 5.2: Serum and haematological variables identify an immunosuppressive phenotype both pre- and post-operatively which is associated with the development of subsequent perioperative infections

Whole blood and serum was taken from patients undergoing major surgery at induction of anaesthesia (pre-op; PO) and 24 hours post-operatively (D1) and analysed by clinical laboratory evaluation whole blood monocyte count (a.i), lymphocyte count (a.ii), neutrophil count (a.iii.) and neutrophil/lymphocyte ratio (NLR) (a.iv.), and CRP (b.i.). The following serum levels were measured by ELISA: TNF-α (b.ii), IL-1β (b.iii), IL-10 (b.iv), PD-1 (c.i.), IL-7 (c.ii.), PD-L1 (c.iii.) and IL-6 (c.iv.)

Data expressed as either count or concentration. Dots represent individual patients, horizontal bar the median, box the interquartile range and whisker the range. Data analysed using mixed-effects two-way ANOVA. Data are presented as differences over time, between groups, and the difference in the change over time between the 2 groups (interaction term). Only p-values <0.05 shown.

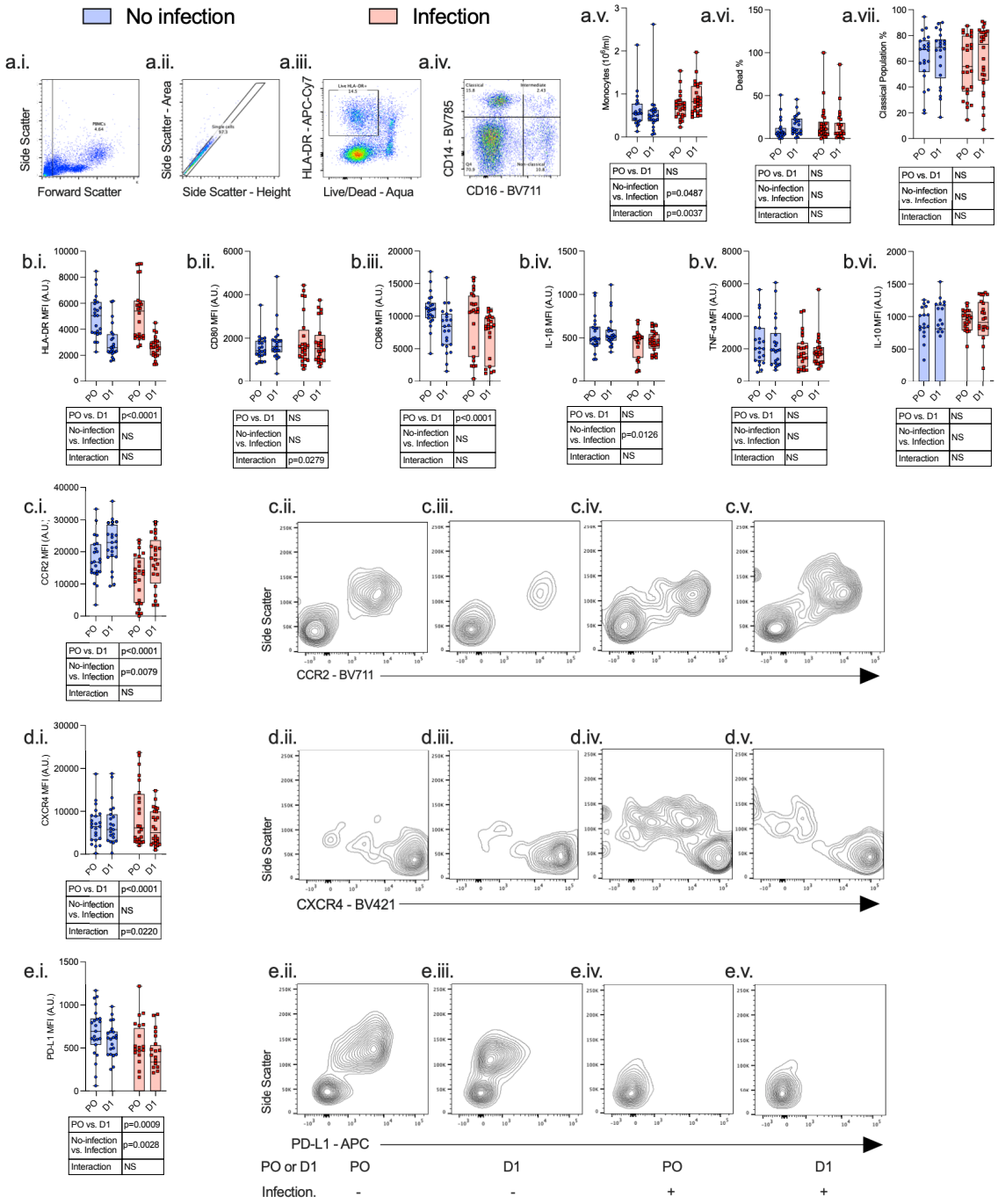

Figure 5.3: Monocytes express an immunosuppressive phenotype both pre- and post-operatively which is associated with the development of subsequent perioperative infections

PBMCs were isolated from patients undergoing major surgery at induction of anaesthesia (pre-op; PO) and 24 hours post-operatively (D1) and classical monocytes analysed by flow cytometry with the following gating strategy: PBMCs, single cells, Live HLA-DR$^+$ cells, CD14/CD16 differentiation (a.i.-a.iv.). The following data was analysed: whole blood monocyte count (a.v), percentage dead (a.vi.), percentage classical population (a.vii.), HLA-DR (b.i.), CD80 (b.ii.), CD86 (b.iii.), IL-1β (b.iv.), TNF-α (b.v.), IL-10 (b.vi.), CCR2 (c.i.-c.v.), CXCR4 (d.i.-d.v.) and PD-L1 (e.i.-e.v.). Data expressed as either median fluorescence intensity (MFI) measured in arbitrary units (A.U.) or percentage of population (%). Dots represent individual patients, horizontal bar the median, box the interquartile range and whisker the range. Data analysed using mixed-effects two-way ANOVA. Data are presented as differences over time, between groups, and the difference in the change over time between the 2 groups (interaction term). Only p-values <0.05 shown. Zebra plots show differences between patients who did not (ii. and iii.) or did (iv. and v.) develop an infection preoperatively (ii. and iv.) and post-operatively (iii. and v.).

Compared to patients who did not have a post-operative infection, patients who developed an infection had a higher monocyte count ($p<0.05$), higher $CD4^+$ lymphocyte IL-7R ($p<0.001$) and $CD8^+$ lymphocyte IL-2R ($p<0.05$) expression, and lower monocyte CCR2 and PD-L1 (both $p<0.01$) expression before and after surgery.

I next assessed surgery-induced immune perturbations across 24 hours in patients who did and did not develop a post-operative infection. Compared to patients who did not have any post-operative infection, patients who developed an infection had an increase in monocyte count ($p<0.01$), $CD4^+$ lymphocyte IL-7R ($p<0.001$), and fall in monocyte CD80 and CXCR4 expression (both $p<0.05$). (Figure 5.3 and Figure 5.4)

Principal component analysis was conducted in 25 of 48 patients for whom full datasets were available. PCA provided reasonable separation between patients with and without subsequent infections with the first two components providing 30% cumulative proportion of variance. Monocyte chemokine receptors (pre- and post-operative monocyte CXCR4), and receptors involved in antigen presentation (CD80) were the greatest discriminators between patients with and without post-operative infections (loading vector coefficient of > 0.8). Post-operative monocyte count was significantly associated with the development of post-operative infection (FDR < 1%; adjusted p-value =0.001) with an AUROC of 0.84 ($p<0.001$). (Figure 5.5)

Co-variates included in the multivariate analysis to assess for risk factors associated with post-operative infection were based on univariate analyses. I included post-operative monocyte count, age, the presence of active cancer and surgical time in the regression analysis. Active cancer (OR=24.6; p=0.056) and post-operative monocyte count (OR=8.9, p=0.056) were associated with increased risk of post-operative infections; albeit not statistically significant. Age (OR=1.019; p=0.593) and surgical time (OR=1.004; p=0.327), however, were not independently associated with post-operative infections.

5.4.1.3 *In vitro* functional capacity of PBMCs before and after surgery

Analysis of immune cell phenotype before and after surgery provided insight into changes associated with surgery. Next, I sought to investigate the effect of surgery on the ability of immune cells to respond to an *in vitro* stimulus (i.e. their functional capacity). PBMCs isolated from healthy volunteers were used as a reference.

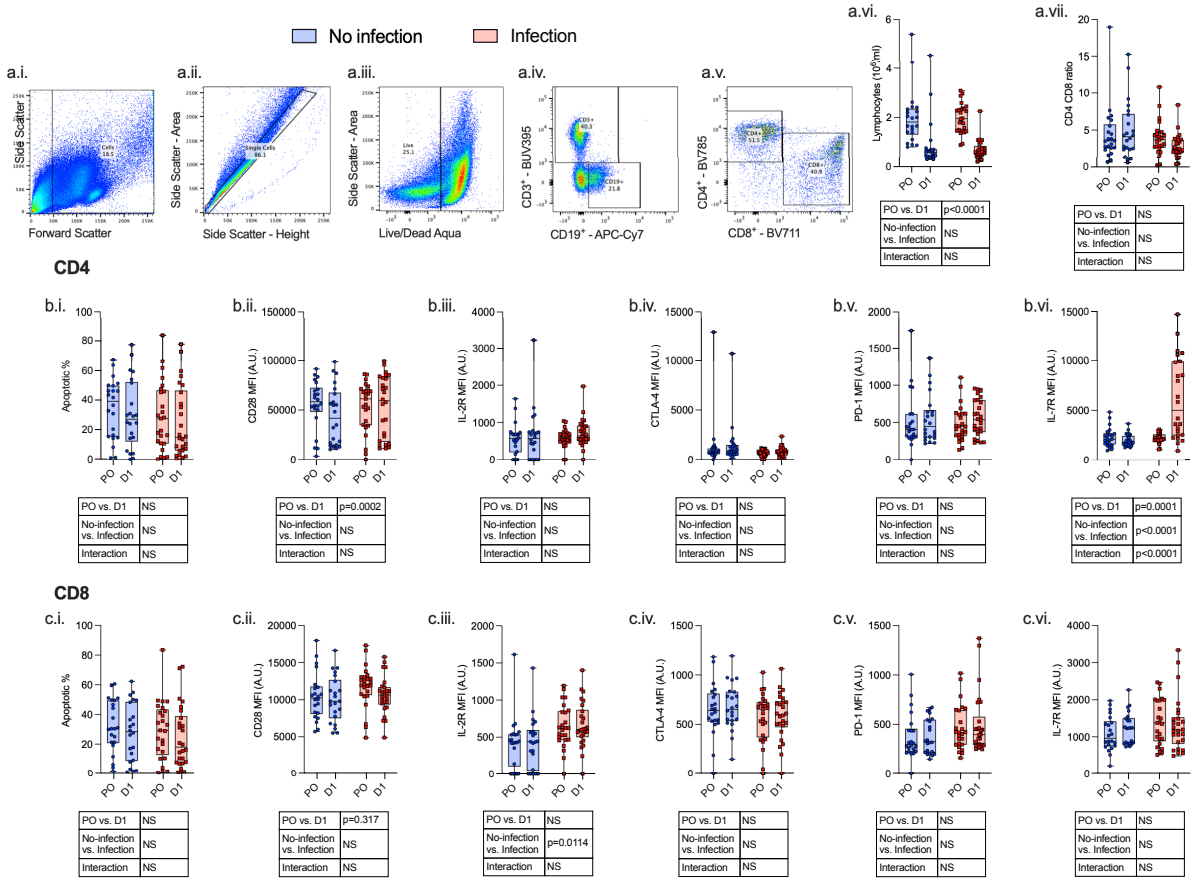

Figure 5.4: Lymphocytes express an immunosuppressive phenotype both pre- and post-operatively which is associated with the development of subsequent perioperative infections

PBMCs were isolated from patients undergoing major surgery at induction of anaesthesia (pre-op; PO) and 24 hours post-operatively (D1) and analysed by flow cytometry with the following gating strategy: Lymphocytes, single cells, $CD3^+$ or $CD19^+$ cells, $CD4^+$/$CD8^+$ differentiation (a.i.-a.v.). The following data was analysed: whole blood lymphocyte count (a.vi), $CD4^+$:$CD8^+$ ratio (a.vii), $CD4^+$ (row b.) and $CD8^+$ (row c.) apoptosis (i.), CD28 (ii.), IL-2R (iii.), CTLA-4 (iv.), PD-1 (v.) and IL-7R (vi.).

Data expressed as either median fluorescence intensity (MFI) measured in arbitrary units (A.U.) or percentage of population (%). Dots represent individual patients, horizontal bar the median, box the interquartile range and whisker the range. Data analysed using mixed-effects two-way ANOVA. Data are presented as differences over time, between groups, and the difference in the change over time between the 2 groups (interaction term). Only p-values <0.05 shown.

5.4.1.4 Monocyte stimulation

Following 24- hour stimulation with HKB, there was an increase in CD86 ($p<0.05$), IL-1β, and TNF-α (both $p<0.01$), and reduction in CXCR4 and HLA-DR (both $p<0.001$) in healthy volunteer monocytes. Among patients without a post-operative infection, there was an increase in monocyte IL-1β ($p<0.05$) and reduction in CXCR4 ($p<0.01$) in pre-operative samples. Following surgery there were no changes in monocyte phenotype following HKB stimulus. Among patients with a post-operative infection, there were no changes in monocyte phenotype following HKB stimulus in pre-operative samples. Following surgery, HKB induced an increase in monocyte PD-L1 expression ($p<0.01$) and reduction in IL-10 ($p<0.05$). (Figure 5.6 and Figure 9.19)

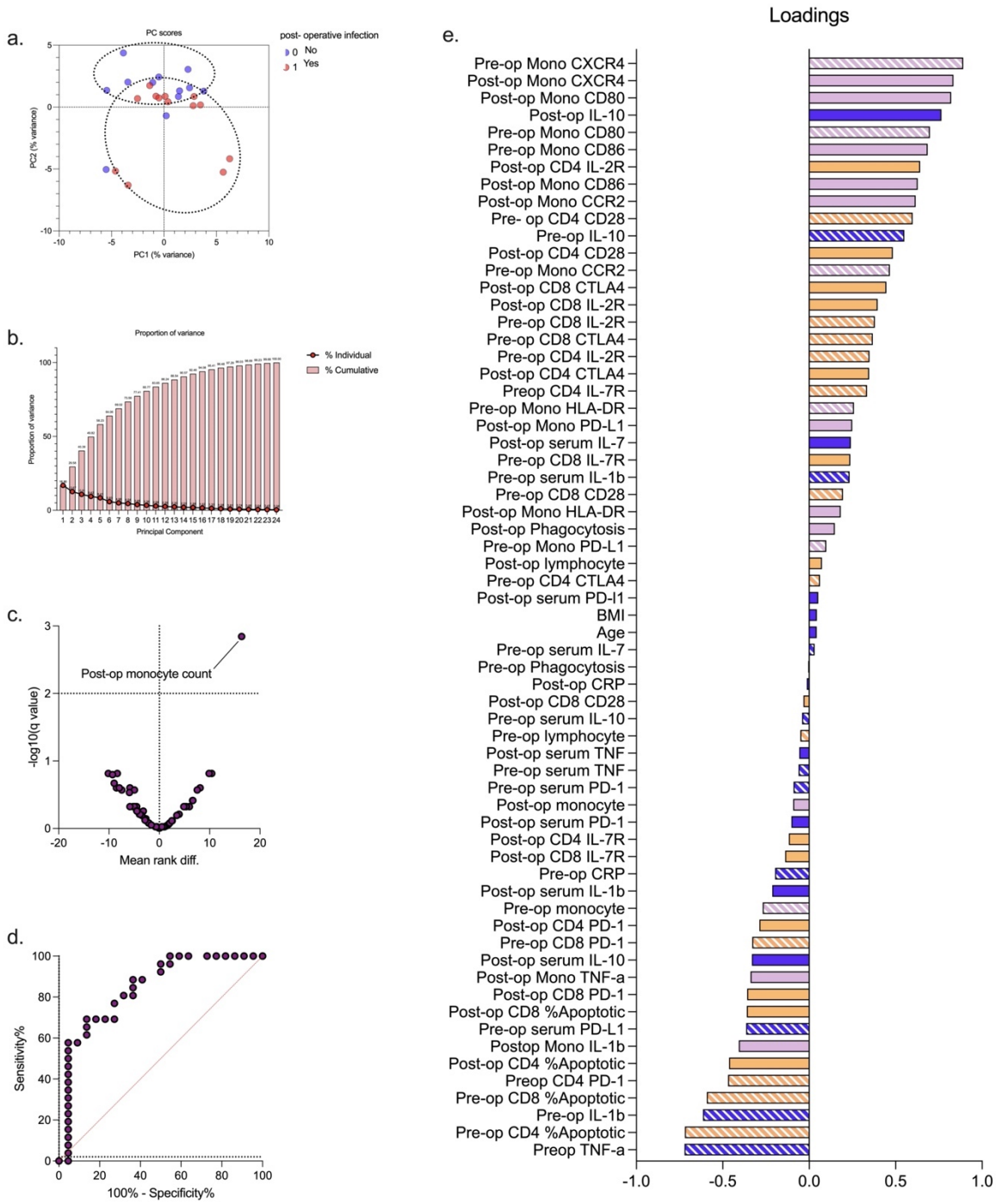

Figure 5.5: Classical monocyte chemokine receptor expression and markers associated with antigen presentation are associated with the development of perioperative infection
Principal component analysis (PCA) demonstrated reasonable separation between patients with and without subsequent infections (a.) with the first two components providing 30% cumulative proportion of variance (b.). The volcano plot demonstrated that post-operative monocyte count correlated with the development of post-operative infection (c.) with an area under the receiving operator characteristic curve (AUROC) of 0.84 ($p<0.0001$). The PCA loadings plot (e.) identified markers of monocyte chemotaxis (pre- and post-operative monocyte CXCR4), and antigen presentation (CD80) were greatest discriminators between patients with and without post-operative infections (loading vector coefficient of > 0.8). Pre-operative variables represented by stripes, post-operative variables by solid fill. Dark blue represents clinical, serum and haematological variables, purple represents monocyte variables, and orange represents T-lymphocyte variables. Data analysed by multiple Mann-Whitney tests (volcano plot) or principal component analysis (remaining plots).

5.4.1.5 $CD4^+$ lymphocyte stimulation

Following PBMC stimulation with CD3/CD28 beads, an increase in $CD4^+$ lymphocyte IL-2R expression and reduction in IL-7R expression was consistent between healthy volunteers, pre-operative and post-operative samples (in patients with and without post- operative infections) ($p<0.05$ for all). An increase in $CD4^+$ lymphocyte CD28 expression was seen in all groups ($p<0.05$ for all) apart from pre-operative samples from patients with post-operative infections. CTLA-4 expression increased only in pre-operative PBMCs isolated from patients with and without (both $p<0.05$) subsequent infections.

An increase in $CD4^+$ lymphocyte PD-1 was limited to healthy volunteers and in pre-operative PBMCs from patients without post-operative infection (both $p<0.01$); although an increase in $CD4^+$ lymphocyte apoptosis was not observed. In contrast, there was in increase in $CD4^+$ lymphocyte % apoptosis ($p<0.01$) among pre-operative samples from patients with post-operative infections, but no increase in PD-1 expression. (Figure 5.6 and Figure 9.20)

5.4.1.6 $CD8^+$ lymphocyte stimulation

Following PBMC stimulation with CD3/CD28 beads, an increase in $CD8^+$ lymphocyte IL-2R expression was consistent between healthy volunteers, pre-operative and post-operative samples (in patients with and without post-operative infections) ($p<0.05$ for all). An increase in $CD8^+$ lymphocyte CD28 expression was seen in patients before and after surgery (with and without post- operative infections) ($p<0.05$ for all), but not in healthy volunteers. In contrast, in increase in CTLA-4 was seen in healthy volunteers ($p=0.0210$) but not in PBMCs isolated from patients before and after surgery. A reduction in IL-7R expression was evident only in healthy volunteers ($p<0.001$) and in pre-operative PBMCs isolated from patients with and without (both $p<0.05$) subsequent infections. An increase in $CD8^+$ lymphocyte PD-1 expression was seen in healthy volunteers, in pre-operative $CD8^+$ lymphocytes from patients without infectious complications and post-operative $CD8^+$ lymphocytes from patients with infectious complications (all $p<0.01$). (Figure 5.6 and Figure 9.20)

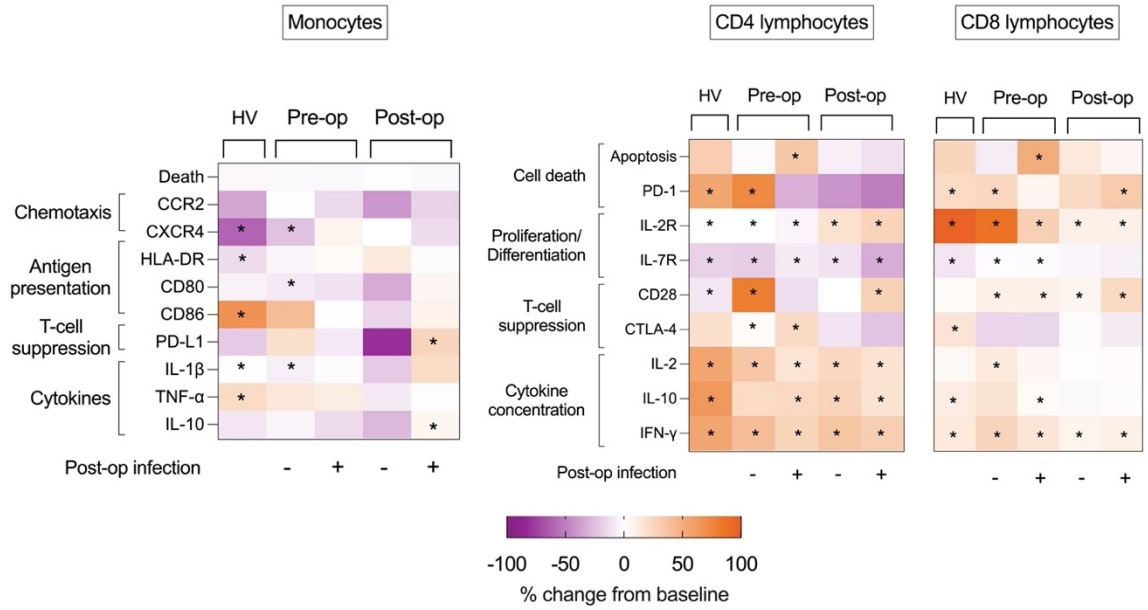

Figure 5.6: Heat maps of effect of *ex vivo* stimulus on volunteer and patient immune cells
Heat maps illustrating percentage changes in immune cell phenotype following HKB (monocyte) or CD3/CD28 bead (lymphocyte) stimulation in PBMCs isolated from heathy volunteers (HV) or from pre- and post- operative samples obtained from patients who did or did not succumb to post-operative infectious complications. Difference between HKB-stimulated compared to unstimulated cells analysed using Mann Whitney U test, and displayed as percentage change, only $p<0.05$ shown indicated by *.

5.4.2 Effect of antibiotics on surgery-induced immunosuppression

5.4.2.1 Clinical data

12 patients were included. Patients had a median age of 61(47-70) and 8 (67%) were male. Nine (75%) underwent upper gastrointestinal, 2 (17%) maxillofacial, and 1 (8%) gynaecological surgery. All patients were being treated for cancer and 8 (67%) had received neoadjuvant chemotherapy. All patients received peri-operative antimicrobial prophylaxis; 9 (75%) received cefuroxime and metronidazole, 2 (17%) received co-amoxiclav and 1 (8%) ciprofloxacin and clindamycin. The median duration of antibiotics was 1(0-1) day. Post-operative infections were diagnosed by the clinical team in 6 (50%) patients and occurred 3 (3-4) days following surgery. Two patients developed pneumonia, 2 developed a wound infection, 1 developed a urinary tract infection and 1 patient had an unknown source of infection. Two of the 6 patients did not satisfy the StEP-COMPAC criteria for post-operative infections. (Table 5.2 and Figure 5.7) Two (17%) patients died during their hospital admission.

PBMCs isolated immediately following surgery were used for the following experiments.

Variable	Patients (n=12)
Age (years)	61 (47-70)
Gender (% male)	8 (67%)
BMI	23.8 (20.6-28.7
Co-morbidities n (%)	
Hypertension	7 (58%)
Cardiovascular disease	3 (25%)
Respiratory disease	3 (25%)
Type 2 diabetes	4 (33%)
ASA Grade	3 (3-3)
Active cancer	12 (100)
Cancer staging	2 (2-3)
Neoadjuvant chemotherapy	8 (67%)
Other immunosuppressive medication	0%
Long term steroids	0%
SORT Score (%)	1.5 (1.2-3.2)
Type of surgery n (%)	
Upper GI	9 (75%)
Maxillofacial	2 (17%)
Gynaecological	1 (8%)
Peri-operative antibiotics	
Prophylaxis administered (%)	100%
Duration of prophylaxis (days)	1 (0-1)
Cefuroxime and metronidazole	9 (75%)
Co-amoxiclav	2 (17%)
Ciprofloxacin and clindamycin	1 (8%)
Intra-operative dexamethasone use (%)	11 (92%)
Operation duration (mins)	229 (199-374)
Blood loss (mls)	200 (200-650)
Peri-operative blood transfusion (%)	1 (8%)
Unplanned ICU readmission (%)	0%
Post-op infection	6 (50%)
Chest	2
Urine	2
Wound	1
Other/Unclear	1
Clavien-Dindo classification	2 (1-2)
Hospital length of stay (days)	12 (9-18)
Death (%)	2 (17%)

Table 5.2: Clinical variables and outcomes of included patients

Abbreviations: BMI: Body mass index, ASA: American Society of Anesthesiologists, SORT: Surgical Outcome Risk Tool, GI: Gastro-intestinal, ICU: Intensive Care Unit

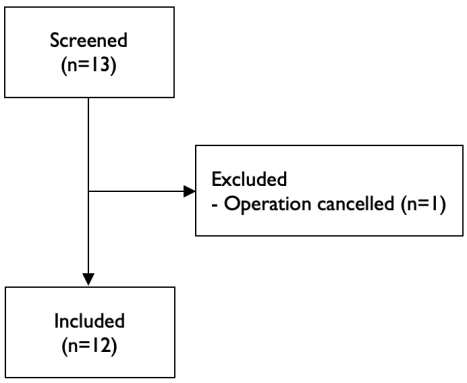

Figure 5.7: Flow chart of included patients

5.4.2.2 *Ex vivo* effect of antibiotics on monocytes and lymphocytes isolated from patients following surgery

In unstimulated PBMCs, amoxicillin caused an increase in monocyte CD80 at low and high doses (both $p<0.05$). (Figure 9.21) High dose amoxicillin caused an increase in $CD4^+$ lymphocyte IL-2 ($p<0.05$) and IFN-γ ($p<0.01$), a decrease in viability and an increase in the T_{reg} population (both $p<0.05$). (Figure 9.22) Amoxicillin at high dose caused a decrease in $CD8^+$ lymphocyte CD28, an increase in CTLA-4 (both $p<0.01$) and IL-2R, an increase IL-2 (both $p<0.05$), and a decrease in viability ($p<0.001$). (Figure 9.23)

Cefuroxime caused an increase in monocyte CCR2 at low dose and a reduction in IL-10 at high dose (both $p<0.05$). (Figure 9.21) It increased $CD4^+$ lymphocyte IL-2R at high dose ($p<0.001$), a dose-dependent increase in IL-2 ($p<0.05$ and $p<0.01$ at low and high dose respectively) and IFN-γ (both $p<0.01$), a dose-dependent decrease in viability (both $p<0.01$), and an increase in the T_{reg} population ($p<0.05$) at high dose. (Figure 9.22) In $CD8^+$ lymphocytes, cefuroxime caused an increase in CD28 ($p<0.01$) at low dose, a dose-dependent decrease in CTLA-4 ($p>0.01$ and $p<0.05$ for low and high dose respectively), an increase in IL-2R ($p<0.01$) and IL-2 ($p<0.05$) at high dose, a dose-dependent increase in IFN-γ ($p<0.05$ and $p<0.01$ for low and high dose respectively), and a dose-dependent decrease in viability (both $p<0.01$). (Figure 9.23)

In monocytes, metronidazole had no effect but caused a dose-dependent increase in $CD4^+$ lymphocyte IFN-γ (both $p<0.05$), a dose-dependent decrease in viability (both $p<0.01$) and an increase in the T_{reg} population ($p<0.05$) at low dose only. (Figure 9.22) In $CD8^+$ lymphocytes it caused a decrease in CTLA-4 and an increase in IFN-γ at low dose, and a dose-dependent decrease in viability (all $p<0.01$). (Figure 9.23)

Combined cefuroxime-metronidazole had no effect on monocytes, but caused an increase in $CD4^+$ lymphocyte IL-2R and IL-2 (both $p<0.01$) at high dose, a dose-dependent increase in IFN-γ ($p<0.05$ and $p<0.001$ at low and high dose respectively), a decrease in IL-10 ($p<0.05$) at low dose, a dose-dependent decrease in viability ($p<0.01$ and $p<0.001$ at low and high dose respectively), and a dose-dependent increase in the T_{reg} population ($p<0.05$ and $p<0.01$ at low and high dose respectively). (Figure 9.22) In $CD8^+$ lymphocytes, it caused an increase in CD28 at high dose ($p<0.01$), a dose-dependent decrease in CTLA-4 (both $p<0.01$), an increase in IL-2R, IL-2 and IFN-γ (all $p<0.001$) at high dose, and a dose-dependent decrease in viability ($p<0.05$ and $p<0.001$ for low and high dose respectively). (Figure 9.23)

5.4.2.3 Dynamic *ex vivo* immune response of monocytes and lymphocytes isolated from patients following surgery

In monocytes, heat-killed E. *coli* caused an increase in CD80 ($p<0.01$), a decrease in CD86 ($p<0.05$), an increase in IL-1ß ($p<0.01$), PD-L1, and a decrease in viability, and an increase in the T_{reg} population (all $p<0.05$). (Figure 9.24)

CD3/CD28 beads in $CD4^+$ lymphocytes caused a decrease in CD28 and IL-7R, an increase in IL-2, IFN-γ (all $p<0.001$), and IL-10 ($p<0.05$), an increase the T_{reg} population and a decrease in viability (all $p<0.05$). (Figure 5.8 and Figure 9.25)

In $CD8^+$ lymphocytes, CD3/CD28 beads caused a decrease in CD28 and IL-7R, an increase in intracellular IL-2, IFN-γ, and IL-10, a decrease in PD-1 (all $p<0.01$) and viability ($p<0.05$). (Figure 5.8 and Figure 9.26)

5.4.2.4 Effect of antibiotics on stimulated monocytes and lymphocytes *ex vivo*

In heat-killed E. *coli* stimulated PBMCs, amoxicillin caused a dose-dependent decrease in monocyte CXCR4 (both $p<0.05$). (Figure 9.24) In CD3/CD28 stimulated PBMCs, amoxicillin caused a decrease in $CD4^+$ lymphocyte PD-1 and viability (both $p<0.05$) at high dose. (Figure 9.25) In $CD8^+$ lymphocytes it caused a decrease in viability at high dose ($p=0.0143$). (Figure 9.26)

Cefuroxime caused a decrease in monocyte viability ($p<0.05$) at high dose. (Figure 9.24) In $CD4^+$ lymphocytes at high dose it caused an increase in IL-2R ($p<0.01$), IL-2 and IFN-γ (both $p<0.001$), and a decrease in viability ($p<0.05$), whilst at low dose, it an increase in an increase the T_{reg} population ($p<0.05$). (Figure 5.8 and Figure 9.25) In $CD8^+$ lymphocytes, high dose cefuroxime caused a decrease in CD28 ($p<0.05$), an increase in IL-2R ($p=0.01$), intracellular IL-2 and IFN-γ (both $p<0.001$), whilst there was a dose-dependent decrease in viability (both $p<0.05$). (Figure 9.26)

Metronidazole had no effect on monocyte function. In $CD4^+$ lymphocytes it caused a decrease in CTLA-4 ($p<0.05$) at low dose. (Figure 9.25) In $CD8^+$ lymphocytes it caused a decrease in CTLA-4 and PD-L1 (both $p<0.01$) at low dose, and a dose-dependent decrease in viability ($p<0.01$ and $p<0.05$ for low and high dose respectively). (Figure 9.26)

Combined cefuroxime-metronidazole caused an increase in monocyte CXCR4 ($p<0.05$) at high dose. (Figure 9.24) In $CD4^+$ lymphocytes it caused a decrease in CTLA-4 at low dose ($p<0.05$), whilst high dose caused an increase in IL-2R, IL-2 and IFN-γ, and a decrease in viability (all $p<0.01$). (Figure 9.25) In $CD8^+$ lymphocytes it caused a decrease in CTLA-4 and PD-L1 and an increase in IL-7R (all $p<0.05$) at low dose, an increase in IL-2R and IFN-γ at high dose (both $p<0.01$) and a dose-dependent decrease in viability ($p<0.05$ and $p<0.01$ for low and high dose respectively). (Figure 9.26)

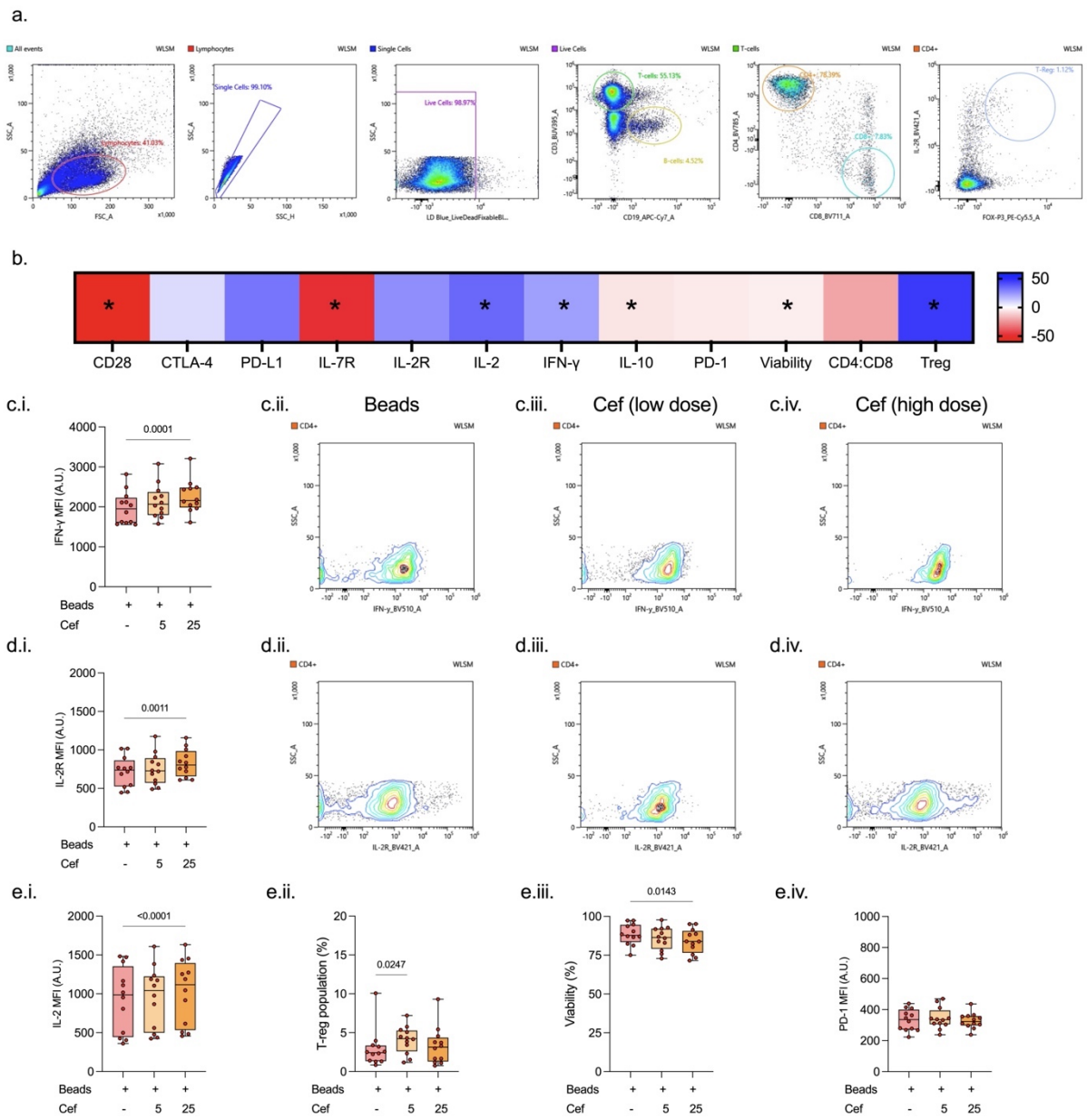

Figure 5.8: Cefuroxime augments postoperative CD4⁺ lymphocyte function

PBMCs isolated from patients immediately postoperatively (n=12) were incubated with CD3/CD28 beads (Beads, red dots) and the effect on $CD4^+$ lymphocyte of cefuroxime (orange), at a concentration of 5 or 25μg/ml was delineated. (a.) Gating strategy for lymphocytes. (b.) Heat-map summary of effect of bead stimulus on $CD4^+$ lymphocyte function. Effect of cefuroxime on (c.) intracellular IFN-γ, (d.) IL-2R expression, (e.i.) intracellular IL-2, (e.iii.) T-reg population (e.iii.). cell viability and (e.iv.) PD-1 expression. Data expressed as median percentage change (heat-map), median fluorescence intensity measured in arbitrary units (MFI (A.U.)), percentage of population (%). Individual points represent individual patients, horizontal line the median, box the interquartile range and whisker the range. Raw MFIs of each antibiotic compared to bead-stimulated cells using Friedman multiple comparisons test without Dunnett's correction, only results with $p<0.05$ indicated by * reported

5.5 Discussion

5.5.1 Effect of surgery on immune function

Major surgery results in significant and prolonged physiological and inflammatory changes. Innate and adaptive cells can mount simultaneous opposing pro- and anti-inflammatory functions.[371-373] I hypothesized that patients who develop infection following surgery demonstrate a marked anti-inflammatory phenotype following surgery (compared to patients who do not develop an infection), representing an impaired ability to mount a pro-inflammatory response to a subsequent infectious challenge. Specific alterations to the immune phenotype within 24 hours of major surgery were associated with the development of a subsequent infection.

A significant reduction in receptors involved in monocyte antigen presentation (CD80) and chemokine receptors (CXCR4) were associated with infectious complications. Monocyte CCR2 expression increased following surgery, in patients with and without post-operative infections. However, patients who developed post-operative infections had lower levels of CCR2 expression pre- and post-operatively compared to patients without post-operative infections. Circulating monocyte counts fell in patients who did not develop any post-operative infections, whereas this was not evident in patients who developed a post-operative infection. Together, these findings suggest that in patients who develop a post-operative infection, circulating monocytes may remain in the peripheral blood and fail to migrate to sites of inflammation due to impairments in chemotaxis. Post-operative complications in high risk and cancer surgery are associated with higher peripheral monocyte counts.[374,375] The mechanism underpinning this observation is unclear, but may represent an impairment in ability of monocytes to migrate to sites of inflammation/ infection akin to impairments in neutrophil chemotaxis associated with post-operative infections.[376-379]

Lymphopenia and impaired lymphocyte function are associated with increased risk of developing post-operative infections.[380] I found a significant reduction in lymphocyte count and expression of lymphocyte co-stimulatory receptor (CD28) following surgery, although this did not discriminate between patients with and without post-operative infections. Alternative pathways associated with lymphocyte dysfunction may be contributary.[380]

Several studies have investigated the change in immunophenotype of patients following surgery.[363,364,371-373,381,382] However, few studies have investigated the effect on the dynamic immune response to a subsequent *in vitro* challenge before and after surgery; and how this differs between patients who develop post-operative infections and those who do not.

In comparison to healthy volunteer monocytes, there was paucity of changes in immune phenotype following *in vitro* HKB stimulus in patients undergoing surgery. Although patients who did not develop post-operative infections demonstrated few changes consistent with healthy volunteers in the pre-operative period, monocytes failed to demonstrate any response to *in vitro* HKB stimulus following surgery; suggestive of decreased monocyte functional reserve seen after other major surgery.[383-385]

Among patients who had a subsequent post-operative infection, there were no changes to monocyte phenotype following HKB stimulus in cells isolated pre-operatively. In contrast, post-operative monocyte PD-L1 expression increased following HKB stimulus in patients with post-operative infections, which was not evident in healthy volunteers or patients without post-operative infections. An increase in monocyte PD-L1 expression is associated with $CD4^+$ lymphocyte inhibition via the PD-1/ PD-L1 pathway suggesting a plausible mechanism.[386] Elevated monocyte PD-L1 expression in critically ill patients is associated with lymphocyte anergy and increased risk of secondary infections.[115,386] Monocyte PD-L1 is less well characterised in surgery although elevated monocyte and serum PD-L1 are associated with increased risk of infection in other inflammatory processes such as pancreatitis.[387,388]

In contrast to monocytes, many changes to $CD4^+$ and $CD8^+$ lymphocyte immune phenotype following CD3/CD28 bead stimulation in healthy volunteer cells was seen in patient cells. This was more evident in pre-operative lymphocytes from patients without post-operative infections. Fewer changes were seen in post-operative samples compared to pre-operative samples, suggestive of reduced functional capacity following surgery. Impaired lymphocyte functional responses may be mediated by a hypometabolic phenotype which occurs following surgery.[380]

A number of approaches have been attempted to reverse post-operative immunosuppression including G-CSF,[389] IFN-γ,[390] and IL-10 pathways,[391] but with no conclusive benefit. Attempted modulation of a single immunomodulatory target is unlikely to yield results as related co-stimulatory or inhibitory pathways may be simultaneously affected.

I acknowledge limitations in this study. Assessment of the trajectory of immune phenotype over a longer duration would provide greater insight into the recovery following surgery. Majority of our patients had underlying solid organ malignancies, a proportion of who received chemotherapy. It is not possible to extrapolate our findings to other cohorts of patients. All *in vitro* experiments were performed using a single concentration and strain of HKB or CD3/CD28 beads. The percentage of T-lymphocyte apoptosis was high compared to healthy populations, however this was consistent with other high-risk surgical cohorts.[392] I did not investigate neutrophil function, which is also known to be impaired in surgery.[376-379] Similarly, I have not investigated the role of B-cells. I have not presented data on intermediate and non-classical monocyte subsets as cell counts from patients were limited.

However, quantification of cell surface markers on monocyte subsets are rarely, if ever, used to stratify immune status in critically ill patients.

I assessed levels of ligands and receptors (e.g., PD-L1 and PD-1) on flow cytometry but were unable to assess their interaction or associated pathways. Specifically, monocyte chemokine receptor expression could be further explored using chemotaxis assays. However, typical cell counts required for such assays exceeds that obtained from patients. The response to an *in vitro* stimulus (HKB of CD3/CD28 beads) may not represent *in vitro* changes in patients with infections.

I found that post-operative monocyte count is by far the most differentiating feature on the volcano plot although not a major discriminator on PCA. This might be explained by the fact that PCA includes only patients with complete datasets. I included 62 immune markers, age, and BMI- full datasets were available in 25 of 47 patients. However, for multiple comparisons using a Mann-Whitney test, data from all patients were used.

I conducted a multivariate analysis to assess the independent effects of different covariates on infectious complications. Due to the relatively small sample size, I was limited in the number of co-variates in our analysis. However, the multivariate analysis supports the findings of our other analyses, demonstrating that post-operative monocyte count may be independently associated with post operative infections.

An unsupervised analysis of a wider panel of markers may reveal other druggable targets. Several studies have assessed the transcriptomic profile of immune cells in the perioperative period, although transcriptional changes may not be reflected in cell surface proteins/ receptor expression, and bulk transcriptomics do not directly assess the phenotype of specific cell subsets.[363,382]

5.5.2 Effect of antibiotics on surgery-induced immunosuppression

Surgery is associated with alterations to immune function which may predispose to the development of post-operative infections.[311] Antibiotics can modulate the immune system,[359] although their effect in the peri-operative cohort is relatively unexplored. I have demonstrated that commonly prescribed antimicrobial prophylaxis modulates the immune response in PBMCs isolated immediately post-operatively.

Lymphocyte differentiation is regulated by IL-2 and its receptor. Levels of both IL-2 and receptor expression fall post-operatively to a nadir around days 3-4, which corresponds with the timing of development of post-operative infections.[393-395] Lymphocytes however retain the ability to increase both IL-2 concentration and IL-2R expression in response to a subsequent stimulus.[311] *Ex vivo*, cefuroxime increases expression of $CD4^+$ lymphocyte IL-2/ IL-2R following surgery. In response to an

ex vivo stimulus, cefuroxime in associated with an increase in $CD4^+$ lymphocyte IL-2R and augments the increase in intracellular IL-2. IL-2 has an autocrine effect, with increased production and receptor binding causing clonal proliferation and cell differentiation of naive to effector cell populations, including helper (T_{h1}) and regulatory (T_{reg}) cells.[396] This is mediated through the JAK-STAT5, PI3K-AKT and RAS-MAP kinase pathways.[397] Beta-lactam antibiotics upregulate activity of the PI3K-AKT pathway in lymphocyte cell lines through DNA damage-induced apoptosis pathways.[281] In stimulated volunteer PBMCs, cefuroxime (at supra-clinical concentrations) alters gene expression encoding transcription factors which regulate lymphocyte differentiation.[345] Additionally, cephalosporins bind directly to the IL-2R causing inhibition at supra-clinical concentrations, but lower doses could cause activation and there lymphocyte differentiation.[344] Proteomics or further experiments using flow cytometry could confirm alteration of proteins and transcription factors involved in this signalling pathway.

T_{h1} cells are vital for facilitating the response to infection by facilitating macrophage-mediated bacterial clearance,[398] however their numbers are reduced post-operatively with reduced IFN-γ release in response to a secondary stimulus consistent with anergy.[395,399] They are the main lymphocyte sub-population which produces IFN-γ. IFN-γ has important roles in tissue homeostasis, immune and inflammatory responses and tumour immunosurveillance.[400] Prolonged high IFN-γ levels can lead to excessive inflammation.[401] Cefuroxime appears to either prevent or reverse this anergy as I found it increased $CD4^+$ IFN-γ. However, specific markers to identify T_{h1} cells were not used (e.g. the transcription factor T-bet and chemokine receptor CCR6). Cephalosporins have been shown to increase IFN-γ release,[402] although inhibition occurs at supra-clinical doses.[344,403,404] This suggests cefuroxime could enhance T_{h1} function which may be protective against post-operative infections and tumour recurrence,[400] although prolonged high levels of IFN-γ are associated with increased inflammation,[401] including formation of adhesions in mouse models.[405]

T_{reg} cells are broadly immunosuppressive and act to limit the inflammatory response after surgery through release of anti-inflammatory cytokines including IL-10, and directly inhibiting antigen presenting cells through CTLA-4.[398,406] Their proportion increases following surgery,[399,407] and whilst their direct effect on the risk of post-operative infection is yet to be delineated, they are implicated in the risk of secondary infections after sepsis.[408] I demonstrate that cefuroxime augments the increase in percentage of T_{reg} cells in stimulated $CD4^+$ lymphocytes, likely mediated by cefuroxime-augmented IL-2 concentration as IL-2 is critical for T_{reg} proliferation and survival.[409] However, as the total percentage itself is small, and there is no associated increase in $CD4^+$ IL-10 concentration, the immunosuppressive effects may be limited.

Lymphopenia is a common immunosuppressive feature seen after surgery, caused by an increase in mitochondrial-dependant apoptosis and decreased proliferation, and is associated with worse outcomes.[380,392] I demonstrate that cefuroxime decreases lymphocyte viability although this is not associated with up-regulation of PD-1. Other studies have also shown that PD-1 expression is relatively unaltered following surgery.[311,410] Activation-induced apoptosis occurs via the Fas signalling pathway and is activated by IL-2, which may explain the increase in lymphocyte death.[411] Cefuroxime, by augmenting IL-2 concentration could therefore reduce cell viability through this mechanism. However, others have shown that post-operative lymphopenia is not associated with alterations in expression of the Fas receptor, although changes in expression of its effector, caspase-8, were not measured.[380]

Whilst I demonstrate increases in T_{reg} populations and imply improved T_{h1} function through IFN-γ concentration, I did not measure lymphocyte proliferation. Cefuroxime has previously been shown to have an inhibitory effect on $CD4^+$ lymphocyte proliferation, although this occurs at supra-clinical concentrations.[272,275,276] Further work is needed to identify whether alterations in IL-2 and IL-2R are associated with increased proliferation, especially as IL-7R expression, a receptor associated with proliferation, was unchanged.

In monocytes, cefuroxime exhibited fewer effects; this could be related to the shorter duration of incubation, and it was the only antibiotic to reduce viability. This could be mediated by the effect of cefuroxime on T_{h1} cytokine production as IFN-γ increases monocyte apoptosis through the Fas receptor *ex vivo*, however IL-2 is protective.[412] I measured intracellular cytokines and further *ex vivo* mechanistic work is needed to assess concentrations of released cytokines.

The relative lack of effect of amoxicillin in comparison to cefuroxime suggests there are in class differences between penicillin and cephalosporin beta-lactams. Penicillins may have opposite effects to cefuroxime on the expression of transcription factors controlling lymphocyte differentiation in stimulated human PBMCs;[345] the differences related to the beta-lactam ring structure. Relatively few changes were seen with metronidazole co-incubation. Nitroimidazoles including metronidazole can cause lymphopenia by causing damage to cellular DNA in *in vivo* models,[368,413] although this was only seen in unstimulated cells. Prolonged culture may have unmasked this effect.

Given the small sample size and large number of variables measured, I have only performed univariate analysis. However, the dose-dependent effect of cefuroxime in multiple components of $CD4^+$ lymphocyte effector function (IFN-γ/ IL-2/ IL-2R/ T_{reg}) in both unstimulated and stimulated cells provide robustness to my findings. Further work is required to identify the mechanism of action of cefuroxime on IL-2 mediated pathways including apoptosis, cytokine release, and differentiation as described above.

I chose to assess the immune response in the immediate postoperative period. However, immune functional changes preoperatively or at later stages in following surgery may have yielded different findings. A significant proportion of patients had active cancer and neoadjuvant chemotherapy, and use of intra-operative dexamethasone. As such, my findings may only be pertinent to this cohort of patients and extrapolation to other patient populations may not be possible. Additionally, all patients received peri-operative antibiotics which may have had some residual effect on PBMCs. All *in vitro* experiments were performed using a single concentration and strain of heat-killed bacteria or CD3/CD28 beads. I was unable to assess the effect of antibiotics on clinical outcomes after surgery, including post-operative infection. A large cohort of patients would be required to investigate this as several confounders need to be accounted for.

In summary, cefuroxime has multiple immunomodulatory effects on $CD4^+$ lymphocyte function which may improve peri-operative lymphocyte function reducing the risk of postoperative infection, including IL-2 dependant cell differentiation and T_{h1}-dependant IFN-γ release. However, cefuroxime was also associated with a decrease in lymphocyte viability and an increase in T_{reg} population. This was potentially mediated by IL-2. Further research is required to explore its mechanism of action, and to evaluate the net effect of these changes on the risk of subsequent infections.

5.6 Chapter summary

I have demonstrated important differences in the early host response to surgery between patients who do and do not develop a subsequent infection. Given the numbers of patients who undergo major surgery globally, and the proportion who develop post-operative infections, my findings warrant further investigations. Specifically, the underlying mechanisms and potential therapeutics to reverse defects in immune cell function require exploration.

Additionally, routine peri-operative antimicrobial prophylaxis has immunomodulatory effects on surgery-induced changes to the immune system, with potential beneficial effects on lymphocytes seen with cefuroxime. Further work is required to identify the mechanisms responsible, and whether the effect is clinically relevant.

6 Immune responses to COVID-19

6.1 Chapter context

Prior to commencing my PhD, I was awarded a National Institute of Health Research Academic Clinical Fellowship. This gave me dedicated research time as part of my clinical training to generate pilot data for my PhD. I developed techniques including cell culture and flow cytometry.

My original plan had been to explore my hypothesis in patients with bacterial sepsis, followed by non-bacterial inflammatory conditions including surgery and viral-induced acute respiratory failure. However, the COVD-19 pandemic delayed me commencing a PhD, due to heavy clinical commitments and inadvertent delays in the PhD enrolment processes.

During the first 'wave' of the pandemic, my research group obtained ethical approval to explore immune responses in acute COVID-19. I therefore investigated the immune changes associated with COVID-19, and the impact of commonly used antibiotics.

6.2 Introduction

Patients with COVID-19 demonstrate a heterogeneous clinical course ranging from mildly symptomatic disease through to acute respiratory distress syndrome (ARDS) and death.[414] Hospital mortality in patients admitted to UK critical care units during the first surge of the COVID-19 pandemic was 42%.[415] The short- and long-term morbidity burden was also significant.[416] There was clearly a need for further effective therapies targeting both virus and host response to improve outcomes.

The approximate 10-day delay between COVID-19 symptom onset and development of critical illness provides an important window of opportunity to intervene.[417,418] The pandemic triggered a precipitous entry of multiple novel therapeutic candidates into clinical trials often without control groups, randomisation, or adequate statistical power. To this long list can be added a re-purposing of existing therapeutic strategies used for other inflammatory or viral illnesses. Suppressing or removing mediators where blood levels are only mild-to-moderately elevated, or boosting endogenously raised levels of mediators to supranormal values, may prove futile or even detrimental. The same mistakes made over decades for sepsis may be repeated.[419]

A search of clinicaltrials.gov on 3rd July 2020 identified 1366 registered trials, of which 279 were RCTs assessing immunomodulatory therapies. These included targets against 39 different immune pathways

and 90 different drugs or therapies. (Figure 6.1). By 1st November 2020, this number had swelled further with 477 randomized trials assessing immunomodulatory therapies being utilised for the treatment of COVID-19.[420] These included targets against 83 different immune pathways and utilised 168 different therapies. The greatest number of registered clinical trials related to the use of convalescent plasma (n=87), anti-IL-6 monoclonal antibodies (n=43), mesenchymal stem cells (n=46), IFN-α or IFN-β agonists (n=14), and IL-1ß antagonism (n=16). Other biological targets included TNF-α, interleukin-7, interleukin-8, IFN-γ, and therapies either augmenting or inhibiting GM-CSF. Only 12 trials specified measurement of the relevant biological target as an inclusion criterion.

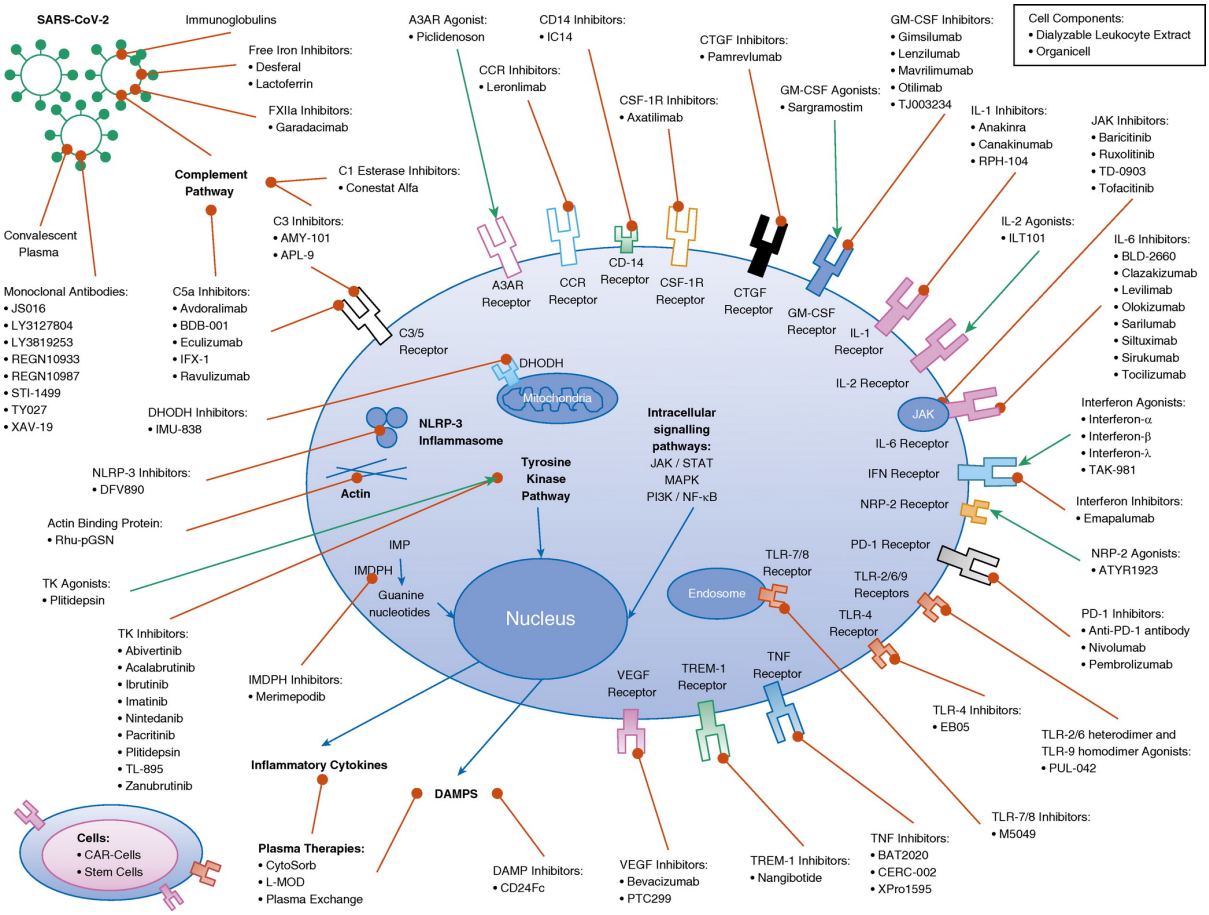

Figure 6.1: Summary of biological therapies undergoing randomised controlled trials in COVID-19

Abbreviations: A3AR - Adenosine A3 receptor; CCR - C-C chemokine receptor; CSF-1R - Colony stimulating factor 1 receptor; CTGF - Connective tissue growth factor; DAMP - Damage-associated molecular patterns; DHODH - Dihydroorotate dehydrogenase; GM-CSF - Granulocyte-macrophage colony-stimulating factor; IMP - Inosine-5′-monophosphate; IMPDH - Inosine-5′-monophosphate dehydrogenase; JAK - Janus kinase; MAPK - Mitogen-activated protein kinase; NF-κB - Nuclear factor-κB; NLRP-3 - NOD-, LRR- and pyrin domain-containing protein 3; NRP-2 - Neuropilin 2; PD-1 - Programmed cell death protein 1; PI3K - Phosphoinositide 3-kinase; STAT - Signal transducer and activator of transcription; TREM-1 - Triggering receptor expressed on myeloid cells-1; VEGF - Vascular endothelial growth factor. Reprinted with permission of the American Thoracic Society. Cite: Snow TAC, Singer M, Arulkumaran N. Immunomodulators in COVID-19: Two Sides to Every Coin. Am J Respir Crit Care Med. 2021 Mar 15;203(6):782. The American Journal of Respiratory and Critical Care Medicine is an official journal of the American Thoracic Society.

Our still incomplete understanding of the COVID-19 disease process, including temporal change, has driven arguably inappropriate, ill-timed or ill-judged interventions, either within trials or compassionate use. Description of the 'cytokine storm' epithet to COVID-19 has driven the application of immunosuppressive therapies. At the time of publication, 47 registered RCTs were evaluating inhibition of interleukin-6 (IL-6), mostly recruiting on clinical criteria alone and without incorporating measurement of circulating IL-6 levels. Although circulating IL-6 levels are higher among COVID-19 non-survivors compared to survivors,[421,422] circulating IL-6 levels in COVID-19 are often 1-2 log-orders lower than other causes of ARDS or viral influenza.[423] While there may indeed be benefit from inhibiting IL-6, timing, dosing and patient selection are key. Outcome improvements in some subsets may be diluted or counterbalanced by lack of effect or harm in others. An acceptable toxicity profile for use in other inflammatory conditions does not necessarily translate to COVID-19, especially in the critically ill subset where both the severity of the disease process and multiple iatrogenic factors magnify immunosuppression and the risk of secondary nosocomial infection. A single dose of the IL-6 inhibitor, tocilizumab, can significantly dampen any C-reactive protein and temperature response for a week.[424] Apart from a potential increased risk of infection, traditional clinical signs may be masked with resulting delays in identification and treatment. The same risk-benefit balance holds for other immunomodulators.

As a further example of scientific uncertainty, therapeutic approaches with directly opposing actions are being promulgated. As an example, with granulocyte-macrophage colony-stimulating factor (GM-CSF), both direct activation and inhibition are being targeted. If modulation in one direction proves successful, the counter-approach may well harm. A further possibility is that both are efficacious, albeit at different timepoints in the disease process; to our knowledge, the critical issue of timing is not being addressed. While the scientific merits behind these contrasting approaches has been eloquently argued, the challenge lies in determining the Goldilocks effect.[425] The intricacy behind the pleiotropic biology of these drug targets and the unknown trade-offs between advantage and detriment in a complex multisystem disease cannot be underestimated.

Publication bias for positive results in small case series may also provide false reassurance of the safety and efficacy of an experimental intervention. Similar issues arise at the other end of the spectrum. While buoyed by the impressive outcome improvements achieved by low-dose dexamethasone within the large-scale RECOVERY study, the explanation for many unexplained findings in this study remained unresolved such as the disparate effects depending on gender, age, illness severity and timing of intervention.[426]

Well-meaning attempts to intervene should not take priority over understanding of the pathogenic mechanisms underlying impaired viral clearance and the development of organ failure. Use of

theranostic biomarkers may identify patients most likely to benefit and to subsequently monitor treatment effects. Risk stratification can also be performed using routinely collected clinical parameters.[427] This will enable trial enrichment, targeting patients most likely to benefit while not exposing those patients unlikely to benefit to potential detriment.

Whilst the potential risk and cost associated with the use of immunomodulatory therapies was being explored in multiple clinical trials, other trials looked at utilising existing low-cost and well-tolerated therapeutic agents. This included several antimicrobial agents, including macrolide antibiotics.

Macrolide antibiotics have immunomodulatory effects,[298] which have theoretical benefits in the management of non-bacterial inflammatory diseases including viral severe acute respiratory illness (SARI).[299] Whilst azithromycin lacks any clinical benefit in the management of COVID-19,[302] a within-class effect may exist with clarithromycin having different immunomodulatory potential. The precise effects of macrolides on T-cell responses to COVID-19, dissimilarities between different macrolides, and synergistic effects with other antibiotics have not been explored.

In this chapter I aim to characterise the immunology of COVID-19 using a cohort of COVID-19 patients recruited at University College Hospital London to evaluate if the therapeutic biomarkers being trialled as COVID-19 therapies discriminated between patients with mild and severe disease or those who subsequently died. Identification of biomarkers which showed discrimination would guide plausible therapeutic strategies and identify those patients who might benefit.

In addition, I will explore whether commonly prescribed antibiotics including macrolides modulate the immune response. Using my patient cohort, I ascertain whether there is a mortality benefit associated with macrolide use compared to other antibiotics on hospital presentation among critically ill patients with COVID-19. I will then characterise these immunomodulatory effects *ex vivo* with a focus on cytokines either with direct antiviral activity or cytokines associated with mortality in COVID-19.

6.3 Methods summary

I performed a retrospective cohort study of adult patients (>17 years of age) presenting with PCR-confirmed COVID-19 admitted via the ED using an assay developed inhouse for the SARS-CoV-2 ORF1a gene.[428] Serum samples taken within the first 5 days of admission which had sample remaining following measurement of routine biochemistry were used. Patients were excluded if they received immunomodulatory treatments.

Samples were analysed using multiplex panels which included the most common therapeutic immune markers undergoing evaluation in clinical trials for COVID-19. Relevant laboratory, clinical, antibiotic

use, immunomodulatory treatments received, and outcome data was also stored and combined with experimental results at study completion.

Volunteer PBMCs were stimulated with SARS-CoV-2 S1 and S2 spike protein and amoxicillin, azithromycin or clarithromycin for 6 hours and the effect on monocyte phagocytosis (assessed using pHRodo bioparticles) and lymphocyte intracellular cytokines was assessed using flow cytometry.

6.4 Results

6.4.1 Effect of COVID-19 on immune function

6.4.1.1 Demographic, clinical and routine laboratory data

90 COVID-19 patients with serum samples were recruited, four were excluded as they had received recent chemotherapy for underlying malignancy, leaving 86 patients in the final analysis. (Table 6.1) There were similar numbers of patients with mild disease (WHO scale <6) (n=44; 51%) and severe disease (WHO scale 6-10) (n=42; 49%) during their hospital stay. The time from hospital admission to blood sample collection was shorter in patients with mild disease compared to those with severe disease (0.5(0-1) vs. 1(0-2.5); $p<0.05$). Healthy volunteers (n=7) consisted of four Caucasians and three from Black or Asian backgrounds. None had pre-morbid illness and their age was 34 (28-49) years.

There were no differences in the proportions of sex or underlying co-morbidities between mild and severe groups. Compared to patients with mild disease, patients with severe illness were older, presented earlier to hospital, had worse oxygenation, and a higher viral load (defined by a lower C_t value). Patients with severe disease had higher admission values of serum creatinine, C-reactive protein and neutrophil counts and lower values of albumin and lymphocyte count. (Table 6.1)

Adjunctive therapies (for this March-June 2020 cohort) throughout the entire length of hospitalisation included steroid use (7/86, 8%), antibiotics (63/86, 73%), and antiviral medications (2/86; 2%). Fourteen (16%) patients needed invasive mechanical ventilation and a further 22 (26%) patients required continuous positive airways pressure. Patients with severe disease were more likely to receive antibiotics and steroids, albeit not in the first week of presentation (Table 6.1). Twenty-one patients (24%) did not survive to hospital discharge.

6.4.1.2 Biomarker and antibody data.

The ability of routinely measured biochemical variables (creatinine, CRP, albumin, neutrophil counts, and lymphocyte count) to predict corresponding biomarker levels was limited; the strongest correlation was between CRP and IL-6 (Spearman's correlation coefficient 0.66; $p<0.001$). (Figure 6.2, Table 6.2, Figure 6.3)

Levels of interferons were elevated in patients with COVID-19 compared to healthy controls. Pro-inflammatory cytokines IL-1ß and TNF- α were lower than seen in controls, albeit within the normal range. Similarly, levels of IL-8 and GM-CSF were lower than seen in healthy controls. Soluble IL-1ra however was significantly elevated in COVID-19 patients compared to controls, as was IL-7, a promoter of lymphocyte development and proliferation. (Figure 6.2, Table 6.2, Figure 6.3)

Between patients with mild or severe disease, levels of GM-CSF, IFN-γ, TNF-α and IL-1ß were similar. Six biomarkers (IL-6, IL-7, IL-8, IFN-α, IFN-ß, IL-1ra) and neutralizing antibody titers) were higher in patients with severe compared to mild disease (all $p<0.05$). Levels of IL-6 on admission correlated well with CRP ($r^2 = 0.398$; $p< 0.001$). On hospital admission, IL-6 discriminated between eventual survivors and non-survivors (AUROC 0.67, $p<0.05$). Admission levels of IL-6 had better discriminatory value for development of critical illness or death compared to patients with mild illness (AUROC 0.78, $p<0.001$). (Figure 6.2, Table 6.2, Figure 6.3, Figure 6.4)

A similar proportion of patients with mild or severe disease had detectable S1 or N IgG antibodies (70% vs. 59%). Among patients with detectable antibodies, there was no difference in S1 or N IgG values between patients with mild or severe disease. Among patients who seroconverted, those with severe disease had a higher serum neutralisation titre compared to patients with mild disease ($p<0.05$). (Table 6.2, Figure 6.2, Figure 6.3)

	Total N=86	Mild N=44	Severe N=42	p-value (mild vs. severe)
Age (years)	61 (48-73)	59 (46-69)	67 (52-75)	0.04
Body mass index (kg/m^2)	25 (23-29)	25 (23-29)	25 (23-30)	0.87
Time from symptoms to hospital (days)	7 (4-11)	10 (5-14)	5 (3-8)	0.02
Time from symptoms to blood sample (days)	1 (0-2)	0.5 (0-1)	1 (0-2.5)	0.12
SpO_2: FiO_2 ratio	438 (378-462)	448 (424-462)	395 (157-452)	<0.001
Respiratory rate (breaths/min)	26 (20-32)	24 (19-31)	28 (21-36)	0.06
Temperature (°C)	37.5 (36.9-38.4)	37.2 (36.8-38.1)	37.7 (37.0-38.8)	0.22
Ct value	37 (32-40)	38 (35-40)	34 (28-39)	0.01
Male (%)	55 (64%)	24 (56%)	31 (74%)	0.08
Diabetes mellitus (%)	18 (21%)	9 (21%)	9 (21%)	0.91
Hypertension (%)	30 (35%)	15 (34%)	15 (38%)	0.70
Smoker (%)	7 (8%)	5 (11%)	2 (5%)	0.26
Creatinine (micromol/L)	88 (68-114)	81 (62-100)	94 (73-140)	0.02
C-reactive protein (mg/L)	114 (52-197)	78 (32-121)	180 (106-266)	<0.001
Albumin (g/L)	39 (33-41)	40 (35-42)	37 (33-40)	0.04
Bilirubin (micromol/l)	10 (7-13)	10 (7-12)	10 (8-13)	0.48
Haemoglobin (g/L)	129 (113-140)	126 (113-141)	132 (114-139)	0.89
Lymphocyte count (10^9/mL)	0.93 (0.62-1.36)	1.14 (0.68-1.52)	0.75 (0.53-1.16)	0.04
Neutrophil count (10^9/mL)	6.42 (4.40-9.08)	5.28 (4.11-7.88)	7.18 (5.56-9.58)	0.03
Platelet count (10^9/mL)	238 (164-290)	245 (174-298)	232 (142-288)	0.33
Steroid use (%)	7 (8%)	1 (2%)	6 (14%)	0.05
Antiviral drug use (%)	2 (2%)	0 (0%)	2 (5%)	0.14
CPAP (%)	34 (40%)	-	34 (81%)	-
Mechanical ventilation (%)	13 (15%)	-	13 (31%)	-
Vasopressors (%)	13 (15%)	-	13 (31%)	-
Renal replacement therapy (%)	3 (3%)	-	3 (7%)	-
Hospital mortality	21 (24%)	0	21 (50%)	-

Table 6.1: Clinical data of patients with COVID-19

Continuous data are presented as median (interquartile range). Mann-Whitney U test and Chi-squared test used to assess differences between patients with mild disease and patients with severe disease or who subsequently died. CPAP: Continuous positive airway pressure; Ct: Threshold cycle

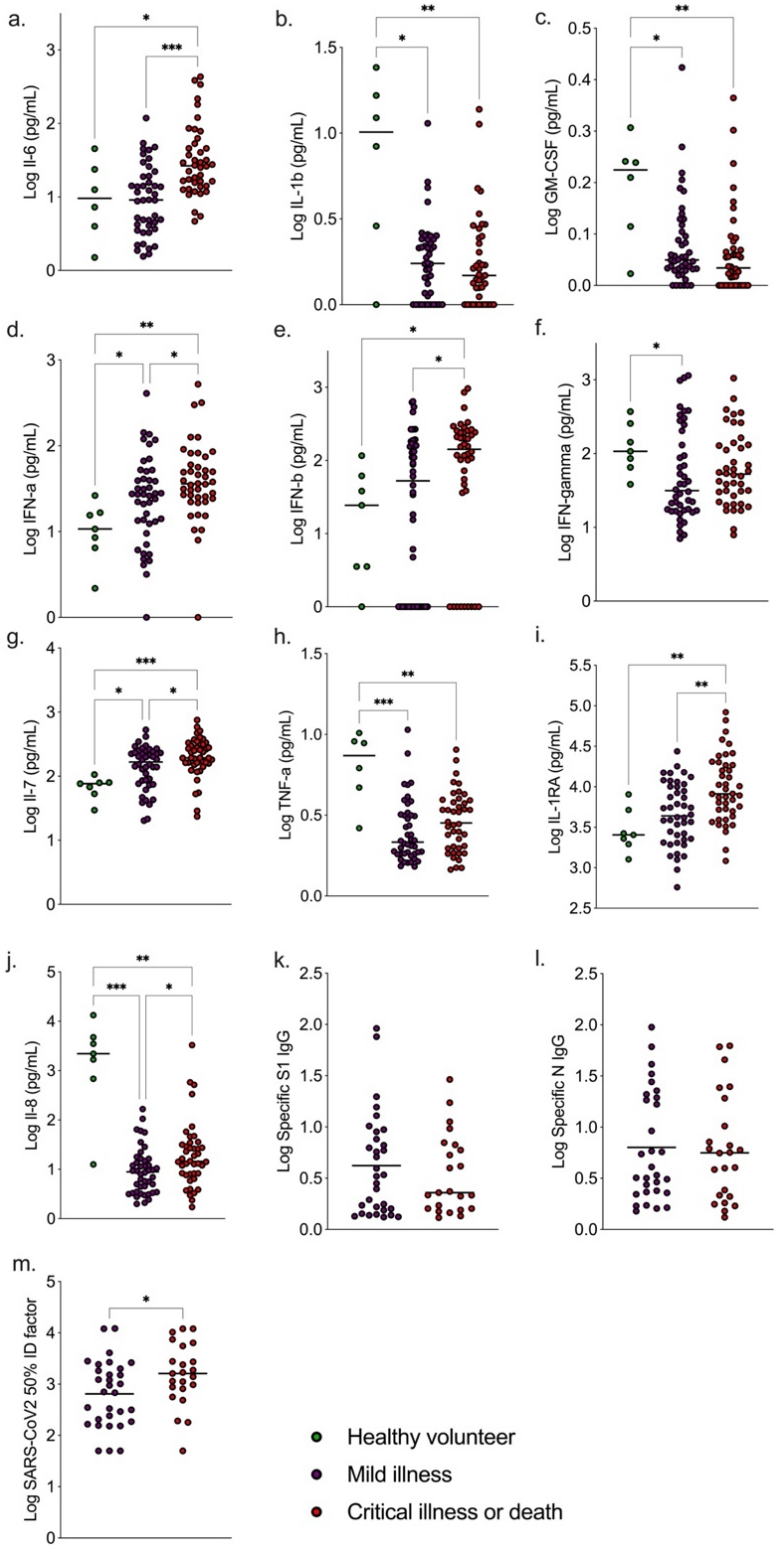

Figure 6.2: Measured biomarkers differentiated between patients with mild or critical COVID-19

Serum levels of IL-6 (a.), IL-1ß (b.), GM-CSF (c.), IFN α (d.), IFN-ß (e.), IFN-γ (f.), IL-7 (g.), TNF-α (h.), IL-1ra (i.), and IL-8 (j.) were compared between healthy controls (n = 7) and patients with both mild (n = 44) and severe COVID-19 (n = 42). Serum levels of anti-S1 antibody (k.), anti-N antibody (l.), and neutralisation titre (m.) were compared between those with mild and severe disease only. Data presented as dots (individual patients or volunteers, horizontal line represents the median and compared using Mann Whitney-U test, * signifies $p<0.05$; ** $p<0.01$, and *** $p<0.001$.

Therapeutic target	Mechanism of therapeutic agent	Levels in mild vs. severe disease	Levels in mild disease	Levels in severe disease	p-value (mild vs. severe)	AUROC (95% CI) (mild vs. severe)
IL-6	Inhibitor	Higher in severe disease	13 (4-29)	22 (14-42)	< 0.001	0.78 (0.68 to 0.88)
IL-1RA	Agonist	Higher in severe disease	5974 (3418 - 12033)	7155 (3642 - 19990)	0.002	0.70 (0.59 to 0.81)
Neutralising antibody	Agonist	Higher in severe disease	823 (190 – 1983)	1612 (810 - 5551)	0.046	0.66 (0.52 to 0.81)
IL-8	Inhibitor	Higher in severe disease	9 (4 – 25)	13 (5 – 26)	0.045	0.66 (0.54 to 0.78)
IL-7	Agonist	Higher in severe disease	196 (120 – 268)	183 (128 – 263)	0.027	0.64 (0.52 to 0.76)
IFN-β	Agonist	Higher in severe disease	95 (0 – 201)	142 (42 – 224)	0.035	0.63 (0.51 to 0.75)
IFN-α2a	Agonist	Higher in severe disease	27 (14 – 49)	42 (22 – 81)	0.043	0.63 (0.51 to 0.74)
TNF-α	Inhibitor	No difference	1.6 (0.9 – 2.8)	1.0 (0.8-2.1)	0.246	0.62 (0.50 to 0.74)
GM-CSF	Inhibitor Agonist	No difference	0.12 (0.05 - 0.25)	0.07 (0.04 – 0.16)	0.398	060 (0.48 to 0.72)
IFN-γ	Inhibitor	No difference	44 (20 – 163)	34 (18 – 86)	0.700	0.58 (0.46 to 0.70)
IL-1β	Inhibitor	No difference	0.6 (0.0 – 6.2)	0.0 (0.0 – 3.0)	0.999	0.54 (0.42 to 0.66)
Convalescent serum	Agonist	No difference				
Anti-N IgG			3.1 (1.3 – 18.2)	3.8 (1.2 – 24.6)	0.924	0.53 (0.37 to 0.69)
Anti-S1 IgG			1.8 (0.6 – 5.7)	2.5 (0.5 – 7.3)	0.793	0.53 (0.38 to 0.68)

Table 6.2: Association with disease severity with different biological targets

Ability of measured biomarkers to differentiate between patients with mild (n=44) and severe (n=42) disease. Continuous data presented as median (interquartile range). AUROC: Area under the receiver operating characteristic curve with (95% confidence interval). Units of cytokines (pg/mL), anti-N-IgG and anti-S1-IgG in microg/mL). The neutralization titre (ID_{50}) was defined as the reciprocal of the serologic reagent dilution that produced a 50% reduction in luminescence (as a proxy of infection) compared to untreated virus control wells.

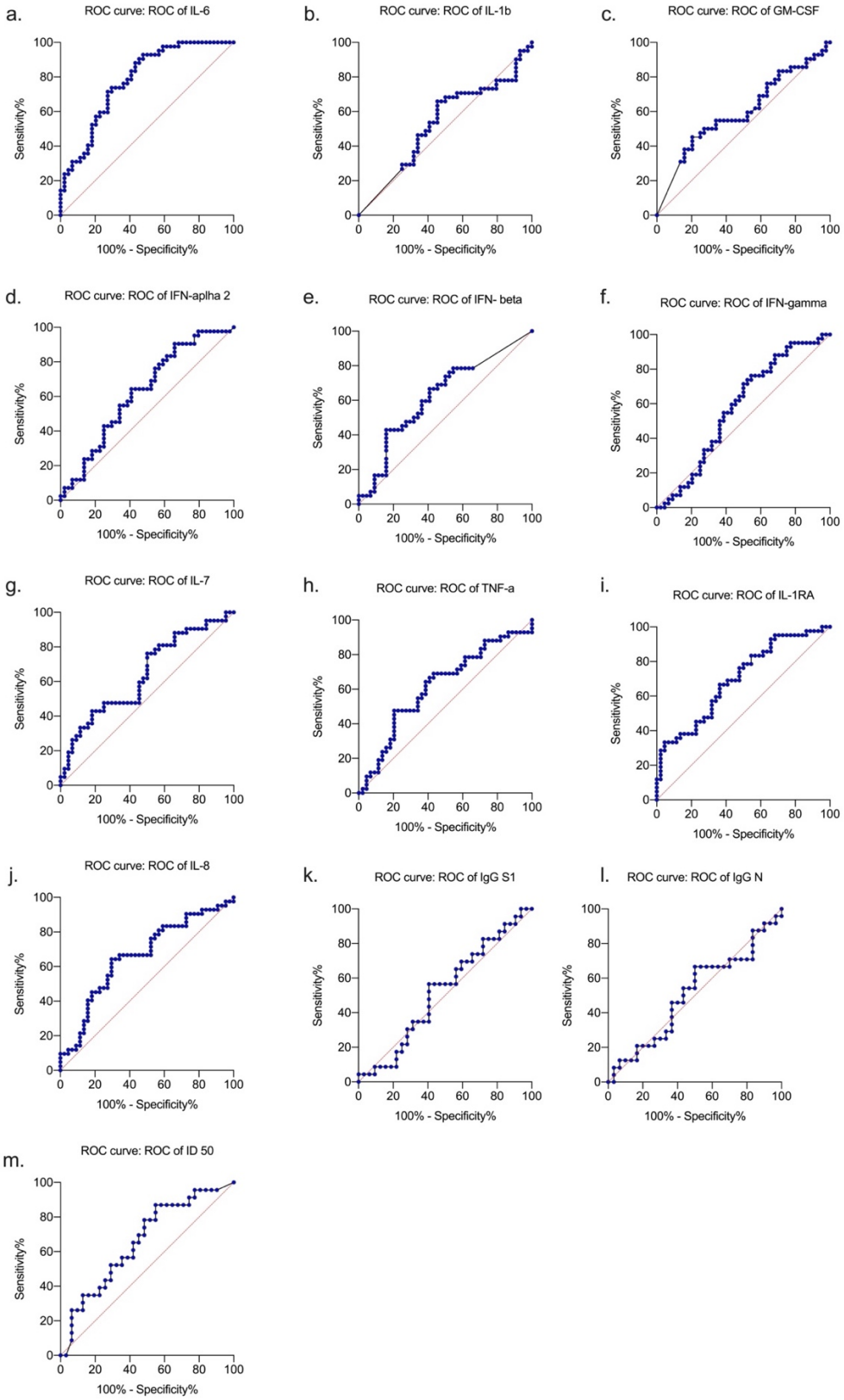

Figure 6.3: Association between measured biomarkers and clinical severity

The association between biomarkers and clinical severity was assessed using area under the receiver operating characteristic curve (AUROC) including IL-6 (a.), IL-1ß (b.), GM-CSF (c.), IFN-a2 (d.), IFN-ß (e.), IFN-γ (f.), IL-7 (g.), TNF-α (h.), IL-1ra (i.), IL-8 (j.), anti-S1 antibody (k.), anti-N antibody (l.), neutralisation titre (m.).

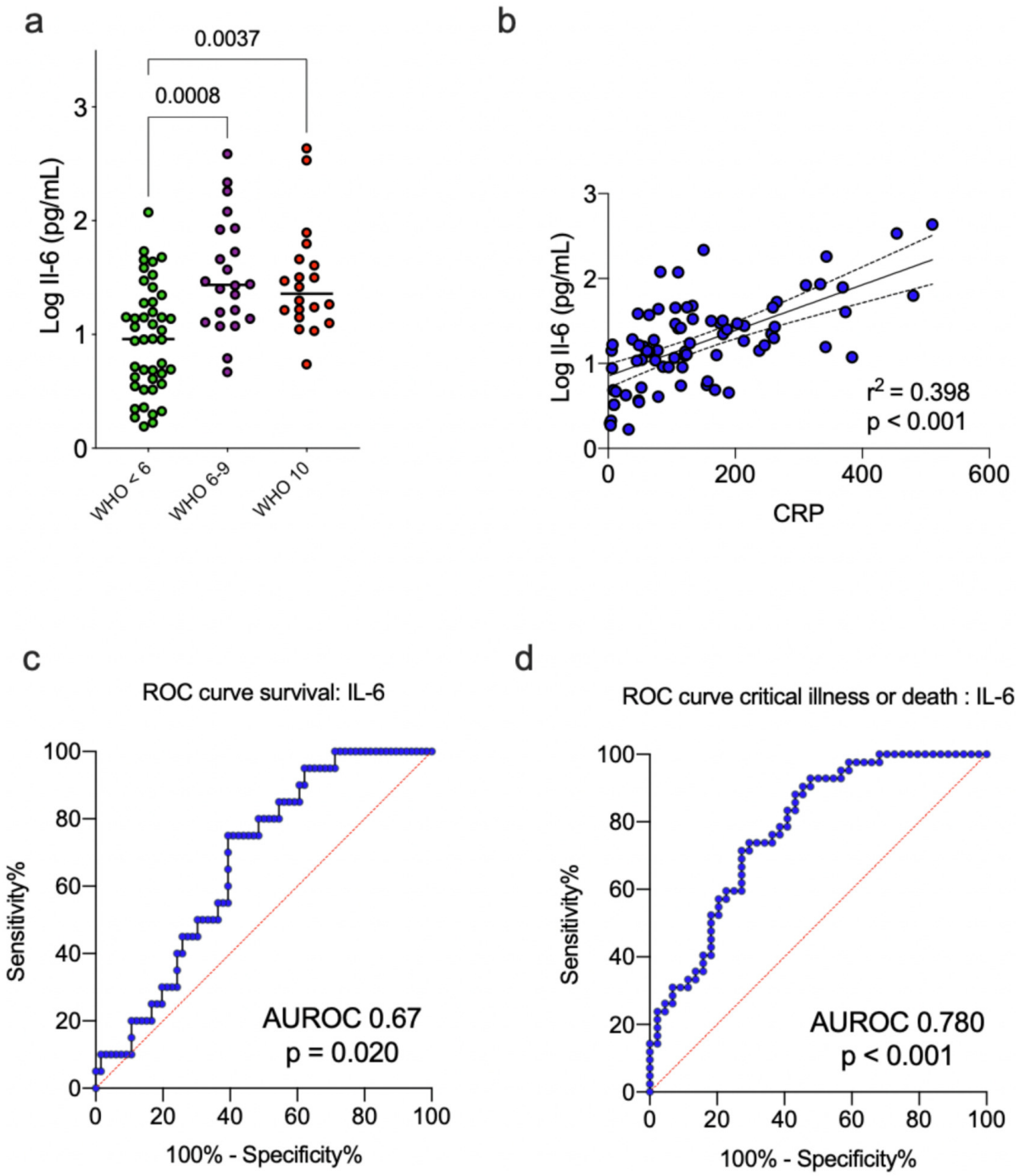

Figure 6.4: Interleukin-6 levels in hospitalized patients with COVID-19

Patients were subdivided by World Health Organisation (WHO) COVID-19 ordinal severity scale. WHO 4–5 (n=44, hospitalized with or without supplemental oxygen via nasal prongs or face mask (mild disease)), WHO 6–9 (n=22, hospitalized requiring non-invasive or invasive respiratory support (critical illness)), and WHO 10 (n=10, died following hospital admission). Data shown includes admission IL-6 levels (a), correlation between IL-6 and CRP (b), area under the curve analysis of IL-6 with survival (c) and progression to critical illness or death (d). Comparison of continuous data between groups was performed using the Kruskal Wallis. Pearsons correlation is used to assess correlation between IL-6 and CRP levels. Area under the receiver operator curve (AUROC) was constructed to ascertain the predictive value of cytokines for mortality.

6.4.2 Effect of antibiotics on COVID-19 -induced inflammation

6.4.2.1 Clinical data

192 patients were identified of whom 90 were excluded (56 were inter-hospital transfers, 14 had active haematological malignancy, 11 received no antibiotics on hospital admission, 8 died within 24 h of admission), and one patient received azithromycin on day 4, leaving 102 patients for the final analysis. At the time the study was conducted, patients were infected with the wild-type virus. No variants of concern were identified in the UK at the time.

Of these patients, 62 received clarithromycin, and 40 received other antibiotic combinations. (Table 6.3) Half the patients received a combination of a macrolide and penicillin. Patients receiving immunomodulatory therapies (e.g. monoclonal antibodies) including early steroids were excluded. Two patients receiving clarithromycin and one patient receiving non-macrolide antibiotics were administered Remdesivir. The dose of clarithromycin administered was 500 mg twice daily. None of the patients received clarithromycin alone.

Group	Antibiotic co-prescribed	N (%)
Macrolide	Co-amoxiclav	41 (66%)
	Amoxicillin	11 (18%)
	Cefuroxime	9 (15%)
	Teicoplanin	1 (2%)
Non-macrolide	Cefuroxime	13 (32%)
	Amoxicillin and cefuroxime	5 (12%)
	Co-amoxiclav	5 (12%)
	Cefuroxime and piperacillin / tazobactam	3 (7%)
	Co-amoxiclav and piperacillin / tazobactam	2 (5%)
	Amoxicillin	1 (2%)
	Doxycycline	1 (2%)
	Piperacillin / tazobactam	1 (2%)
	Teicoplanin	1 (2%)
	Cefuroxime and ciprofloxacin	1 (2%)
	Cefuroxime and clindamycin	1 (2%)
	Cefuroxime and gentamicin	1 (2%)
	Ciprofloxacin and metronidazole	1 (2%)
	Ciprofloxacin and teicoplanin	1 (2%)
	Co-amoxiclav and doxycycline	1 (2%)
	Gentamicin and piperacillin / tazobactam	1 (2%)
	Meropenem and teicoplanin	1 (2%)

Table 6.3: Antibiotic co-prescriptions
Details of antibiotic co-prescriptions in both macrolide and non-macrolide groups.

None of the patients were diagnosed with co-existing atypical pneumonia nor had positive blood cultures within 2 days of admission. Only eight patients had sputum cultures taken on admission as many were unable to expectorate. Fourteen (12%) patients were intubated in the first 48 hours, limiting the number of tracheal or deeper aspirates. A total of 14 sputum/tracheal aspirate samples taken within the first 48 hours, of which four were positive on microbial culture.

Baseline patient characteristics were well matched except for gender, with a greater proportion of males compared to females receiving clarithromycin (82% vs. 29%; p<0.01). (Table 6.4) The median duration of macrolide antibiotics and non-macrolide antibiotics were similar (3(2–4) vs. 3(2–4) days). None of the patients received immunomodulatory therapies,[429] as the cohort were admitted prior to publication of the RECOVERY trial results,[426,430] although 43 patients received steroids late (>10 days) after initial presentation as rescue therapy for non-resolving ARDS; with no difference between patients who received macrolide and non-macrolide antibiotics (44% vs. 39%).

Three days following antibiotic initiation, the change in CRP, temperature, neutrophil, and lymphocyte count were similar between patients receiving macrolide and non-macrolide antibiotics. Unadjusted hospital survival was better among patients receiving macrolide compared to non-macrolide antibiotics (30% vs. 70%; p<0.05 on log-rank), but similar between patients receiving amoxicillin compared to non-amoxicillin antibiotics (32% vs. 68%).

Following adjustment for gender, age, CRP, macrolide use, penicillin use, and duration of antibiotic exposure, advancing age (HR = 1.072 (1.040–1.105); p<0.001), and higher CRP (HR = 1.003 (1.001–1.006); p<0.05) were associated with increased mortality risk. Longer duration of antibiotics (HR = 0.839 (0.705–0.997); p<0.05) and macrolide use (HR = 0.540 (0.275–1.079); p<0.1) were associated with a decreased mortality risk, although the latter did not reach statistical significance. Neither male gender (HR = 1.48 (0.755–2.754)) nor amoxicillin use (HR = 1.092 (0.548–2.244)) was associated with mortality.

	Total cohort (n = 102)	Macrolide (n = 62)	Non-macrolide (n = 40)	p-value
Demographics				
Age (years)	67 (54–74)	65 (55–73)	67 (54–74)	0.615
Male	74 (73%)	51 (82%)	23 (59%)	0.008
Body mass index (BMI) (kg. m^{-2})	28 (25–32)	27 (24–32)	30 (26–32)	0.117
Symptom onset to hospital admission (days)	8 (5–10)	8 (5–10)	7 (4–12)	0.497
Co-morbidities				
Hypertension	57 (55%)	33 (53%)	24 (59%)	0.596
Diabetes	28 (27%)	17 (27%)	11 (27%)	0.947
Smoker	4 (4%)	2 (3%)	2 (5%)	0.671
Chronic obstructive pulmonary disease (COPD)	7 (7%)	4 (7%)	3 (7%)	0.864
Ethnicity				
White	50 (49%)	32 (52%)	18 (45%)	0.731
Other/Unknown	23 (23%)	13 (21%)	10 (25%)	
Asian	15 (15%)	10 (16%)	5 (12%)	
Black	14 (14%)	7(11%)	7 (18%)	
Admission variables				
C-reactive protein (mg/dl)	188 (106–285)	203 (110–298)	169 (89–275)	0.325
Haemoglobin	133 (119–144)	135 (123–146)	131 (117–141)	0.189
Bilirubin	11 (7–14)	10 (8–14)	11 (7–14)	0.674
Albumin	37 (35–40)	37 (36–40)	37 (35–41)	0.765
Creatinine	91 (73–112)	92 (79–110)	90 (73–113)	0.816
Lymphocytes ($10^{6\cdot}$ mL^{-1})	0.75 (0.53–1.10)	0.76 (0.59–1.00)	0.74 (0.51–1.21)	0.914
Neutrophils ($10^{6\cdot}$ mL^{-1})	7.56 (4.97–10.36)	7.59 (4.78–10.35)	7.08 (5.24–10.64)	0.613
Ratio	9.57 (5.63–14.19)	9.55 (5.22–13.35)	9.57 (5.66–17.06)	0.657
Platelets (10^9 mL^{-1})	231 (173–308)	229 (178–303)	231 (158–315)	0.962
Temperature (°C)	38.0 (37.1–38.7)	38.0 (37.2–38.6)	38.0 (37.1–38.8)	0.606
SpO_2:FiO_2 ratio	139 (95–362)	142 (100–313)	138 (93–375)	0.975
Treatments used				
Duration of antibiotics (days)	3 (2–4)	3 (2–4)	3 (2–4)	0.530
Continuous positive airway pressure (CPAP)	92 (89%)	56 (90%)	36 (88%)	0.686
Invasive mechanical ventilation	34 (33%)	21 (34%)	13 (32%)	0.819
Renal replacement therapy	11 (11%)	7 (11%)	4 (10%)	0.805
Noradrenaline use	36 (35%)	22 (36%)	14 (34%)	0.889
Steroid use	43 (41%)	27 (44%)	16 (39%)	0.649

Table 6.4: Baseline characteristics of patient cohort

Data expressed as median (inter-quartile range) if continuous, or number (n) (%).

6.4.2.2 Lymphocyte intracellular cytokines

Median healthy volunteer PBMC viability was 95% (93%–97%) with no differences on stimulation with spike protein or co-incubation with antibiotics. Stimulation of PBMCs with spike protein resulted in an increase in $CD8^+$ IL-2 ($p<0.01$), TNF-α and IFN-α; $CD4^+$ IL-2, and IL-6; and $CD19^+$ IL-6 and IFN-γ (all $p<0.05$). (Figure 6.5, Figure 6.6, Figure 6.7)

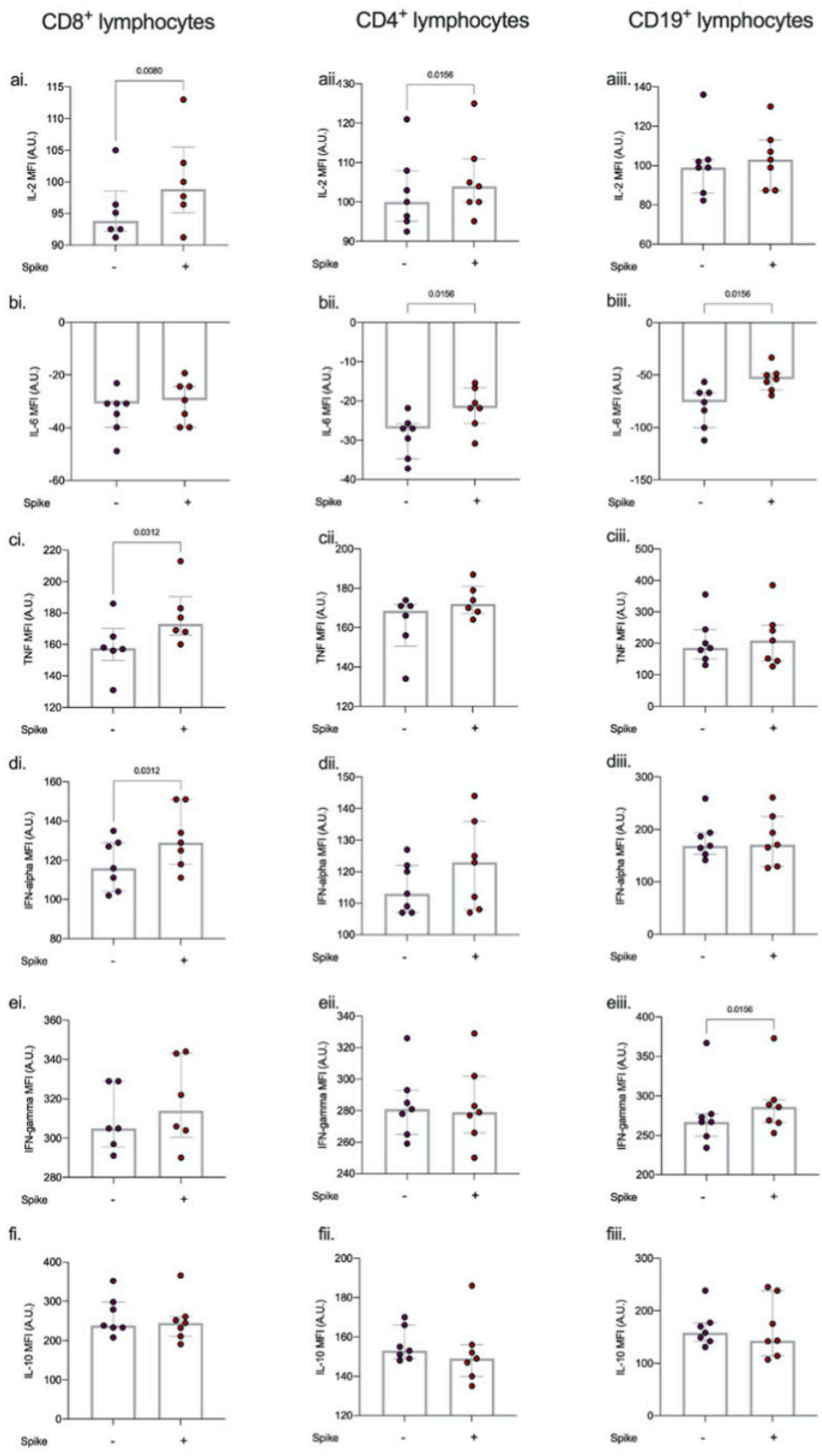

Figure 6.5: Effect of spike protein on lymphocyte intracellular cytokine production
Intracellular cytokines a) IL-2, (b). IL-6, (c). TNF-α, (d). IFN-α, (e). IFN-γ and (f). IL-10 in CD4 (i.), CD8 (ii.), and CD19 (iii.) cells following 6 hours spike protein stimulation *ex vivo*. Intracellular cytokine concentration is expressed as median fluorescent intensity (MFI) measured in arbitrary units (A.U.). Dots represent individual replicate values (n = 7), column heights represent the median of the replicates, bars show inter-quartile range Differences between groups were compared using Mann- Whitney *U* test.

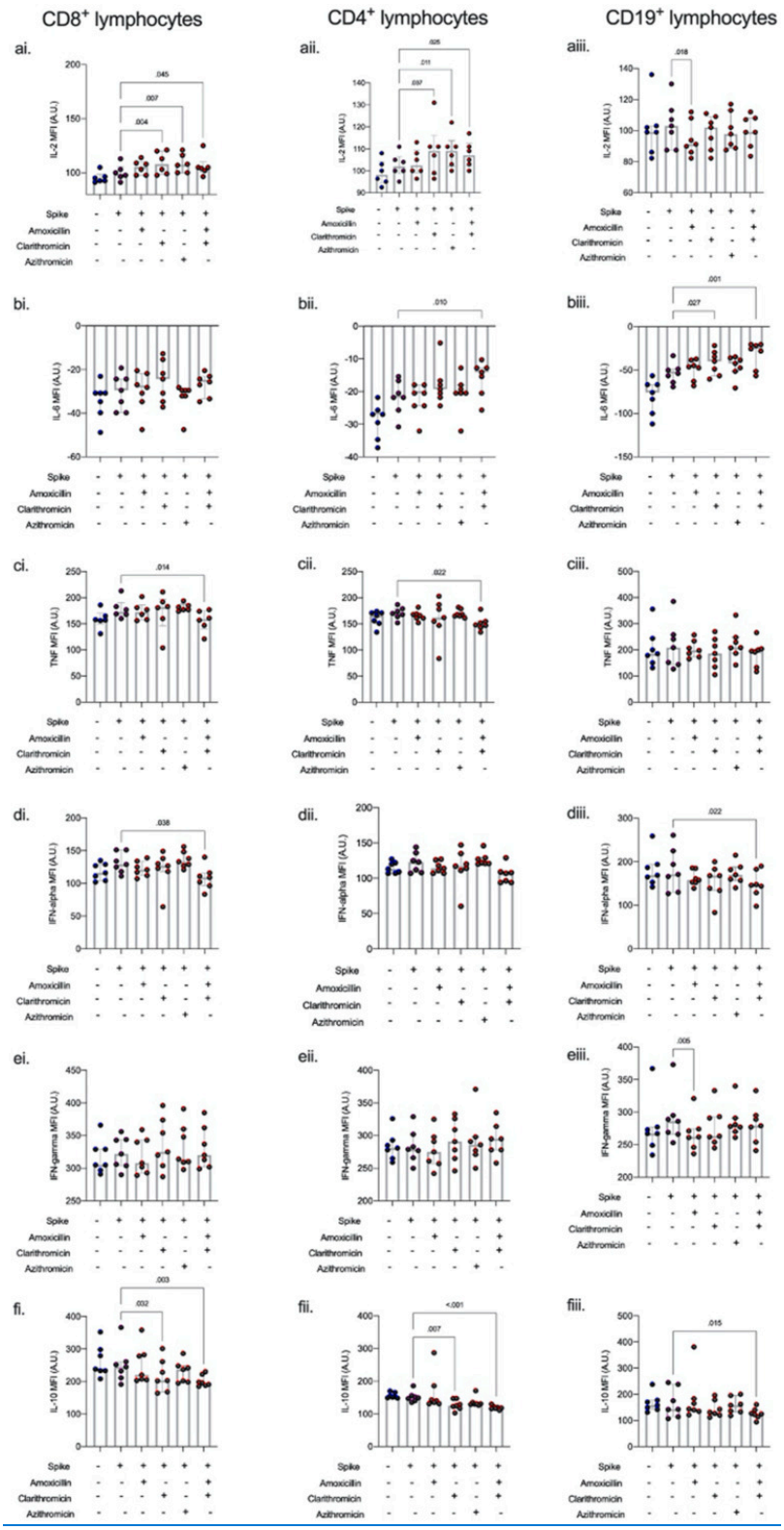

Figure 6.6: Effect of antibiotics on spike protein-induced intracellular cytokine concentration

Intracellular cytokines (a) IL-2, (b). IL-6, (c). TNF-α, (d). IFN-α, (e). IFN-γ, and (f). IL-10 in CD4 (i.), CD8 (ii.), and CD19 (iii.) cells following 6 hours spike protein stimulation ± antibiotic co-incubation *ex vivo*. Intracellular cytokine concentration is expressed as median fluorescent intensity (MFI) measured in arbitrary units (A.U.). Dots represent individual replicate values (n=7), column heights represent the median of the replicates, and bars show inter-quartile range. Differences between groups were compared using a non-parametric Kruskal- Wallis test without Dunnett's correction.

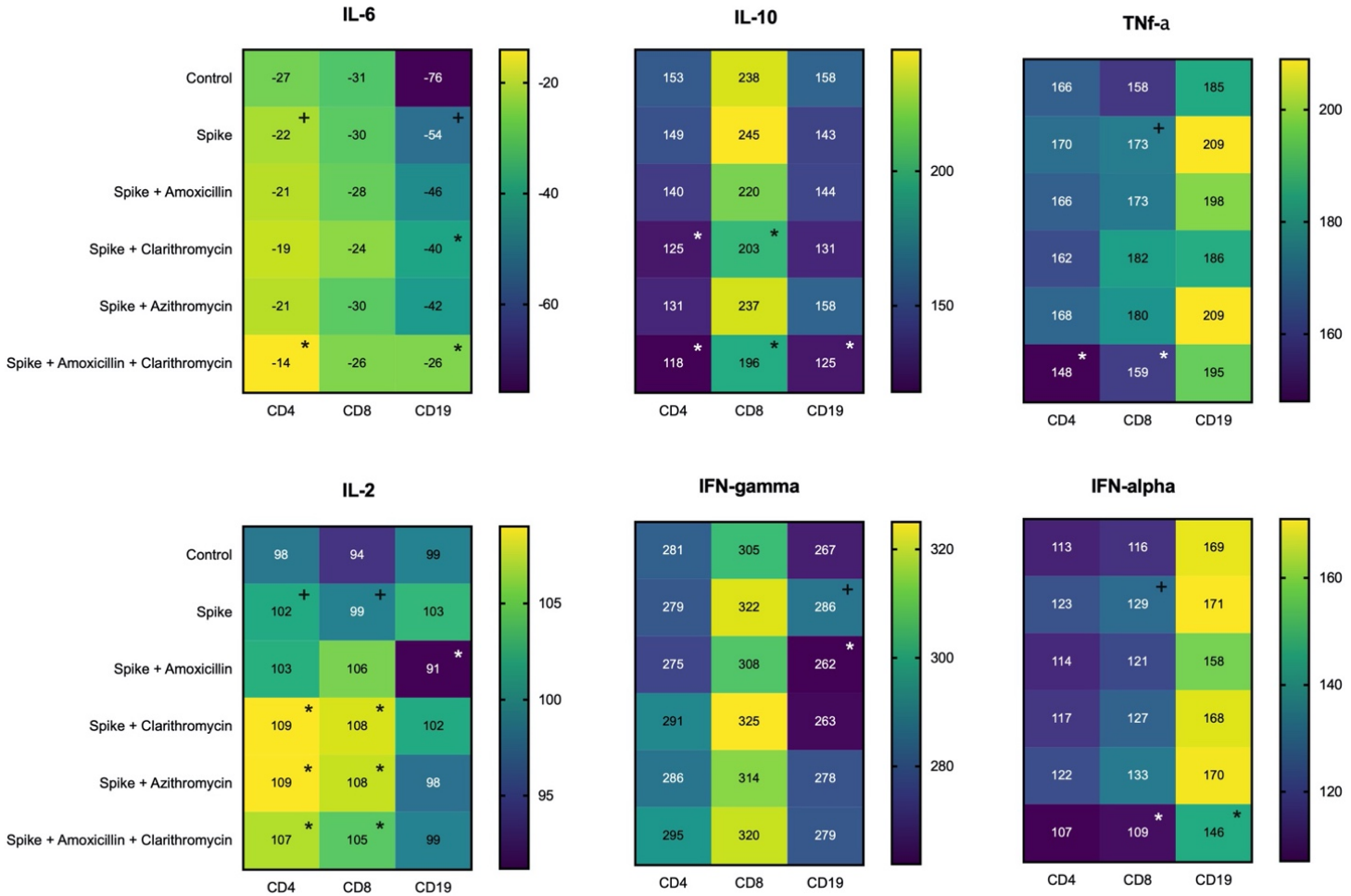

Figure 6.7: Heat map of intracellular cytokines

Numbers within cells represent intracellular cytokine concentration, expressed as median fluorescent intensity (MFI) measured in arbitrary units (A.U.).

[+] Indicates $p<0.05$ compared to control sample. Differences between groups were compared using Mann- Whitney U test.

* Indicates $p<0.05$ compared to spike protein stimulated sample. Differences between groups were compared using a non-parametric Kruskal- Wallis test without Dunnett's correction.

Co-incubation with azithromycin resulted in an increase in $CD8^+$ ($p<0.01$) and $CD4^+$ ($p<0.05$) IL-2. Similarly, co-incubation with clarithromycin increased $CD8^+$ and $CD4^+$ IL-2 (both $p<0.01$). In addition, co-incubation with clarithromycin resulted in an increase in $CD19^+$ IL-6 ($p<0.05$) and decrease in $CD4^+$ ($p<0.01$) and $CD8^+$ ($p<0.05$) IL-10. Co-incubation of spike protein-stimulated cells with amoxicillin resulted in a decrease in $CD19^+$ IFN-γ ($p<0.01$). The combination of amoxicillin and clarithromycin had synergistic effects on spike-protein stimulated lymphocytes. A significant decrease in IL-10 was seen in $CD4^+$ ($p<0.001$), $CD8^+$ ($p<0.01$), and $CD19^+$ ($p<0.05$) lymphocytes. Additionally, TNF-α was reduced in both $CD4^+$ and $CD8^+$ (both $p<0.05$) lymphocytes and IFN-α was decreased in $CD8^+$ and $CD19^+$ (both $p<0.05$) lymphocytes. (Figure 6.5, Figure 6.6, Figure 6.7)

6.4.2.3 Monocyte phagocytosis

Co-incubation of PBMCs with spike protein alone resulted in an increase in phagocytic capacity among 4 of 6 individuals, although not statistically significant. Addition of antibiotics to PBMCs treated with spike protein did not affect monocyte phagocytosis. In the absence of spike protein, phagocytosis of pHRodo red *S. aureus* bioparticles resulted in a decrease in monocyte surface HLA-DR expression ($p<0.01$). Co-incubation with both clarithromycin and amoxicillin resulted in a small but statistically significant reduction in surface HLA-DR expression. (Figure 6.8)

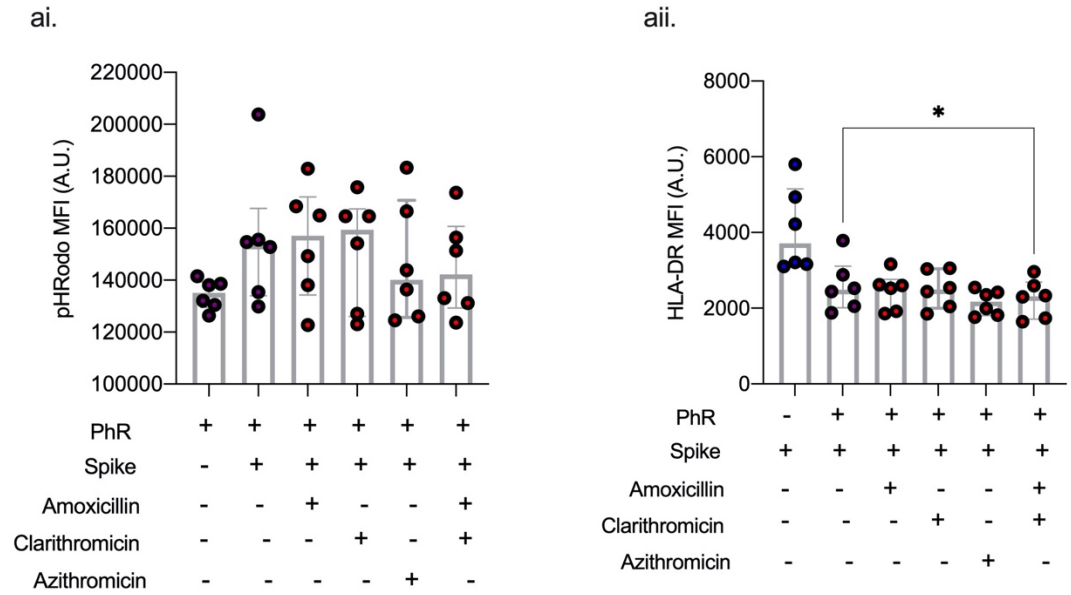

Figure 6.8: Effect of antibiotics on spike protein-induced monocyte phagocytosis
Phagocytic capacity (ai.) and HLA-DR expression (aii.) of classical monocytes was assessed following 6 hours spike protein stimulation ± antibiotic co-incubation *ex vivo*. Phagocytic capacity as measured by pHRodo and HLA-DR surface expression are shown as median fluorescent intensity (MFI) measured in arbitrary units (A.U.). Dots represent individual replicate values (n=6), column heights represent the median of the replicates, and bars show inter-quartile range. Differences between groups were compared using a non-parametric Kruskal- Wallis test without Dunnett's correction.

6.5 Discussion

6.5.1 Defining potential therapeutic targets in COVID-19

Multiple mechanisms have been proposed to explain disease severity in COVID-19 including an impaired host response to the virus and a dysregulated host inflammatory response including immunosuppression, endothelial injury and a pro-thrombotic state.

In this experiment I assessed plasma levels of twelve of the most frequently investigated targets based on my search of clinicaltrials.gov. Five (TNF-α, IL-1β, GM-CSF, IFN-γ, and anti-SARS-CoV-2 antibodies) did not differentiate between patients with mild or severe disease, challenging the validity of modulating these immune mediators in the treatment of COVID-19, and potentially increasing patient risk. Seven (IFN-α, IFN-β, IL-6, IL-7, IL-8, IL-1ra, and neutralising antibody titres) were increased in patients with severe disease. However, despite some cytokines being significantly higher among patients with severe disease, the absolute change in cytokines and chemokines above that seen in healthy individuals was modest in many cases. Inflammatory cytokine elevations in patients with severe or critical COVID-19 disease were markedly lower than those reported in patients with sepsis, ARDS unrelated to SARS-CoV-2 infection, and chimeric antigen receptor (CAR) T cell-induced cytokine release syndrome.[431]

In my patient cohort, IL-1ra levels were significantly higher in the severe patient subset while levels of IL-1ß did not differentiate between mild or severe COVID-19. Of interest, anakinra, a recombinant and modified version of the human IL-1ra protein, is being investigated in 16 trials. Interferon ß1 levels were also similar in our mild and severe disease groups. The SOLIDARITY trial recently reported no survival benefit from interferon ß1 in 4100 patients.[432] As a further example of scientific ambiguity, I also detected no differences in GM-CSF levels between mild and severe groups yet ongoing studies are directly conflicting, either giving exogenous GM-CSF or blocking its effects.[425]

While more biological rationale might be attached to a target that does show severity-related differences, this is not a *sine qua non*. A raised biomarker level may simply be an epiphenomenon, reflecting the underlying disease process but with no impact on survival. It is also uncertain if raised serum levels of an inflammatory mediator represent an adaptive/protective host response, especially when levels are only modestly elevated. In this case, targeted blockade may be ineffective or even counter-productive. A similar approach of targeting mediators associated with mortality in sepsis have not yielded any successful therapies.[433]

As confirmed by others, levels of IL-6 were elevated among patients with severe COVID-19,[434] yet these are often 1-2 log-orders lower than other causes of ARDS, sepsis or critical illness, and often

barely elevated above values measured in normal subjects.[431] Despite this, IL-6 was able to discriminate between patient with mild and severe disease. Furthermore, observational reports describing the physiological response to Tocilizumab in COVID-19 patients support the biological plausibility of Tocilizumab use in COVID-19.[435]

IL-6 is a key regulator of CRP production and fever. The well-established association between elevated CRP and illness severity in COVID-19 raises the possibility of a mortality benefit with IL-6 blockade in the sickest patients.[427] Indeed, a mortality benefit of IL-6 blockade was seen in the RECOVERY study,[430] and REMAP-CAP study in which ICU admission and advanced respiratory support was a pre-requisite for trial enrolment.[436] This benefit was demonstrated in a subsequent meta-analysis and Tocilizumab was adopted into clinical practice.[437]

I found a good correlation between serum IL-6 and CRP; IL-6 being a key regulator of CRP production. However, co-interventions, particularly the use of corticosteroids, affect CRP levels. This may explain the lack of association between the treatment effect of tocilizumab with baseline CRP in clinical trials.[437] Furthermore, a significant proportion of our patients had IL-6 levels that were only marginally elevated, consistent with other studies.[431] No clinical trial investigating the use of IL-6 receptor antagonists have stratified patients based on circulating levels of IL-6 levels.[437]

The association between higher viral load and disease severity has been reported elsewhere.[438] The higher viral load among our patients with severe disease or who subsequently die supports early diagnosis and the early use of a direct-acting-antiviral especially in individuals with risk factors as shown in our data. With the emergence of the novel variants which appear linked to infections with higher virus load, our observation is of critical importance although further data will be required to confirm it.[439]

I found type I interferon levels were as expected, elevated in critically ill patients with higher viral loads. Critically ill patients have a higher viral load and higher interferon levels; the latter which may be an adaptive response. However, neutralizing immunoglobulin G (IgG) autoantibodies against type I interferons have been described in a proportion of critically ill COVID-19 patients, which may render elevated interferon levels ineffective.[440] Further augmenting this host response in all COVID-19 patients is thus of questionable benefit.

Several studies have also highlighted an association between higher SARS-CoV-2 reactive antibody responses and disease severity, however these have predominantly compared mild or asymptomatic infection to severe disease.[441,442] Furthermore, the trend towards higher titres in severe disease could be a result of an increased duration of infection leading to greater antibody maturation.[443] Importantly, our study covers an earlier window (~10 days of infection) than most other studies and I saw no

evidence of an association between anti-N or anti-S1 responses and disease severity in this cohort.[444] Thus, while early antibody levels do not predict outcome in this cohort it remains an unanswered question as to whether disease severity and associated higher antigen load drives higher antibody titers or *vice versa* at later stages of the disease. Clinical trials investigating convalescent plasma in COVID-19 have not demonstrated any clinical benefit, even among studies with a minimum threshold of specific SARS-CoV-2 IgG antibody titres in infused plasma.[445,446] Meta-analysis has subsequently demonstrated no benefit.[447]

Among patients who seroconverted, the 50% inhibitory dilution factors (ID_{50}) against SARS-CoV-2 pseudotyped virus was higher among patients with severe illness than in patients with mild illness, which may reflect greater antigen burden and thus more extensive antibody maturation. With monoclonal antibody trials ongoing and whilst REGN-COV2, an antibody cocktail containing two SARS-CoV-2–neutralizing antibodies, had no clinical benefit in non-hospitalized patients with COVID-19,[448] its potential benefit in preventing seronegative hospitalised patients from progressing to critical illness is unknown.

Limitations of my study include the relatively small number of patients and the lack of serial data to evaluate the association between biomarker trajectory and outcome. Cytokine differences between patients with mild illness and those with critical illness or who died may reflect the expected trajectory of inflammatory markers rather than the nature of disease. Published studies on proinflammatory cytokine trajectory demonstrate that the highest levels are seen in the first few days following presentation.[449] The samples measured represent a subset of our entire patient cohort and were selected based on availability of residual serum. The time from hospital admission to blood sample collection was shorter in patients with mild disease and those with severe disease by 0.5 days. Although statistically significant, a difference in 0.5 days is unlikely to have any clinical significance. The numbers of healthy volunteers are small, and not matched to the patient demographics. However, our main comparison is between patients with mild disease and those who progresses to critical illness or death. Data from healthy volunteers were included to provide context to patient data.

Furthermore, the Spike protein specific antibody titres were generated using the S1 subunit not whole Spike, assessment of which may provide additional information regarding different outcomes.[450] However, the neutralisation titres were generated against virus pseudotyped with whole Spike. My findings were consistent with those of others that include modest elevations in cytokine levels among COVID-19 patients compared to other conditions.[431] The effect of viral load on host immune response and cytokine levels requires further evaluation. My study included patients prior to the results of the RECOVERY study were published 477 randomized trials, including 168 different therapies against 83

different pathways were identified.[426] Therefore, majority of patients did not receive steroids and the effect of steroid therapy on the biomarkers measured in COVID-19 is unknown.

The functional impact of pleotropic cytokines, including IL-6, may not be reflected in the absolute level of the cytokine measured in serum.[451] Further understanding of the impact of soluble mediators in the context of their diverse immune and non-immune functions remains a challenge. Understanding of the pathogenic mechanisms underlying impaired viral clearance and the development of organ failure should precede well-meaning efforts to intervene. Use of therapeutic and prognostic biomarkers may identify appropriate therapeutic targets, patients most likely to benefit (e.g. those individuals with markedly elevated and potentially pathological cytokine levels), and to subsequently monitor treatment effects.

I demonstrated that many of the immunomodulatory agents selected to undergo evaluation in randomised control trials were targeting biomarkers either not significantly raised compared to non-infected individuals nor did not discriminate between those patients with mild and severe disease. This suggested that many of these agents were predestined to fail and could cause harm to patients. However, given my cohort recruitment was unrestricted, there was still potential that certain subgroups might benefit from therapies compared to others.

6.5.2 Beneficial ex vivo immunomodulatory and clinical effects of clarithromycin in COVID-19

I showed that on stimulation with SARS-CoV-2 spike protein, the effect of azithromycin and clarithromycin on lymphocytes differed. Azithromycin had relatively limited immunomodulatory properties in comparison to clarithromycin. Whilst amoxicillin alone had minimal immunomodulatory properties, the combination of amoxicillin and clarithromycin had synergistic effects. Immunomodulatory properties vary between macrolides. As examples, suppressed T-cell activation with azithromycin only occurs at high concentrations of clarithromycin,[452] and suppressed monocyte cytokine release occurs with azithromycin but not clarithromycin.[306]

Whilst a number of non-immune cells are able to secrete cytokines, I chose to measure lymphocyte – associated cytokines as lymphopenia and lymphocyte dysfunction is common in COVID-19,[443] suggesting immunomodulation of lymphocytes is important. The increase in lymphocyte IL-2 associated with macrolide use may facilitate resolution of lymphopenia and improves survival in severe viral illness. Whilst raised IL-6 is associated with mortality in COVID-19,[422] the elevated intracellular levels of IL-6 associated with clarithromycin (*ex vivo*) may facilitate viral clearance. The suppression of TNF-α in COVID-19 may seem beneficial, given the degree of systemic inflammation. INF-α is important for

viral clearance.[453] It is unclear if reduction in $CD8^+$ and $CD19^+$ lymphocyte intracellular IFN-α associated with clarithromycin/amoxicillin results in any functional impairment.

As patients with COVID-19 may present with bacterial co-infection,[454] I also explored the *ex vivo* effect of antibiotics on bacterial phagocytosis with and without the presence of COVID-19 spike protein 1 + 2. I found the immunomodulatory effects of macrolides were limited to lymphocytes, with no effect on monocyte phagocytosis or antigen presentation.

Compared to non-macrolide antibiotics, clarithromycin use in critically ill patients with COVID-19 was associated with a significant survival benefit on unadjusted analysis, albeit this significance was lost following adjustment for covariates. Longer antibiotic duration was associated with improved survival, suggesting that there might have been a survival benefit associated with greater exposure to clarithromycin. As expected, advancing age and elevated CRP were also associated with higher mortality risk.

Despite mechanistic and observational data supporting the use of macrolides in COVID-19, clinical trials have yet to show survival benefit. This may be related to patient selection and timing of treatment. Azithromycin has been the most commonly studied macrolide in COVID-19. However, our data supports a role for clarithromycin.[455] The dose of macrolide required to achieve adequate immunomodulatory effects *in vivo* is unknown. Many *ex-vivo* studies demonstrate immunomodulation occurs at higher doses than might be achievable clinically.[456]

A significant proportion of critically ill patients with COVID-19 are treated with empirical antibiotics, as exclusion of bacterial co-infection is often vexatious. The median duration of antibiotics in our centre was 3 days, reflecting the Surviving Sepsis Guidelines recommendation that initial empirical antibiotic therapy is continued until further microbiology results become available.[457] Although the absolute difference in antibiotic duration may not appear significant (3(2–4) days), the relative difference in antibiotic duration between patients is not insignificant (e.g. twice as much antibiotic exposure with a 4-day compared to two-day course). The association between lower mortality and longer course of antibiotics may be explained by the treatment of an undiagnosed bacterial co-infection rather than the immunomodulatory effect of antibiotics.

Macrolides have demonstrated numerous potentially beneficial immunomodulatory properties in the context of non-COVID-19 viral infections, gram-negative sepsis and ventilator associated pneumonia (VAP).[298] However, the *ex-vivo* effect of macrolides on immune function in SARS-CoV-2 is relatively unknown.

As with all retrospective analyses, I acknowledge the possibility of residual confounding, and that results are associative. Only a minority of patients had sputum cultures or tracheal aspirates taken on admission as many were unable to expectorate. I did not include patients who did not receive antibiotics on admission to hospital as their illness severity was milder and thus not comparable.

All data have been performed *ex vivo* on healthy volunteer PBMCs using a single concentration of both spike protein and antibiotics. I have not demonstrated if a similar effect occurs *in vivo*, or at different concentrations or timepoints. Assessment of intracellular protein (cytokine) synthesis *ex vivo* following cell stimulation requires use of monensin to prevent protein transport beyond the Golgi apparatus. However, beyond 6 hours, monensin is cytotoxic, precluding longer incubation times.

Although I demonstrate alterations in intracellular cytokines, the underlying mechanisms have not been explored. Our *ex-vivo* model utilises the SARS-CoV-2 S1+2 domain of the spike protein; other parts of the SARS-CoV-2 virus may have different immunogenic properties. I was unable to model the effect of a prolonged viral infection prior to commencement of antibiotics in keeping with clinical COVID-19 infection. Additionally, the effect of antibiotics on SARS-CoV-2 S1+2 – stimulated non-immune cells is unknown. My healthy volunteers were younger than the clinical cohort, although the sex distribution was similar.

Crucially, empirical antibiotic use at my centre was for limited duration (<4 days) and I do not advocate clinicians take a *carte blanche* attitude to prescribing antibiotics for theoretical benefits. Clarithromycin has immunomodulatory properties over and above azithromycin. Amoxicillin in addition to clarithromycin is associated with synergistic *ex vivo* immunomodulatory properties. The potential benefit of clarithromycin in critically ill patients with COVID-19 and other viral pneumonitis merits further exploration.

6.6 Chapter summary

With the worst of the COVID-19 pandemic (hopefully) over thanks to the prevalence of vaccination and declining virulence, my initial concern regarding the rationale for the plethora of immunomodulatory treatments for COVID-19 was well placed. Only five therapeutics targeting four different pathways demonstrated enough benefit to be incorporated into clinical practice, the corticosteroid dexamethasone, IL-6 antagonists (tocilizumab), JAK inhibitors (baricitinib) and monoclonal antibodies (casirivimab and imdevimab, or sotrovimab). Ironically, this number does indeed represent approximately 5% of immune pathways assessed, suggesting that these cures could have been stumbled upon fortuitously among the various heterogenous study designs and interventions.

Whilst IL-6 blockade has been shown to have biological plausibility for its effect, including in the work described in this chapter, concerns regarding its use in subgroups persist especially in females, where subgroup analysis does not demonstrate a benefit. This is unsurprising given levels of serum IL-6 are lower in females and appear to demonstrate better correlation with other cytokines compared to males suggesting reduced immune dysregulation. Most clinical trials did not measure the level of biomarker they were attempting to modulate thus it remains unclear whether benefits are related to the patient's serum levels.

Finally, the potential benefits I identified of clarithromycin as an immunomodulatory agent for COVID-19 in a healthy volunteer ex vivo model, have shown to be replicated in small open-label clinical trails only. Randomised trials assessing other macrolides, particularly azithromycin however have not demonstrated any clinical benefit. This suggests that any immunomodulatory effect is likely to be small and the risks of inappropriate antibiotic use outweigh any potential benefits.

7 Future directions

I have investigated how commonly prescribed antibiotics alter the immune response to several critical illnesses, including sepsis, surgical trauma, and COVID-19. I first characterised the immune response in each critical illness, and subsequently investigated how antibiotics modulate this response *ex vivo*. This thesis adds to our current knowledgebase regarding these inflammatory clinical syndromes commonly encountered in the intensive care unit, and the immunomodulatory role of antibiotics. I have identified key areas which need further work.

7.1 Sepsis

In this chapter I confirmed that low monocyte HLA-DR is associated with poor outcomes in sepsis. This suppression relates to the severity of illness and is evidenced even in mild or early illness. More importantly, whilst it is one of the best characterised features of sepsis-induced immunosuppression, HLA-DR suppression represents just one of several impairments in monocyte antigen presentation pathways, with defects uncovered in co-stimulatory receptor expression, and other related pathways including phagocytosis and LPS-induced cytokine release. Lymphocyte effector cell function may also be impaired, with increased markers of cell apoptosis and reduced markers of proliferation. Further work is therefore required to elucidate the following:

a. What is the mechanism responsible for suppressed HLA-DR and associated antigen presentation pathway impairments? – Key potential pathways that regulate antigen presentation include transcription factors (CIITA), receptors and proteins involved in intracellular processing (CLIP and HLA-DM), and pathways responsible for receptor cell surface cycling and endocytosis (IL-10). These pathways could be investigated either using a targeted approach with flow cytometry, or a broader approach combining transcriptomics and proteomics.
b. Is sepsis-induced lymphocyte dysfunction a consequence a direct effect of sepsis, or mediated by impaired antigen presenting cell function? – Research using cell separating chambers and incubation of reconditioned healthy volunteer monocytes with septic patient lymphocytes could suggest that this is a direct cell-cell mediated effect. This could be investigated in two ways; (i.) using an animal model of sepsis *in vitro* to assess immune cell signalling and function, isolating and separating monocytes and lymphocyte pre- and post- a septic insult before co-incubating the isolated cells (monocytes and lymphocytes pre, monocytes pre with lymphocytes post, monocytes post with lymphocyte pre, and monocytes and lymphocytes post) and assessing function using flow cytometry, or (ii.) in a healthy volunteer *in vivo* model of infection e.g. an E. *coli* blister or LPS model, isolating PBMCs and co-incubating as described for (i.).

c. What is the timing of changes in HLA-DR expression following an infection that leads to suppression? – Preliminary work by myself and others suggests that in healthy volunteers, HLA-DR expression rises immediately after an infectious stimulus *in vitro*, but becomes suppressed at a later timepoint. I had tried to investigate this *in vitro* by comparing monocyte HLA-DR expression on heat-killed bacteria or LPS stimulated PBMCs. However, the immune phenotype of the unstimulated monocyte control changed in culture media over the time course making a comparison difficult. This may be best investigated in a volunteer *in vivo* model as described above, or in patients undergoing surgery albeit the stimulus would be different.
d. Which features of sepsis-induced immunosuppression predispose to persistent or secondary infections? – Whilst I have described changes in immune function early in the course of infection or sepsis, it is unclear how these changes evolve over time. This could be partly solved in the experiments described in (c) above, however an additional time course in patients recovering from sepsis would add further information, particularly by comparing the changes in those who do and do not develop secondary infections.
e. Do therapeutic agents targeting monocyte HLA-DR improve global monocyte function? – Several therapeutic agents have been demonstrated to increase monocyte HLA-DR both *in vitro* and *in vivo* but have not demonstrated a benefit in clinical trials. Given I have identified that reduced HLA-DR is just one feature of an immunosuppressed monocyte phenotype, these agents may be increasing HLA-DR but not improving other monocyte pathways. This could be investigated *in vitro* by co-incubating volunteer and septic PBMCs with various therapeutic agents and an infectious stimulus before using flow cytometry to assess the changes induced in monocyte function.

In an extended mechanistic spectral flow cytometry panel, I investigated the effect of beta-lactam antibiotics on the immune function of patients with infections. They exacerbated features of sepsis-induced immunosuppression particular in respect to antigen presentation pathways but not related to the mechanisms postulated in (a) above. Further work is therefore required to identify:

a. What is the responsible mechanism? – Given the lack of changes seen in CIITA or CLIP/HLA-DM, receptor mediated endocytosis mediated by IL-10 is the next most likely candidate although this does not appear to be related to an autocrine effect of intrinsic monocyte IL-10. Candidate responsible pathways could be identified using single cell transcriptomics. If the IL-10 pathways is confirmed, isolated monocytes could be incubated with beta-lactams, LPS and either recombinant IL-10 or a receptor inhibitor and the effect on monocyte antigen presentation and IL-10 signalling pathways (Jak1/Tyk2/STAT3 pathway) assessed using flow cytometry or proteomics.
b. Is there an *in vivo* effect of beta-lactams on immune function? – *In vitro* effects may not always translate *in vivo* but could be investigated in a healthy volunteer model of infection e.g. LPS blister, with co-administration of antibiotics. An animal model of inflammation e.g. zymosan, or infection,

could be considered although the lack of HLA-DR in rodent models would be a limitation. Immune cell function would be assessed using flow cytometry and HPLC would enable measurement of serum antibiotic concentration.

c. If an *in vivo* effect is demonstrated, does this translate to a clinical benefit? – Identification of a clinical benefit may be difficult given the multiple confounders including the different immunological effects on stimulus, co-morbidities, and effect of other co-prescribed medications. As such, a randomised trial of different beta-lactams (e.g. narrow versus broad) assessing immune function may not be feasible or cost-effective An observational study investigating therapeutic drug monitoring of beta-lactams could however be designed to identify a dose-dependent effect on immune function *in vivo*.
d. Do other antibiotics have a similar immune effect on antibiotics? – Many other antibiotic classes modulate the immune system by various mechanisms, some of which may be beneficial. This could be investigated using a chemistry-based approach to identify the structure-function relationship of each antibiotic on various immune cell receptors given work in allergic patients has shown direct T-cell receptor binding to amoxicillin.

7.2 Surgery

Surgery induced many changes in immune function similar to those seen in sepsis. This included reduction in monocyte HLA-DR and cytokine release. However, some key differences included altered monocyte chemokine receptor expression and reduced T-cell suppression. Additionally, surgery caused impairments in monocyte response to *in vitro* stimulus highlighting an immunocompromised state induced by sterile inflammation. Lymphocyte function however remained relatively intact. Changes in monocyte chemokine receptor and T-cell suppression expression predicted which patients would develop a post-operative infection, although many of the effects may be related to patient factors including cancer and use of neo-adjuvant chemotherapy. Further work is therefore required to elucidate:

a. What is the mechanism responsible for reduced HLA-DR and associated antigen presentation pathway impairments? – This could be investigated as per (a) in the sepsis section described above. The benefit of patients undergoing elective surgery is the use of a preoperative sample which can act as baseline control to identify changes in receptor and protein expression.
b. Do changes in chemokine receptor expression which predict postoperative infections correlate with altered monocyte chemotaxis? – Chemotaxis of monocytes isolated pre- and post-operatively could be assessed using Boyden chamber or agarose gel chemotaxis assays and alterations correlated with changes in chemokine receptors. The effect of a secondary stimulus on chemotaxis could also be assessed.

c. What is the time course of immune cell impairment both pre- and post-operatively? – It is unclear whether the impairments seen in the immediate preoperative period are present prior to this timepoint. A group of collaborators are currently running a clinical study to assess whether whole body metabolism correlates with immune cell function which could aid in answering this part of question. This may be most relevant in the population who receive neoadjuvant chemotherapy and therefore do not proceed to expedited surgery. Additionally, a postoperative time course is required to assess whether further defects in immune cell function occur later in the postoperative recovery period as per (c) in the sepsis section above.
d. Do therapeutic agents targeting monocyte antigen presentation improve global monocyte function? – This could be assessed as per (e) in the sepsis section above. However, given many of the impairments are demonstrated preoperatively, the optimal timing of therapeutic intervention preoperatively would need to be delineated.

Contrary to my expectations cefuroxime, but not other commonly prescribed antibiotics, appeared to have a protective role on perioperative immune cell function, enhancing lymphocyte cytokine release and markers of differentiation. To investigate this further the following would be required:

a. Does the effect of cefuroxime on receptors associated with cell differentiation cause alterations in $CD4^+$ lymphocyte populations? – This could be investigated using the mechanistic spectral flow cytometry panel as per (a) in the sepsis section above.
b. What is the mechanism of action of cefuroxime on lymphocyte differentiation? – There are differences in the effect of cefuroxime on lymphocyte function in patients with sepsis or surgery suggesting different mechanisms of action. Experimental design would be guided by the findings of (a) above.
c. Are the effects of cefuroxime a drug effect or a cephalosporin effect? – I would repeat experiments in (a and b) above with other cephalosporins, including those of other generations to identify whether this is a drug or class effect.
d. Do the potential immunomodulatory benefits of cefuroxime *in vitro* translate to a clinical benefit? – A clinical study could compare use of cefuroxime (or other cephalosporins) with other antibiotics (e.g. beta-lactams) as antimicrobial prophylaxis on immune function *in vivo*, and correlate and functional changes in immune cells with postoperative outcomes.

7.3 COVID-19

I assessed serum levels of multiple immune biomarkers which were undergoing therapeutic modulation in clinical trials of COVID-19 patients. Of the markers I assessed, only seven differentiated between those with mild and severe disease and the absolute levels of most were lower than other

hyperinflammatory conditions. COVID-19 has become one of the most extensively studied diseases. Future viral pandemics are likely to occur. Lessons learned from COVID-19 should be taken forwards in the investigation of other viruses likely to cause pandemics (particularly other coronaviruses and influenzas).

I also investigated the immunomodulatory effects of clarithromycin seen in my patient cohort *in vitro* demonstrating potential benefits on viral protein stimulated healthy volunteer lymphocyte cytokine release. Further work to explore this includes:

a. Does prolonged incubation alter the immunomodulatory effects? – I incubated PBMCs for a single 6 hour timepoint. Other effects of macrolides on monocyte and lymphocyte function may be elucidated with prolonged incubations similar to my sepsis and surgery models given the mechanism of action of macrolides on inhibition of protein synthesis.
b. Does the choice of viral protein used as a stimulus alter the immunomodulatory response? – I used spike protein in my model as that was commercially available at the time. Other proteins are now available and may alter the immune response.
c. Do the effects translate into patients? – I did not have ethical approval at the time to collect and store COVID-19 patient PBMCs so was unable to assess the effect of macrolides *in vivo*. Most clinical trials assessed azithromycin, not clarithromycin with which I demonstrated an immunomodulatory effect. *In vivo* evidence of this may have helped guide drug choices for ongoing clinical research.

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9 Supplemental Material

Immune/ Non-immune cell	Reference/ PMID	Author, Year	Antibiotic	In vivo, in vitro	Animal/ human	Stimulus/ disease	Cell type	Effect	Mechanism
Immune: Basophils	[458]	Abuaf N, 2008	Amoxicillin	In vivo	Human	Allergy	Basophils	Activation - Increased CD203c	-
Immune: Bone marrow	[459]	Leach KL, 2007	Oxazolidinones	In vitro	Human	-	K562 lymphoblast cell line	Mechanistic	Inhibited protein synthesis by crosslinking ribosomal RNA in the peptidyl transfer centre of mitochondrial, but not cytoplasmic, ribosomes
Immune: Dendritic cells	[244]	Lima CMF, 2021	Amoxicillin Penicillin	In vitro	Human	Amoxicillin allergy	Dendritic cells	Cytokine release - Increased IL-6	-
Immune: Dendritic cells & Lymphocytes	[266]	Rodriguez-Pena R, 2006	Amoxicillin	In vivo	Human	Delayed-type hypersensitivity reaction	Dendritic cells T-cells	Antigen presentation– upregulation of HLA-DR & CD86/80 Lymphocyte proliferation – increased proliferation, Cytokine release - Decreased IFN	Bidirectional signalling between dendritic cells & T-cells
Immune: Dendritic cells & Lymphocytes	[245]	Juanola O, 2016	Norfloxacin	In vivo	Human Mice	Cirrhosis Bacterial peritonitis	Dendritic cells T-cells	Antigen presentation - Decreased CD80& CD86 Cytokine release - Increased IL-10 Population – Increased Tregs	Treg population changes related to increases in rag1 gene (T-cell receptor processing)
Immune: Eosinophils	[201]	Kohyama T, 1999	Erythromycin Clarithromycin Josamycin Tetracycline Cefazolin	In vitro	Human	Atopy	Eosinophils	Cytokine release – Reduced IL-8 by 14-member macrolides	Effect occurred post-transcriptionally & related to structure of macrolide
Immune: Eosinophils	11408771	Cui CH, 2001	Roxithromycin	In vitro	Human	-	Eosinophils	ROS release – Reduced	-
Immune: Eosinophils	10383596	Shoji T, 1999	Roxithromycin	In vivo	Human	Asthma	Peripheral & pulmonary eosinophils	Counts - reduced eosinophils Leukotrienes - reduced	-
Immune: Eosinophils	10875487	Amayasu H, 2000	Clarithromycin	In vivo	Human	Asthma	Peripheral & pulmonary eosinophils	Counts - reduced eosinophils Leukotrienes - reduced	-
Immune: Langerhans cells	[206]	Ohshima A, 1998	Roxithromycin	In vitro	Mouse	-	Langerhans cells	Antigen presentation – Reduced HLA-DR expression Cytokine release – Reduced IL- 1β	-
Immune: Lymphocyte	[60]	Lawrence JW, 1996	Ciprofloxacin	In vitro	Mouse	-	L1210 lymphocyte cell line	Cell cytotoxicity	Interfered with mitochondrial topoisomerase II resulting in a loss of mtDNA.
Immune: Lymphocytes	[283]	Koziel R, 2006	Ciprofloxacin	In vitro	Human	-	Jurkat lymphocyte cell line	Cell cytotoxicity	60% reduction of mtDNA content, inhibition of the

									respiratory chain, & a significant decrease in mitochondrial membrane potential
Immune: Lymphocytes	[272]	Banck G, 1979	Penicillins Cephalosporins Aminoglycosides Chloramphenicol Sulfamethoxazole Trimethoprim Nalidixic acid 5-fluorocytosine Erythromycin Clindamycin Rifampin Fusidic acid Nitrofurantoin Doxycycline	In vivo	Human	-	Lymphocytes	Lymphocyte Proliferation – Impaired by erythromycin, clindamycin, & rifampin. No effect with penicillins, cephalosporins, aminoglycosides, chloramphenicol, sulfamethoxazole, trimethoprim, nalidixic acid, & 5-fluorocytosine,	Protein synthesis inhibited
Immune: Lymphocytes	[209]	Strzepa A, 2016	Enrofloxacin	In vivo	Mouse	Ovalbumin	Lymphocytes	Cytokine release - Production of type-1 (IFN-γ), type-2 (IL-4, IL-5, IL-10, IL-13) & Th17-associated (IL-17A) cytokines was inhibited	-
Immune: Lymphocytes	[212]	Konno S, 1992	Roxithromycin	In vivo	Mouse	Concanavalin A	Lymphocytes	Cytokine release - Initial increase (<14 days) in IL-1/2 but inhibited after 42 days Blastogenesis - Increased	-
Immune: Lymphocytes	[213]	Konno S, 1993	Roxithromycin	In vivo	Mouse	Concanavalin A	Lymphocytes	Cytokine release - Initial increase (<7 days) in IL-1/2 but inhibited after 28 days Blastogenesis – Increased Specific inhibitor of Th2 cells	-
Immune: Lymphocytes	36891994	Ghorab MM, 2023	Quinazolinone Benzenesulfonamide	In vivo	Mouse	-	Lymphocytes	Activation - Increased	-
Immune: Lymphocytes	[243]	Park SJ, 2004	Erythromycin	In vivo	Human	Diffuse panbronchiolitis	Pulmonary lymphocytes	Cytokine release – Reduced IL-2 & IFN-γ, increased IL-4, IL-5, IL-13 Shift from Th1 to Th2 phenotype	-
Immune: Lymphocytes & Monocytes	[275]	Pu-Iverer G, 199-2	Cefodizime Cefotaxime	In vitro	Mouse	-	Leukocytes Monocytes	Lymphocyte proliferation – Inhibited by Cefotaxime	-
Immune: Lymphocytes & Neutrophils	[173]	Stamatiou R, 2023	Colistin	In vivo	Rat	Emphysema LPS	Neutrophils Lymphocytes	Cytokine release – Reduce IL-1β, no effect on TNF-α Migration/chemotaxis – Increased Proliferation – No effect Cell death - Increased	Cell death mediated by increased by caspase-3
Immune: Lymphocytes & Neutrophils	[248]	Taw-fik AF, 1919-1	Vancomycin Teicoplanin Daptomycin Coumermycin	In vitro In vivo	Human Mouse	-	Leukocytes Volunteer PBMCs	Phagocytosis - no effect	-

Immune: Lymphocytes & Neutrophils	[190]	Gialdroni Grassi G, 1984	Ceftriaxone	In vitro In vivo	Human	S aureus C albicans	Volunteer neutrophils & lymphocytes	Chemotaxis – Inhibited in vitro Phagocytosis - No effect	-
Immune: Lymphocytess	[281]	Smith DM, 2002	Lactam I	In vivo	Human	-	Jurkat lymphocyte cell line	Lymphocyte Apoptosis - Increased	Induced DNA damage & inhibited DNA replication. Caused p38 mitogen-activated protein kinase activation, S phase arrest, & apoptotic cell death. p38 was found to be a central player in beta-lactam-induced apoptosis & resided downstream of DNA damage but upstream of caspase activation. Accompanying caspase-8 activation was cleavage of the pro-apoptotic Bcl-2 family protein Bid, & release of the mitochondrial cytochrome c. This was also associated with activation of caspase-9 & -3.
Immune: Macrophages	[220]	Yan M, 2017	Danofloxacin	In vivo	Pig	LPS	Alveolar macrophages	Cytokine release - Decreased IL-1β, TNF-α, IL-6, NO (nitric oxide), & PGE2. Increased IL-10	Effects occur pre-translationally
Immune: Macrophages	[260]	Hodge S, 2006	Azithromycin	In vitro	Human	COPD	Alveolar macrophages	Phagocytosis - increased Cytokine release - Deceased	Inhibition of phagocytosis mediated by phosphatidylserine pathway caused inhibition suggesting pathway
Immune: Macrophages	[208]	Liu S, 2023	Doxycycline	In vivo	Mouse	-	Bone marrow derived macrophages	Inflammasome assembly – Inhibited NLRP3 Cytokine release - Reduced IL- 1β Apoptosis – Inhibited caspase-1	Inhibited mitochondrial translation
Immune: Macrophages	[267]	Lino Y, 2001	Clarithromycin Roxithromycin	In vivo	Human	Chronic sinusitis	Macrophages	Antigen presentation - increased CD80, no change in HLA-DR/CD54	Number of rings in the structure causes effect
Immune: Macrophages	[171]	Miyata T, 1998	Ampicillin Cephalexin Cefotiam Amikacin Clindamycin Tetracycline Bleomycin	In vitro	Rat	-	Macrophages	Phagocytosis – Reduced by all except ampicillin Chemotaxis - Reduced	-
Immune: Macrophages	[210]	Ogino H, 2009	Ciprofloxacin Gatifloxacin Norfloxacin Levofloxacin	In vivo In vitro	Mouse	LPS	Peritoneal macrophages	Cytokine release - Ciprofloxacin, gatifloxacin, & norfloxacin inhibited both TNF- & IL- 1β production. Levofloxacin inhibited IL- 1β production only. LPS stimulated IL-6 production was inhibited only by norfloxacin.	Greater effect seen with those with cyclopropyl group at the N1 position &/or a piperazinyl group at the C7 position

Immune: Macrophages	[169]	Nunez RM, 1989	Carbopenem	In vitro In vivo	Mouse	C albicans	Peritoneal macrophages	Chemotaxis – Increased Phagocytosis - Increased	-
Immune: Macrophages	[170]	Barriga C, 1996	Teicoplanin Vancomycin	In vivo	Mouse	C albicans	Peritoneal macrophages	Phagocytosis – Enhanced Chemotaxis - Enhanced	-
Immune: Macrophages	[203]	Eswarappa SM, 2008	Folimycin	In vitro	Mouse	LPS	Peritoneal macrophages	Cytokine release – No effect on TNF NO production – Reduced NF-κB - Inhibited	Inhibits V-ATPases, alters intra-Golgi pH, which in turn causes defective processing & reduced surface expression of TLR4 NO inhibited pre-translationally, potentially through NF-κB
Immune: Macrophages & Lymphocytes	[207]	Ortega E, 2004	Erythromycin Azithromycin Josamycin	In vivo	Mouse	-	Peritoneal macrophages Splenic lymphocytes	Phagocytosis -Impaired in macrophages Cytokine release – Decreased macrophage IL-12 but increased IL-18 & lymphocyte IL-4	-
Immune: Macrophages & Neutrophils	[254]	Yamaryo T, 2003	Clarithromycin Erythromycin Roxithromycin Oleandomycin Josamycin Spiramycin Clindamicin Azithromycin Ampicillin Cefaclor	In vitro	Human	LPS	Neutrophils Alveolar macrophages	Apoptosis – increased in neutrophils Phagocytosis – Increased in macrophages	Effect only seen in 14-member & 15-member macrolides
Immune: Macrophages & Neutrophils	[211]	Ianaro A, 2000	Roxithromycin Clarithromycin Erythromycin Azithromycin	In vitro In vivo	Rat	Carrageenin pleurisy	Lung neutrophils J774 macrophage cell line	Cytokine release - Decreased prostaglandins & TNF-α	Inhibition of cyclooxygenase-2 & inducible nitric oxide synthase protein expression
Immune: Macrophages & Neutrophils	[280]	Plekhova NG, 2015	Maxifloxacin	In vitro	Mouse	Sterile beef broth S pneumoniae	Peritoneal neutrophils & macrophages	Cell death – Increased ROS production - Increased	Effect reversed by immunomodulation with tinrostim & licopid
Immune: Macrophages & PBMCs	[249]	Mato R, 1992	Lomefloxacin	In vitro	Human Mouse	C albicans	Volunteer PBMCs Peritoneal macrophages	Phagocytosis - no effect	-
Immune: Mast cells	11001175	Sugimoto, 2000	Everniomicin Teicoplanin Vancomycin Concanavalin A	In vitro	Rat	-	Peritoneal mast cells	Histamine release – Increased by vancomycin & teicoplanin	-
Immune: Mast cells	10757422	Toyoguchi T, 2000	Vancomycin Miconazole Fluconazole Fosfomycin Cilastin Fluconazole	In vitro	Rat	-	Peritoneal mast cells	Histamine release – Increased by vanc & miconazole	-
Immune: Monocyte & Promyelocyte	[166]	Milosevic TV, 2018	Linezolid Tedizolid	In vitro	Human	-	HL-60 promyelocyte & THP-1 monocyte cell line	Mechanistic	Inhibition of CYTox I expression, cytochrome *c*-oxidase activity, & spare respiratory capacity, causing swelling of the mitochondrial matrix & loss of their cristae

Immune: Monocytes	[231]	Bailly S, 1990a	Ciprofloxacin Pefloxacin Ofloxacin	In vitro	Human	LPS	Volunteer isolated monocytes	Cytokine release - Decreased TNF & IL-1	Impaired protein synthesis rather than impaired release, potentially mediated by quinolone-induced accumulation of intracellular cAMP
Immune: Monocytes	[234]	Khan AA, 1998	Trovafloxacin	In vitro	Human	LPS Heat-killed S aureus	Monocytes	Cytokine synthesis - Reduced IL-1, 6, 10 TNF	-
Immune: Monocytes	[246]	Spyridaki A, 2012	Clarithromycin	In vivo	Human	Ventilator-associated pneumonia & sepsis	Monocytes	Antigen presentation – Decreased CD86 Cytokine release - Decreased TNF, increased IL-10	-
Immune: Monocytes	[204]	Bode C, 2015	Linezolid Vancomycin Daptomycin	In vitro	Human	LPS	THP-1 monocyte cell line	Cytokine release - Linezolid increase IL-1, 6 &10, & TNF. Vancomycin increased IL-6, 10, TNF. Daptomycin increased IL-6/10 but decreased IL-1 TLR expression – Upregulated by linezolid & vancomycin, downregulated daptomycin. Phagocytosis – Increased by vancomycin	Pre-translation effect
Immune: Monocytes	[221]	Ives TJ, 2003	Grepafloxacin	In vitro	Human	S. aureus Zymogen A	THP-1 monocyte cell line	Cytokine release - Reduced IL-1, IL6, IL-8, TNF release ROS production - Reduced	-
Immune: Monocytes	[250]	Muenster S, 2015	Amphotericin Itraconazole Anidulafungin	In vitro	Human	LPS	THP-1 monocyte cell line	Cytokine release - ambisome decreases TNF, itraconazole increases TNF & IL-1, anidulafungin increases IL-1 Phagocytosis – Suppressed by ambisome & Itraconazole	Pre-translation
Immune: Monocytes	[154]	Bailly S, 1990b	Ciprofloxacin	In vitro	Human	LPS	Volunteer isolated monocytes	Cytokine release - Decreased monocyte IL-1	Post-transcriptional inhibition
Immune: Monocytes & Neutrophils	[187]	Fietta A, 1986	Teicoplanin Vancomycin	In vitro	Human	S aureus	Volunteer neutrophils & monocytes	Chemotaxis/adherence – No effect Phagocytosis - No effect Killing – Enhanced in monocytes	-
Immune: Monocytes & Neutrophils	[222]	Franks Z, 2013	Linezolid Vancomycin	In vitro In vivo	Human Mouse	MRSA LPS	Volunteer neutrophils & isolated monocytes Mice	Cytokine release - Reduced release of IL- 1β, IL-6 & TNF-α	-
Immune: Monocytes & PBMCs	[205]	Bode C, 2014	Piperacillin Doxycycline Erythromycin Moxifloxacin Gentamicin	In vitro	Human	LPS Cardiac bypass	THP-1 monocyte cell line PBMCs	Cytokine release - Erythromycin, moxifloxacin & doxycycline increased IL- 1β, 6 TLR expression - Erythromycin, moxifloxacin & doxycycline increased TLR-1,2,4,6	Pre-translation

								Phagocytosis – Inhibited by piperacillin, doxycycline & moxifloxacin	
Immune: Neutrophils	[175]	Sugita K, 1995	Ampicillin Methicillin Oxacillin Benicillin Sulbenicillin Ticarcillin Piperacillin Cefotaim Cefoperazone Ceftizoxime Cefmenoxime Ceftazadime Ceftriaxone Cefpimizole Cefuzonam Cefsulodin Cefmetazole Cefbuperazone Latamoxef Flumoxef Erythromycin Josamycin Midekamycin Rokitamycin Tetracycline Doxycycline Minocycline Gentamicin Tobramycin Amikacin Sisomycin Piromidic acid Cinoxacin Norfloxacin Ofloxacin Enoxacin Ciprofloxacin Rifampicin Chloramphenicol Fosfomycin Lincomycin Clindamycin	In vitro	Human	Volunteers	Neutrophils	Chemotaxis – Inhibited by Minocycline & doxycycline	Chelation of Ca-ions
Immune: Neutrophils	[179]	Naess A, 2006	Linezolid	In vitro	Human	Zymosan	Neutrophils	Chemotaxis – No effect Phagocytosis – No effect Respiratory burst – No effect	-
Immune: Neutrophils	[197]	Suzuki H, 1997	Roxithromycin	In vivo	Human	Chronic sinusitis	Neutrophils	Chemotaxis/recruitment - reduced	Impaired IL-8
Immune: Neutrophils	[257]	Herrera-Insua, 1997	Qinupristin Dalfopristin Sparfloxacin	In vitro	Human	E faecium	Neutrophils	Phagocytosis - enhanced	Strain dependant, phagocytosis impaired if vancomycin-resistant strain used

Immune: Neutrophils	[261]	Noma T, 1998	Roxithromycin Cefaclor Ofloxacin Aztreonam	In vivo	Human	Seriously handicapped with severe mental retardation	Neutrophils	Phagocytosis – Enhanced by roxithromycin Bactericidal – Enhanced by roxithromycin	-
Immune: Neutrophils	7759458	Kamoi H, 1995	Roxithromycin	In vivo	Human	Asthma	Neutrophils	ROS production - reduced	-
Immune: Neutrophils	36713462	Pereiro P, 2023	Sulfamethoxazole Clarithromycin	In vivo	Zebra fish & larvae	Carp virus	Neutrophils	Counts - Reduced	Altered transcription of complement components
Immune: Neutrophils	38147695	Rieder JC, 2023	Doxycycline	In vitro	Dog	S aureus	Neutrophils	ROS production – Reduced NET release - Increased	-
Immune: Neutrophils	[194]	Sakito O, 1996	Erythromycin Roxiflomycin	In vivo	Human	Diffuse panbronchiolitis	Patient & volunteer neutrophils	Chemotaxis/migration – Reduced Cytokine release – TNF-α & IL- 1β reduced	Impaired IL-8 release
Immune: Neutrophils	[202]	Scaglione F, 1993	Clarithromycin	In vivo	Human	Chronic bronchitis	Patient & volunteer neutrophils	Phagocytosis – enhanced Chemotaxis – no effect	-
Immune: Neutrophils	[193]	Kadota J, 1993	Erythromycin	In vivo	Human Mice	Diffuse panbronchiolitis	Patient, volunteer & mice pulmonary neutrophils	Chemotaxis/migration - reduced	Impaired chemotactic gradient (IL-8)
Immune: Neutrophils	[195]	Banerjee D, 2004	Clarithromycin	In vivo	Human	COPD	Pulmonary neutrophils	Chemotaxis – Reduced Cytokine release - No effect on IL-8/TNF	-
Immune: Neutrophils	[191]	Oda 1994	Erythromycin	In vivo	Human	Diffuse panbronchiolitis	Pulmonary neutrophils	Chemotaxis - Inhibited	Impairs chemokine gradient
Immune: Neutrophils	[192]	Oda H, 1995	Erythromycin	In vivo	Human	Diffuse panbronchiolitis	Pulmonary neutrophils	Chemotaxis/migration - Inhibited	Inhibits chemokine leukotriene B4 production
Immune: Neutrophils	[242]	Simpson JL, 2007	Clarithromycin	In vivo	Human	Asthma	Sputum neutrophils	Counts – Reduced Cytokines – Reduced IL-8	IL-8 mediated drop in numbers
Immune: Neutrophils	[196]	Piacentini GL. 2007	Azithromycin	In vivo	Human	Paediatric asthma	Sputum neutrophils	Count - Reduced	-
Immune: Neutrophils	[253]	Pasqui AL, 1995	Imipenem	In vitro In vivo	Human	Elderly Diabetic	Volunteer & patient neutrophils	Phagocytosis - increased Oxidative burst – Increased	-
Immune: Neutrophils	[252]	Scheffer J, 1992	Cefaclor Cefetamet Ro 40-6890	In vitro	Human	E coli P aeruginosa P mirabilis	Volunteer neutrophils	Phagocytosis – Increased (not Ro) Bactericidal – Enhanced Leukotriene release - Decreased	In class differences in actions
Immune: Neutrophils	[178]	Matera G, 1995	Meropenem	In vitro	Human	PMA LPS	Volunteer neutrophils & monocytes	Phagocytosis - Reduced ROS production - Reduced Chemotaxis - no effect Cytokine release - reduced TNF (but not IL-1/6/8)	-
Immune: Neutrophils	[273]	Kushiya K, 2005	Azithromycin Rokitamycin Vancomycin Teicoplanin Arbekacin Linezolid	In vivo	Human	Toxic shock syndrome toxin-1	Volunteer neutrophils	Cytokine release - Macrolides reduced production, Vancomycin, teicoplanin, linezolid, & arbekacin,, no effect Proliferation – No effect	-
Immune: Neutrophils	[229]	-Reato G, 1-999	Co-amoxiclav	In vitro	Human	-	Volunteer neutrophils	Cytokine release - enhanced IL-8 & IL- 1β release Phagocytosis - enhanced	-

Immune: Neutrophils	[230]	Lankelma JM, 2017	Ciprofloxacin Vancomycin Metronidazole	In vivo	Human	LPS S pneumoniae K pneumonia E coli	Volunteer neutrophils	Cytokine release - no effect Chemotaxis/migration – No effect	-
Immune: Neutrophils	[238]	Yoshimura T, 1996	Levofloxacin	In vitro	Human	PHA	Volunteer neutrophils	Cytokine release - Increased IL-2, reduced IL- 1β, no effect on IL-8	-
Immune: Neutrophils	[174]	Anderson R, 1989	Erythromycin Roxithromycin	In vitro	Human	-	Volunteer neutrophils	Chemotaxis – Increased ROS production - decreased	Enhance neutrophil migration by an antioxidant mechanism that is not due to inhibition of transductional events involved in the activation of NADPH-oxidase or to oxidant scavenging properties
Immune: Neutrophils	[176]	Belsheim JA, 1981	Benzylpenicillin Ampicillin Mecillinam Cefuroxime Cefoxitin Cefotaxime Ceftriaxone Lymecycline Doxycycline Gentamycin Amikacin	In vitro	Human	E coli P aeruginosa	Volunteer neutrophils	Chemotaxis – inhibited by aminoglycosides & tetracycline	-
Immune: Neutrophils	[177]	Fietta A, 1983	Carbenicillin Piperacillin Thienamycin Cefotetan Ceftazidime Moxalactam	In vitro	Human	S aureus	Volunteer neutrophils	Chemotaxis – Inhibited by cephalosporins	-
Immune: Neutrophils	[180]	Ballesta S, 2003	Linezolid	In vitro	Human	S aureus E faecalis	Volunteer neutrophils	Phagocytosis – No effect Chemotaxis – No effect	-
Immune: Neutrophils	[181]	Labro MT, 1986	Cefotaxime Cefodizime	In vitro	Human	S aureus	Volunteer neutrophils	Chemotaxis – no effect ROS production - Increased	In class differences in effect on ROS production
Immune: Neutrophils	[183]	Rodriguez AB, 1993	Cefoxitin	In vitro	Human	C albicans	Volunteer neutrophils	Chemotaxis/adherence – Increased Phagocytosis - Increased	-
Immune: Neutrophils	[184]	Rodriguez AB, 1991	Cefmetazole	In vitro	Human	-	Volunteer neutrophils	Chemotaxis – Increased Phagocytosis – Increased ROS production - Increased	-
Immune: Neutrophils	[185]	Burgaleta C, 1987	Cefotaxime Cefoxitin Ceftazidime Latamoxef Amikacin Sisomicin Tobramycin	In vitro	Human	C albicans	Volunteer neutrophils	Chemotaxis/migration – Impaired by cephalosporins Phagocytosis – No effect	In class effects on migration
Immune: Neutrophils	[186]	Capodicasa E, 1991	Teicoplanin Vancomycin	In vitro	Human	C albicans	Volunteer neutrophils	Chemotaxis/adherence – Inhibited Phagocytosis – Inhibited	High doses only

Immune: Neutrophils	[188]	Moran FJ, 1991	Teicoplanin Vancomycin	In vitro	Human	C albicans	Volunteer neutrophils	Chemotaxis – Inhibited Phagocytosis – no effect	-
Immune: Neutrophils	[189]	Schultz MJ, 2000	Erythromycin	In vivo	Human	S pneumoniae	Volunteer neutrophils	Cytokine release - reduced chemokine (IL-8) production	-
Immune: Neutrophils	[251]	Wenisch C, 1996	Azithromycin Clarithromycin Roxithromycin	In vitro	Human	E coli	Volunteer neutrophils	Phagocytosis – Impaired by azithromycin & clarithromycin ROS production – Decreased by azithromycin	-
Immune: Neutrophils	[255]	-Braga PC, 1-997	Rokitamycin	In vitro	Human	-	Volunteer neutrophils	Phagocytosis – no effect ROS production - reduced	-
Immune: Neutrophils	[256]	Lianou PE, 1993	Ciprofloxacin	In vivo in vitro	Human	-	Volunteer neutrophils	Phagocytosis – enhanced Chemotaxis – no effect Bacterial killing – No effect	-
Immune: Neutrophils	[258]	Forsgren A, 1985	Ciprofloxacin Norfloxacin Ofloxacin	In vitro	Human	Zymosan S aureus Chemotactic peptide	Volunteer neutrophils	Phagocytosis – No effect Killing - Enhanced	Enhanced killing by direct bacterial effect only
Immune: Neutrophils	[259]	Gruger T, 2008	Pipemidic acid Cinoxacin Norfloxacin Lomefloxacin Enoxacin Ciprofloxacin Ofloxacin Levofloxacin Enrofloxacin Moxifloxacin Gatifloxacin Sparfloxacin Garenoxacin	In vitro	Human	C albicans	Volunteer neutrophils	Phagocytosis – Inhibited at high dose by Ciprofloxacin, Garenoxacin, Moxifloxacin, Enoxacin Oxidative burst – Inhibited at high dose by Ciprofloxacin, Garenoxacin, Moxifloxacin Activation – Increased CD11b expression at high dose by Ciprofloxacin, Garenoxacin, Moxifloxacin Killing – Increased at high dose by norfloxacin & sparfloxacin	Effect related to structure of fluroquinolones with effects seen in those with a cyclopropyl-moiety at position N1 only
Immune: Neutrophils	8560094	Mitsuyama T, 1995	Erythromycin	In vitro	Human	fMLP PMA	Volunteer neutrophils	Neutrophil ROS - Decreased	Cyclic AMP-dependent protein kinase (PKA), H-89 dependant
Immune: Neutrophils	[225]	Foca A, 1993	Teicoplanin	In vitro	Human	LPS	Volunteer neutrophils	Cytokine release - reduced IL-1β, IL-8 TNF	-
Immune: Neutrophils	[226]	Schultz MJ, 1998	Erythromycin Penicillin	In vitro	Human	Heat-killed S pneumoniae	Volunteer neutrophils	Cytokine release - Erythromycin decreased TNF & IL-6, & IL-10, IL-12 & IFN-γ at high dose	Il-6 inhibition was mediated by TNF inhibition
Immune: Neutrophils & PBMCs	[182]	Fietta A, 1994	Cefixime Cefdinir	In vitro	Human	Zymosan	Volunteer neutrophils & PBMCs	Phagocytosis – Enhanced by cefdinir Chemotaxis – no effect ROS production – no effect	-
Immune: PBMCs	[239]	Roche Y, 1988	Ciprofloxacin Ofloxacin Pefloxacin	In vivo	Human	Phytohemagglutinin (PHA)	PBMCs	Proliferation – Decreased Cytokine release – Increased IL-2 IL-2R – No change	Independent of DNA synthesis
Immune: PBMCs	[232]	Roche Y, 1987	Ciprofloxacin Ofloxacin Pefloxacin	In vivo	Human	Phytohemagglutinin (PHA)	PBMCs	Proliferation – decreased Cytokine release - IL-1 decreased	-
Immune: PBMCs	[235]	Mori S, 2010	Ciprofloxacin	In vivo	Human	Glyceraldehyde-derived AGE	PBMCs	Lymphocyte proliferation – Inhibited	Enhance COX-2 expression increasing cAMP

								Monocyte adhesion – reduced expression Cytokine release - Reduced TNF/IFN	
Immune: PBMCs	[268]	Karakike E, 2022	Clarithromycin	In vivo	Human	Sepsis ARDS	PBMCs	Antigen presentation - upregulated monocyte HLA-DR	Upregulation in genes involved in cholesterol homeostasis
Immune: PBMCs	[223]	Garcia-Roca P, 2006	Erythromycin Linezolid	In vitro	Human	LPS	Volunteer PBMCs	Cytokine release- Reduced monocyte IL-1β, TNF-α & IL-6	-
Immune: PBMCs	[224]	Stevens DL, 1995	Clindamycin Penicillin	In vitro	Human	LPS	Volunteer PBMCs	Cytokine release - clindamycin reduced TNF	Inhibits protein synthesis
Immune: PBMCs	[227]	Vickers IE, 2006	Ciprofloxacin Ceftazidime Cotrimoxazole Piperacillin-tazobactam	In vitro	Human	Heat-killed S maltophila	Volunteer PBMCs	Cytokine release - Co-trimoxazole inhibited TNF secretion at all doses, ciprofloxacin & ceftazidime inhibited at high dose.	-
Immune: PBMCs	[228]	Picherean S, 2012	Vancomycin Trimethoprim/sulfamethoxazole Tigecycline Daptomycin Linezolid Clindamycin Azithromycin	In vitro	Human	S. aureus toxic shock syndrome toxin-1 (TSST-1) Staphylococcal enterotoxin A (SEA) α-toxin Panton-Valentine leucocidin (PVL)	Volunteer PBMCs	Cytokine release - Decreased IL-6 & IFNy by tigecycline, decreased TNF-α & IL-8 by linezolid, increased IL-8 by trimethoprim. IL-1β, IL-6, IL-8, IFN-γ & TNF-α decreased by all antibiotics at v high concentration (>25mg/ml)	-
Immune: PBMCs	[233]	Riesbeck K, 1990	Ciprofloxacin	In vitro	Human	-	Volunteer PBMCs	Cytokine release - increased lymph IL-2 but no lymph IFN-γ or monocyte IL- 1β & TNF-α	-
Immune: PBMCs	[236]	Ono Y, 2000	Grepafloxacin	In vitro	Human	-	Volunteer PBMCs	Cytokine release - Reduced IL-1, 6, 8, TNF	Occurs at the transcriptional level
Immune: PBMCs	[265]	Roche Y, 1987	Pefloxacin Ciprofloxacin	In vitro	Human	LPS PHA	Volunteer PBMCs	Antigen presentation – No effect Cytokine release - decreased monocyte IL-1 Proliferation - Decreased	-
Immune: Spleen cells	[263]	Asano K, 2001	Roxithromycin	In vitro	Mouse	Haemocyanin absorbed to aluminium hydroxide	Spleen cells	Antigen presentation - Supressed CD80 & CD866	-
Immune: Spleen cells	[262]	Suzuki M, 2002	Roxithromycin	In vitro In vivo	Mouse	Haemocyanin absorbed to aluminium hydroxide	Splenic B-cells	Antigen presentation – Suppressed CD86 & CD80 (but only after 4weeks)	-
Immune: Spleen cells	[274]	Karrow NA, 2001	Clarithromycin	In vivo	Mouse	-	Splenic macrophages, NK & lymphocytes	Proliferation - no effect	-
Immune: Spleen cells	28957452	Cheng RY, 2017	Vancomycin Ceftriaxone	In vitro	Mouse	-	Splenic Treg cells	Differentiation -ceftriaxone decreased splenic Tregs	Modulated via gut microbiome
Immune: Spleen cells	[264]	Kawazu K, 2000	Roxithromycin	In vivo	Mouse	Ovabumin	Splenocytes	Antigen presentation - no effect on CD80/86	-
Immune: T-cells	[271]	Schmid DA 2006	Ciprofloxacin Norfloxacin Moxifloxacin	In vitro	Human	Delayed hypersensitivity reactions	T-cells	Proliferation – Increased by all	Cross reactivity with t-cell receptor causing direct stimulation

Immune: T-cells	[237]	Kaminshi MM, 2010	Ciprofloxacin	In vitro	Human	Atopic dermatitis	T-cells	Cytokine release – Reduced IL-2 & IL-4 ROS production - Reduced	Caused a loss of mtDNA & decreased activity of complex I. Leads to reduction in NF-κB & AP-1 transcription factors
Mixed: Immune: Bone marrow & Lymphocyte Non-immune: Chick embryo	[276]	Neftel KA, 1986	Amoxicillin Azthreonam 6-Aminopenicillanic acid 7-Desacetoxycephalosporanic acid Carbenicillin Ticarcillin Piperacillin Methicillin Penicillin-G Mezlocillin Azlocillin Cloxacillin Oxacillin Flucloxacillin 7-Desacetylcephalosporanic acid Ceftriaxone Cefoxitin Moxalactam 7-Cephalosporanic acid Cefmenoxime Ceftizoxime N-formimidoyl-Thienamycin Cephalothin Clavulanic acid Ceftazidime Cefazolin Cephalexin Cefuroxime Cefotaxime Cefotiam	In vitro In vivo	Human Chickens Mouse	Orthopaedic surgery	Bone marrow cells Chick embryo liver cells Mouse lymphoma YAC-1 & EL4 cell lines	Lymphocyte proliferation – All inhibited in a dose dependant fashion	-
Mixed: Immune: Bone marrow Non-immune: Renal & ovarian	[460]	Nagiec EE, 2005	Eperezolid	In vitro	Human Hamster	-	K562 erythroleukemia cells, HEK renal, & CHO ovarian cell lines	Cell proliferation - inhibited	Decrease in mitochondrial cytochrome oxidase subunit I levels, consistent with an inhibition of mitochondrial protein synthesis.
Mixed: Immune: PBMCs Non-immune: Skin nerve fibres	[278]	Garrabou G, 2017	Linezolid	In vitro	Human	Joint infections	PBMCs Skin nerve fibres	Mitochondrial-dependant apoptosis - Increased	Reduced mitochondrial protein levels, complex IV activity, & mitochondrial mass. Certain mitochondrial polymorphisms more susceptible
Mixed: Immune: Bone marrow	[165]	McKee EE, -2006	Chloramphenicol Tetracycline Erythromycin Azithromycin Clindamycin	In vitro	Rat Rabbit	-	Isolated heart, liver, & bone marrow mitochondria	Mitochondrial toxicity - Oxazolidinones chloramphenicol & tetracycline inhibit mitochondrial protein	-

Non-immune: Cardiac, Hepatic, Renal			Kasugamycin Lincomycin Streptomycin Eperezolid Linezolid					synthesis. Macrolides, lincosamides, & aminoglycosides no effect	
Non-immune: Bladder	[285]	Aranha O, 2002	Ciprofloxacin	In vitro	Human	-	HTB9 bladder cell line	Mitochondrial Induced apoptosis - Increased	Mitochondrial depolarisation disruption of calcium homeostasis, cytochrome C release, caspase-3 activation, mitochondrial swelling & Bcl-2 dependant redistribution of Bax to the mitochondrial membrane
Non-immune: Brain	[218]	Mike JK, 2023	Azithromycin	In vivo	Sheep	Hypoxic-encephalopathy	Brain tissue	Cytokine release - Reduced il-6	-
Non-immune: Breast	[282]	Chen D, 2008	Lactam I	In vivo	Mouse	-	Breast cancer cell line	Apoptosis - Increased	induction of DNA damage leading to apoptosis
Non-immune: Breast	[269]	Yu M, 2016	Levofloxacin	In vivo	Human	-	Breast cancer cell line	Proliferation – Inhibited Apoptosis - Increased s	Deactivation of PI3K/Akt/mTOR & MAPK/ERK pathways
Non-immune: Cochlear	[168]	Desa DE, 2018	Gentamicin	In vitro	Mouse	-	Isolated cochlear explants	Mitochondrial dysfunction - Increased	The rapid conversion of highly reactive O2.- to H2O2 occurs during the acute stage of ototoxic antibiotic exposure & the endogenous antioxidant system is significantly altered.
Non-immune: Colon & Hepatic	[284]	Herold C, 2002	Ciprofloxacin	In vitro	Human	-	CC-531, SW403, HT-29 colon & HepG2 cell lines	Mitochondrial Induced apoptosis - Increased Cell proliferation - Reduced	Suppressed mtDNA synthesis, increased upregulation of Bax & of the activity of caspases 3, 8 & 9, & decreased mitochondrial membrane potential
Non-immune: Enzyme	[461]	Morris JC, 1996	Gentamicin Kanamycin A G418	Organochemistry	*Bacillus cereus*	-	Isolated phosphatidylinositol phospholipase	Mechanistic	Act as allosteric activators of phospholipase c
Non-immune: Hepatic, Muscle, Renal	[279]	De Vriese AS, 2006	Linezolid	In vivo	Human Rat	Linezolid induced optic neuropathy, encephalopathy, skeletal myopathy, lactic acidosis, & renal failure	Muscle, liver, & kidney tissue	Mechanistic	Inhibits mitochondrial protein synthesis with no effect on mtDNA
Non-immune: Histology & Serum	29406285	Takahashi E, 2017	Clarithromycin	In vivo	Mouse	Influenza A	Serum Lung histology	Migration/chemotaxis – Reduced Cytokine release – No effect on IL-6, MCP-1, IFN-γ, TNF-α, MIP-1α	--
Non-immune: Lung	[270]	Song M, 2016	Levofloxacin	In vitro	Human	-	A549, H3255, NCL-69 & H460 lung cell lines	Proliferation - Inhibited Mitochondrial-dependant apoptosis - Increased	Inhibits activities of mitochondrial electron transport chain complex I & III, leading to inhibition of mitochondrial respiration & reduction of ATP production
Non-immune: Mechanistic	[159]	Hong S, 2015	Apramycin Gentamicin Kanamycin A	Organochemistry	Human	-	Isolated mitochondrial 23S rRNA	Mechanistic	Direct binding of aminoglycosides to helix 69 of human ribosomal RNA

			Hygromycin B						
Non-immune: Multiple: Renal & Hepatic	[160]	O'Reilly M, 2019	Gentamicin	In vitro	Rat	-	Isolated renal cortical & hepatic cell mitochondria	Mitochondrial dysfunction - Increased	Gentamicin behaves as an uncoupler of the electron transport chain (ETC) Stimulates State 4 & inhibits State 3u mitochondrial respiration leading to collapse of mitochondrial membrane potential & reduced ROS production
Non-immune: Multiple: Renal & Hepatic	[161]	Simmons CF, 1980	Gentamicin	In vitro	Rat	-	Isolated renal cortical & hepatic cell mitochondria	Mitochondrial dysfunction - Increased	Inhibits Stage 3 mitochondrial respiration. Drop in whole kidney ATP concentration
Non-immune: Multiple: Renal & Hepatic	[163]	Weinberg JM, 1980, b	Gentamicin Neomycin Kanamycin Streptomycin	In vitro	Rat	-	Isolated renal cortical & hepatic cell mitochondria	Mitochondrial dysfunction - Increase	Stimulates State 4 mitochondrial respiration & inhibits State 3 & DNP-uncoupled respiration The potency of the aminoglycosides in producing these effects strongly correlated with the number of ionizable amino groups present on the aminoglycoside molecule suggesting that cationic charge is an important molecular determinant of aminoglycoside-induced mitochondrial toxicity.
Non-immune: Multiple: Renal & Hepatic	[164]	Yang CL, 1995	Gentamicin	In vitro	Rat	-	Isolated renal cortical cell mitochondria	Mitochondrial dysfunction - Increased	Enhanced superoxide anion & hydroxyl radical generation
Non-immune: Renal	[158]	Weinberg JM, 1980, a	Gentamicin	In vitro	Rat	-	Isolated renal cortical cell mitochondria	Mitochondrial dysfunction - Increased	Increased mitochondrial Stage 4 respiration. Enhanced uptake of sodium- & potassium- acetate enhancing energy-dependant swelling
Non-immune: Renal	[162]	Ueda N, 1993	Gentamicin	In vitro	Rat	-	Isolated renal cortical cell mitochondria	Mitochondrial dysfunction - Increased	Increased hydrogen peroxidase production mobilised mitochondrial iron release
Non-Immune: Renal	[286]	Denamur S, 2016	Gentamicin	In vitro	Pig	-	LLC-PK1 renal cell line	Mitochondrial-dependant apoptosis - Increased	ROS dependant increase in p53 levels resulted in accumulation of p21 & of phospho-eIF2α. These effects could be related to an impairment of proteasome as we demonstrated an inhibition of trypsin- & caspase-like activities. Moderate endoplasmic reticulum stress could also participate to cellular toxicity induced by gentamicin, with activation of caspase-12 without change in GRP74 & GRP98.
Non-Immune: Renal	[287]	Servais H, 2005	Gentamicin	In vitro	Pig	-	LLC-PK1 renal cell line	Mitochondrial Induced apoptosis -Increased	Within 2 h, gentamicin induced a partial relocalisation [from

									lysosomes to cytosol] of the weak organic base acridine orange followed by a loss of mitochondrial membrane potential, release of cytochrome c from granules to cytosol, & the activation of caspase-9 (as from 12 h, & increase in caspase-3
Non-immune: Renal	[167]	Morales AI, 2010	Gentamicin	In vivo In vitro	Rat	Metformin	Renal cortical cells	Mitochondrial dysfunction - Increased	Gentamicin depleted respiratory components (cytochrome c, NADH), probably due to the opening of mitochondrial transition pores & increased reactive oxygen species production from the electron transfer chain.
Non-immune: Respiratory fluid	[214]	Breslow-Deckman JM, 2013	Linezolid	In vivo	Mouse	Influenza then S. pneumonia	BAL fluid	Cytokine release - Decreased IFN-γ & TNF-α	-
Non-immune: Respiratory fluid	[172]	Jacqueline C, 2014	Linezolid Vancomycin	In vivo	Mouse	MRSA	BAL fluid	Cytokine release – Linezolid decreased IL- 1β MIP2 & TNF-α	-
Non-immune: Respiratory fluid	[215]	Kaku N, 2016	Tedizolid Linezolid Vancomycin	In vivo	Mouse	MRSA	BAL fluid	Cytokine release – Linezolid & tedizolid decreased TNF-α, IL-6 & MIP-2,	-
Non-immune: Respiratory fluid	[216]	Yanagihara K, 2009	Linezolid	In vivo	Mouse	MRSA	BAL fluid	Cytokine release - Decreased	-
Non-immune: Respiratory fluid	[217]	Verma AK, 2019	Linezolid	In vivo	Mouse	Influenza then MRSA	BAL fluid	Cytokine release - Decreased	-
Non-Immune: Respiratory fluid	[198]	Cervin A, 2008	Clarithromycin	In vitro	Human	Chronic rhinosinusitis	Nasal lavage	Cytokine release – Reduced IL-8	-
Non-Immune: Respiratory fluid	[199]	Wallwork B, 2006	Roxithromycin	In vivo	Human	Chronic sinusitis	Nasal lavage	Cytokine release – Reduced IL-8	-
Non-Immune: Respiratory fluid	[200]	Yamada T, 2000	Clarithromycin	In vivo	Human	Chronic sinusitis	Nasal lavage	Cytokine release – reduced IL-8	-
Non-Immune: Respiratory fluid	[241]	Fonseca-Aten M, 2006	Clarithromycin	In vivo	Human	Paediatric asthma	Nasaopharyngeal aspirates	Cytokine release - Reduced TNF-α, IL- 1β, IL-10	-
Non-immune: Respiratory fluid & Serum	[219]	Luna CM, 2009	Linezolid	In vivo	Pig	MRSA	Serum & BAL cytokines	Cytokine release – No effect	-
Non-immune: Respiratory Fluid & Serum	[240]	Cameron EJ, 2013	Azithromycin	In vivo	Human	Smokers with asthma	Sputum aspirates & serum	Cytokine release – No effect	-
Non-immune: Serum	26917573	Van Opstal E, 2016	Vancomycin	In vivo	Mouse	C. difficile	Serum	Humoral immunity - Reduced antibodies (IgG/M)	-

Non-immune: Skeletal	26657404	Protti A, 2016	Linezolid	In vitro	Human	Linezolid-induced lactic acidosis	Skeletal muscle	Lactic acidosis	Diminished global oxygen consumption & extraction reflective of selective inhibition of mitochondrial protein synthesis (probably translation) with secondary mitonuclear imbalance.
Non-immune: Zebra fish larvae	36427668	Liu S, 2023	Chlortetracycline Oxytetracycline	In vivo	Zebra fish larvae	-	Larvae		Increased NF-κB regulated gene expression

Table 9.1: Summary of evidence for antibiotic-induced immunomodulation

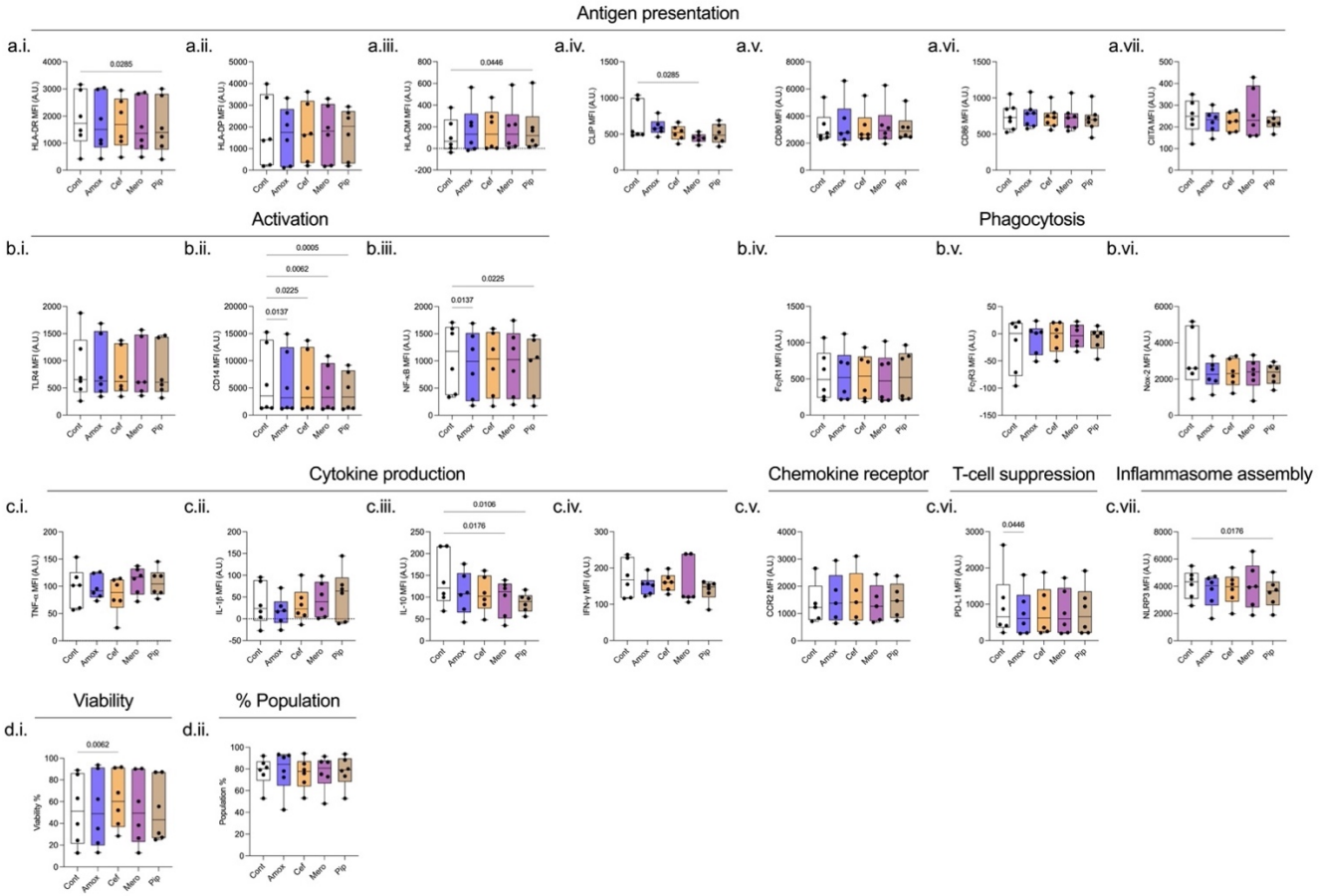

Figure 9.1: Effect of beta-lactams on unstimulated classical monocyte function in bacterial infection

PBMCs from ED patients (n=12) presenting with bacterial infection were incubated for 24 hours alone (white box) or with clinically relevant doses of amoxicillin (blue, 25μg/ml), cefuroxime (orange, 25μg/ml), meropenem (purple, 60μg/ml) and piperacillin (brown, 250μg/ml). Effects were measured on classical monocyte markers associated with (a.) antigen presentation (i. HLA-DR, ii. HLA-DP, iii. HLA-DM, iv. CLIP, v. CD80, vi. CD86, vii. CIITA), (b.) activation (i. TLR4, ii. CD14, iii. NF-κB) and phagocytosis (iv. FcγR1, v. FcγR3, vi. NOX-2), (c.) cytokine production (i. TNF-α, ii. IL-1β, iii. IL-10, iv. IFN-γ), chemokine receptors (v. CCR2), T-cell suppression (vi. PD-L1), inflammasome assembly (vii. NLRP3) and (d.) viability (i. percentage live) and population (ii. percentage of total monocyte population). Data expressed as median fluorescent intensity (MFI) measured in arbitrary units (A.U.) or percentage population (%) and each antibiotic compared to control using Friedman multiple comparison test without post hoc correction and displayed as individual patients represented with dots, horizontal line median, box interquartile range and whisker range. Only values with $p<0.05$ are shown. Six patients were excluded due to cell counts <10.

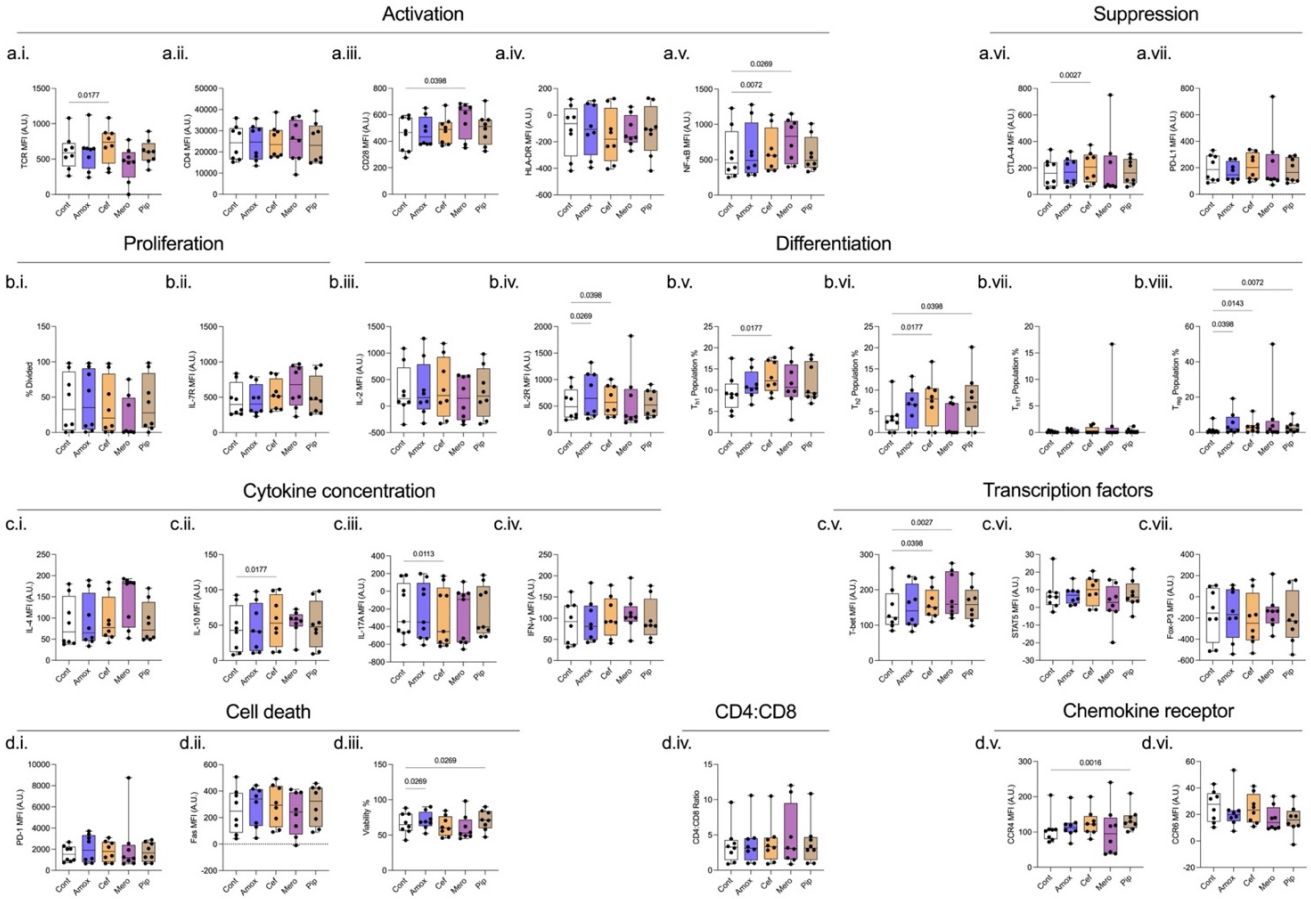

Figure 9.2: Effect of beta-lactams on unstimulated CD4⁺ lymphocytes in bacterial infection

PBMCs from ED patients (n=12) presenting with bacterial infection were incubated for 72 hours alone (white box) or with clinically relevant doses of amoxicillin (blue, 25μg/ml), cefuroxime (orange, 25μg/ml), meropenem (purple, 60μg/ml) and piperacillin (brown, 250μg/ml). Effects were measured on $CD4^+$ lymphocyte markers associated with (a.) activation (i. TCR (CD3), ii. CD4, iii. CD28, iv. HLA-DR, v. NF-κB) and suppression (vi. CTLA-4, vii. PD-L1), (b.) proliferation (i. percentage divided cells, ii. IL-7R) and differentiation (iii. IL-2, iv. IL-2R, v. percentage T_{h1} population, vi. percentage T_{h2} population, vii. percentage T_{h17} population, viii. percentage T_{reg} population), (c.) cytokine production (i. IL-4, ii. IL-10, iii. IL-17A, iv. IFN-γ) and transcription factors (v. T-bet, vi. STAT5, and vii. Fox-P3), and (d.) cell death (i. PD-1, ii. Fas, iii. percentage viable), iv. CD4:CD8 ratio and chemokine receptors (v. CCR4 and Vi. CCR6). Data expressed as median fluorescent intensity (MFI) measured in arbitrary units (A.U.) or percentage population (%) and each antibiotic compared to control using Friedman multiple comparison test without post hoc correction and displayed as individual patients represented with dots, horizontal line median, box interquartile range and whisker range. Only values with $p<0.05$ are shown. Four patients were excluded due to cell counts <10.

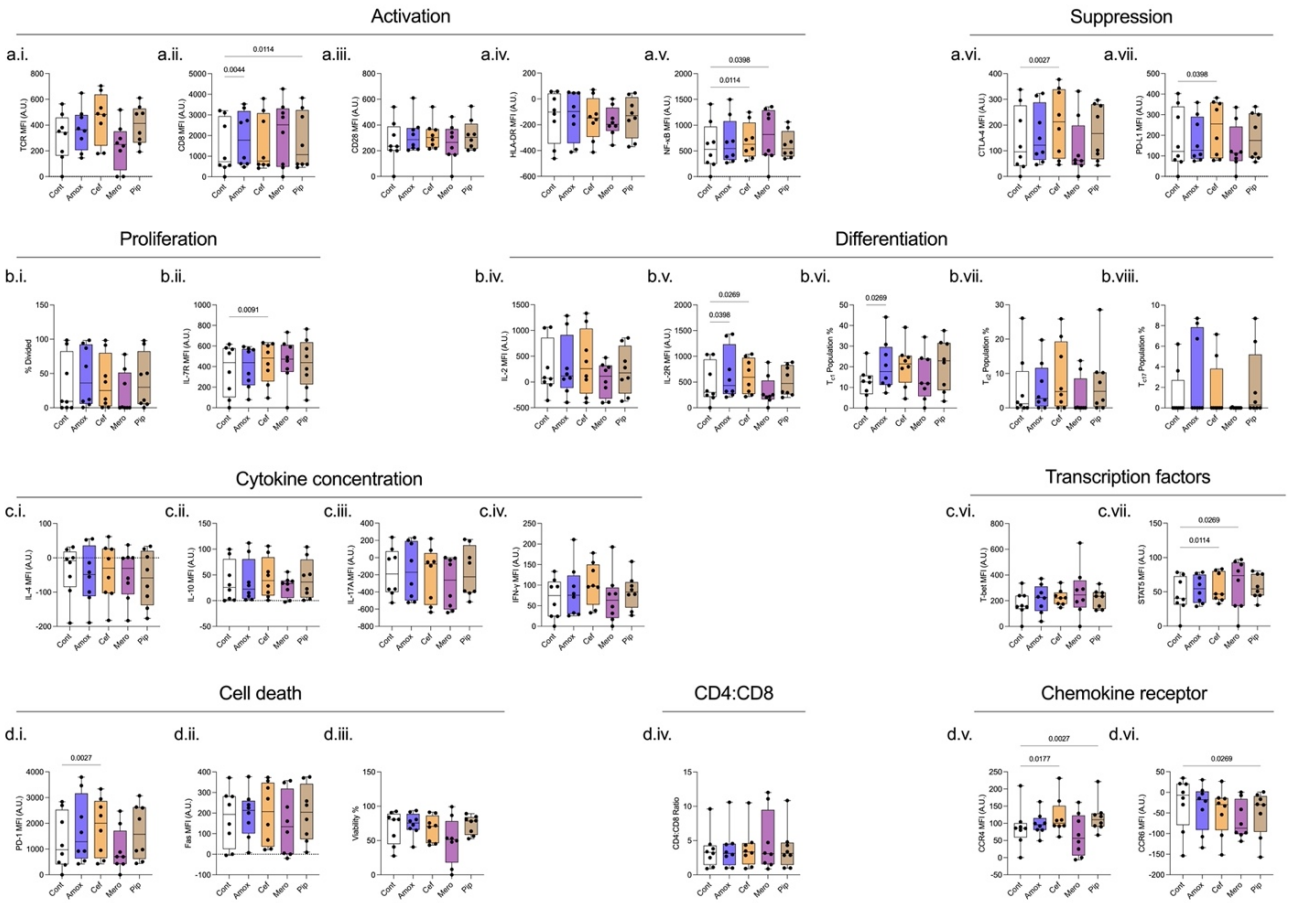

Figure 9.3: Effect of beta-lactams on unstimulated CD8⁺ lymphocytes in bacterial infection

PBMCs from ED patients (n=12) presenting with bacterial infection were incubated for 72 hours alone (white box) or with clinically relevant doses of amoxicillin (blue, 25μg/ml), cefuroxime (orange, 25μg/ml), meropenem (purple, 60μg/ml) and piperacillin (brown, 250μg/ml). Effects were measured on $CD8^+$ lymphocyte markers associated with (a.) activation (i. TCR (CD3), ii. CD8, iii. CD28, iv. HLA-DR, v. NF-κB) and suppression (vi. CTLA-4, vii. PD-L1), (b.) proliferation (i. percentage divided cells, ii. IL-7R) and differentiation (iii. IL-2, iv. IL-2R, v. percentage T_{c1} population, vi. percentage T_{c2} population, vii. percentage T_{c17} population), (c.) cytokine production (i. IL-4, ii. IL-10, iii. IL-17A, iv. IFN-γ) and transcription factors (v. T-bet, vi. STAT5), and (d.) cell death (i. PD-1, ii. Fas, iii. percentage viable), iv. CD4:CD8 ratio and chemokine receptors (v. CCR4 and Vi. CCR6). Data expressed as median fluorescent intensity (MFI) measured in arbitrary units (A.U.) or percentage population (%) and each antibiotic compared to control using Friedman multiple comparison test without post hoc correction and displayed as individual patients represented with dots, horizontal line median, box interquartile range and whisker range. Only values with $p<0.05$ are shown. Four patients were excluded due to cell counts <10.

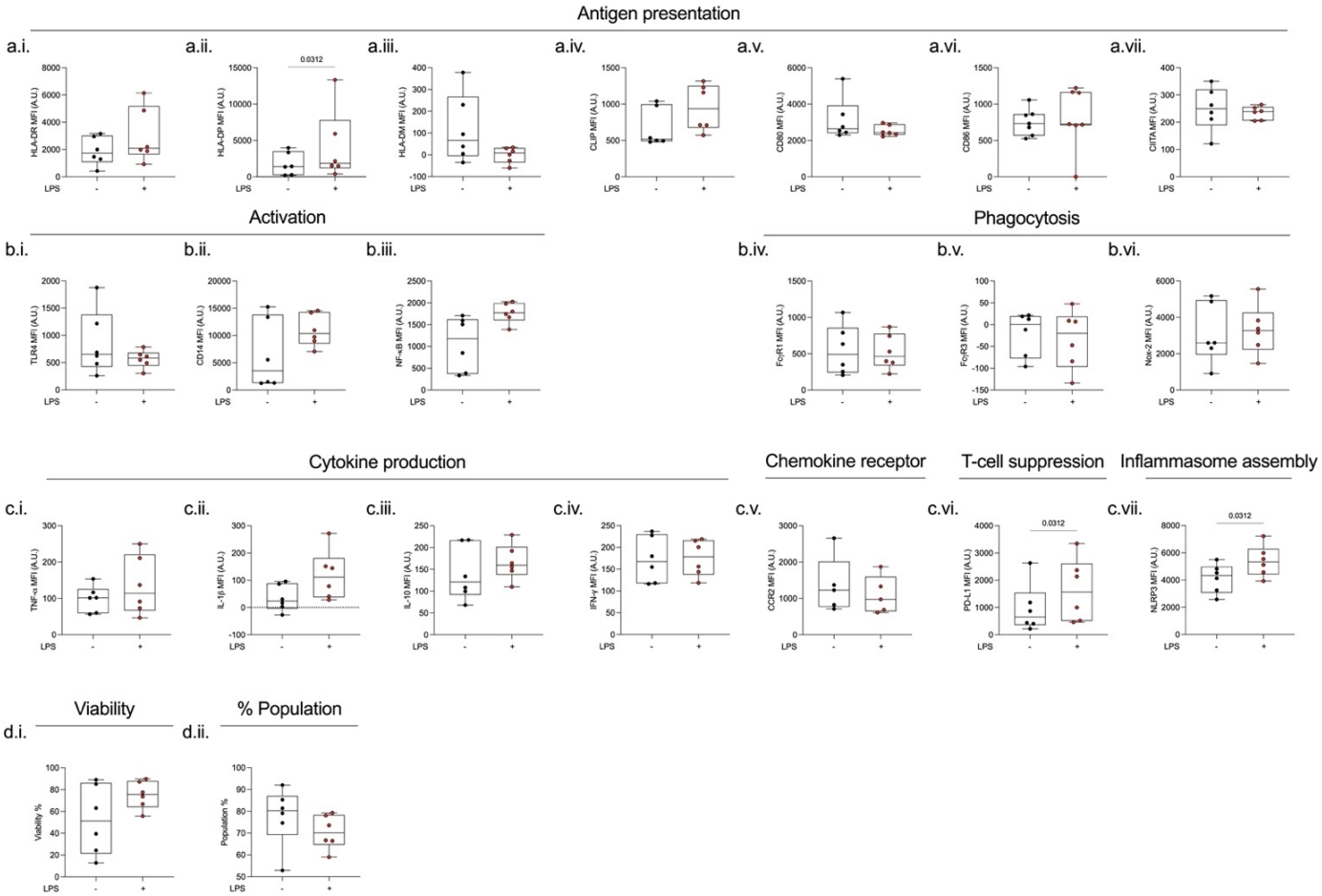

Figure 9.4: Effect of LPS stimulation on monocytes in bacterial infection

PBMCs from ED patients (n=12) presenting with bacterial infection were incubated for 24 hours alone (black dots) or stimulated with LPS (red dots, 100ng/ml) and effect measured on classical monocyte markers associated with (a.) antigen presentation (i. HLA-DR, ii. HLA-DP, iii. HLA-DM, iv. CLIP, v. CD80, vi. CD86, vii. CIITA), (b.) activation (i. TLR4, ii. CD14, iii. NF-κB) and phagocytosis (iv. FcγR1, v. FcγR3, vi. NOX-2), (c.) cytokine production (i. TNF-α, ii. IL-1β, iii. IL-10, iv. IFN-γ), chemokine receptors (v. CCR2), T-cell suppression (vi. PD-L1), inflammasome assembly (vii. NLRP3) and (d.) viability (i. percentage live) and population (ii. percentage of total monocyte population). Data expressed as median fluorescent intensity (MFI) measured in arbitrary units (A.U.) or percentage population (%) and compared using Wilcoxon test and displayed as individual patients represented with dots, horizontal line median, box interquartile range and whisker range. Only values with $p<0.05$ are shown. Six patients were excluded due to cell counts <10.

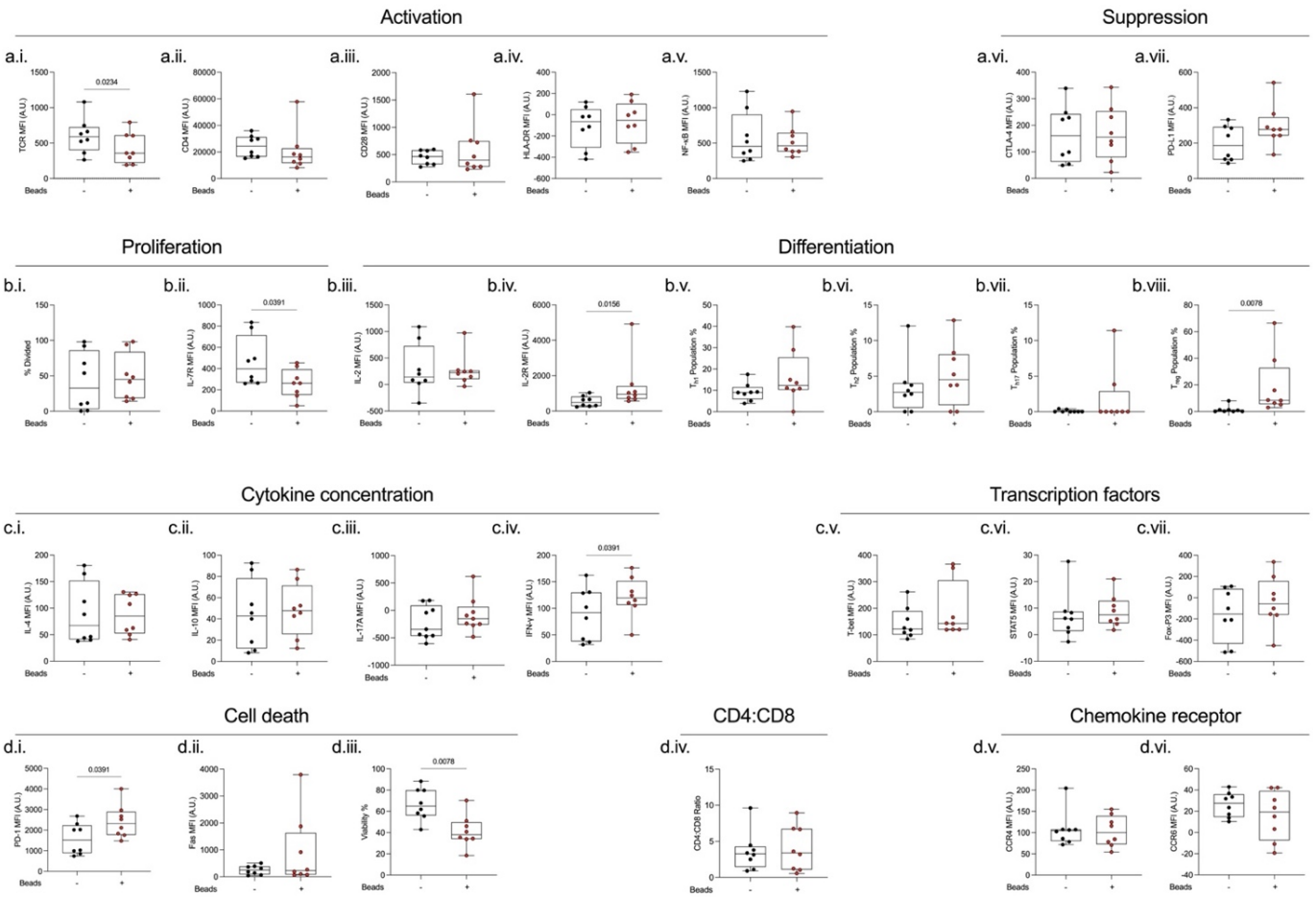

Figure 9.5: Effect of CD3/CD28 bead stimulation on CD4⁺ lymphocytes in bacterial infection

PBMCs from ED patients (n=12) presenting with bacterial infection were incubated for 72 hours alone (black dots) or stimulated with CD3/CD28 beads (red dots, 4:1 beads:PBMCs) and effects were measured on $CD4^+$ lymphocyte markers associated with (a.) activation (i. TCR, ii. CD4, iii. CD28, iv. HLA-DR, v. NF-κB) and suppression (vi. CTLA-4, vii. PD-L1), (b.) proliferation (i. percentage divided cells, ii. IL-7R) and differentiation (iii. IL-2, iv. IL-2R, v. percentage T_{h1} population, vi. percentage T_{h2} population, vii. percentage T_{h17} population, viii. percentage T_{reg} population), (c.) cytokine production (i. IL-4, ii. IL-10, iii. IL-17A, iv. IFN-γ) and transcription factors (v. T-bet, vi. STAT5, and vii. Fox-P3), and (d.) cell death (i. PD-1, ii. Fas, iii. percentage viable), iv. CD4:CD8 ratio and chemokine receptors (v. CCR4 and Vi. CCR6). Data expressed as median fluorescent intensity (MFI) measured in arbitrary units (A.U.) or percentage population (%) and compared using Wilcoxon test and displayed as individual patients represented with dots, horizontal line median, box interquartile range and whisker range. Only values with $p<0.05$ are shown. Four patients were excluded due to cell counts <10.

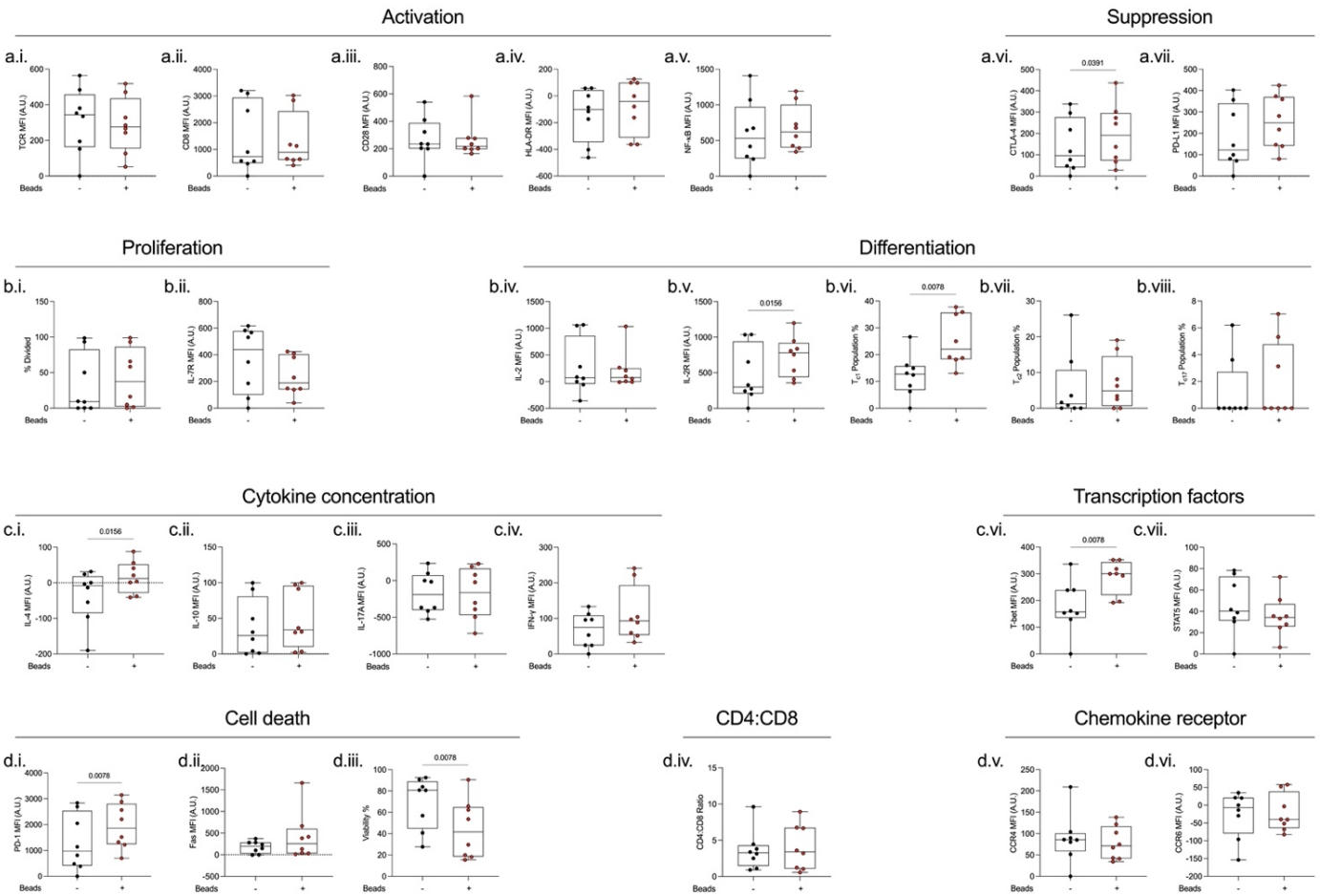

Figure 9.6: Effect of CD3/CD28 bead stimulation on CD8⁺ lymphocytes in bacterial infection

PBMCs from ED patients (n=12) presenting with bacterial infection were incubated for 72 hours alone (black dots) or stimulated with CD3/CD28 beads (red dots, 4:1 beads:PBMCs) and effects were measured on $CD8^+$ lymphocyte markers associated with (a.) activation (i. TCR, ii. CD8, iii. CD28, iv. HLA-DR, v. NF-κB) and suppression (vi. CTLA-4, vii. PD-L1), (b.) proliferation (i. percentage divided cells, ii. IL-7R) and differentiation (iii. IL-2, iv. IL-2R, v. percentage T_{c1} population, vi. percentage T_{c2} population, vii. percentage T_{c17} population), (c.) cytokine production (i. IL-4, ii. IL-10, iii. IL-17A, iv. IFN-γ) and transcription factors (v. T-bet, vi. STAT5), and (d.) cell death (i. PD-1, ii. Fas, iii. percentage viable), iv. CD4:CD8 ratio and chemokine receptors (v. CCR4 and Vi. CCR6). Data expressed as median fluorescent intensity (MFI) measured in arbitrary units (A.U.) or percentage population (%) and compared using Wilcoxon test and displayed as individual patients represented with dots, horizontal line median, box interquartile range and whisker range. Only values with $p<0.05$ are shown. Four patients were excluded due to cell counts <10.

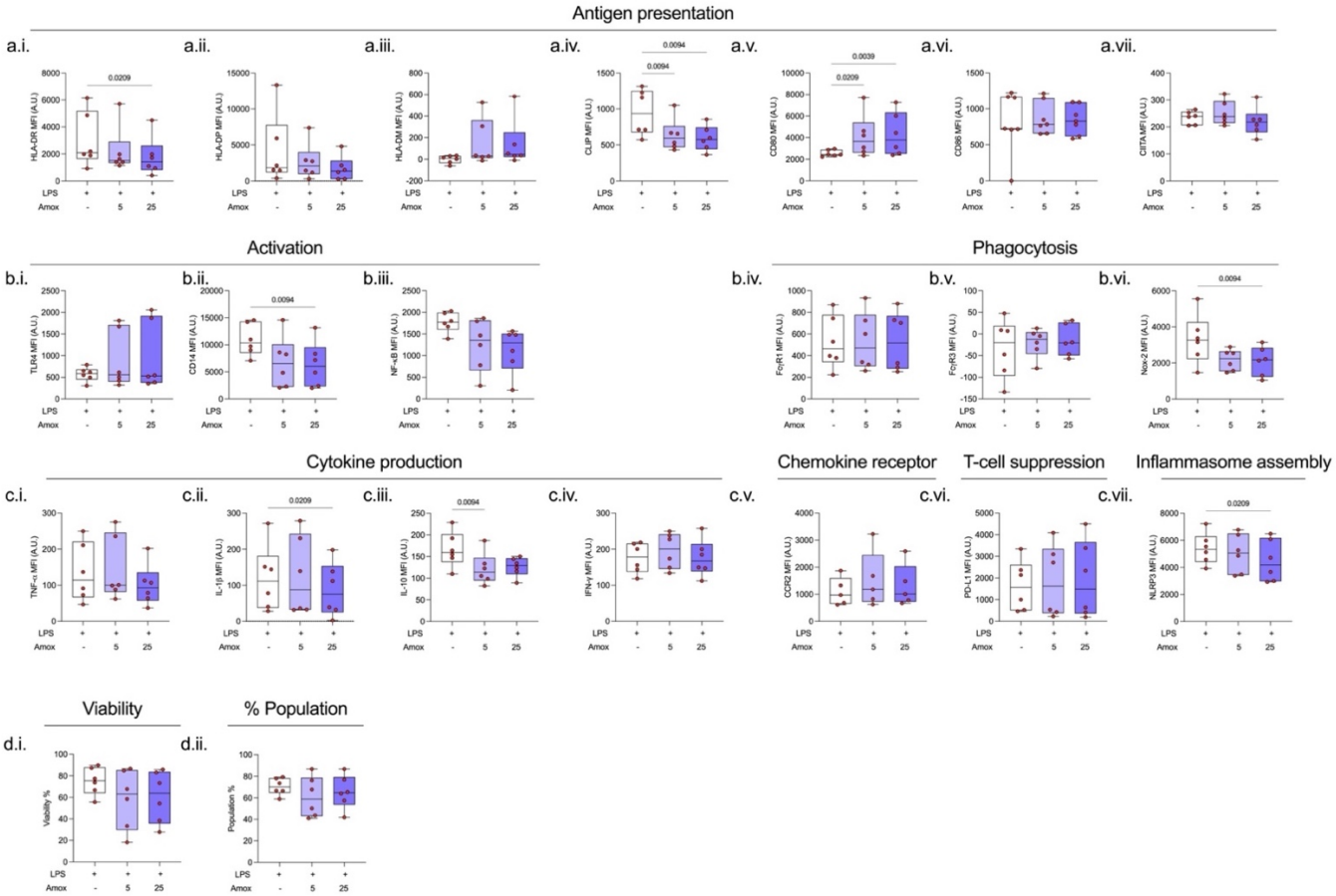

Figure 9.7: Effect of amoxicillin on LPS-stimulated classical monocytes in bacterial infection

PBMCs from ED patients (n=12) presenting with bacterial infection were stimulated for 24 hours with LPS (red dots, 100ng/ml) alone (white box) or with the addition of low or high clinically relevant doses of amoxicillin (blue boxes, 5 and 25μg/ml). Effects were measured on classical monocyte markers associated with (a.) antigen presentation (i. HLA-DR, ii. HLA-DP, iii. HLA-DM, iv. CLIP, v. CD80, vi. CD86, vii. CIITA), (b.) activation (i. TLR4, ii. CD14, iii. NF-κB) and phagocytosis (iv. FcγR1, v. FcγR3, vi. NOX-2), (c.) cytokine production (i. TNF-α, ii. IL-1β, iii. IL-10, iv. IFN-γ), chemokine receptors (v. CCR2), T-cell suppression (vi. PD-L1), inflammasome assembly (vii. NLRP3) and (d.) viability (i. percentage live) and population (ii. percentage of total monocyte population). Data expressed as median fluorescent intensity (MFI) measured in arbitrary units (A.U.) or percentage population (%). Each antibiotic concentration is compared to LPS alone using Friedman multiple comparison test without post hoc correction and displayed as individual patients represented with dots, horizontal line median, box interquartile range and whisker range. Only values with $p<0.05$ are shown. Six patients were excluded due to cell counts <10.

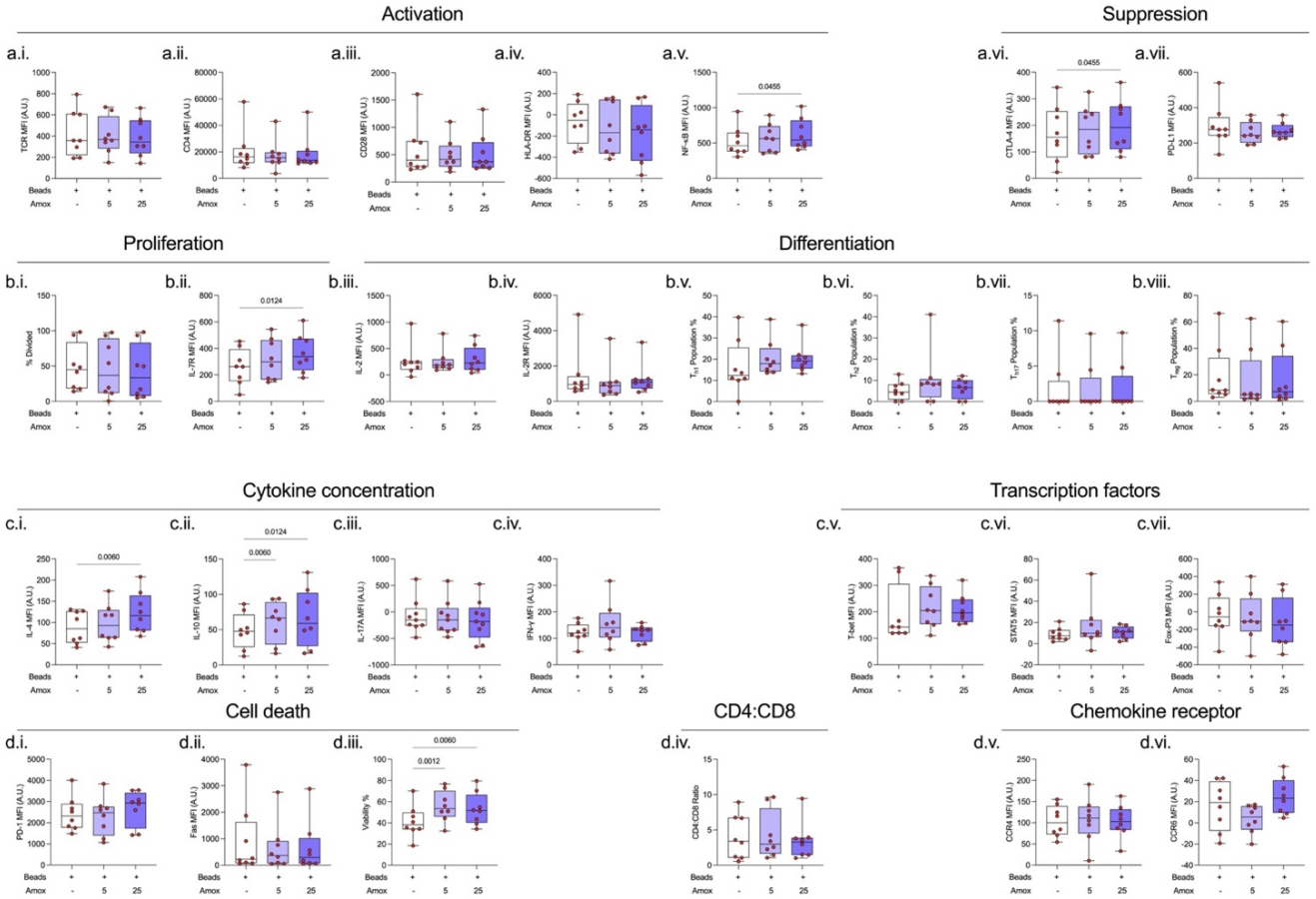

Figure 9.8: Effect of amoxicillin on bead-stimulated CD4⁺ lymphocytes in bacterial infection

PBMCs from ED patients (n=12) presenting with bacterial infection were stimulated for 72 hours with CD3/CD28 beads (red dots, 4:1 beads:PBMCs) alone (white box) or with the addition of low or high clinically relevant doses of amoxicillin (blue boxes, 5 and 25μg/ml). Effects were measured on $CD4^+$ lymphocyte markers associated with (a.) activation (i. TCR, ii. CD4, iii. CD28, iv. HLA-DR, v. NF-κB) and suppression (vi. CTLA-4, vii. PD-L1), (b.) proliferation (i. percentage divided cells, ii. IL-7R) and differentiation (iii. IL-2, iv. IL-2R, v. percentage T_{h1} population, vi. percentage T_{h2} population, vii. percentage T_{h17} population, viii. percentage T_{reg} population), (c.) cytokine production (i. IL-4, ii. IL-10, iii. IL-17A, iv. IFN-γ) and transcription factors (v. T-bet, vi. STAT5, and vii. Fox-P3), and (d.) cell death (i. PD-1, ii. Fas, iii. percentage viable), iv. CD4:CD8 ratio and chemokine receptors (v. CCR4 and Vi. CCR6). Data expressed as median fluorescent intensity (MFI) measured in arbitrary units (A.U.) or percentage population (%). Each antibiotic concentration is compared to beads alone using Friedman multiple comparison test without post hoc correction and displayed as individual patients represented with dots, horizontal line median, box interquartile range and whisker range. Only values with $p<0.05$ are shown. Four patients were excluded due to cell counts <10.

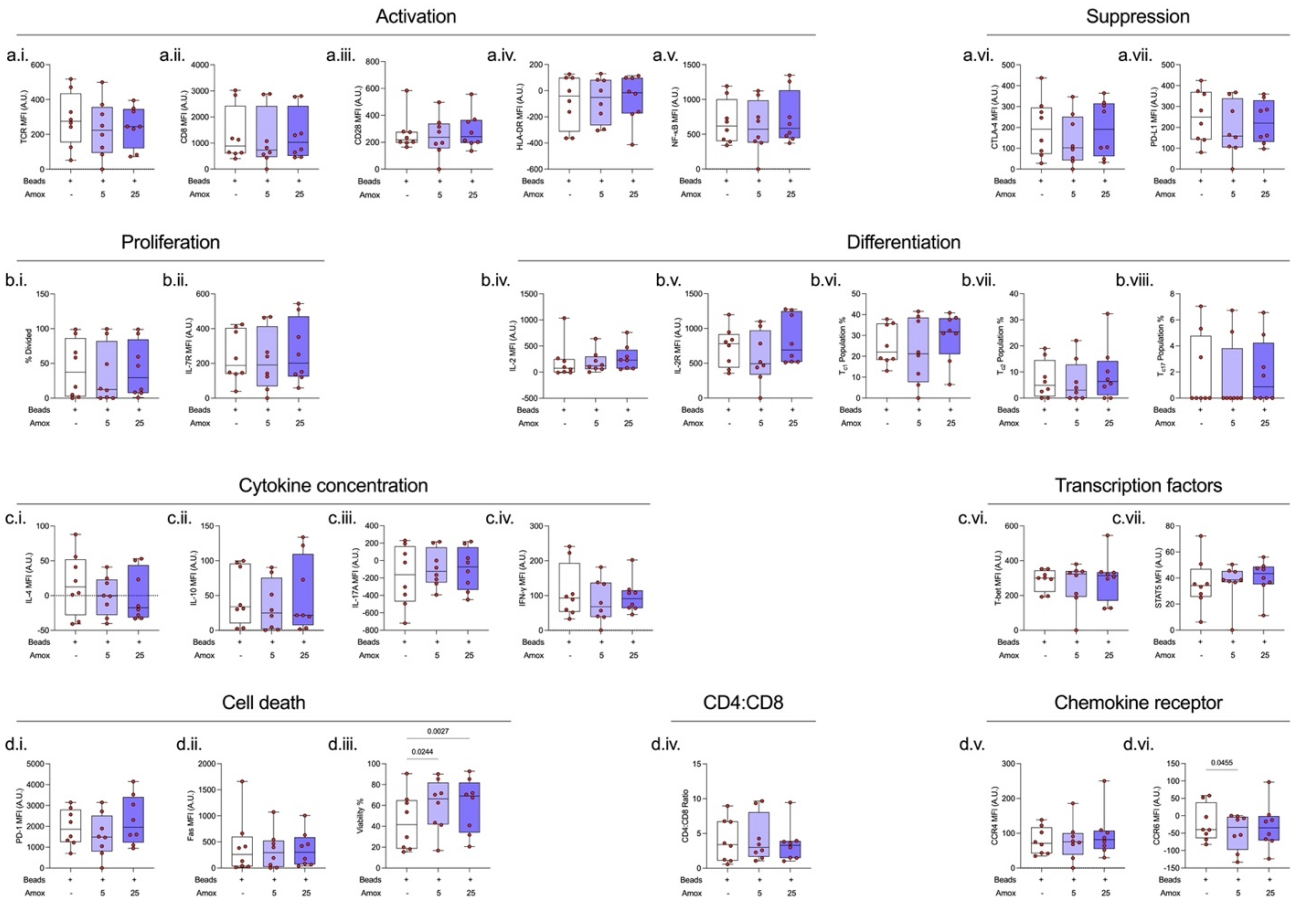

Figure 9.9: Effect of amoxicillin on bead-stimulated CD8⁺ lymphocytes in bacterial infection

PBMCs from ED patients (n=12) presenting with bacterial infection were stimulated for 72 hours with CD3/CD28 beads (red dots, 4:1 beads:PBMCs) alone (white box) or with the addition of low or high clinically relevant doses of amoxicillin (blue boxes, 5 and 25μg/ml). Effects were measured on $CD8^+$ lymphocyte markers associated with (a.) activation (i. TCR, ii. CD4, iii. CD28, iv. HLA-DR, v. NF-κB) and suppression (vi. CTLA-4, vii. PD-L1), (b.) proliferation (i. percentage divided cells, ii. IL-7R) and differentiation (iii. IL-2, iv. IL-2R, v. percentage T_{h1} population, vi. percentage T_{h2} population, vii. percentage T_{h17} population, viii. percentage T_{reg} population), (c.) cytokine production (i. IL-4, ii. IL-10, iii. IL-17A, iv. IFN-γ) and transcription factors (v. T-bet, vi. STAT5, and vii. Fox-P3), and (d.) cell death (i. PD-1, ii. Fas, iii. percentage viable), iv. CD4:CD8 ratio and chemokine receptors (v. CCR4 and Vi. CCR6). Data expressed as median fluorescent intensity (MFI) measured in arbitrary units (A.U.) or percentage population (%). Each antibiotic concentration is compared to beads alone using Friedman multiple comparison test without post hoc correction and displayed as individual patients represented with dots, horizontal line median, box interquartile range and whisker range. Only values with $p<0.05$ are shown. Four patients were excluded due to cell counts <10.

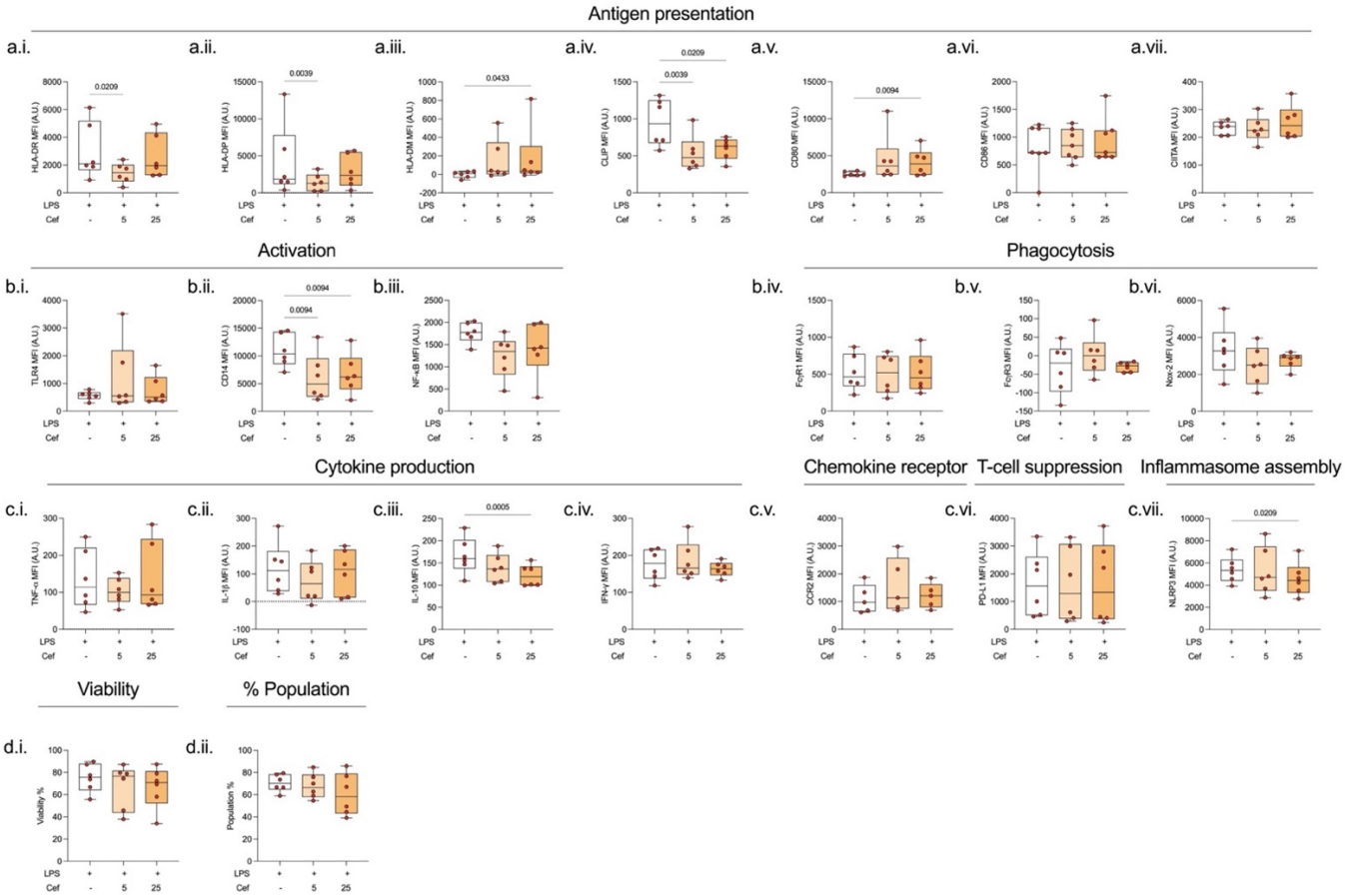

Figure 9.10: Effect of cefuroxime on LPS-stimulated classical monocytes in bacterial infection

PBMCs from ED patients (n=12) presenting with bacterial infection were stimulated for 24 hours with LPS (red dots, 100ng/ml) alone (white box) or with the addition of low or high clinically relevant doses of cefuroxime (orange boxes, 5 and 25μg/ml). Effects were measured on classical monocyte markers associated with (a.) antigen presentation (i. HLA-DR, ii. HLA-DP, iii. HLA-DM, iv. CLIP, v. CD80, vi. CD86, vii. CIITA), (b.) activation (i. TLR4, ii. CD14, iii. NF-κB) and phagocytosis (iv. FcγR1, v. FcγR3, vi. NOX-2), (c.) cytokine production (i. TNF-α, ii. IL-1β, iii. IL-10, iv. IFN-γ), chemokine receptors (v. CCR2), T-cell suppression (vi. PD-L1), inflammasome assembly (vii. NLRP3) and (d.) viability (i. percentage live) and population (ii. percentage of total monocyte population). Data expressed as median fluorescent intensity (MFI) measured in arbitrary units (A.U.) or percentage population (%). Each antibiotic concentration is compared to LPS alone using Friedman multiple comparison test without post hoc correction and displayed as individual patients represented with dots, horizontal line median, box interquartile range and whisker range. Only values with $p<0.05$ are shown. Six patients were excluded due to cell counts <10.

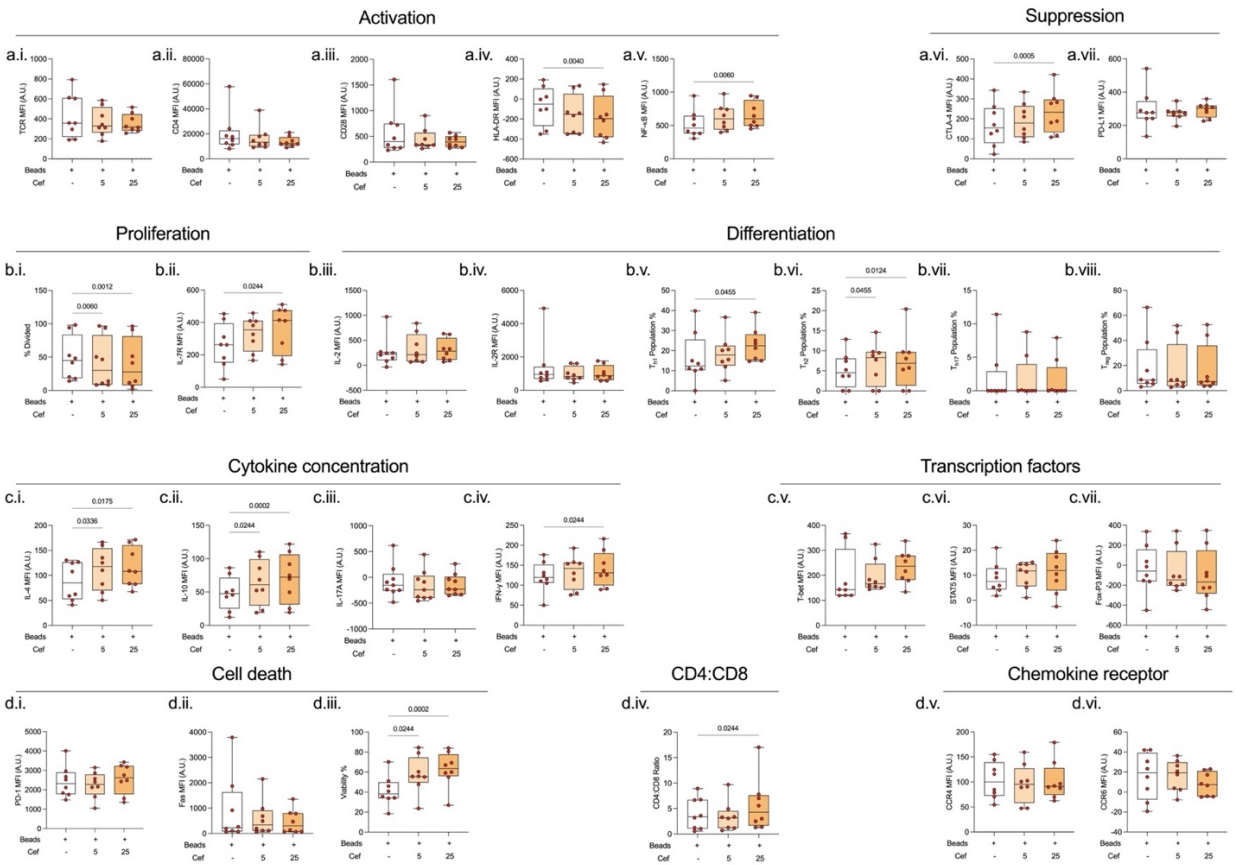

Figure 9.11: Effect of cefuroxime on bead-stimulated CD4⁺ lymphocytes in bacterial infection

PBMCs from ED patients (n=12) presenting with bacterial infection were stimulated for 72 hours with CD3/CD28 beads (red dots, 4:1 beads:PBMCs) alone (white box) or with the addition of low or high clinically relevant doses of cefuroxime (orange boxes, 5 and 25μg/ml). Effects were measured on $CD4^+$ lymphocyte markers associated with (a.) activation (i. TCR, ii. CD4, iii. CD28, iv. HLA-DR, v. NF-κB) and suppression (vi. CTLA-4, vii. PD-L1), (b.) proliferation (i. percentage divided cells, ii. IL-7R) and differentiation (iii. IL-2, iv. IL-2R, v. percentage T_{h1} population, vi. percentage T_{h2} population, vii. percentage T_{h17} population, viii. percentage T_{reg} population), (c.) cytokine production (i. IL-4, ii. IL-10, iii. IL-17A, iv. IFN-γ) and transcription factors (v. T-bet, vi. STAT5, and vii. Fox-P3), and (d.) cell death (i. PD-1, ii. Fas, iii. percentage viable), iv. CD4:CD8 ratio and chemokine receptors (v. CCR4 and Vi. CCR6). Data expressed as median fluorescent intensity (MFI) measured in arbitrary units (A.U.) or percentage population (%). Each antibiotic concentration is compared to beads alone using Friedman multiple comparison test without post hoc correction and displayed as individual patients represented with dots, horizontal line median, box interquartile range and whisker range. Only values with $p<0.05$ are shown. Four patients were excluded due to cell counts <10.

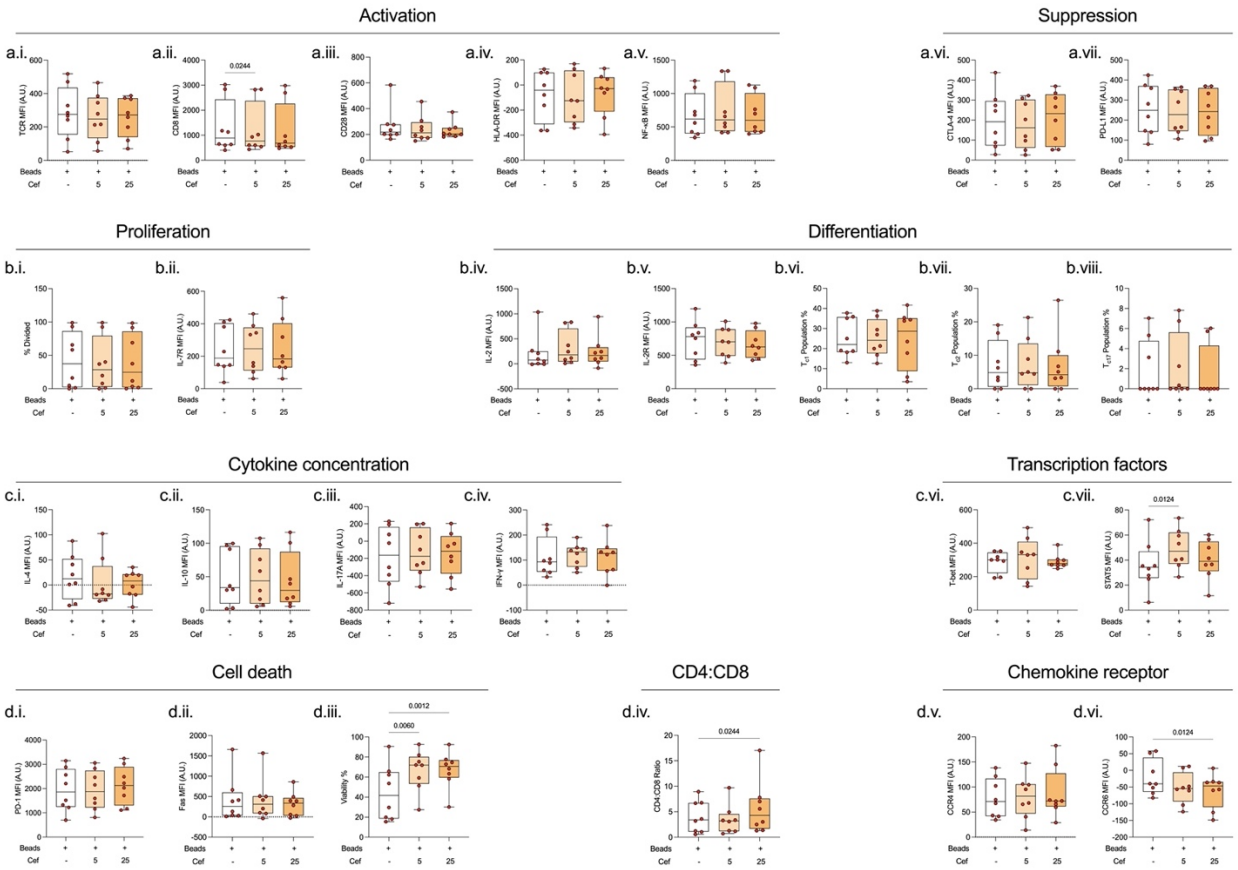

Figure 9.12: Effect of cefuroxime on bead-stimulated CD8⁺ lymphocytes in bacterial infection

PBMCs from ED patients (n=12) presenting with bacterial infection were stimulated for 72 hours with CD3/CD28 beads (red dots, 4:1 beads:PBMCs) alone (white box) or with the addition of low or high clinically relevant doses of cefuroxime (orange boxes, 5 and 25μg/ml). Effects were measured on $CD8^+$ lymphocyte markers associated with (a.) activation (i. TCR, ii. CD4, iii. CD28, iv. HLA-DR, v. NF-κB) and suppression (vi. CTLA-4, vii. PD-L1), (b.) proliferation (i. percentage divided cells, ii. IL-7R) and differentiation (iii. IL-2, iv. IL-2R, v. percentage T_{h1} population, vi. percentage T_{h2} population, vii. percentage T_{h17} population, viii. percentage T_{reg} population), (c.) cytokine production (i. IL-4, ii. IL-10, iii. IL-17A, iv. IFN-γ) and transcription factors (v. T-bet, vi. STAT5, and vii. Fox-P3), and (d.) cell death (i. PD-1, ii. Fas, iii. percentage viable), iv. CD4:CD8 ratio and chemokine receptors (v. CCR4 and Vi. CCR6). Data expressed as median fluorescent intensity (MFI) measured in arbitrary units (A.U.) or percentage population (%). Each antibiotic concentration is compared to beads alone using Friedman multiple comparison test without post hoc correction and displayed as individual patients represented with dots, horizontal line median, box interquartile range and whisker range. Only values with $p<0.05$ are shown. Four patients were excluded due to cell counts <10.

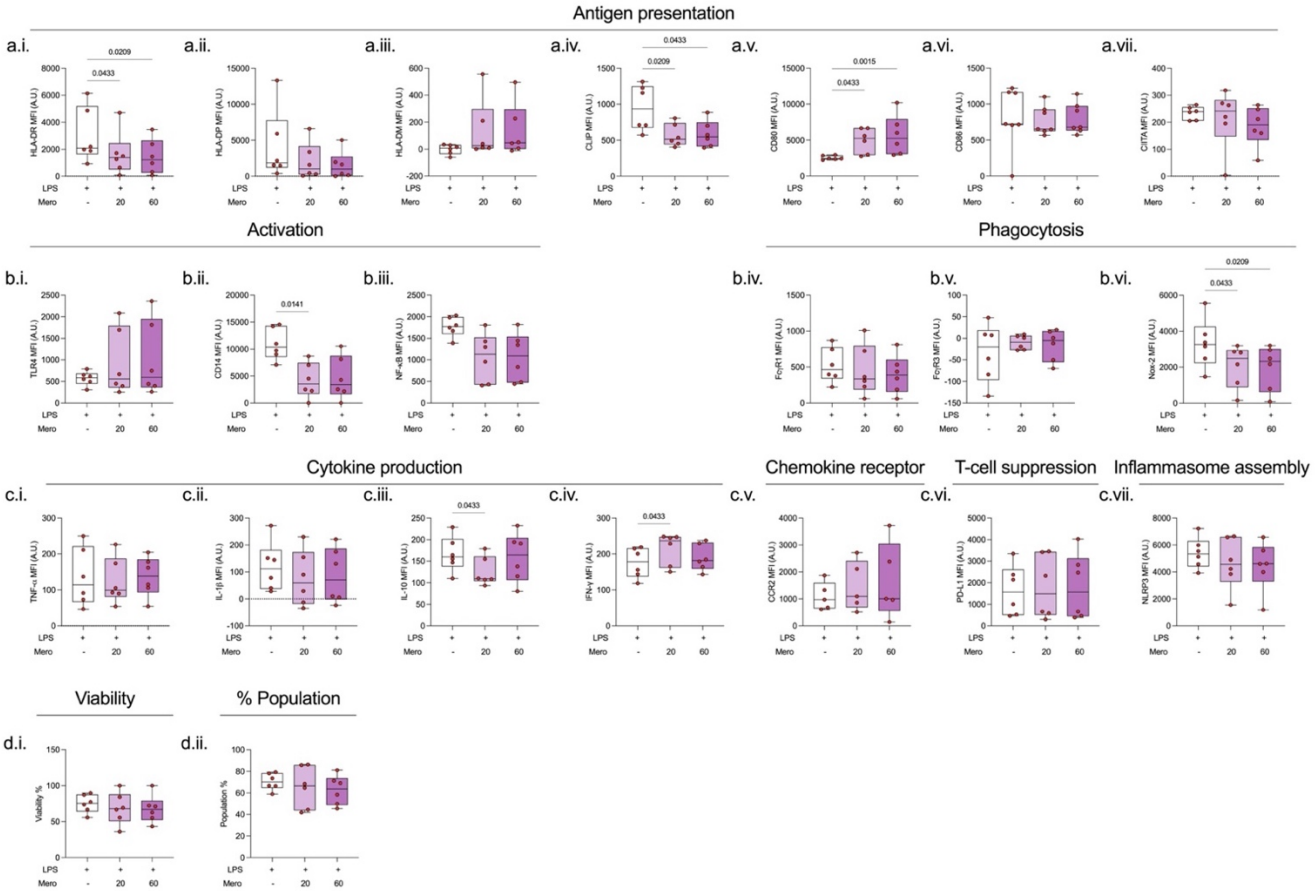

Figure 9.13: Effect of meropenem on LPS-stimulated classical monocytes in bacterial infection

PBMCs from ED patients (n=12) presenting with bacterial infection were stimulated for 24 hours with LPS (red dots, 100ng/ml) alone (white box) or with the addition of low or high clinically relevant doses of meropenem (purple boxes, 20 and 60μg/ml). Effects were measured on classical monocyte markers associated with (a.) antigen presentation (i. HLA-DR, ii. HLA-DP, iii. HLA-DM, iv. CLIP, v. CD80, vi. CD86, vii. CIITA), (b.) activation (i. TLR4, ii. CD14, iii. NF-κB) and phagocytosis (iv. FcγR1, v. FcγR3, vi. NOX-2), (c.) cytokine production (i. TNF-α, ii. IL-1β, iii. IL-10, iv. IFN-γ), chemokine receptors (v. CCR2), T-cell suppression (vi. PD-L1), inflammasome assembly (vii. NLRP3) and (d.) viability (i. percentage live) and population (ii. percentage of total monocyte population). Data expressed as median fluorescent intensity (MFI) measured in arbitrary units (A.U.) or percentage population (%). Each antibiotic concentration is compared to LPS alone using Friedman multiple comparison test without post hoc correction and displayed as individual patients represented with dots, horizontal line median, box interquartile range and whisker range. Only values with $p<0.05$ are shown. Six patients were excluded due to cell counts <10.

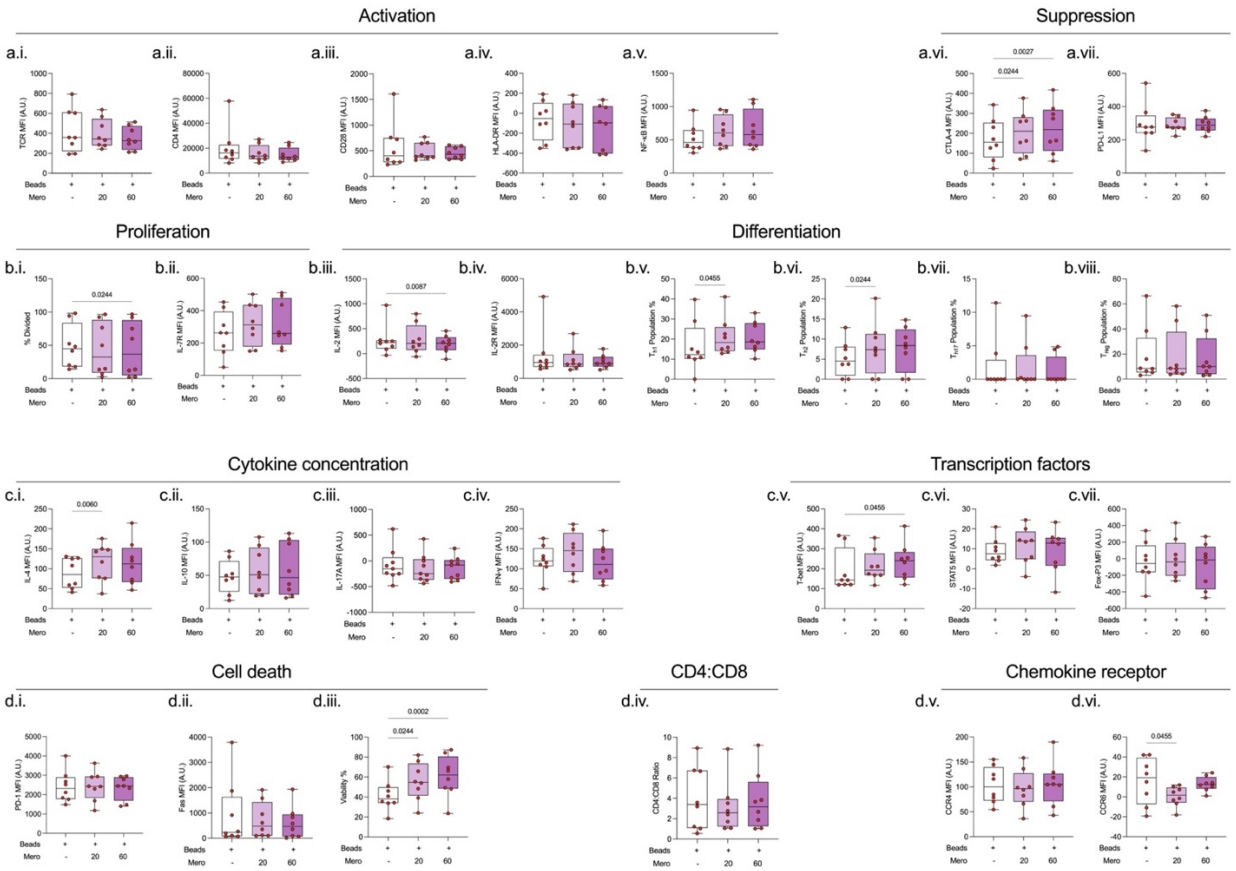

Figure 9.14: Effect of meropenem on bead-stimulated CD4⁺ lymphocytes in bacterial infection

PBMCs from ED patients (n=12) presenting with bacterial infection were stimulated for 72 hours with CD3/CD28 beads (red dots, 4:1 beads:PBMCs) alone (white box) or with the addition of low or high clinically relevant doses of meropenem (purple boxes, 20 and 60μg/ml). Effects were measured on $CD4^+$ lymphocyte markers associated with (a.) activation (i. TCR, ii. CD4, iii. CD28, iv. HLA-DR, v. NF-κB) and suppression (vi. CTLA-4, vii. PD-L1), (b.) proliferation (i. percentage divided cells, ii. IL-7R) and differentiation (iii. IL-2, iv. IL-2R, v. percentage T_{h1} population, vi. percentage T_{h2} population, vii. percentage T_{h17} population, viii. percentage T_{reg} population), (c.) cytokine production (i. IL-4, ii. IL-10, iii. IL-17A, iv. IFN-γ) and transcription factors (v. T-bet, vi. STAT5, and vii. Fox-P3), and (d.) cell death (i. PD-1, ii. Fas, iii. percentage viable), iv. CD4:CD8 ratio and chemokine receptors (v. CCR4 and Vi. CCR6). Data expressed as median fluorescent intensity (MFI) measured in arbitrary units (A.U.) or percentage population (%). Each antibiotic concentration is compared to beads alone using Friedman multiple comparison test without post hoc correction and displayed as individual patients represented with dots, horizontal line median, box interquartile range and whisker range. Only values with $p<0.05$ are shown. Four patients were excluded due to cell counts <10.

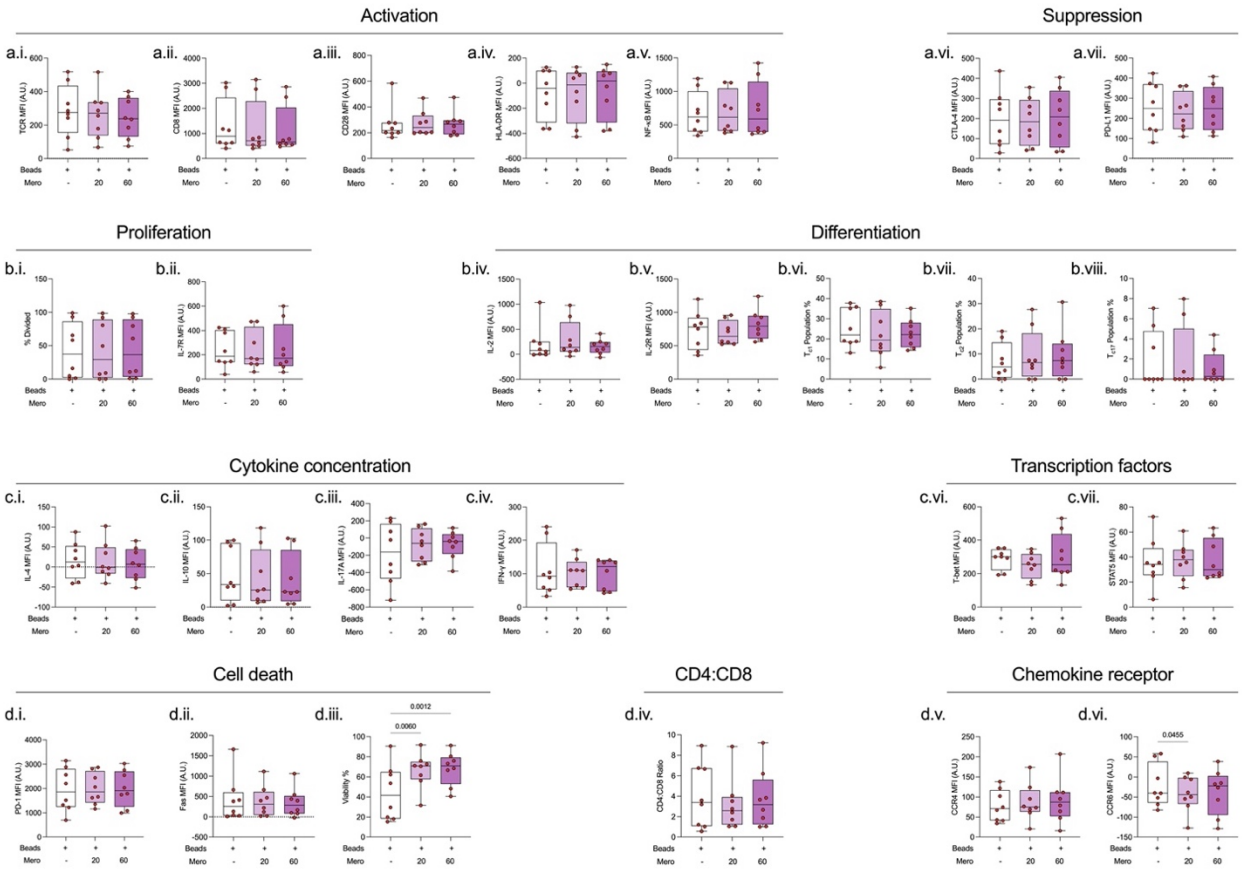

Figure 9.15: Effect of meropenem on bead-stimulated CD8⁺ lymphocytes in bacterial infection

PBMCs from ED patients (n=12) presenting with bacterial infection were stimulated for 72 hours with CD3/CD28 beads (red dots, 4:1 beads:PBMCs) alone (white box) or with the addition of low or high clinically relevant doses of meropenem (purple boxes, 20 and 60μg/ml). Effects were measured on $CD8^+$ lymphocyte markers associated with (a.) activation (i. TCR, ii. CD4, iii. CD28, iv. HLA-DR, v. NF-κB) and suppression (vi. CTLA-4, vii. PD-L1), (b.) proliferation (i. percentage divided cells, ii. IL-7R) and differentiation (iii. IL-2, iv. IL-2R, v. percentage T_{h1} population, vi. percentage T_{h2} population, vii. percentage T_{h17} population, viii. percentage T_{reg} population), (c.) cytokine production (i. IL-4, ii. IL-10, iii. IL-17A, iv. IFN-γ) and transcription factors (v. T-bet, vi. STAT5, and vii. Fox-P3), and (d.) cell death (i. PD-1, ii. Fas, iii. percentage viable), iv. CD4:CD8 ratio and chemokine receptors (v. CCR4 and Vi. CCR6). Data expressed as median fluorescent intensity (MFI) measured in arbitrary units (A.U.) or percentage population (%). Each antibiotic concentration is compared to beads alone using Friedman multiple comparison test without post hoc correction and displayed as individual patients represented with dots, horizontal line median, box interquartile range and whisker range. Only values with $p<0.05$ are shown. Four patients were excluded due to cell counts <10.

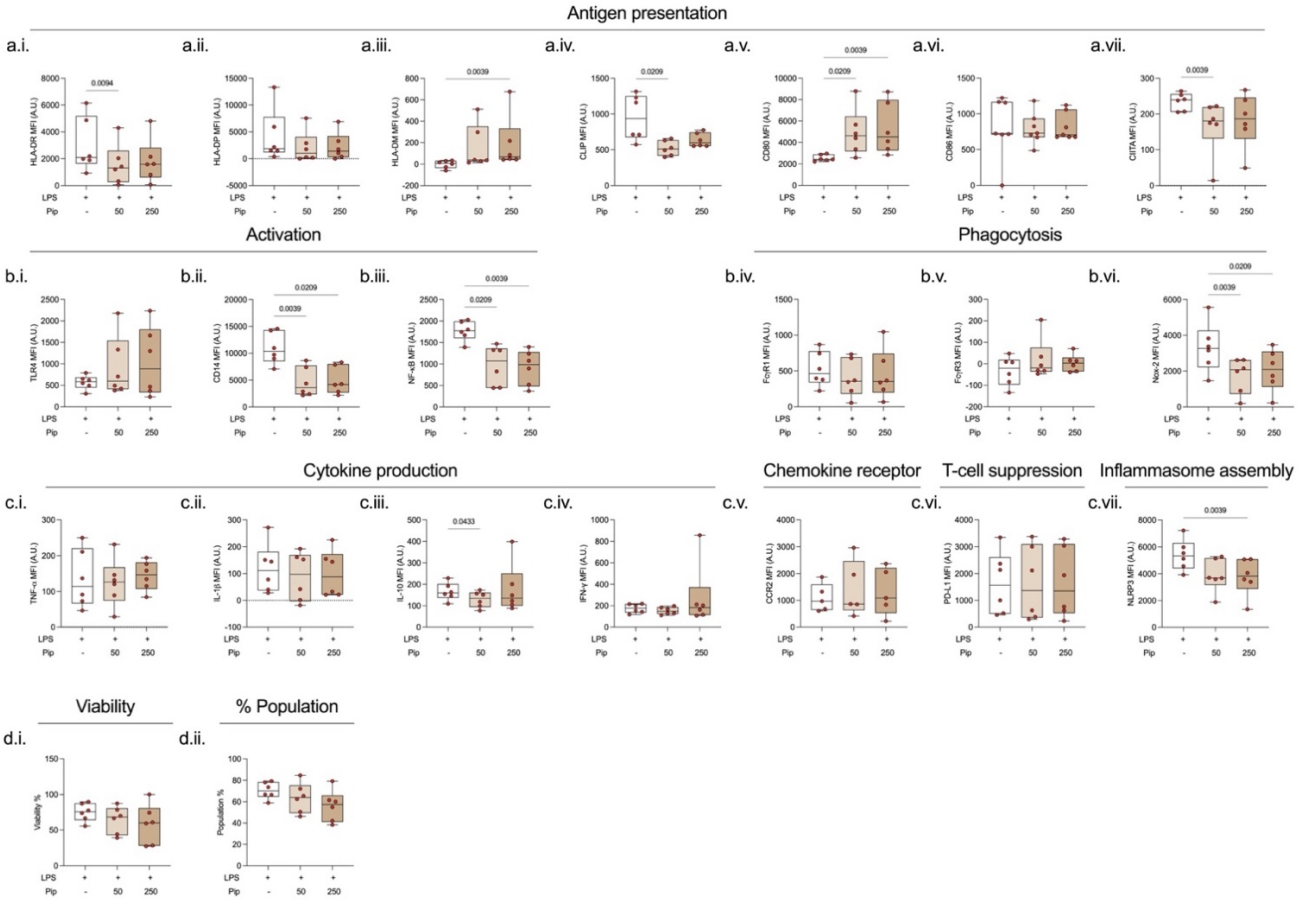

Figure 9.16: Effect of piperacillin on LPS-stimulated classical monocytes in bacterial infection

PBMCs from ED patients (n=12) presenting with bacterial infection were stimulated for 24 hours with LPS (red dots, 100ng/ml) alone (white box) or with the addition of low or high clinically relevant doses of piperacillin (brown boxes, 50 and 250μg/ml). Effects were measured on classical monocyte markers associated with (a.) antigen presentation (i. HLA-DR, ii. HLA-DP, iii. HLA-DM, iv. CLIP, v. CD80, vi. CD86, vii. CIITA), (b.) activation (i. TLR4, ii. CD14, iii. NF-κB) and phagocytosis (iv. FcγR1, v. FcγR3, vi. NOX-2), (c.) cytokine production (i. TNF-α, ii. IL-1β, iii. IL-10, iv. IFN-γ), chemokine receptors (v. CCR2), T-cell suppression (vi. PD-L1), inflammasome assembly (vii. NLRP3) and (d.) viability (i. percentage live) and population (ii. percentage of total monocyte population). Data expressed as median fluorescent intensity (MFI) measured in arbitrary units (A.U.) or percentage population (%). Each antibiotic concentration is compared to LPS alone using Friedman multiple comparison test without post hoc correction and displayed as individual patients represented with dots, horizontal line median, box interquartile range and whisker range. Only values with $p<0.05$ are shown. Six patients were excluded due to cell counts <10.

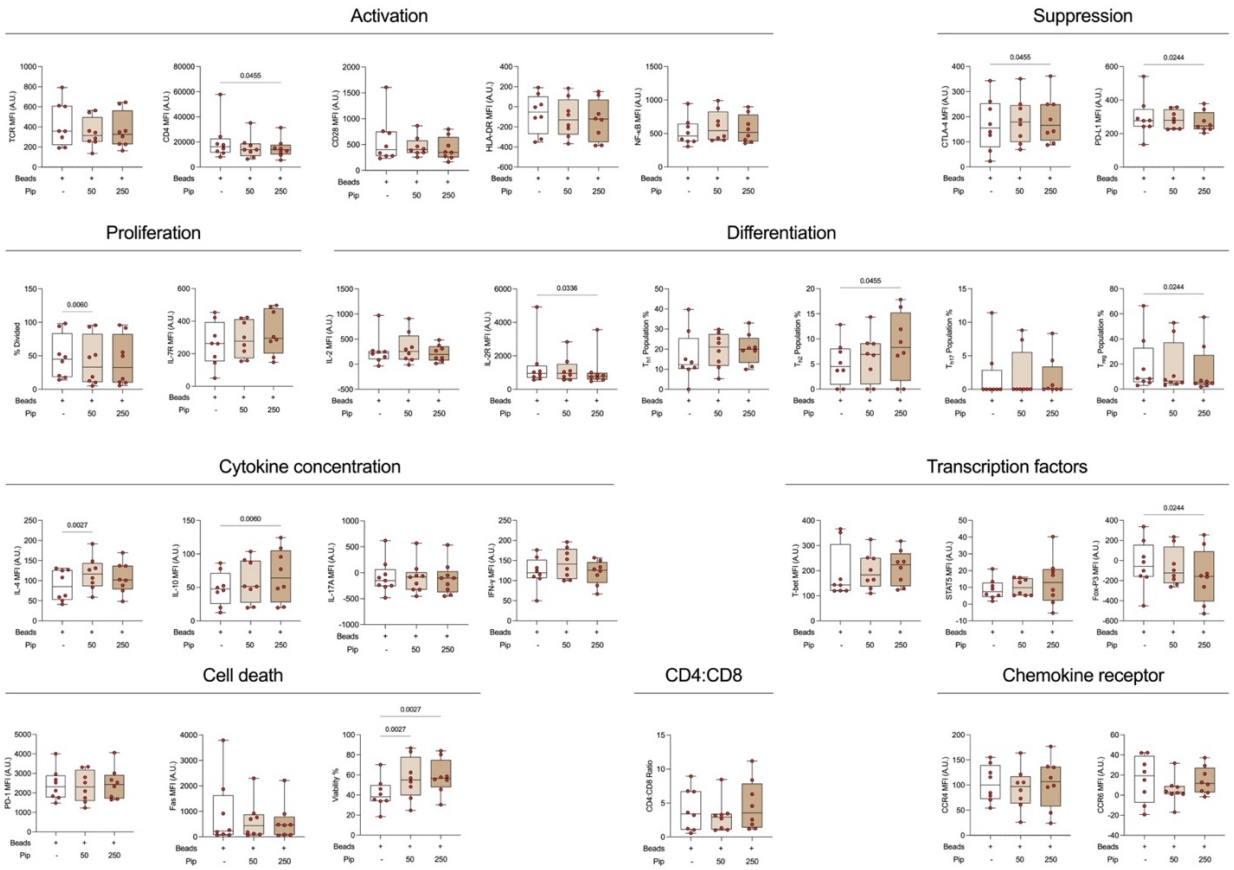

Figure 9.17: Effect of piperacillin on bead-stimulated CD4⁺ lymphocytes in bacterial infection

PBMCs from ED patients (n=12) presenting with bacterial infection were stimulated for 72 hours with CD3/CD28 beads (red dots, 4:1 beads:PBMCs) alone (white box) or with the addition of low or high clinically relevant doses of piperacillin (brown boxes, 50 and 250μg/ml). Effects were measured on $CD4^+$ lymphocyte markers associated with (a.) activation (i. TCR, ii. CD4, iii. CD28, iv. HLA-DR, v. NF-κB) and suppression (vi. CTLA-4, vii. PD-L1), (b.) proliferation (i. percentage divided cells, ii. IL-7R) and differentiation (iii. IL-2, iv. IL-2R, v. percentage T_{h1} population, vi. percentage T_{h2} population, vii. percentage T_{h17} population, viii. percentage T_{reg} population), (c.) cytokine production (i. IL-4, ii. IL-10, iii. IL-17A, iv. IFN-γ) and transcription factors (v. T-bet, vi. STAT5, and vii. Fox-P3), and (d.) cell death (i. PD-1, ii. Fas, iii. percentage viable), iv. CD4:CD8 ratio and chemokine receptors (v. CCR4 and Vi. CCR6). Data expressed as median fluorescent intensity (MFI) measured in arbitrary units (A.U.) or percentage population (%). Each antibiotic concentration is compared to beads alone using Friedman multiple comparison test without post hoc correction and displayed as individual patients represented with dots, horizontal line median, box interquartile range and whisker range. Only values with $p<0.05$ are shown. Four patients were excluded due to cell counts <10.

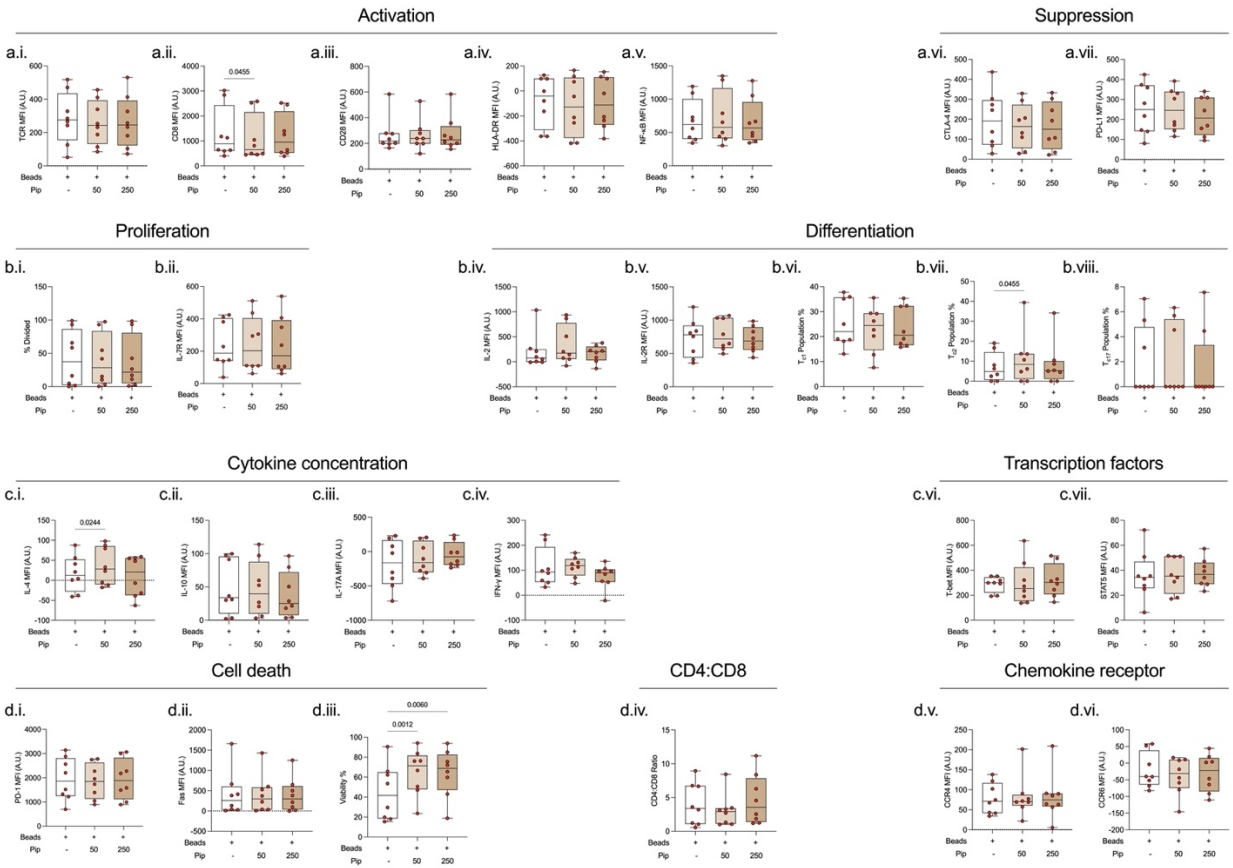

Figure 9.18: Effect of piperacillin on bead-stimulated CD8⁺ lymphocytes in bacterial infection

PBMCs from ED patients (n=12) presenting with bacterial infection were stimulated for 72 hours with CD3/CD28 beads (red dots, 4:1 beads:PBMCs) alone (white box) or with the addition of low or high clinically relevant doses of piperacillin (brown boxes, 50 and 250μg/ml). Effects were measured on $CD8^+$ lymphocyte markers associated with (a.) activation (i. TCR, ii. CD4, iii. CD28, iv. HLA-DR, v. NF-κB) and suppression (vi. CTLA-4, vii. PD-L1), (b.) proliferation (i. percentage divided cells, ii. IL-7R) and differentiation (iii. IL-2, iv. IL-2R, v. percentage T_{h1} population, vi. percentage T_{h2} population, vii. percentage T_{h17} population, viii. percentage T_{reg} population), (c.) cytokine production (i. IL-4, ii. IL-10, iii. IL-17A, iv. IFN-γ) and transcription factors (v. T-bet, vi. STAT5, and vii. Fox-P3), and (d.) cell death (i. PD-1, ii. Fas, iii. percentage viable), iv. CD4:CD8 ratio and chemokine receptors (v. CCR4 and Vi. CCR6). Data expressed as median fluorescent intensity (MFI) measured in arbitrary units (A.U.) or percentage population (%). Each antibiotic concentration is compared to beads alone using Friedman multiple comparison test without post hoc correction and displayed as individual patients represented with dots, horizontal line median, box interquartile range and whisker range. Only values with $p<0.05$ are shown. Four patients were excluded due to cell counts <10.

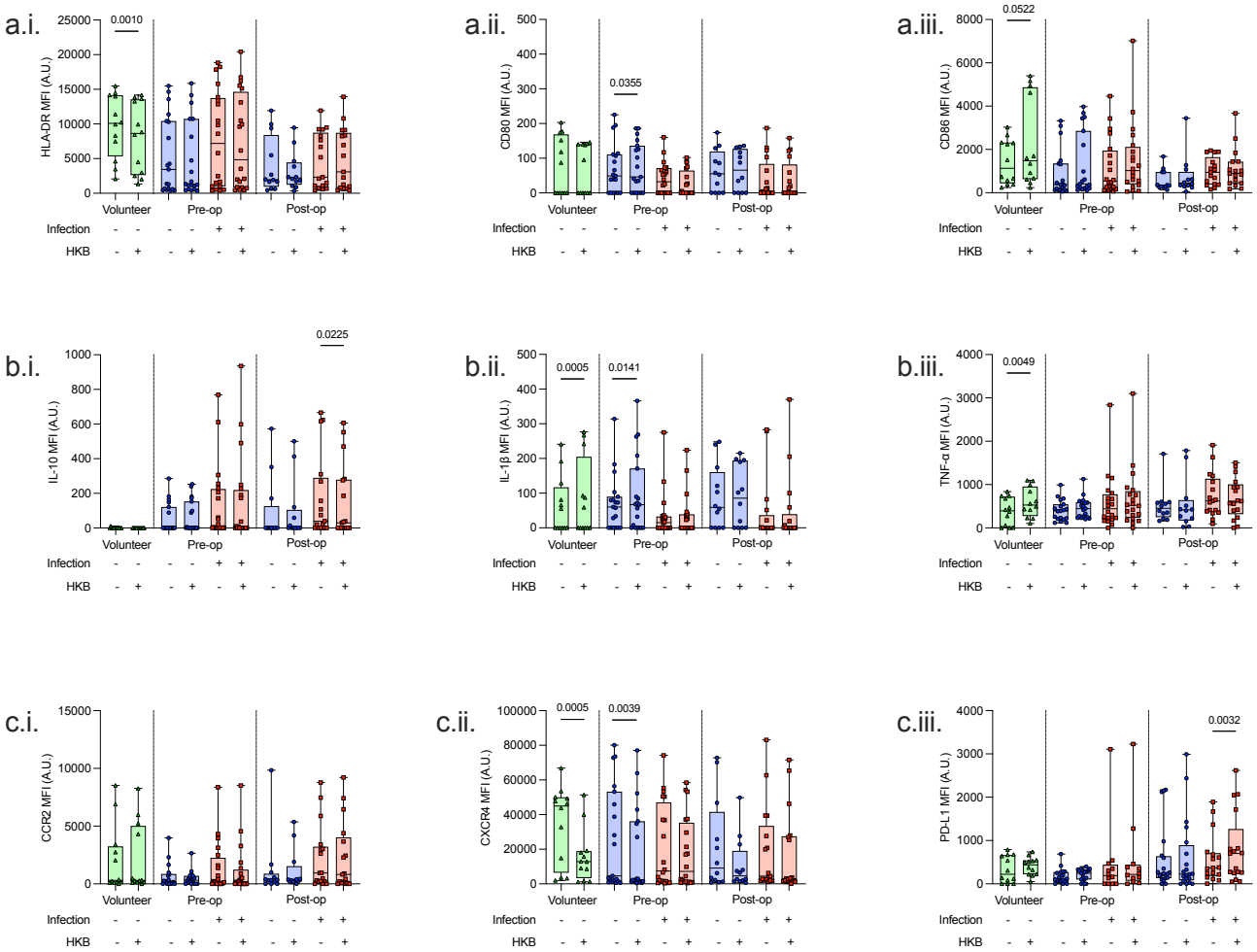

Figure 9.19: Effect of heat-killed bacteria on classical monocytes isolated from volunteers and patients undergoing major surgery

PBMCs were isolated from healthy volunteers (green, n=16) and patients undergoing major surgery both pre- and 24 hours post-operatively who did not (blue, n=22) and did (red, n=26) develop a post-operative infection and the effect of stimulation with or without heat-killed bacteria (HKB, 10^8/ml) for 24 hours on monocyte HLA-DR (a.i.), CD80 (a.ii.), and CD86 (a.iii.) expression, IL-10 (b.i.), IL-1β (b.ii), and TNF-α (c.iii) concentration, and CCR2 (c.i.), CXCR4 (c.ii.) and PD-L1 (c.iii.) expression assessed. Data expressed as median fluorescent intensity (MFI) measured in arbitrary units (A.U.). Each individual is represented by a dot, horizontal line represent median, box the interquartile range and whisker the range. Difference between unstimulated and HKB-stimulated cells compared using multiple t-tests, only $p<0.05$ shown.

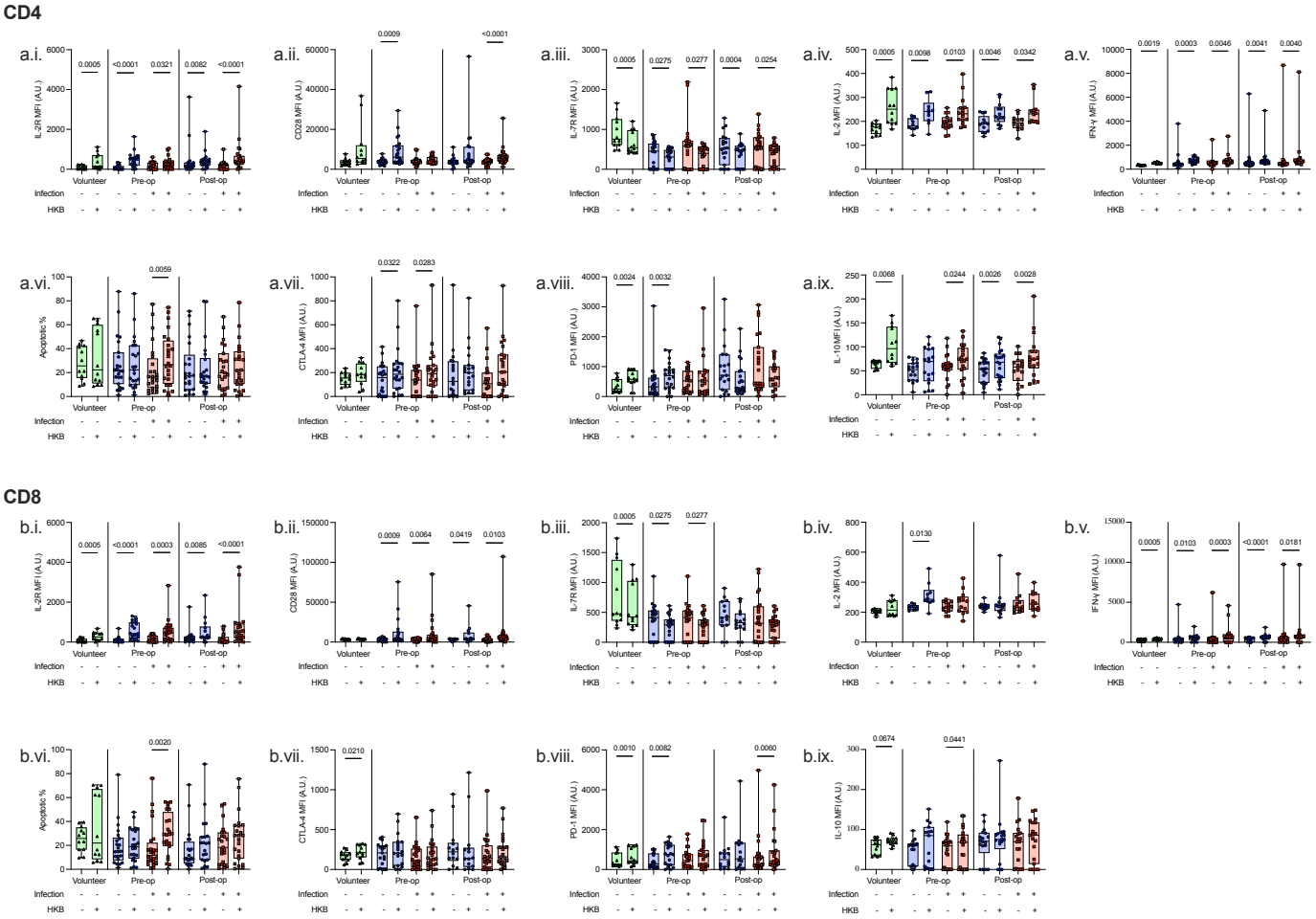

Figure 9.20: Effect of heat-killed bacteria lymphocytes isolated from volunteers and patients undergoing major surgery

PBMCs were isolated from healthy volunteers (green, n=16) and patients undergoing major surgery both pre- and 24 hours post-operatively who did not (blue, n=22) and did (red, n=26) develop a post-operative infection and the effect of stimulation with heat-killed bacteria (HKB, 10^7/ml) for 48 hours on $CD4^+$ (a.) and $CD8^+$ (b.) lymphocyte IL-2R (i.), CD28 (ii.), and IL-7R (iii.) expression, IL-2 (iv.) and IFN-γ (v.) concentration, percentage of apoptotic cells (vi.), CTLA-4 (vii.) and PD-1 (viii.) expression and IL-10 (ix.) concentration assessed. Data expressed as median fluorescent intensity (MFI) measured in arbitrary units (A.U.) or percentage (%) of population. Each individual is represented by a dot, horizontal line represent median, box the interquartile range and whisker the range. Difference between unstimulated and HKB-stimulated cells compared using multiple t-tests, only $p<0.05$ shown.

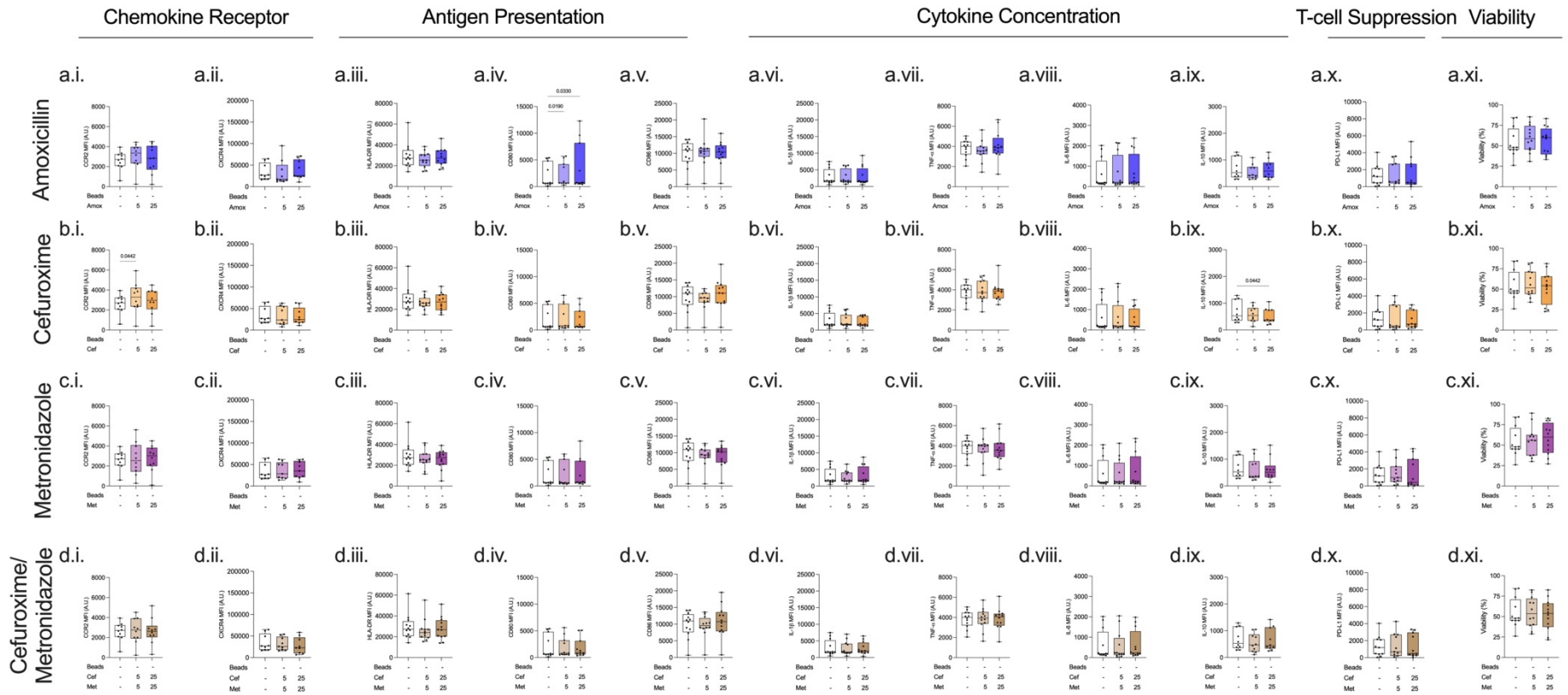

Figure 9.21: Effect of antibiotics on unstimulated classical monocytes after surgery

PBMCs isolated from patients immediately postoperatively (n=12) were incubated for 24 hours with amoxicillin (blue, row a), cefuroxime (orange, row b), metronidazole (purple, row c) and cefuroxime/metronidazole (brown, row d) at a concentration of 5 or 25μg/ml and the effect on classical monocyte immune phenotype delineated. Immune markers measured include those associated with chemokine receptor expression (CCR2, column i, and CXCR4, column ii.) antigen presentation (HLA-DR, column iii., CD80, column iv., CD86, column v), intracellular cytokine concentration (IL-1ß column vi., TNF-α, column viii., IL-6, column viii., and IL-10, column ix), T-cell suppression (PD-L1, column x.), and monocyte viability (column xi.). Data expressed as median fluorescence intensity measured in arbitrary units (MFI (A.U.)) or percentage of population (%). Each antibiotic concentration is compared to control alone using Friedman multiple comparison test without post hoc correction and displayed as individual patients represented with dots, horizontal line median, box interquartile range and whisker range. Only values with $p<0.05$ are shown.

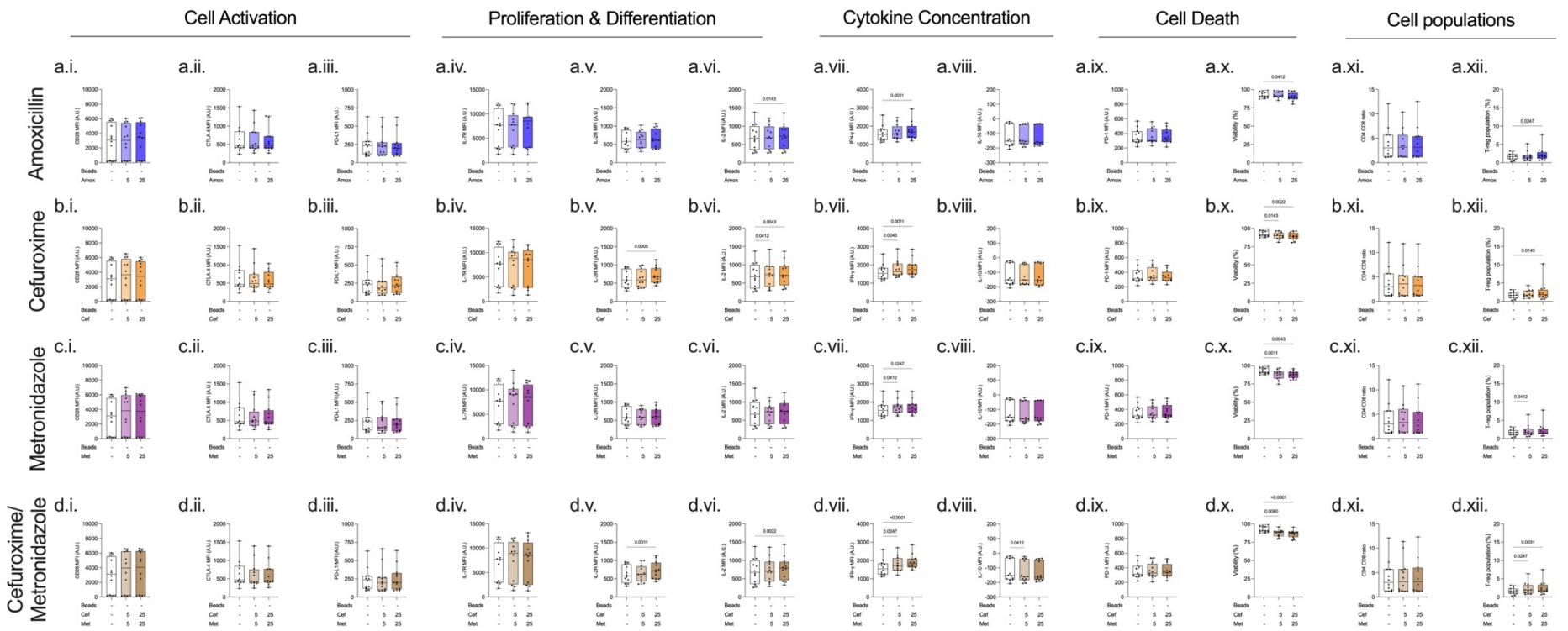

Figure 9.22: Effect of antibiotics on unstimulated CD4⁺ lymphocytes after surgery

PBMCs isolated from patients immediately postoperatively (n=12) were incubated for 48 hours with amoxicillin (blue, row a), cefuroxime (orange, row b), metronidazole (purple, row c) and cefuroxime/metronidazole (brown, row d) at a concentration of 5 or 25 μg/ml and the effect on $CD4^+$ lymphocyte immune phenotype delineated. Immune markers measured include those associated with cell activation (CD28, column i, CTLA-4, column ii., and PD-L1, column iii.), proliferation and differentiation (IL-7R, column iv., IL-2R, column v.), intracellular cytokine concentration (IL-2 column vi., IFN-γ, column viii., and IL-10, column viii.), cell death (PD-1, column ix., and cell viability, column x.), CD4:CD8 ratio (column xi.), and T_{reg} population (column xii.). Data expressed as median fluorescent intensity (MFI, A.U.), percentage of population (%) or ratio. Each antibiotic concentration is compared to control alone using Friedman multiple comparison test without post hoc correction and displayed as individual patients represented with dots, horizontal line median, box interquartile range and whisker range. Only values with $p<0.05$ are shown.

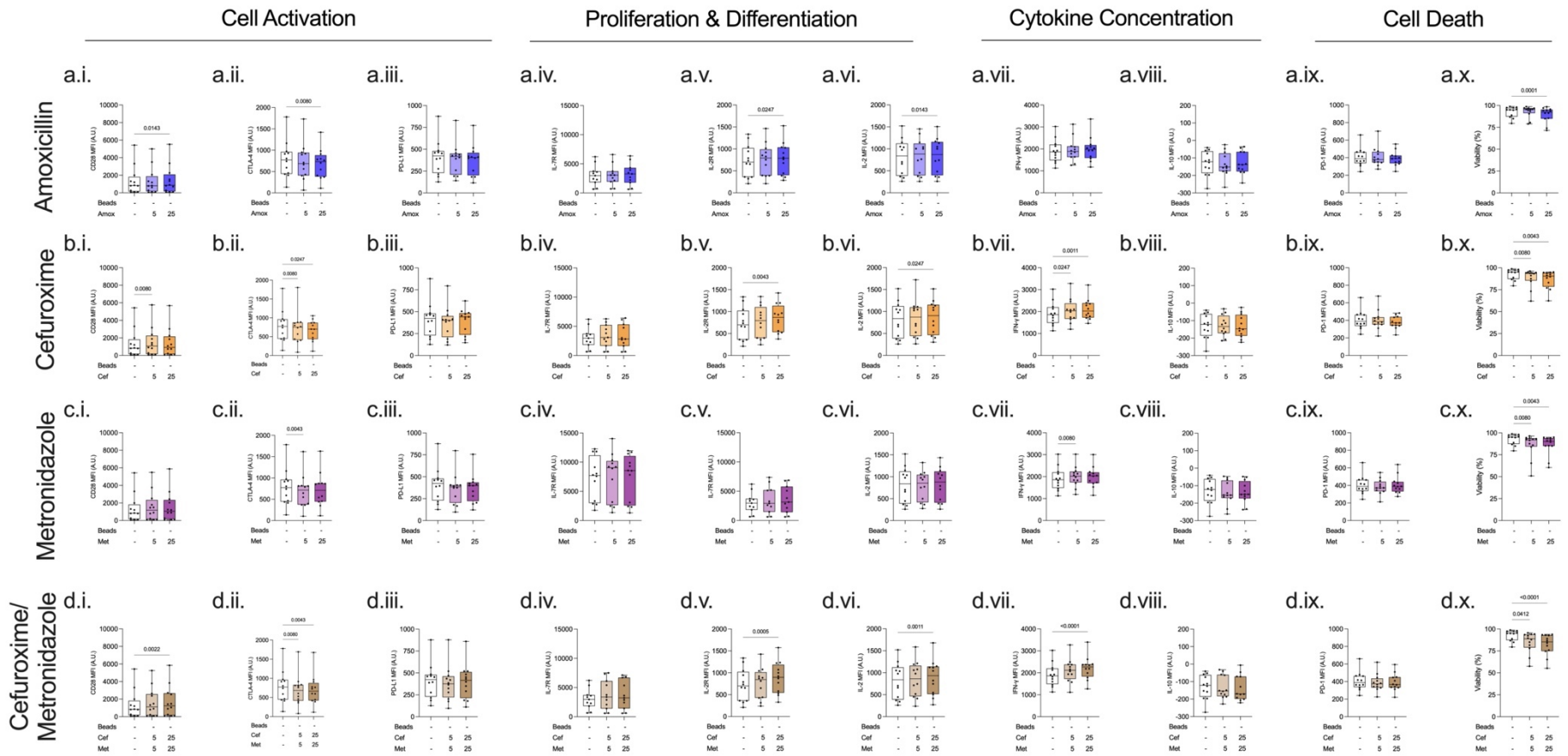

Figure 9.23: Effect of antibiotics on unstimulated CD8⁺ lymphocytes after surgery

PBMCs isolated from patients immediately postoperatively (n=12) were incubated for 48 hours with amoxicillin (blue, row a), cefuroxime (orange, row b), metronidazole (purple, row c) and cefuroxime/metronidazole (brown, row d) at a concentration of 5 or 25μg/ml and the effect on $CD8^+$ lymphocyte immune phenotype delineated. Immune markers measured include those associated with cell activation (CD28, column i, CTLA-4, column ii., and PD-L1, column iii.), proliferation and differentiation (IL-7R, column iv., IL-2R, column v.), intracellular cytokine concentration (IL-2 column vi., IFN-γ, column viii., and IL-10, column viii.), cell death (PD-1, column ix., and cell viability, column x.). Data expressed as median fluorescent intensity (MFI, A.U.) or percentage of population (%). Each antibiotic concentration is compared to control alone using Friedman multiple comparison test without post hoc correction and displayed as individual patients represented with dots, horizontal line median, box interquartile range and whisker range. Only values with $p<0.05$ are shown.

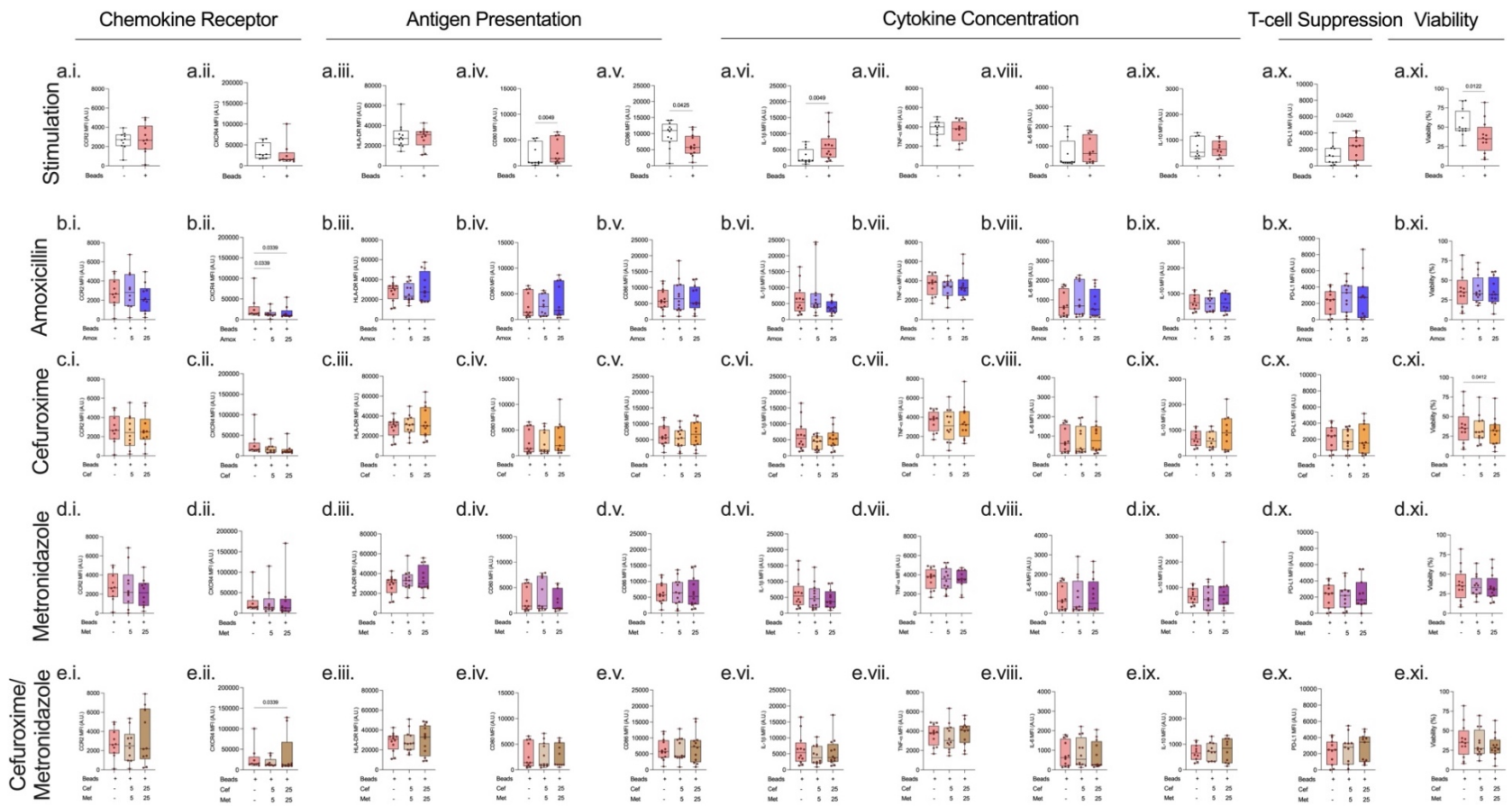

Figure 9.24: Effect of antibiotics on HKB-stimulated classical monocytes after surgery

PBMCs isolated from patients immediately postoperatively (n=12) were incubated for 24 hours with (red dots) and without (black dots) heat-killed E. *coli* (HKB) and the effect of amoxicillin (blue, row a), cefuroxime (orange, row b), metronidazole (purple, row c) and cefuroxime/metronidazole (brown, row d) at a concentration of 5 or 25μg/ml on classical monocyte immune phenotype delineated. Immune markers measured include those associated with chemokine receptor expression (CCR2, column i, and CXCR4, column ii.) antigen presentation (HLA-DR, column iii., CD80, column iv., CD86, column v), intracellular cytokine concentration (IL-1ß column vi., TNF-α, column viii., IL-6, column viii., and IL-10, column ix), T-cell suppression (PD-L1, column x.), and monocyte viability (column xi.). Data expressed as median fluorescence intensity measured in arbitrary units (MFI (A.U.)) or percentage of population (%). HKB compared to control using Wilcoxon test whilst each antibiotic concentration is compared to HKB alone using Friedman multiple comparison test without post hoc correction and displayed as individual patients represented with dots, horizontal line median, box interquartile range and whisker range. Only values with $p<0.05$ are shown.

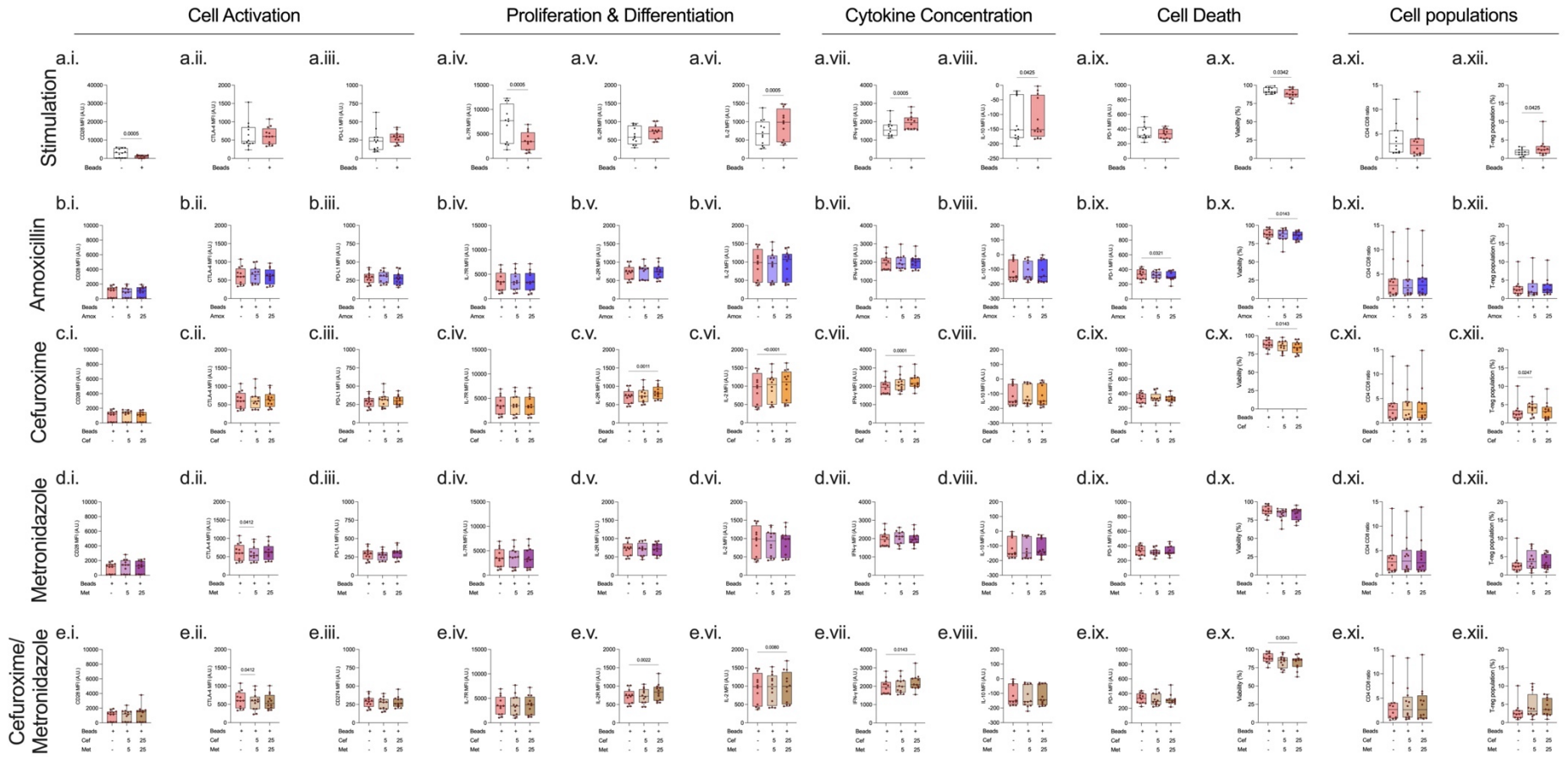

Figure 9.25: Effect of antibiotics on bead-stimulated CD4⁺ lymphocytes after surgery

PBMCs isolated from patients immediately postoperatively (n=12) were incubated for 48 hours with (red dots) and without (black dots) CD3/CD28 beads (beads) and the effect of amoxicillin (blue, row a), cefuroxime (orange, row b), metronidazole (purple, row c) and cefuroxime/metronidazole (brown, row d) at a concentration of 5 or 25μg/ml on $CD4^+$ lymphocyte immune phenotype delineated. Immune markers measured include those associated with cell activation (CD28, column i, CTLA-4, column ii., and PD-L1, column iii.), proliferation and differentiation (IL-7R, column iv., IL-2R, column v.), intracellular cytokine concentration (IL-2 column vi., IFN-γ, column viii., and IL-10, column viii.), cell death (PD-1, column ix., and cell viability, column x.), CD4:CD8 ratio (column xi.), and T_{reg} population (column xii.). Data expressed as median fluorescent intensity (MFI, A.U.), percentage of population (%) or ratio. HKB compared to control using Wilcoxon test whilst each antibiotic concentration is compared to HKB alone using Friedman multiple comparison test without post hoc correction and displayed as individual patients represented with dots, horizontal line median, box interquartile range and whisker range. Only values with $p<0.05$ are shown.

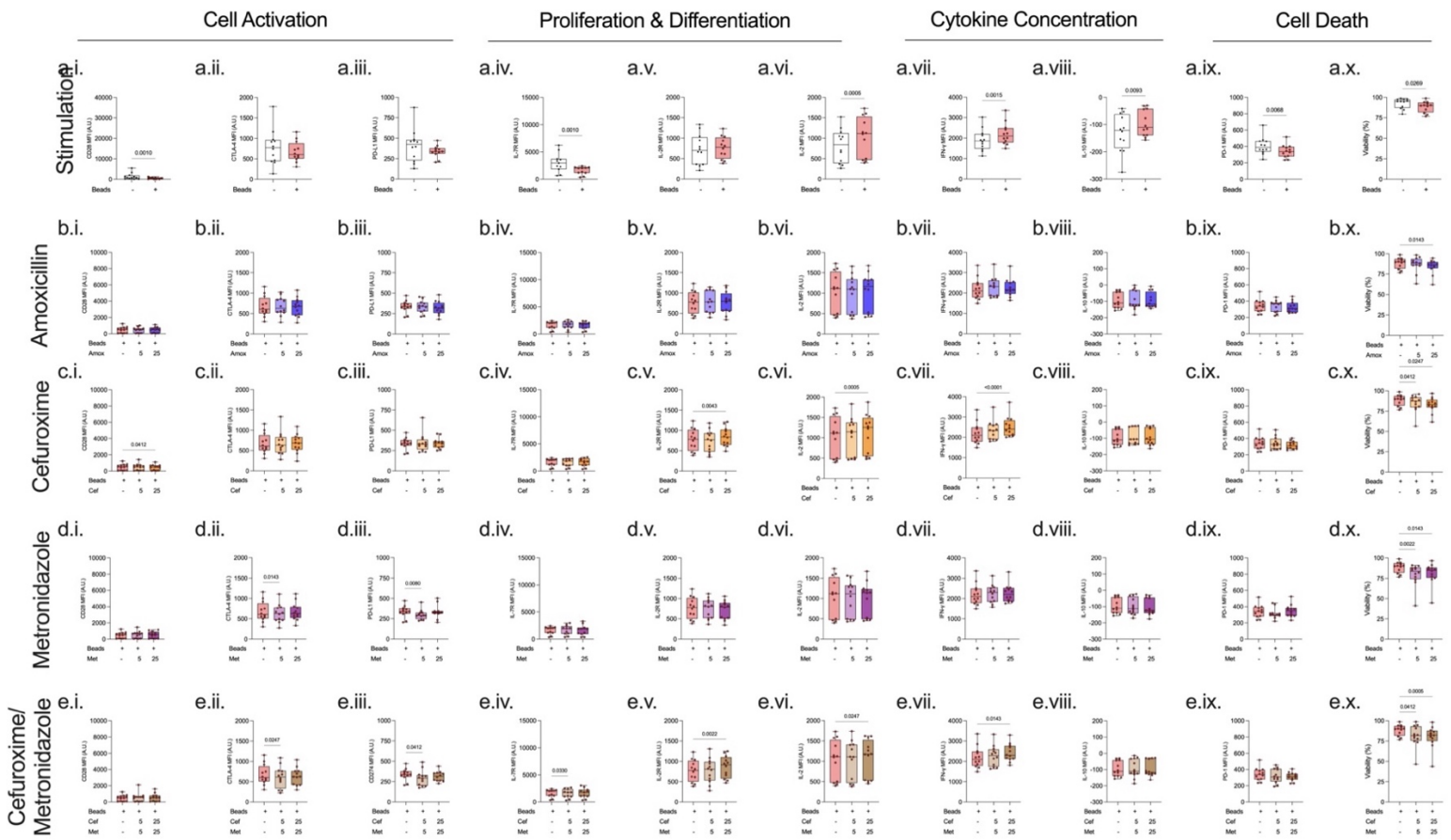

Figure 9.26: Effect of antibiotics on bead-stimulated CD8⁺ lymphocytes after surgery

PBMCs isolated from patients immediately postoperatively (n=12) were incubated for 48 hours with (red dots) and without (black dots) CD3/CD28 beads (beads) and the effect of amoxicillin (blue, row a), cefuroxime (orange, row b), metronidazole (purple, row c) and cefuroxime/metronidazole (brown, row d) at a concentration of 5 or 25μg/ml on $CD8^+$ lymphocyte immune phenotype delineated. Immune markers measured include those associated with cell activation (CD28, column i, CTLA-4, column ii., and PD-L1, column iii.), proliferation and differentiation (IL-7R, column iv., IL-2R, column v.), intracellular cytokine concentration (IL-2 column vi., IFN-γ, column viii., and IL-10, column viii.), cell death (PD-1, column ix., and cell viability, column x.), CD4:CD8 ratio (column xi.), and T_{reg} population (column xii.). Data expressed as median fluorescent intensity (MFI, A.U.), percentage of population (%) or ratio. HKB compared to control using Wilcoxon test whilst each antibiotic concentration is compared to HKB alone using Friedman multiple comparison test without post hoc correction and displayed as individual patients represented with dots, horizontal line median, box interquartile range and whisker range. Only values with $p<0.05$ are shown.