

The Role of Prostaglandin E₂ in Critical Illness-Induced Immune Dysfunction

A Thesis Submitted for the degree of Doctor of
Philosophy, University College London

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Submitted: September 2015

Corrected: February 2016

DECLARATION

'I, James Nicholas Fullerton confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis'

"nullius in verba"

Dysregulation of the inflammatory profile in magnitude or duration following severe infectious or sterile insults including sepsis, burn and trauma is associated with a period of immunoparalysis, the acquisition of hospital acquired-infections and an associated increase in mortality. Prostaglandin E₂ (PGE₂), a cyclooxygenase (COX)-derived eicosanoid classically regarded as pro-inflammatory, regulates multiple aspects of the immune response and has been ascribed a causal role in immunoparalysis during alternate disease states. Systematic review of the clinical literature relating COX-inhibiting non-steroidal anti-inflammatory drugs (NSAIDs) use with either susceptibility to or outcome from acute infection revealed epidemiological evidence of benefit from NSAID administration during severe inflammatory states (sepsis), but not minor infection, providing a rationale for investigation of a mechanistic contribution to critical illness-induced immune dysfunction (CIID).

PGE₂, at pathophysiologically relevant concentrations (IC₅₀ 317pg/mL, 95% CI 105 – 959pg/mL), suppressed *ex vivo* whole blood (WB) cytokine secretion: a validated measure of clinically relevant immune dysfunction. EP4 receptor-mediated increase in intracellular cyclic adenosine monophosphate (cAMP) was determined as the principal pathway, antagonism of which afforded an alternate immunorestorative strategy to established immunoadjuvant agents (interferon- γ and granulocyte-macrophage colony stimulating factor). A complementary *in vitro* bioassay of PGE₂-mediated monocyte deactivation employing 1 α , 25 dihydroxycholecalciferol differentiated (vitamin D₃, 10ng/ml) Mono Mac 6 (MM6), a human cell line, mirrored this response. Pre-clinical evaluation of an association between PGE₂ release during the systemic inflammatory response syndrome (SIRS) and subsequent immunoparalysis using the human intravenous endotoxin model (2ng/kg), however neither confirmed this link, nor refuted it, failing to replicate key immunological features of CIID (sustained reduction in monocyte HLA-DR expression, WB cytokine secretion and absolute lymphocyte count). Mass spectroscopic analysis of plasma revealed significant elevation of COX-derived PGF_{2 α} , thromboxane A₂ and PGE₂, the latter peaking at 3hours, 7.8x higher than baseline values (10pg/mL compared to 1.3pg/mL). These did not suppress MM6 cytokine release.

Compelling arguments suggest PGE₂ contributes to CIID. Alternative or adapted techniques will be required to determine the validity of this premise, potentially identifying a novel therapeutic immunorestorative strategy in the critically ill.

ACKNOWLEDGEMENTS

I am hugely grateful for the help of a large number of individuals who have helped and supported me throughout the course of this degree. Firstly, I would like to thank Prof Derek Gilroy for his guidance, unending enthusiasm and tolerance in letting me follow my own path. I am sure I haven't always been the easiest student but it has been a pleasure to work in your group. Secondly, Dr Alastair O'Brien for starting me on my way, supporting my ideas and showing me how a clinician can contribute to a basic science laboratory (and of course reaffirming the need to be 'robust!'). I would also like to place on record my thanks to the others in my council of elders: Prof Raymond MacAllister, Prof Mervyn Singer, Dr Simon Yona, Dr Richard Day and many others who I have sought out for advice at different points.

In particular I would like to express my gratitude to the other researchers in the Gilroy Group. As they know too well if I were to write down every bit of help provided or question answered this would be the longest chapter in my thesis. Whilst I will not list you all now I hope you are aware of how much your support has meant (and indeed still means) to me. I am especially indebted to those individuals who selflessly helped undertake the endotoxin model – Roel de Maeyer and Alex Maini – providing both technical support and 'good humoured' company at any time of day or night. Finally and most importantly I would like to acknowledge the key role of Dr Elisabetta Segre who assisted with the second half of the participant group and almost single-handedly kept me sane through this period. No statement here will adequately recognise the contribution you have all made to this work and my development; all I can say again is thank you.

Outside of UCL I would like to thank the Wellcome Trust and Pfizer for providing funding to undertake the work and the UCLH/UCL Clinical Research Facility for hosting my study. I would also like to thank the National Institutes of Health Clinical Center for making the EC-6 endotoxin freely available. Lastly, it would be impossibly remiss to not acknowledge the support of my friends and family, most notably my wife Dr Lucy Fullerton and parents Richard and Amanda Fullerton. I can only apologize for the weekends and holidays ruined, promise to try and attain a work/life balance and thank you from the bottom of my heart for your love and tolerance.

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LIST OF ABBREVIATIONS

AC	Apoptotic Cell
CARS	Compensatory Anti-Inflammatory Response Syndrome
CCRE	Clinical Center Reference Endotoxin (Lot EC-6)
CD	Cluster of Differentiation
CI	Critical Illness
CIID	Critical Illness-Induced Immune Dysfunction
COX	Cyclooxygenase
DC	Dendritic Cell
FACS	Fluorescence Activated Cell Sorting
HAI	Hospital Acquired Infection
HBSS	Hanks Balanced Salt Solution
IFN	Interferon
IL	Interleukin
ICU	Intensive Care Unit
LOX	Lipoxygenase
LT	Leukotriene
LX	Lipoxin
M-CSF	Macrophage Colony-Stimulating Factor
MDM	Monocyte-derived macrophage
MDSC	Myeloid-Derived Suppressor Cell
MFI	Median Fluorescence Intensity
MM6	Mono Mac 6
MOF	Multi-Organ Failure

PBS	Phosphate Buffered Saline
PBMC	Peripheral Blood Mononuclear Cell
PG	Prostaglandin
PMA	Phorbol 12-myristate 13-acetate
PMN	Polymorphonuclear Neutrophils
RV	Resolvin
SD	Standard Deviation
SEM	Standard Error of the Mean
SIRS	Systemic Inflammatory Response Syndrome
T-Reg	Regulatory T-Cell
TXA ₂	Thromboxane A ₂
TNF α	Tumour Necrosis Factor α

CHAPTER 1: Introduction

Publications:

Fullerton JN, O'Brien AJ and Gilroy DW. Pathways mediating resolution of inflammation: when enough is too much. *Journal of Pathology* 2013;213:8-20

Fullerton JN, O'Brien AJ and Gilroy DW. Lipid mediators in immune dysfunction after severe inflammation. *Trends in Immunology* 2014;35:12-21

1.1 Critical Illness

Protean in its manifestations, defined variably by type and complexity of disease, severity of organ dysfunction and expected outcome, critical illness (CI) remains an enigma^{1,2}. Key CI syndromes include sepsis, acute lung injury, adult respiratory distress syndrome and multi-organ failure (MOF). These lack pathognomonic 'hallmarks', being diagnosed instead by consensus derived clinical, laboratory, radiologic and physiologic amalgams. United by their therapeutic requirement for organ support on intensive care units (ICU), these syndromes in-turn represent the clinical end-points of a multitude of disparate disease states.

In the US and UK the predominant reasons for admission to ICU (excluding cardiac diagnoses) are sepsis, respiratory insufficiency/failure, trauma, peri-operative care, post-cardiac arrest, neurologic pathology and poisoning^{3,4}. Despite the heterogeneity of the CI population they share a largely common set of clinical sequelae⁵. Prolonged hospitalization, physical⁶⁻⁹, iatrogenic¹⁰ and psychiatric complications¹¹ are common during and after CI and admission to ICU. In particular the acquirement of nosocomial infections with resultant episodes of sepsis pose a particular problem, contributing significantly to excess morbidity and mortality^{12,13}.

The incidence and demand for CI is increasing both in the developed¹⁴⁻¹⁸ and developing world^{1,19}. Already consuming over 13% of hospital costs and 0.5% of the US gross domestic product - sepsis alone accounting for in excess of \$14 billion per annum²⁰ - the expense of caring for CI patients may soon become prohibitive²¹⁻²³. As such, the importance of understanding, delineating and identifying the pathogenic processes mutual to all CI patients and developing therapeutic tools to modify them is increasingly imperative.

1.1.1 Critical Illness: Definitions and Epidemiology

The systemic inflammatory response syndrome (SIRS) is inducible by infectious (sepsis) and non-infectious pathology (ischaemia/reperfusion, trauma, burn, haemorrhage, surgery) alike. Originally defined over 20 years ago by common physiological and haematological abnormalities resulting from severe inflammation (2 or more of: temperature $<36^{\circ}\text{C}$ or $>38^{\circ}\text{C}$; white cell count $<4 \times 10^9/\text{L}$ or $>12 \times 10^9/\text{L}$ or $>10\%$ immature forms; heart rate $>90\text{bpm}$; respiratory rate >20 or arterial $\text{pCO}_2 <4.3\text{kPa}$) it serves as a sensitive, non-specific descriptor that unites these distinct pathological entities (see Table t1.1)²⁴⁻²⁶. Arising from infectious and non-infectious causes with near equally frequency⁵, and having both high incidence and prevalence in ICU patients, the SIRS both reflects and contributes to CI.

SIRS criteria however fail to capture the range of organ specific and biochemical abnormalities that may follow a severe, generalised insult to the host. In an attempt to better represent these, modifications to the original definitions, specifically made to improve the diagnosis and categorisation of sepsis, were thus made in 2001²⁷ (see Table t1.1). Whilst producing a more adequate, holistic description of the sequelae of systemic inflammation, the breadth and complexity of these refined parameters mean that they remain non-specific (being neither unique to, nor pathognomonic of infection). Further, they may fail to capture important differences between subsets of patients relating to site of infection, demographic diversity, co-morbidity burden, endocrine and immunological function etc. potentially leading to the inappropriate grouping of what remains a heterogeneous population. These concerns have led to the current consensus that further refinement of the definitions surrounding CI and, in particular sepsis, are required²⁸⁻³¹. An additional key metric is severity of illness.

Term	Criteria
SIRS*	2 out of the 4 following criteria:
	Temperature >38 °C or <36 °C
	Heart rate >90/min
	Hyperventilation evidenced by respiratory rate >20/min or arterial CO ₂ lower than 32 mmHg
	White blood cell count >12 000 cells/μL or lower than 4000 cells/μL
Sepsis	SIRS criteria with presumed or proven infection
Severe sepsis	Sepsis with organ dysfunction
Septic shock	Sepsis with hypotension despite adequate fluid resuscitation

Note: *SIRS, systemic inflammatory response syndrome.

Term	Criteria
Sepsis	Documented (or suspected) infection with any one of the following clinical or laboratory criteria
General parameters	Fever, hypothermia, tachycardia, tachypnea, altered mental status, arterial hypotension, decreased urine output, significant peripheral edema, or positive fluid balance
Inflammatory parameters	Leukocytosis, leukopenia, hyperglycemia, increased C-reactive protein, procalcitonin, or creatinine, coagulation abnormalities, increased cardiac output, reduced mixed venous oxygen saturation
Hemodynamic parameters	Hypotension, elevated mixed venous oxygen saturation, elevated cardiac index
Organ dysfunction parameters	Arterial hypoxemia, acute oliguria, increase in creatinine level, elevated international normalized ratio or activated partial thromboplastin time, ileus, thrombocytopenia, hyperbilirubinemia
Tissue perfusion parameters	Hyperlactatemia, decreased capillary refill, or mottling

Table t1.1 Evolving definitions of the systemic inflammatory response syndrome and sepsis.

Left Panel: 1991 American College of Chest Physicians and Society of Critical Care Medicine Consensus Conference definitions. **Right Panel:** 2001 Consensus Conference by the Society of Critical Care Medicine / European Society of Intensive Care Medicine / American College of Chest Physicians / American Thoracic Society / Surgical Infection Society modified definitions. Tables reproduced with permission from Mayr et al 2014²⁰.

The terms severe sepsis and septic shock were designed to reflect the increased mortality in those with organ dysfunction and refractory hypotension, being envisaged as part of a continuum of worsening inflammation. Rough current estimates of mortality in each group are 10-20% in those with sepsis, 20-50% when compounded by organ failure (severe sepsis) and 40-80% in septic shock³². Whilst evidence that the risk of dying from sepsis has decreased from near 30% to below 20% over the

past 30 years has accumulated from both large epidemiological studies¹⁵ and analyses of temporal trends in clinical trials³³, mortality rates have remained static for the past ten years¹. Further, given changing demographics and advances in parallel fields (surgery, oncology, transplantation) the actual number of people dying from sepsis – itself highly correlated with overall ICU outcomes given its prevalence - appears to be increasing year on year, now exceeding 200,000 in the US alone, similar to myocardial infarction³². Whilst some of this recorded increase may be attributable to organisational (documentation, coding) factors, it is clear that these syndromes continue to pose both a considerable clinical challenge and economic burden³⁴.

One of the principle reasons why further in-roads have not been made into reducing the incidence, prevalence, morbidity and mortality from CI is that novel management techniques and pharmacological interventions have disappointingly proven largely ineffective^{2,35,36}. It is not yet clear whether this has been the result of employing strategies that target central processes common to all CI in ill-defined populations (reducing the signal to noise ratio), or failing to develop agents that remedy specific aberrations in identifiable patient subsets.

1.1.2 Inflammation Unites Critical Illness

The primary trigger of the systemic inflammatory response has classically been ascribed key importance in delineating pathology, classic divisions being into sterile or infective insults, and within the latter into species and Gram type.

Epidemiological studies have collectively demonstrated an increase in the rate of Gram-positive bacterial sepsis to near parity with Gram-negative infection – the classic cause – with the recent EPIC II study reporting rates of 46.8% and 62.2% respectively^{15,37} (see for review²⁰). However one-third of all patients with severe sepsis never have a positive blood culture³⁸. Whilst bacterial species has been related to outcome this is inevitably confounded by site of infection and additional host factors (e.g. infection with less virulent bacteria likely indicates a greater degree of underlying immuno-compromise)³⁹. Further, as first recognised by Osler, and reiterated by Thomas in his classic review ‘Germs’, there is growing acceptance that the key to the pathogenesis of CI is not the trigger per se but instead that ‘it is our response that makes the disease’⁴⁰.

Irrespective of initiating stimuli, evidence has accumulated that the human inflammatory response appears largely constant. Gene expression profiling in CI has repeatedly demonstrated a surprisingly consistent pattern of genomic re-prioritisation

regardless of aetiology⁴¹⁻⁴³ or pathogen⁴⁴⁻⁴⁶, with few exceptions⁴⁷, which seems to indicate that at least qualitatively, there exists a fundamental human transcriptomic response to severe inflammatory stress⁴². This appears to be due to commonalities in the molecular signalling and transduction pathways that underlie inflammation.

Cellular disruption, whether infective or sterile, releases common motifs - damage-associated molecular patterns (DAMPs) or alarmins - into the circulation⁴⁸⁻⁵². DAMPS such as free-DNA and histones, via evolutionarily conserved similarities to pathogen-associated molecular patterns (PAMPs), bind and activate the same families of cell surface pattern-recognition receptors (e.g. Toll-like receptors [TLR] and nucleotide-binding oligomerisation domain [NOD]-like receptors [NLR]), instituting common signal transduction and downstream processing^{50,53} (see Box 1). These may thus act as a universal 'alarm signals' to tissue injury regardless of the injurious stimuli^{26,42,54-56}. Discrete TLR signalling via TLR-2 and 4 (in response to Gram-positive and Gram-negative bacteria respectively) may also elicit overlapping response elements⁵⁷⁻⁶⁰ with receptors displaying extensive cross-tolerance⁶¹. Thus both distinct ligands and signal transducers may derive a largely indistinguishable cellular response.

Resultant cytokine production at the primary inflammatory site may initially establish SIRS⁶²⁻⁶⁵, however immune-alterations and organ-dysfunction, with sufficient provocation, may rapidly become established independent of this^{66,67}. A compartmentalized cascade through distant tissues has been repeatedly demonstrated in different CI aetiologies. Mediators including bacterial products - endotoxin⁶⁸⁻⁷⁰ (from gut translocation^{71,72} or pulmonary sources⁷³), DNA⁷⁴ and peptidoglycan-associated lipoprotein⁷⁵ - and host derived cytokines (TNF α , IL-1, IL-6), anaphylotoxins (especially C5a), IFN- γ , high-mobility group protein B1 (HMGB-1), macrophage migration inhibition factor (MIF) and others (see^{76,77} for review), are universally released, which, aided by increases in vascular dilatation and epithelial permeability, cause compartment cross-talk and inflammatory contagion. Indeed, organ systems experimentally isolated from plasma that has circulated past inflamed tissues are 'protected'⁷⁸ highlighting this 'domino' effect.

In short, CI, at least in terms of the inflammatory component, may not be as heterogeneous as previously suspected, with a common transcriptomic, biochemical and clinical signature emerging. Therapeutically this is highly relevant as the ability to manipulate or regulate unifying inflammatory processes deemed harmful will potentially have applicability across the pantheon of CI patients.

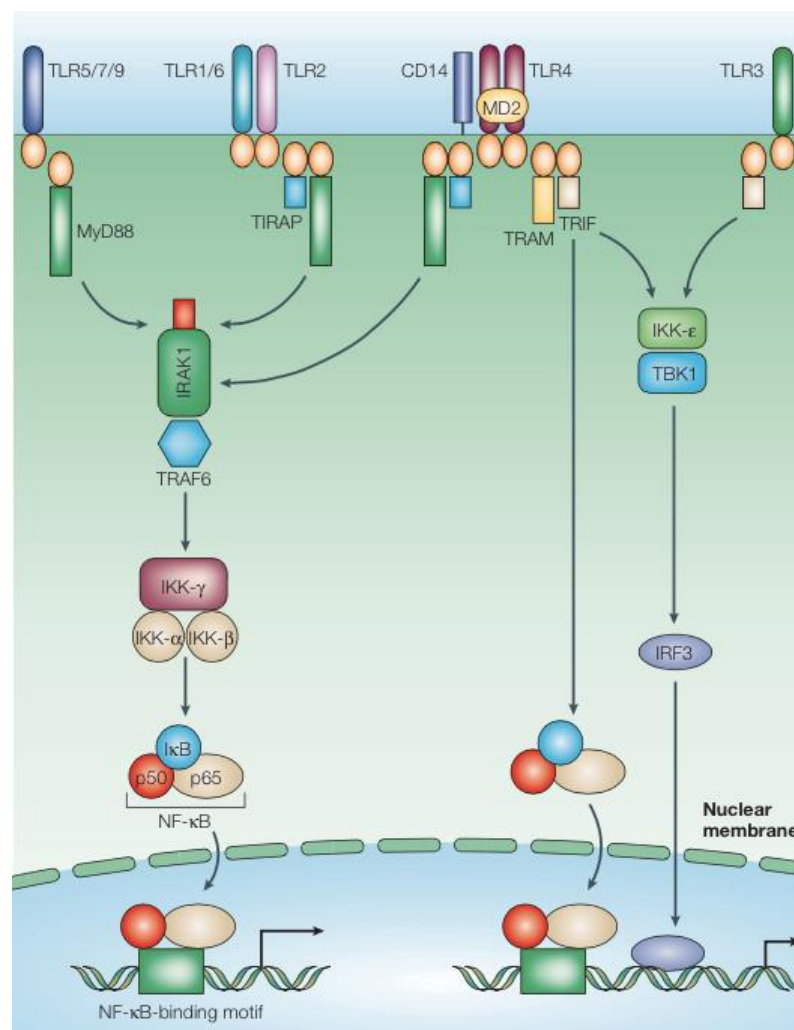
Box 1: Pattern-Recognition Receptors

We are constantly exposed to potentially pathogenic microbes. In order to afford a rapid, effective, but non-specific response to this challenge both cells of innate immune system and non-professional immune cells possess receptors directed towards invariant molecular constituents of infectious agents, or, as more recently appreciated, moieties released as a result of the local tissue damage that may accompany invasion: pattern-recognition receptors (PRR)^{56,79}. These frequently target structures essential for the survival of the microbe (PAMPs), for instance the conserved lipid A of lipopolysaccharide (LPS) or peptidoglycan, or elements normally present only intracellularly, for instance histones (DAMPs). Multiple families of these receptors exist including the cell membrane TLR and C-type lectin receptors (CLRs), and the intracellular cytoplasmic NLR and retinoic acid-inducible gene [RIG]-1-like receptors (RLRs). These last three families have been reviewed extensively and will not be further described here⁸⁰.

TLRs are characterized by N-terminal leucine-rich repeats and a transmembrane region followed by a cytoplasmic Toll/IL-1R homology (TIR) domain. In humans ten separate receptors have been identified that sense discrete but overlapping components of pathogens either independently or via the formation of heterodimers e.g. TLR2 with either TLR1 or TLR6⁸¹. Accessory molecules may additionally be involved with their binding and or signalling. TLR4 for example recognises LPS together with myeloid differentiation factor 2 (MD2), two complexes of TLR4-MD2-LPS interacting symmetrically to form a homodimer⁸². CD14, a soluble or membrane-anchored (on myeloid cells) glycoprotein, is another, being capable of binding a variety of microbial and endogenous products and enhancing the ability of these ligands to activate TLRs. With regards to LPS, CD14 appears to chaperone LPS from LPS-binding protein to TLR4-MD2 at the cell surface, and to be required for both TRIF (TIR domain-containing adaptor protein inducing IFN β) and MyD88 (myeloid differentiation primary-response protein 88) signalling (see⁸³ for review).

Recognition of PAMPs or DAMPs by TLRs leads to transcriptional up-regulation of distinct (but frequently overlapping) genes, depending on the TLR and cell types involved. This is principally determined by the TIR domain-containing adaptor molecules recruited. Whilst five such adaptors exist, signalling is roughly divided into two distinct pathways depending on the usage of the adaptor molecules TRIF and MyD88⁸⁰. Whilst both eventually result in the translocation of NF- κ B to the nucleus and activate the expression of pro-inflammatory cytokine genes (see accompanying

image) they may additionally activate discrete signalling cascades e.g. MAP kinase, which form alternate translational complexes, and possess both different kinetics and regulating factors. These are reviewed in full here^{80,81,84}. Multiple elements of the TLR signalling response from the receptors themselves, their co-factors and accessory molecules and the subsequent signalling cascade are being actively explored as therapeutic targets in the treatment of CI^{85,86}.



Reproduced with permission from Akira and Takeda 2004⁸⁷

1.1.3 Critical Illness: An Immunosuppressive State

Inflammation represents a vital, stereotyped response designed to facilitate containment and removal of noxious stimuli prior to initiation of tissue repair and re-establishment of homeostasis⁸⁸. It involves both a local and systemic response and represents a careful and subtle balance between complex networks of pro (amplifying) and anti (down-regulating)-inflammatory signals⁷⁶. The desired inflammatory response is one with sufficient potency to eliminate the triggering stimuli, yet limited in temporal and spatial dimensions to avert excessive host tissue

damage. Dysregulation of the normally highly conserved inflammatory process has clear pathogenic consequences⁸⁹.

In CI, and sepsis in particular, an excessive pro-inflammatory response has classically been the prime concern^{24,90}. Unbridled activation of the innate immune system and subsequent 'cytokine storm' has long been known to result in physiological collapse (shock) and MOF⁹¹⁻⁹⁴, and pro-inflammatory mediators, variously described as accelerators⁸⁸ or 'go' signals⁹⁵ consequently ascribed as the primary determinants of pathology. However the repeated failure of their pharmacological blockade^{22, 23}, or more recently antagonism of their receptor signalling⁹⁶, to improve clinical outcomes in sepsis - alongside improvements in supportive care over the past 20 years - have either obviated the need for this therapeutic avenue to be pursued (aside from in key exceptions e.g. *Neisseria meningitidis* infection), or rendered it of lower importance^{30,97}. Instead, a predominantly anti-inflammatory, immunosuppressive period, commonly portrayed (and perhaps falsely so⁴²) as the second stage of a serial bi-phasic inflammatory response – the compensatory anti-inflammatory response syndrome (CARS) – may now be regarded as the primary clinical concern^{26,98-100 97,101}.

This phase is associated with well-recognized inter-connected negative pathogenic physiological aberrations including microcirculatory dysfunction¹⁰², coagulopathy¹⁰³, catabolic predominance¹⁰⁴, bioenergetic failure^{105,106} and ultimately multi-organ failure^{107,108}. However, it is principally marked by vulnerability to nosocomial (hospital-acquired infection, HAI) infection and repeated episodes of sepsis^{12,13,109,110}.

1.1.4 Immunoparalysis: Evidence and Definitions

CI patients may fail to remove the initial injurious stimuli¹¹¹ and are rendered vulnerable both to secondary infection with less virulent, multi-drug resistant opportunistic organisms^{12,112} and to re-activation of latent pathogens^{113,114}. Indeed, 2-15% of those post multiple-trauma^{115,116}, nearly 20% after burn injury¹¹⁷ and 1-4% of those undergoing surgery (elective or emergency) ^{118 119} acquire sepsis. In total, nearly 30% of intensive care patients will develop at least one nosocomial infection, a rate 6-times higher than that on standard wards^{12,120}. Acquisition of an infection on ICU has been shown to independently confer a two to three-fold increased risk of in-patient mortality over non-septic patients in large recent point-prevalence studies^{37,116}.

Whether adaptive¹²¹ or pathogenic⁹⁷ the immunological consequences of CARS, variably described as immunoparalysis, anergy, leukocyte re-programming or as here

critical-illness induced immune dysfunction (CIID)¹²²⁻¹²⁴, appear to pose an equal if not greater threat to the host than the initial 'cytokine storm'. Now understood to spatially and temporarily parallel pro-inflammatory signalling, it is the magnitude, extension and establishment of anti-inflammatory networks that appear to drive mortality and morbidity^{42,125,126}. The suggested mechanisms involved are complex, multi-level and likely vary between both organ system and individuals. Loss of key effector cells especially via apoptosis, a shift in cytokine production from a pro-inflammatory Th1 to an anti-inflammatory Th2 profile, alteration in cell-surface receptor expression (increased co-inhibitory [e.g. PD-1] and decreased co-stimulatory receptors e.g. monocyte HLA-DR) and multiple other alterations have been reported. These have been extensively reviewed elsewhere and will not be fully explored here^{97,98,100,123,127-129}.

Recognition of this phase of immunoparalysis has led to a paradigm shift in the use of immunomodulatory therapies in sepsis from targeted blockade of pro-inflammatory mediators to augmentation of the immune system via immunoadjuvant agents including granulocyte macrophage colony-stimulating factor (GM-CSF) and interferon γ (IFN γ)¹³⁰. This approach has demonstrated initial promise in selected septic patients, with evidence of improved infective clearance, reduced time on ventilator, ICU and hospital stay, but not yet 28-day mortality, in early trials^{131,132}.

Key to such an immunorestorative strategy is patient stratification: firstly identifying individuals with infective pathology if employed as primary therapy and secondly those with clinically meaningful immunosuppression who may benefit from intervention (and equally to restrict its use in those without). Whilst multiple biomarkers of immunosuppression have been described (including IL10/TNF α ratio, T-cell PD-1 and neutrophil CD88 expression etc., see for review¹³³), to date, two markers have been repeatedly demonstrated to predict acquisition of nosocomial infection and mortality – flow cytometrically determined monocyte human leukocyte antigen DR (mHLA-DR) expression¹³⁴⁻¹³⁶ and *ex vivo* lipopolysaccharide (LPS)-stimulated whole blood (WB) TNF α release¹³⁷⁻¹³⁹.

Based on these metrics tentative definitions of 'immunoparalysis' have been suggested including prolonged (variably employed: routinely accepted as either on 2 consecutive days or ≥ 3 days) mHLA-DR $< 30\%$ ¹³¹ or reduction in WB TNF α release $< 200\text{pg/mL}$ ¹⁴⁰. Whilst employed in the aforementioned clinical trials these definitions have been neither extensively validated nor compared to alternate cut-offs. Further, the proposed markers are not independent of each other (both pertaining to

monocyte function), may be more accurate in combination (including with other metrics), and their kinetics may be more informative than single values¹³³. Considerable work remains in demonstrating not only that those who do acquire HAI or die on ICU do routinely have immunosuppression (case-control vs. the cohort methodologies employed to date), but in proving both a causal relationship between the two and describing optimal, clinically relevant, cut-offs by which to diagnose and thus treat immunoparalysis.

One neglected but potentially important contributing factor to the CIID phenotype may be the pathways and mediators that regulate the resolution of inflammation¹⁴¹. Capable of multi-modally modulating immune function and demonstrated to be immunosuppressive in alternate disease states¹⁴², a greater understanding of their role in CI may afford both additional means of patient stratification and novel therapeutic targets.

1.2 Resolution of Inflammation

Inflammation does not resolve passively through removal of the injurious stimuli and catabolism of pro-inflammatory mediators alone, but involves a program of unique active pathways, signalling molecules and cell subtypes, ending with completion of tissue repair and re-establishment of homeostasis^{88,143,144}. Key steps in this process include clearance of the inciting stimuli, withdrawal of local survival signals, normalisation of chemokine gradients, apoptosis of infiltrating polymorphonuclear cells (PMN, neutrophils), their efferocytosis by tissue and monocyte-derived macrophages (MDM) and either the incorporation of these myeloid cells into the local population or their recirculation via lymph or blood⁸⁸. Small molecule bioactive lipid mediators (LM) are pivotal in this cascade: determining the switch from the onset of inflammation to resolution and orchestrating these separate but spatially, temporally and functionally linked events¹⁴⁵⁻¹⁴⁷.

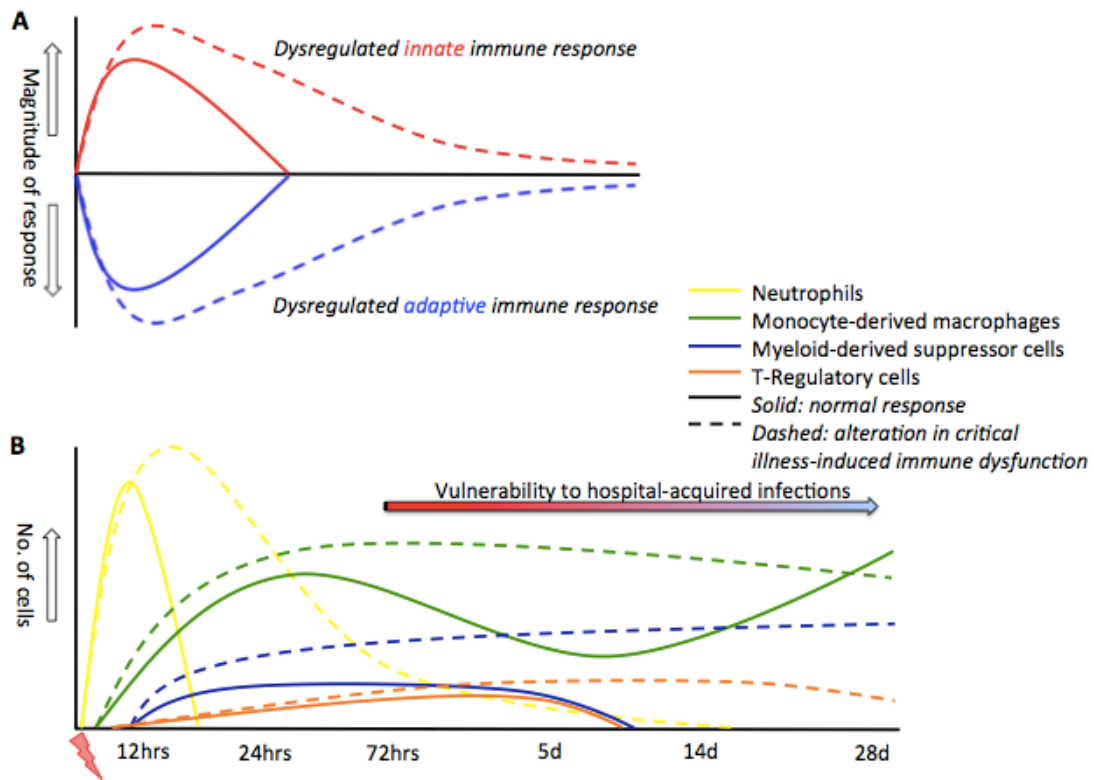


Figure f1.1: *Dysregulation of immune responses in critical illness.* (A) The current paradigm describes simultaneous up-regulation of pro-inflammatory and suppression of adaptive immune responses after significant damage to host tissues, regardless of aetiology. Patients experiencing adverse outcomes (prolonged hospital stay, secondary infection, in-patient mortality) display exaggerated, prolonged dysregulation of the immune-inflammatory state (dashed line); adapted from Xiao *et al* [5]. (B) Illustrative summation of observed alterations in cell numbers resulting from failure of resolution pathways in critical illness. Delayed neutrophil apoptosis, failure of macrophage efferocytotic function and drainage/recirculation with over-representation of MDSCs and T-Regs is proposed as a mechanistic driver of critical illness-induced immune dysfunction (CIID). Functional impairment accompanies and exacerbates numerical/proportional changes (see Figure f1.2). Reproduced with permission from Fullerton *et al*¹⁴¹.

Clinically, failure of resolution has classically been discussed in terms of either an insufficiency or inadequacy leading to chronic inflammation, tissue damage, and dysregulated tissue healing (see ^{89,148,149}). The opposite perspective that these regulatory pathways may exert excessive influence with equally deleterious consequences – what may be termed ‘injurious resolution’ – has not however received significant attention. This concept represents an acceptance that active resolution of inflammation is not a panacea, and that, as with all biological systems, it may become dysregulated with pathogenic consequences. In this hypothetical situation an excessive immuno-regulatory effect is exerted by either the processes or

mediators designed to bring about resolution in magnitude, duration or location leading to a blunting of the inflammatory response and a susceptibility to secondary, predominantly infective, insults – a case of ‘gamekeeper turned poacher’.

In the following sections evidence that regulatory LM released during inflammation, in particular prostaglandin E₂ (PGE₂), may contribute to immune dysfunction is discussed. It is argued that in the context of critical illness-induced severe inflammation the subset of patients experiencing an excessive, dysregulated inflammatory profile - known to have a higher rate of adverse outcomes (including nosocomial infection acquisition⁴²) - do so as a result of either LM excess or deficiency. It is recognised however that LM represent only one class of signalling molecule and that the impact of both alternate mediators and parallel cellular, vascular, metabolic etc, processes to this phenotype is significant. Detailed descriptions of the pathogenic contribution of resolution processes to CIID including cell death (apoptosis vs. necrosis) and clearance (efferocytosis), and the potential involvement of specialized regulatory cell subsets (T-regs and myeloid-derived suppressor cells [MDSC]) are not provided here, however have been elaborated in Fullerton et al¹⁴¹ and are summarised in Figures f1.1 and f1.2.

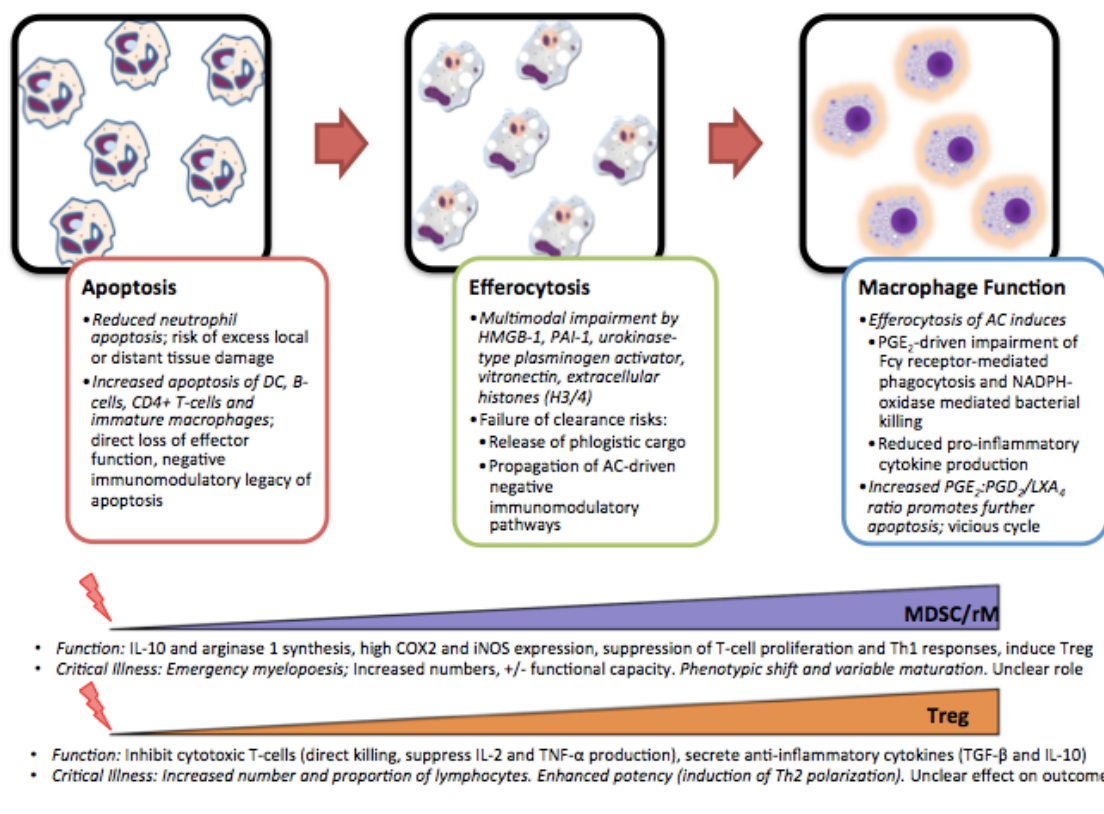


Figure f1.2: Contribution of inflammatory resolution processes to critical illness-induced immune dysfunction (CIID). Active resolution pathways promoting the apoptosis of infiltrating neutrophils, their efferocytosis by both tissue-resident and monocyte-derived macrophages, associated humoral mediators (eicosanoids, particularly PGE₂) and specialized regulatory cell subtypes may all impair innate responses to secondary challenges, a process we term 'injurious resolution'. Reproduced with permission from Fullerton et al¹⁴¹.

1.3 Inflammation-Regulating Lipid Mediators Contribute to Immune Dysfunction

Lipid mediators (LM) including eicosanoids and the more recently discovered 'specialized pro-resolution lipid mediators' (SPM) represent vital endogenous biochemical determinants of inflammatory kinetics and are the principle mediators of inflammatory resolution¹⁴⁹. Arachidonic acid (AA, 20:6, omega-6), docosahexaenoic acid (DHA, 22:6, omega-3) and eicosapentaenoic acid (EPA, 20:5, omega-3) are polyunsaturated fatty acids that form the substrates for the enzymatic generation of several groups of bioactive LM. Eicosanoids – the generic term for a group of bioactive lipids containing 20 carbons derived from AA – are divided into several separate mediator families, the major groups being the prostaglandins (PG, see Box 2), leukotrienes and lipoxins. More recently discovered omega-3 derived SPM families include resolvins, protectins and maresins. The formation, metabolism and signalling of these and other lipid families are comprehensively reviewed here¹⁴⁹⁻¹⁵¹.

The ability of NSAIDs to reduce the primary symptoms and signs of inflammation via cyclooxygenase (COX) inhibition and hence PG suppression has led to a common assumption that they, and LM in general, are universally pro-inflammatory. This represents a grossly simplified view. These signalling molecules are variably constitutive and inducible, expressed widely yet in a cell-type and tissue-specific manner, and their actions are diverse, multi-faceted and vary down to the receptor level. Individual LM have been shown to variably exert pro- and anti-inflammatory effects along with pro-resolution properties in a context-dependent manner.

Given their immuno-modulatory potency and diversity of action, for an effective and self-limited inflammatory reaction to be facilitated LM generation must be localized, balanced, proportionate and timely. Disturbance in any of these dimensions in isolation, or more likely combination, may contribute adversely to disease states. Several pathogenic aberrations may be hypothesized:

- 1) Location of action.
 - Compartment leakage or altered distribution of generation. Endocrine as opposed to typical autocrine or paracrine activity.
- 2) Increased or decreased concentration.
 - Altered synthesis, via host, pathogen or iatrogenic intervention, through modulation of substrate or enzymatic process.
 - Promotion or loss of catabolism (local or systemic) or the failure of feedback loops. Altered bioavailability or protein-binding (e.g. albumin).
- 3) Deranged temporal profile of production.
 - Failure or dysregulation of eicosanoid 'class switching'^{147,152}.
- 4) Up or down regulation of receptors, alteration in receptor profile, including distribution.
- 5) Modification of action (e.g. co-stimulation – additive, synergistic or anergic) by other stimuli/mediators in the surrounding inflammatory milieu

In broad terms and as alluded to earlier, two key patterns may result from such aberrations, either contributing to the anti-inflammatory immunosuppressive phenotype of CIID. Firstly, deficient or failed resolution where either an insufficient concentration of SPM are available to facilitate inflammatory function and termination, or their action is inadequate. This has been recently discussed in varying inflammatory conditions¹⁴⁹. Alternatively, a state of excessive immunoregulation may

exist driven by LM (principally PGs) involved in the initiation or control of resolution, rendering host defences locally or systemically compromised¹⁴¹.

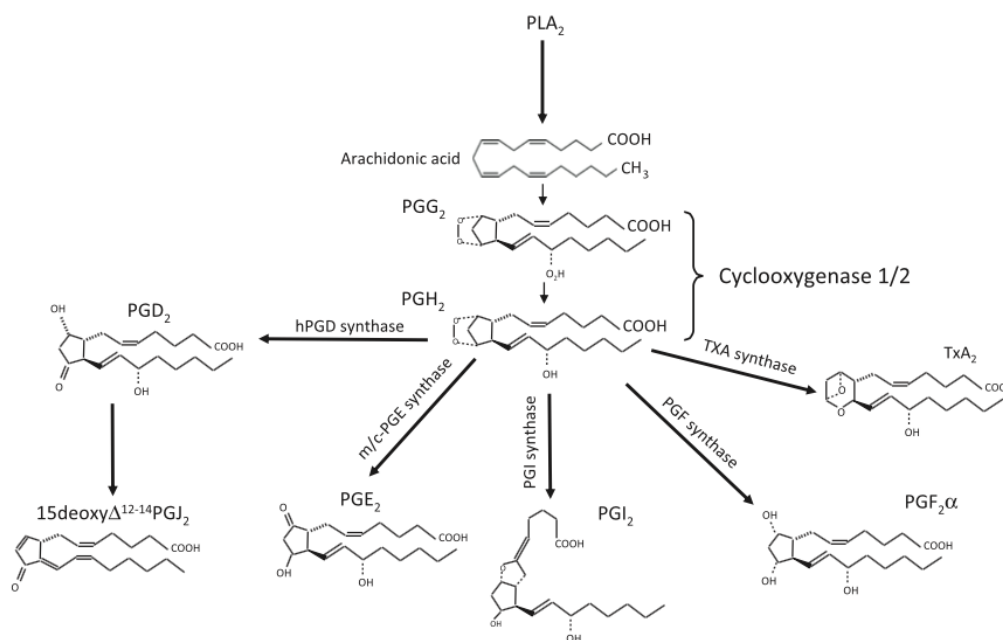
Box 2: Generation of Prostaglandins

AA is released from cell membrane phospholipids through the activity of several phospholipase enzymes, predominantly phospholipase A₂ (PLA₂). Whilst PLA₂ is expressed in numerous different isoforms it is the 85 kDa cytosolic PLA₂ which predominantly supplies AA for PG synthesis. This form requires calcium and calmodulin for activation, and is inducible by various cellular agonists including receptor-mediated agonists (e.g. IL-8, platelet activating factor), microorganisms or DAMPS¹⁵³. Once in the cytosol, AA can be metabolised (oxygenated) via three principal enzymes to form eicosanoids: lipoxygenase (LOX), the cytochrome P450 family, and COX – the enzymes responsible for PG production (see¹⁵¹ for full review of the LOX and P450 pathways).

COX exists as two predominant isoforms: constitutively expressed COX-1 and the usually undetectable COX-2, which is rapidly induced upon exposure to inflammatory stimuli¹⁵⁴. The pathways regulating expression are numerous and complex, varying between cell type and stimulus. Both are membrane-bound, residing in the endoplasmic reticulum after synthesis, have a similar molecular mass (70kDa) and are identical in length, however differ critically in their substrate binding sites and catalytic region. The substitution of Ile in COX-1 for Val in COX-2 in two positions results in COX-2 possessing a larger and more flexible substrate channel. COX-1 further demonstrates negative allosterism at low concentration of AA, suggesting that COX-2 may out compete COX-1 for AA (see¹⁵⁵ for review). COX-1 and 2 are bifunctional enzymes, first catalysing the bisoxygenation and cyclisation of AA to PGG₂, before the peroxidation of PGG₂ to PGH₂. It is this latter molecule that forms the substrate for multiple synthases to generate the biologically active prostanoids PGD₂, PGE₂, PGF_{2α}, PGI₂ (prostacyclin) and thromboxane (TXA₂) (see accompanying schematic).

Individual synthases are expressed in a tissue and cell-type selective manner. Their expression may further be modulated by the prevailing inflammatory state leading to the preferential release of specific prostanoids. As such, the 'end' prostanoid profile of a given cell is resultant on both basal and induced synthase expression and is shaped by biochemical mechanisms including preferential coupling with COX isoforms, the intracellular physical compartmentalisation of COX isoforms and

terminal synthases, and differences in the substrate affinity and kinetics of the various synthases¹⁵⁶⁻¹⁵⁸. Diversity in biological action is further afforded downstream by the binding of individual prostanoids to multiple cell surface receptors with discrete signalling responses.



Reproduced with permission from Stables and Gilroy 2011¹⁵¹

1.3.1 Specialised pro-resolution mediator deficiency

Omega-3 derived SPM from different series appear to have individually separate yet collectively beneficial effects on multiple modalities of immune function. Evidence indicates that a paucity of these LM contributes to derangement of the inflammatory profile and CIID, with therapeutic replacement restoring or augmenting immune function. Data relating to specific LM of the resolvins and protectins series, and later the lipoxins and leukotriene families will be discussed in interventional animal models of infection/inflammation.

Defining features of SPM bio-action include the ability to: i) counter-regulate mediators that summon leukocytes, in particular polymorphonuclear cells [PMN, neutrophils], to an inflamed site; ii) dampen pain; iii) stimulate non-phlogistic monocyte recruitment; and iv) activate macrophages to efferocytose apoptotic granulocytes and clear both pathogens and tissue debris¹⁴⁹. Whilst being part of the endogenous 'anti-inflammatory' process via action i) (with associated prevention of inflammatory amplification) it is attributes iii) and iv) in tandem with promotion of

phagocyte trafficking to lymph nodes¹⁵⁹ that distinguishes them from classical anti-inflammatory mediators such as interleukin (IL)-10 or IL-1 receptor antagonist¹⁴⁹.

SPM have repeatedly been demonstrated to lack an immunosuppressive action, and indeed to augment host-directed anti-microbial defenses¹⁶⁰. These molecules stimulate mucosal production of bactericidal peptides¹⁶¹ and enhance bacterial phagocytosis by PMNs and macrophages, working synergistically with antibiotics, to increase their therapeutic action and hence bacterial clearance¹⁶². They have further been shown to suppress nuclear viral mRNA transcript export, and hence replication, reducing mortality from influenza infection¹⁶³: a potentially novel therapeutic addition to standard antivirals, focused on modifying host immune capability, avoiding the problems posed by these infectious agents diversity, variability and capacity to evolve.

Resolvins and Protectins

Resolvin E1 (RvE1) administered prior to a murine model of aspiration pneumonia (hydrochloric acid with subsequent *Eschenderia coli* challenge) was associated with a reduction in pro-inflammatory cytokines, decreased pulmonary PMN accumulation, enhanced bacterial clearance and improved survival¹⁶⁴. El Kebir and colleagues have further described RvE1's ability to promote resolution of established infective and sterile models of murine lung injury¹⁶⁵. Mechanistically RvE1 was noted to enhance NADPH-oxidase reactive oxygen species generation and promote phagocytosis-induced neutrophil apoptosis (with subsequent efferocytosis by macrophages) via the leukotriene B₄ (LTB₄) receptor BLT1. Increased activation of caspase-8 and caspase-3 in tandem with attenuation of both ERK and Akt-mediated apoptosis-suppressing signals shifting the balance of pro-/anti-survival information toward apoptosis via induction of mitochondrial dysfunction. In addition, RvE1, at concentrations as low as 1nM, enhances macrophage phagocytosis, with the products of its metabolism continuing to exert pro-resolution properties but with reduced bioactivity *in vivo*¹⁶⁶.

Resolvin D1 (RvD1) pre-treatment prior to LPS-induced acute lung injury is protective, improving pathological changes and survival¹⁶⁷. The central mechanism appears to be suppression of NF-kB activation in a partly PPAR γ -dependent manner, with associated reduction in downstream signaling/transcriptomic alteration¹⁶⁸. Resolvin D2 (RvD2) but not its isomer *trans*-RvD2, has been shown specifically to improve survival in murine polymicrobial sepsis (caecal-ligation and puncture, CLP). Its actions appear multifaceted - modulating leukocyte-endothelial interactions in a direct (adhesion receptor expression) and indirect manner (endothelial NO

production), altering the cytokine profile (reduced IL-17, IL-10, PGE₂ and LTB₄), enhancing bacterial phagocytosis and intraphagosomal vacuolar production of reactive oxygen species¹⁶⁰. More recently the ability of RvD2 to restore neutrophil directionality, prevent CIID and thus increase survival from a secondary septic challenge post-burn injury has been demonstrated¹⁶⁹.

Discrete specialized pro-resolution mediators are unlikely to be produced in isolation and have overlapping pro-resolving actions. RvE1, aspirin-triggered (ATL, 15-epi-lipoxin A₄) and protectin D1 may independently rescue COX and lipoxygenase (LOX)-derived 'resolution deficits' *in vitro* and *in vivo*, with actions extending to promotion of phagocyte trafficking away from the primary inflammatory site¹⁵⁹. The ability to bind and act as agonists on alternate SPM receptors (e.g. RvD1 on the lipoxin A₄ (LXA₄) receptor¹⁶⁷) may provide one pharmacological explanation for this phenomenon. However, despite their common actions the source of different classes of SPM in inflammation appears diverse. Recent evidence suggests that RvE1 and 2 are synthesized by PMNs via the 5-LOX pathway¹⁷⁰, whilst eosinophils are responsible for generation of 12/15-LOX-derived mediators protectin D1 and the newly discovered resolvin E3^{171,172}. Deficiency of these cell types in the resolution phase may lead to impaired biosynthesis with deleterious consequences¹⁷¹. The same may be true of poly-unsaturated fatty acids at the inflammatory site.

Experimentally, the omega-3 resolvin precursors EPA and DHA have been demonstrated to increase in exudates during the resolution phase, being both plasma (partially bound to leaked albumin) and locally derived^{149,173}. Whilst early indirect evidence to support the therapeutic benefit of increasing SPM series concentration in humans came from the physiological and clinical benefits observed in randomised controlled trials of fish oil addition to parenteral or enteral nutrition in septic patients^{174,175}, this effect has not been confirmed in recent meta-analyses^{176,177}. There is additionally no proof to date that dietary omega-3 supplementation is directly correlated with SPM formation in humans during inflammation¹⁷⁸.

Leukotrienes and Lipoxins

Therapeutic use of the AA-derived lipoxin series may also be beneficial. Post-insult treatment with LXA₄ has been demonstrated to limit inhaled LPS-induced lung injury¹⁷⁹, and to reduce pro and anti-inflammatory cytokine production, enhance macrophage recruitment, reduce blood bacterial load and improve mortality in a rat CLP model¹⁸⁰. In this later study macrophage recruitment was increased without impairing phagocytic function, and systemic inflammation reduced without increasing

bacterial spread, mirroring the previously described observations with other SPM
160,164.

A similar paradoxical relationship between an attenuated innate immune response (PMN trafficking to the infected site), yet efficacy of the overall inflammatory process, as determined by survival, has been demonstrated in both wild-type mice treated with MK 886 (a 5-lipoxygenase[5-LOX] inhibitor) and in 5-LOX-deficient mice¹⁸¹. This effect could be partially replicated if antagonists of cysteinyl-leukotrienes (cysteinyl-LTs, a family including LTC – F₄) were given, but crucially not with antagonism of the classically pro-inflammatory LTB₄. This elegantly demonstrates the hierarchical, multi-faceted and often opposing effects of eicosanoids in sepsis. In this setting it appears that the prevention of the cysteinyl-LTs deleterious effects on the vasculature, hence host haemodynamics, assumes primacy as the main cause of benefit in 5-LOX antagonism. In contrast selective LTB₄ inhibition prior to and post-CLP appeared to have little effect on vascular tone and permeability, but may have blunted the innate immune response - specifically neutrophil trafficking - exacerbating the infective insult^{182,183}.

The complex interplay between AA-derived LMs in sepsis has also been highlighted in recent data showing flavocoxid, a dual COX-2 and 5-LOX inhibitor, reduced the expression of NF-κB, COX-2 and 5-LOX with resultant improved survival in a murine-CLP model¹⁸⁴. Plasma IL-10 and LXA₄ concentrations were increased whilst TNF-α, IL-6, LTB₄ and PGE₂ were decreased. Whether the observed improvement in outcome is due to enhanced pro-resolution effects driven by increased LXA₄, decreased cytokine storm (TNF-α and IL-6), augmentation of the immune response via reducing PGE₂ and 5-LOX derived LTs (discussed below), selective shunting of AA down the COX or LOX pathways, or a combination of the above is unclear.

Separately, biologically active ATL and aspirin-triggered resolvins have been noted to have pro-resolution effects, inhibiting leukocyte trafficking in a NO-dependent manner in both murine¹⁸⁵ and human inflammation¹⁸⁶, and down-regulating superoxide production in neutrophils along with macrophage inflammatory peptide 2 and IL-1β production¹⁸⁷. The importance of ATL and LXA₄ inhibitory stimulation has been previously demonstrated in CI; its absence leading to unbridled inflammation and elevated mortality in animal models of infection due to DC hypersensitivity¹⁸⁸.

Thus, accumulating evidence supports the notion that SPM are both necessary to the host immune response and beneficial in severe inflammatory states. Increasing lipoxin, resolvins, and protectin concentrations augments host defence and improves

survival in pre-clinical models of CI via multi-modally enhancing innate immune function and ameliorating CIID. The immunomodulatory actions of SPM on inflammatory resolution processes are summarized in Figure f1.3.

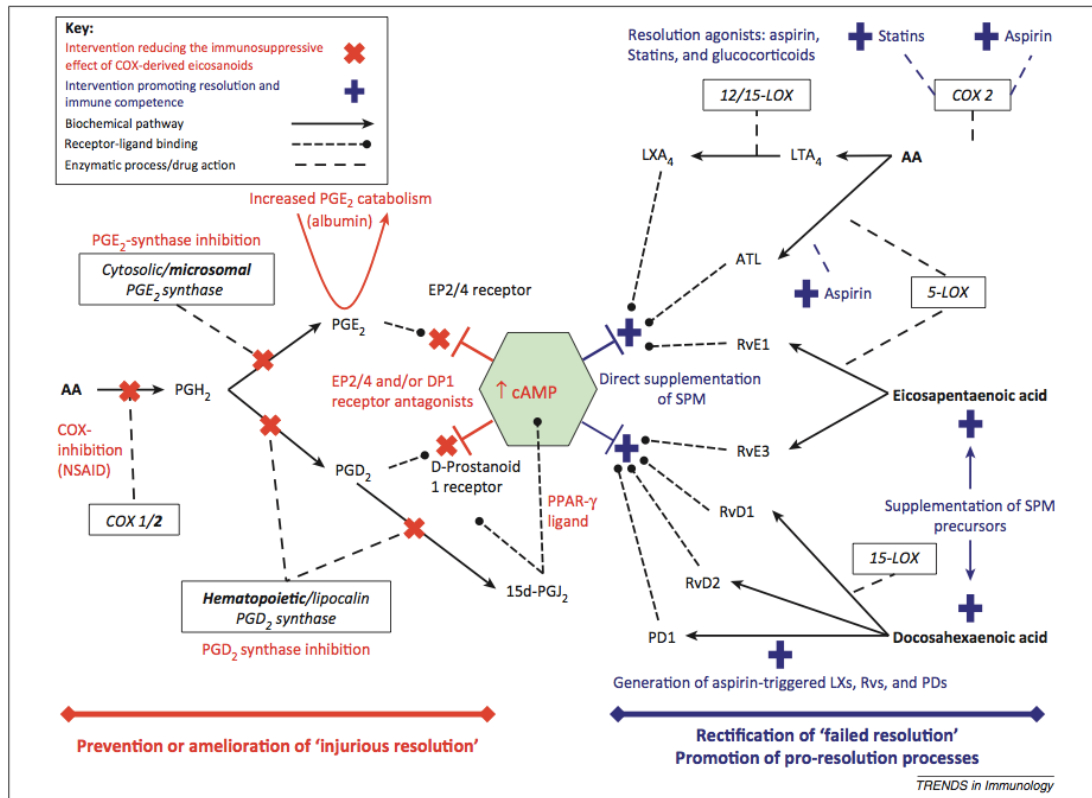


Figure f1.3: Potential therapeutic interventions to modify resolution defects and improve innate effector cell functionality in critical illness. Targets on the left of the diagram (red) describe means of reducing excessive or prolonged production of immunosuppressive COX-generated PGs or antagonizing their action. Targets on the right (blue) indicate means of supplementing and/or augmenting levels of SPMs that nonphlogistically enhance multiple effector modalities. AA, arachidonic acid; ATL, aspirin-triggered lipoxin (15-epi-LXA₄); COX, cyclooxygenase; EP2/4, E prostanoid 2/4 receptor; LOX, lipoxygenase; LT, leukotriene; LX, lipoxin; PD, protectin; PG, prostaglandin; PPAR, peroxisome proliferator-activated receptor; Rv, resolvin; SPM, specialized proresolving lipid mediator. Reproduced with permission from Fullerton et al.¹⁸⁹

1.3.2 Cyclooxygenase-derived eicosanoid excess

Pre-Clinical Evidence

The majority of the interventional studies described above have used a paradigm of substrate supplementation, direct molecule addition or aspirin-augmented biosynthesis to highlight the benefits of SPM in CI. Their pathogenic contribution to severe inflammatory states thus appears to be one of absence or insufficiency. Conversely, excess (absolute or relative) or persistence of early-phase LM, specifically prostaglandins, may be equally deleterious to host immune function. Indication of their role in CI is consequently derived from the reverse methodology - their reduction or antagonism.

Like humans, severe inflammatory stress in mice is associated with a predominantly anti-inflammatory state and relative immunosuppression following the initial pro-inflammatory response. This is characterized by increased susceptibility to and worse outcomes from infection¹⁹⁰⁻¹⁹², mimicking clinical observations¹⁹³ and thus providing a model to explore CIID. Vulnerability to secondary infective challenge (replicating HAI) is dependent on time post-initial insult, decreasing with increased temporal separation^{191,192}, and may be reduced by therapeutic strategies designed to restore immune function¹⁹¹. PGE₂ appears to be a key mediator of this phenomenon.

Bronchoalveolar lavage fluid in mice subjected to pulmonary *Aspergillus fumigatus conidia* challenge post CLP contains higher amounts of PGE₂, the production of which seems dependent upon both alveolar macrophages and epithelial cells¹⁹⁴. Treatment with ketoprofen (a non-selective COX-inhibitor) after CLP but prior to fungal challenge reduced the PGE₂ concentration leading to enhancement in neutrophil recruitment, macrophage phagocytosis and pro-inflammatory cytokine secretion, with a consequent four-fold increase in survival. The central mechanism underlying suppression of innate immune effector cell function was elevation of intracellular cyclic adenosine monophosphate (cAMP) concentration via the E-prostanoid (EP) 4 receptor¹⁹⁴. The efficacy of COX-2 inhibition (via NS-398) in attenuating critical illness-induced PGE₂-mediated immune suppression has also been demonstrated in response to alternate severe inflammatory stressors: mortality from infective challenge post trauma/haemorrhage being reduced by NS-398 administration (a selective COX2 inhibitor)^{195,196}.

In addition to signalling via the EP4 receptor, other G protein-coupled EP receptors have also been implicated as mediators of immune suppression. Aronoff and co-workers demonstrated that selective knockout of the G_i-coupled EP3 leads to

increased survival after intra-pulmonary *Streptococcus pneumoniae* or intra-peritoneal LPS challenge¹⁹⁷. EP3^{-/-} mice exhibited increased pulmonary clearance of bacteria despite reduced accumulation of lung neutrophils, lower number of circulating leucocytes and an impaired febrile response to infection, the key mechanism being augmented alveolar macrophage phagocytic and bactericidal capacity. Interestingly, alveolar macrophages may additionally be compromised by a PGE₂-G_s-coupled EP2-cAMP axis, with both specific EP2 receptor antagonists or EP2-deficient mice preventing PGE₂-induced impairment of Fcγ receptor-dependent phagocytosis and H₂O₂-mediated, NADPH oxidase-generated bacterial killing^{198,199}. These phenomena appear to be related to PGE₂-induced alterations in scavenger receptor and microRNA-155 expression²⁰⁰, and inhibition of the p40phox subunit of NADPH oxidase respectively²⁰¹. Indeed the most frequently observed and reproducible immunosuppressive pathway in macrophage populations appears to be either EP2 or EP4-mediated increase in cAMP with subsequent activation of protein kinase A (PKA) and associated downstream effects. These have demonstrated relevance to clinical infection outside the respiratory tract^{202,203}.

Beyond functional deficits, PGE₂ may additionally impair microbial sensing. PGE₂, again via the G_s-coupled EP2 and EP4 receptors and unanchored type 1 PKA has recently been shown to reduce the key PAMP recognition receptor TLR4 at the translational level, having similar kinetics to cycloheximide in rat alveolar macrophages²⁰⁴. Such alterations may compound the pleiotropic effects PGE₂ exerts on the nascent TLR response post-ligation²⁰⁵, and represents an additional mechanism to previously established effects on transcription, proteolysis and secretion of proteins²⁰⁶. It further represents a novel explanation for the reduction in pro-inflammatory cytokine release observed in LPS-stimulated monocytes or macrophages pre-treated with PGE₂²⁰⁷⁻²⁰⁹.

Temporal Correlation and Source of Prostanoid Generation

Alterations in COX-2 expression and PGE₂ synthesis correspond with the observed temporally defined window of immunosuppression following CI. Higher circulating plasma concentrations of PGE₂ have been recorded on both day 1 and 7 post-severe inflammatory stress in mice compared to controls, suggesting exaggerated and prolonged prostanoid production¹⁹⁶. Patients who have experienced major trauma were reported to have plasma PGE₂ concentrations that peaked 5-7 days post-insult at 400% of control, the source being identified as circulating 'inhibitory' monocytes. If PGE₂-release was ablated in *ex-vivo* culture of patient's PBMCs by the addition of an NSAID, immune competence, as determined by IL-2 release, was restored²¹⁰. This

result was replicated *in-vivo* in a small clinical trial of IV indomethacin (a non-specific COX-inhibitor) administration following major surgery, where multimodal improvements in cell-mediated immunity were observed including monocyte kinetics and lymphocyte co-stimulatory receptor expression²¹¹. Crucially, it has also been shown that PGE₂-mediated immune cell suppression may not merely arise from increased concentration of this LM but secondary to amplified sensitivity to it, likely through an alteration in receptor profile: burn patient's displaying both elevated levels of PGE₂ and a proportionally greater functional reduction at any given concentration²¹².

With regards to the source of circulating PGE₂ it is likely multi-focal. Splenic macrophages were found to have increased COX-2 mRNA expression in response to LPS stimulation even at day 7 post-severe inflammatory stress and are thus one potential source of endocrine PGE₂^{195,196}. Alternatives may be liver (Kupffer cells) and alveolar macrophages, or peripheral blood mononuclear cells (PBMCs), all of which demonstrated increased COX-2 expression via real-time PCR in either murine models or patient samples¹⁴². Increased PGE₂ concentrations may additionally arise downstream and independent of COX, stemming from selective induction of microsomal-PGE₂ synthase 1 (mPGES-1) by CI states¹⁹⁴. In comparison, peritoneal neutrophils from burn-injured animals have been reported to exhibit a late-phase decrease in COX-2 expression and PGE₂ synthesis coupled with a lack of induction to secondary infective challenge²¹³, likely indicating the compartmentalization of the inflammatory response and/or potential compensatory downregulation in the face of elevated circulating concentrations of PGE₂^{76,214}.

As intimated above there is considerable evidence that CI also modifies AA release and its COX-mediated metabolism in humans as well as mice. In patients with fracture or burn injury, peripheral blood mononuclear cells exhibit increased COX-2 mRNA and PGE₂ synthesis in response to LPS stimulation²¹⁵. In contrast, COX-2 gene expression in septic patients has been reported as reduced such that both basal circulating concentrations of its metabolites (including PGE₂) and those induced by LPS-stimulation of blood leukocytes *ex-vivo*, were lower than in healthy controls²¹⁶. Further, the degree of AA-metabolism derangement was associated with disease severity (greater in septic shock vs. severe sepsis), and failure of its recovery between admission to ICU and day 3 post-admission was predictive of adverse clinical outcome (prolonged admission or death). The authors speculate that reduced prostanoid generation forms part of the anti-inflammatory CIID phenotype.

This seemingly opposing data may be potentially be rationalized by two inter-linked hypotheses: 1) that altered AA-metabolism and PGE₂ generation in the blood compartment either represents an adaptive change to prevent systemic inflammation^{76,121}; or 2) a response to excess production extra-vascularly (i.e. the primary infective/inflammatory site). Up- and down-regulation of prostanoid receptors has been observed in CI humans²¹⁵ along with alterations in COX-2 and mPGES-1 expression²¹⁶ indicating a dynamic, responsive system. In mice the EP2 and EP4 receptors are differentially expressed on monocytes depending on activation status^{217,218}, and may contribute to a negative feedback loop that suppresses COX-2 and thus PGE₂ synthesis upon ligand binding²¹⁹. Late-phase immunosuppressive mediators are thought to arise from tissue-resident macrophages *in vivo*²²⁰. It would thus not be surprising if endocrine release of PGE₂ into the circulation from macrophages local to the primary event (or indeed in distal tissue beds) induced compensatory adaptations in blood leukocytes.

Immune Dampening by Apoptotic Cells and Efferocytosis is PGE₂-Mediated

Autopsy studies in septic patients have demonstrated profound apoptosis-induced loss of follicular dendritic cells (DC), B-cells, and CD4+ T-cells in secondary lymphoid organs^{193,221-223}. Whilst mature macrophage numbers and MHC II expression may be relatively maintained²²³, immature or developing macrophages have been shown to be at particular risk (murine CD68+CD11b^{hi}F4/80^{inter})²²⁴. Increased numbers of circulating apoptotic lymphocytes and mononuclear cells are seen in septic patients, levels correlating with disease severity, being higher initially in septic shock than sepsis alone^{225,226}. Failure to restore the undamaged lymphocyte count (notably CD8+ T cells) by day 6 post-insult in CI patients, implying on-going excessive apoptosis, is predictive of in-patient mortality²²⁶. These observations have led to dysregulation of apoptosis being accepted as a key contributor to immunosuppression^{123,227}. Animal studies support this supposition.

Adoptive transfer of AC prior to induction of sepsis is associated with a significant increase in mortality compared to controls, whereas transfer of necrotic cells is protective, an effect in which IFN γ appears pivotal²²⁸. Multiple other studies have also demonstrated the benefits of modulating (reducing) excess apoptosis to restore both innate and adaptive immune function via TNF-related apoptosis-inducing ligand (TRAIL)^{229,230}, IL-15²³¹⁻²³², IL-7²³³, caspase-1 inhibition²³⁴, Fas/Fas-ligand²³⁵ and programmed cell death protein 1(PD-1)^{236,237} in models of CI. Rectification of the apoptotic-inflammatory imbalance may thus represent an attractive therapeutic avenue^{238,239}.

Mechanistically, aside from the consequences of direct effector cell loss, AC exert independent multimodal immuno-modulatory effects. Uptake of AC by macrophages and dendritic cells (DC) – efferocytosis - triggers the release of anti-inflammatory IL-10, transforming growth factor (TGF)- β , nitric oxide (NO) and PGE₂ in addition to reducing TLR-4 mediated pro-inflammatory cytokine secretion in response to subsequent LPS challenge²⁴⁰⁻²⁴³. Purported mechanisms include both translational down-regulation and transcriptional inhibition by autocrine/paracrine mediators including TGF- β ²⁴⁴, PGE₂ and platelet-activating factor²⁴¹. Down-regulation of co-stimulatory molecule expression by DC after phagocytosing AC induces immune tolerance²⁴⁵⁻²⁴⁷ and may in part be responsible for alterations in the adaptive immune response in CI^{248,249}.

Medeiros and colleagues¹⁹⁸ have shown that the efferocytosis of AC by activated macrophages causes suppression of Fc γ receptor-mediated phagocytosis and bacterial killing by an autocrine/paracrine PGE₂ mechanism in the lung. EP2 receptor-mediated elevation of intracellular cAMP appeared to be the primary pathway in AC-induced functional impairment, being reversed with aspirin or indomethacin pre-treatment and direct EP2-receptor antagonists¹⁹⁸. This finding reinforces a previously described immunosuppressive role for the PGE₂-EP2-cAMP axis, being separately demonstrated to reduce both phagocytosis²⁵⁰ and NADPH oxidase-mediated killing¹⁹⁹ in a dose dependent manner. Other authors have implicated TGF- β ²⁵¹, potentially via PGE₂ again (directly or indirectly²⁵²) or 15-LOX derived lipoxins A₄ (LXA₄)²⁵¹, and an IFN γ /NO mechanism²⁵³ as the soluble immunosuppressive mediators involved in the effect of AC on phagocytes. Such heterogeneity in data indicates that AC have pleiotropic effects, the predominant pathway likely depending on the subset, phenotype and activation state of the efferocytosing phagocyte^{198,254}.

Evidence that AC induced PGE₂ may not only contribute to failure of primary pathogen clearance but CIID and susceptibility to subsequent nosocomial challenge also exists. Intra-pulmonary pre-treatment with apoptotic thymocytes leads to reduced PMN recruitment and greater lung and bloodstream bacterial burden after *Streptococcus pneumoniae* challenge, an effect not seen in EP2^{-/-} KO mice¹⁹⁸. This mirrors work showing that alveolar macrophages derived from these mice display enhanced *ex-vivo* pro-inflammatory cytokine production (TNF- α , MIP-2), phagocytosis, intracellular killing and reactive oxygen species generation, functions which translate to a reduced bacterial burden and improved survival on *in-vivo* challenge²⁵⁵. Part of this additional anti-microbial capacity may derive from greater

macrophage maturity. PGE₂ was recently demonstrated to restrain macrophage maturation *in-vitro* via EP2-mediated protein kinase A activation with septic EP2^{-/-} mice exhibiting a higher percentage of F4/80^{high}/CD11b^{high} cells and greater expression of macrophage colony-stimulating factor receptor in both the blood and the inflammatory site²⁵⁶.

The immunosuppressive pathogenicity of PGE₂ in CIID may be extended by its regulatory role in apoptosis of phagocytes. Early studies demonstrated a mixed effect of PGE₂ in this capacity, being demonstrated to either induce apoptosis during T-cell differentiation²⁵⁷, or inhibit apoptosis in PMNs²⁵⁸ through a cAMP mediated mechanism^{258,259}. Recent data indicates that PGE₂, or its metabolites PGA₂ (via non-enzymatic dehydration) and 15-keto PGE₂ (catalyzed by 15-hydroxyprostaglandin dehydrogenase) promote apoptosis in several cell types through Bcl-2-associated X protein (Bax). PGD₂ may however inhibit PGE₂ binding to Bax and hence PGE₂-mediated apoptosis, the ratio determining cell fate²⁶⁰. Comparative levels of other eicosanoids to PGE₂, particularly LXA₄, may additionally influence whether phagocytes undergo necrosis or apoptosis, PGE₂ again representing the pro-apoptotic factor via EP2/4 signaling²⁶¹.

In summary, this data indicates that, contrary to popular conceptions, COX-derived eicosanoids, and in particular PGE₂, have significant anti-inflammatory and immunosuppressive effects (see Figure 1.3). In both animal and human studies, CI induces alteration of prostanoid dynamics either as part of the primary inflammatory cascade or as part of resolution-regulating programmes. Sustained or excessive production of these resolution-regulating eicosanoids alone or in conjunction with other 'vulnerability factors' (e.g. reduction in serum albumin¹⁴²) may be pathogenic, contributing to CIID, increasing susceptibility to secondary infection and reducing patient's ability to clear primary infections. The efficacy displayed by COX-inhibition and PGE₂ antagonism in pre-clinical work clearly warrants further investigation²⁶².

Non-Immune Benefits of PGE₂ Suppression or Antagonism

PGE₂'s actions are multi-faceted, impacting on all aspects of Virchow's classic signs of inflammation - calor, dolor, rubor and tumour – by virtue of the wide but varied distribution and different signalling pathways of its four receptors²¹⁹. Pursuance of a strategy of PGE₂ ablation or, ideally, selective EP receptor antagonism, may therefore have additional benefits in the CI aside from restoration of immune competence.

CI states are frequently marked by recalcitrant hypotension necessitating the use of vasoactive inotropic drugs and excessive vascular leak with resultant protein-rich tissue fluid and clinical oedema, secondary to multiple mechanisms and mediators^{263,264}. In animal models PGE₂ has been shown to exert diverse effects on vascular tone dependent on the vascular bed tested, concentration employed and presence or absence of alternate agents (e.g. noradrenaline, potassium)²⁶⁵. This appears to relate to the relative expression of EP receptor subtypes: EP3 being more prevalent centrally (e.g. aortic rings) and promoting contraction, whilst EP4 is found in distal vessels and causes dilatation (e.g. mesenteric arteries and tail)²⁶⁶ - the central mechanism of which appears to be endothelium-dependent stimulation of eNOS activity that results in cGMP-mediated vasorelaxation²⁶⁷. It is thus theoretically possible that the central/peripheral split in PGE₂'s effect could be exploited; either using pan-blockade to achieve central vasodilation and peripheral vasoconstriction to maximise vital organ support, or employing selective EP4 antagonism as a selective peripheral vasopressor to maintain blood pressure²⁶⁸. With regards to the latter strategy this may also have benefits on vascular leak, Omori and colleagues recently demonstrating that both EP2 and EP4 signalling increases blood flow *and* vascular permeability²⁶⁹. Whilst EP3 was observed to exert the opposite effect in this paper – enhancing the endothelial barrier – a conflicting role for the receptor in mediating mast cell activation, histamine release and hence inducing permeability has been described²⁷⁰. Whilst it is clear that further mechanistic and proof-of-principle work needs to be conducted, the benefits of a novel strategy to reduce both vasodilation and vascular permeability would be profound, being exemplified by the success of IFN β1a in reducing mortality from acute respiratory distress syndrome (ARDS) in a preliminary trial via increasing lung CD73 expression and hence endothelial barrier protective adenosine release²⁷¹.

Conceptually, further benefits may arise from antagonism of both PGE₂'s role in temperature regulation and nociception. PGE₂ is central to the generation of fever, acting in the median pre-optic nucleus via the EP3 receptor to disinhibit central heat-conserving and heat-generating pathways²⁷². The potential benefits and hazards of therapeutic temperature control (or not) is currently a key topic in the management of the CI, with various strategies proposed and debated^{273,274}. Whilst there is a strong theoretical case for the benefit for fever suppression in the critically ill, particularly with regards to physiological work, the failure of both the classic ibuprofen in sepsis study and a recent trial of paracetamol in the same context to demonstrate benefit casts doubt on whether this promise will translate to reality^{275,276}. Finally, PGE₂ is also

a key mediator of inflammatory pain both at peripheral sites and select dorsal root ganglion neurons, primarily through the EP4 receptor^{277,278}. Reduction of visceral and neuropathic pain in CI may serve to both reduce sympathetic outflow (with attendant physiological benefits) and ameliorate patient discomfort, reducing sedation and analgesic requirements, potentially impacting psychological outcomes. As with all the above modes of action, proof of clinical action requires to be established

1.3.3. *Feast and Famine: A single or dual defect?*

It is unlikely that either an excess of immunosuppressive eicosanoids or a deficiency of SPM exists independently in CI, their production being inextricably inter-linked. Over the course of the inflammatory response, lipid-mediator profiles undergo a 'class switch' from initial-phase COX-derived PGs and LTs to specialized pro-resolution mediators of the lipoxin, resolvin and protectin series, with PGE₂ playing a controlling role^{147,152}. In particular there is now evidence to suggest that different phagocytic cell types and their subpopulations display specific eicosanoid profile signatures that are dynamically altered at defined intervals throughout inflammation, influenced by the surrounding milieu and ingested material²⁵⁴.

Recent data demonstrate that severe inflammatory stress undermines these normally tightly regulated resolution programs. In a murine peritonitis model contrasting low dose, self-resolving inflammation with high-dose, non-resolving inflammation (10mg vs. 1mg zymosan), sustained high amounts of PGE₂ and LTB₄ (>5x normal) were reported in the high-dose exudates along with persistently compromised SPM production (LXA₄, protectin D1 and resolvins ~3-fold less than normal concentrations)²⁷⁹. Alterations in microRNA (miRNA) expression, specifically miR-219-2, and subsequent target gene expression were implicated. This situation appears analogous to the dysregulated transcriptomic and inflammatory response associated with adverse outcomes in CI⁴². Humans display different inflammatory response profiles to set stimuli that are largely determined by resolution-processes and mediators, broad categories of 'resolution phenotype' being established pre-clinically²⁸⁰ and tentatively in various clinical settings^{281,282}. I hypothesise that inflammatory stress of sufficient magnitude, in individuals rendered susceptible via their resolution phenotype, may trigger higher and persistent amounts of initial-phase eicosanoids with concomitant failure of SPM generation, resulting in both injurious *and* failed resolution (see Figure f1.4). Such dysregulation of lipid mediator synthesis may both exacerbate the acute systemic inflammatory response (SIRS) and subsequently contribute mechanistically to late-phase CIID¹⁴¹.

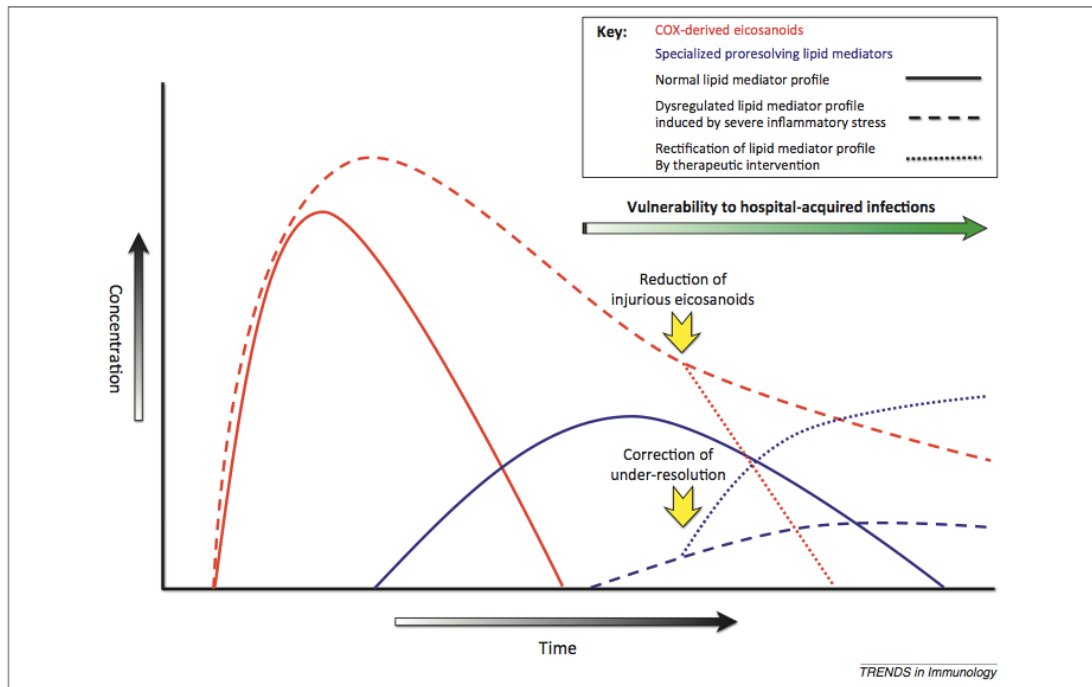


Figure f1.4: Proposed dysregulation of resolution-phase lipid mediators in critical illness. Solid lines indicate the normal inflammatory profile, with an early rise in cyclooxygenase (COX)-derived prostaglandins (PGs) and 5-lipoxygenase-derived leukotriene B₄, which trigger a subsequent rise in specialized proresolving lipid mediators (SPMs) including lipoxins, resolvins, and protectins. Dashed lines display the altered profile of eicosanoids in critical illness-induced immune dysfunction, with persistence of early-phase lipid mediators that exert negative immunomodulatory effects and a paucity or relative insufficiency of SPMs, which non-phlogistically augment several key resolution pathways including bacterial clearance. The combination of this dual defect contributes to vulnerability to hospital-acquired infection. Identification of aberrant eicosanoid profiles in critically ill patients may allow their therapeutic correction or antagonism to ameliorate effector cell functional impairment as indicated by the dotted lines. Reproduced with permission from Fullerton et al¹⁸⁹.

1.4 Hypothesis

Prostaglandin E₂ mechanistically contributes to critical-illness induced immune dysfunction and represents both a predictive biomarker and potential therapeutic target.

1.5 Aims

In order to address this hypothesis I intend to:

- a) Conduct a scoping systematic review exploring the clinical literature for evidence of either a beneficial or detrimental effect of non-steroidal anti-inflammatory drugs (NSAIDs, including aspirin) in acute infection. This will include both altered incidence, presentation or progression of infection in those taking NSAIDs for alternate indications, and their ability to modify outcomes from infection when prescribed acutely.
- b) Characterise the effect of PGE₂ in clinically validated assays of immune dysfunction, describing novel bioassays that determine this lipid mediator's contribution to observed alterations in pre-clinical and clinical samples.
- c) Employ intravenous endotoxemia to elucidate the time-course of immune dysfunction and the plasma lipid mediator profile elicited by systemic inflammation in humans. Ex-vivo assays characterised in b) will be employed to quantify the functional contribution of PGE₂ to observed changes.

1.6 Expected Outcome

Implication of PGE₂ as a key driver of immune dysfunction in critically ill patients experiencing excessive dysregulated inflammation, with consequent identification of a novel pathway through which to restore immune competence and reduce acquisition of nosocomial infection.

CHAPTER 2: General Materials and Methods

2.1 Human Blood

2.2 Mono Mac 6 Cell Line

2.3 Assays

2.4 Flow Cytometry

2.5 Intravenous Endotoxemia

2.6 Statistical Analysis

2.7 Ethical Approval

Publications:

Fullerton JN, Segre E, De Maeyer RPH, Maini AAN and Gilroy DW. Intravenous endotoxin challenge in healthy humans: An experimental platform to investigate and modulate systemic inflammation. *Journal of Visualised Experimentation*. *In Press*

2.1 Human Blood

Whole blood, primary cells (peripheral blood mononuclear cells [PBMCs], monocyte-derived macrophages [MDMs]) and both plasma and serum were obtained from healthy, consenting male and female volunteers (aged 21 - 50 years).

2.1.1 Human Plasma and Serum

Whole blood was obtained from the median cubital vein using a 20g butterfly needle and aseptic non-touch technique (ANTT). BD Vacutainer® tubes pre-filled with different anti-coagulants (ethylenediaminetetraacetic acid [EDTA], lithium and sodium heparin [LH, 17 IU/mL] and sodium citrate [0.129M]) were employed to collect plasma. After repeated inversion tubes were immediately centrifuged (2000g/10mins/20°C) and the layer of platelet rich plasma aspirated and stored at -80°C. Serum was obtained via BD Vacutainer® serum separator tubes (SST). After repeated inversion tubes were left on the bench top for 30mins to allow clotting. They were subsequently centrifuged (2000g/10mins/20°C) and serum removed via pipette and stored as above.

2.1.2 Peripheral Blood Monocyte-Derived Macrophage Isolation (1)

Different techniques may be employed to isolate cell sub-populations from total blood leukocytes. The overall aim is to maximise recovery (yield), viability, and functionality of cells to ensure downstream assay consistency and reliability. Broadly, PBMC may be separated via either their physical characteristics – most commonly by density-gradient centrifugation and/or adherence to different substances – or their cell surface marker profile. The latter may employ positive (selecting for markers associated with the sub-population e.g. using fluorescence-activated cell sorting) or negative (removing cells possessing lineage markers inconsistent with the desired population e.g. antibody-bound magnetic beads) selection. Isolated cells may be then be further sub-divided using the above methods (in combination) or via incubation with reagents known to cause a shift in the phenotypic or maturation profile. Techniques involving separation by physical characteristics, whilst less specific than antibody-dependent methodologies, are felt to preserve the *in vivo* functional state, being less likely to ‘activate’ the target cell population or have off-target effects. Consequently this strategy was employed initially.

Monocytes were isolated from heparinized venous blood (5U/mL) by density-gradient centrifugation (800g/30mins/20°C/no brake) with Lymphoprep (Axis-Shield). The isolated PBMC layer was extracted, washed twice in sterile phosphate buffered saline (PBS, Gibco, Grand Island NY), and re-suspended in RPMI 1640 (Gibco).

10mL 5×10^6 cells/mL cell suspension was plated onto sterile 8cm² tissue-culture plates (Nunc™), incubated for 2 hours (37°C/5% CO₂) before being washed with PBS to remove non-adherent cells, and adherent cells re-covered with 10mL RPMI 1640 (Gibco, Grand Island, NY) supplemented with 1% Penicillin/Streptomycin (Gibco) and 10% fetal calf serum (FCS, Invitrogen™) and incubated for four days (37°C/5% CO₂) to allow differentiation. Adherent cells (MDMs) were scraped, centrifuged (250g/5mins/20°C) and re-suspended in 1mL X-Vivo (Lonza, UK) prior to counting. MDMs were diluted in X-Vivo to a concentration of 1×10^6 cells/mL, plated in sterile 96-well (Corning®Costar®) tissue culture plates at 100,000 cells/well (in 100µL) and incubated overnight (37°C/5% CO₂). The following day all media was aspirated and replaced in preparation for reagent addition. Protocol adapted from Smith and co-workers²⁸³.

Yield and cell purity was assessed via microscopic examination of morphology, vital dye exclusion (2.1.6) and flow cytometry (2.4). Whilst acquired cells were responsive to stimulation (5.2.2), MDM yield was variably between 2 and 30×10^6 MDM from 200mL of blood rendering experimental planning challenging ($\sim 1 \times 10^7$ cells required per 96-well plate at 100,000 cells/well). Yield was both inter and intra-volunteer dependent. Additionally, purity of the acquired MDM population was inconsistent and frequently well below the accepted cut-off of 95%: CD14+ cells (monocytes/macrophages) comprising 25-50% of acquired cells, the remainder being predominantly CD3+ or CD19+ (T or B-cells respectively). In light of these considerations an alternate methodology, employing both physical characteristic and antibody-mediated selection, for acquiring MDM was trialled.

2.1.3 Peripheral Blood Monocyte-Derived Macrophage Isolation (2)

100mL of blood taken in EDTA-coated Vacutainers® was stained with 12.5µL/mL of RosetteSep™ Human Monocyte Enrichment Cocktail (StemCell Technologies, Cambridge, UK) for 20min under constant agitation. RosetteSep™ represents a specific antibody cocktail (CD2, CD3, CD8, CD19, CD56, CD66b, CD123 and glycophorin A) that crosslinks un-wanted cells to erythrocytes to form immunorosettes, facilitating purification via negative selection. Monocytes were isolated by density-gradient centrifugation (1000g/40mins/20°C/no brake) with Ficoll-Paque™ (GE Healthcare), the cell layer being aspirated via pipette, transferred to a new vial, and centrifuged (300g/10min/10°C). Cells were re-suspended in ACK lysis buffer (2.1.5) to remove residual erythrocyte contamination, washed twice in 30mL HBSS and centrifuged at 120g (10mins/20°C) to reduce platelet contamination. After counting (2.1.6) monocytes were plated at 4million cells/well in complete X-Vivo 15

medium (10% pooled human serum) in a 6-well polystyrene plate and allowed to adhere over 2 hours, prior to medium aspiration and replacement to reduce lymphocyte contamination. Media was supplemented with 20 ng/mL macrophage-colony stimulating factor (M-CSF) and cells incubated (37°C/5% CO₂) with media replacement every 3 days to allow differentiation.

After 6 days media was aspirated and replaced with 4 mg/ml lidocaine and 10 mM EDTA in PBS and incubated for 20 mins (37°C/5% CO₂) prior to gentle scraping to obtain monocyte-derived macrophages. After washing twice in HBSS, cells were assessed for purity, counted and seeded in 96 well tissue culture-treated plates (Corning CoStar, Corning, NY) at 50,000 cells/well in 50 µL complete X-Vivo 15 supplemented with 20 ng/mL M-CSF. After incubation overnight cells were stimulated as per desired experimental conditions.

This technique was found to exhibit greater consistency of yield (~10x10⁶ cells from 100 mL blood) and purity (MDM routinely exceeding 98%), as indicated above. Viability was universally >95%.

2.1.4 Peripheral Blood Mononuclear Cell Isolation

20 mL whole blood (BD EDTA Vacutainer®) was layered over SepMate 50™ tubes (Stemcell Technologies™, Cambridge, UK) pre-filled with 15 mL of density gradient medium (Ficoll-Paque™), topped up to 50 mL total volume with Hank's Balanced Salt Solution (HBSS, Gibco) and centrifuged at 1200g (10 min/20°C/high break). The resultant PBMC-enriched supernatant was rapidly decanted into a second tube, topped up to 50 mL with HBSS to wash the cells, and spun as previously to leave a pellet of purified PBMC. Residual erythrocyte contamination was, if required, reduced via ACK lysis (as per 2.1.5).

2.1.5 Ammonium-Chloride-Potassium Erythrocyte Lysis

Leukocytes were isolated from whole blood via the addition of Ammonium-Chloride-Potassium (ACK) lysis buffer. EDTA anti-coagulated blood or previously isolated cell populations with residual erythrocyte contamination were mixed at a ratio of 1 part to 9 parts ACK lysis buffer and allowed to stand until transparent (~10 min) before centrifugation (300g/5 min/20°C). The resultant leukocyte pellet was washed twice in an appropriate buffered salt solution prior to analysis.

2.1.6 Cell Counting and Viability

Vital dye exclusion method was employed to determine overall leukocyte or isolated cell population number, live cell count and percentage cell viability. Cells were re-suspended in a known volume, and either with or without an optional dilution step

(e.g. 100:1, 10:1) dependant on expected number, mixed 1:1 with 0.4 % Trypan Blue (Sigma). The cell/dye suspension was added to a cell counting chamber and evaluated using a Countess™ automated cell counter (Invitrogen™).

2.2 Mono Mac 6 Cell Line

Mono Mac 6 (MM6) were obtained as a frozen culture from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Germany).

MM6 are a human cell line established from the peripheral blood of a 64-year-old man with relapsed acute monocytic leukemia (AML FAB M5) following myeloid metaplasia²⁸⁴. Morphologically they are single, round/multiformed cells or small clusters of cells in suspension that are occasionally loosely adherent. 1-5% are giant cells. Cell surface marker expression has been reported as CD3 -, CD4 -, CD13 +, CD14 +, CD15 +, CD19 -, CD33 +, CD34 -, cyCD68 +²⁸⁴. CD14 expression is highly dependent on cultivation conditions²⁸⁵.

2.2.1 Culture Conditions

Mono Mac 6 cells were cultured under LPS free conditions in RPMI 1640 (Gibco) containing 10% FCS (Invitrogen™), 200U/mL penicillin (Gibco), 200µg/mL streptomycin (Gibco), 2mM L-glutamine, 1mM sodium pyruvate (Gibco), 1mM oxaloacetic acid (Sigma), 1x MEM non-essential amino acids (Gibco) and 9 µg/mL human insulin (Sigma) in accordance with standard practice²⁸⁴. After addition of the aforementioned supplements, the medium was ultra-filtered and stored at 4°C.

After removal of MM6 from cryostorage cells were cultured initially for 1 week in culture medium alone in 24 well plates (Orange Scientific, Belgium) at a density of 2×10^5 cells/mL (2mL/well) and passaged every 48 hours. Doubling time was initially, 40-50 hours decreasing to 30-40 hours, with cell viability increasing from 86-88% to $\geq 95\%$. MM6 were subsequently maintained in T75 flasks (25mL media), passaged every 48 hrs with seeding at 2×10^5 cells/mL (5×10^6 /flask). Morphology was regularly monitored microscopically (Figure f2.1) to evaluate for any apparent shift in phenotype (increased giant cells, increased multinucleated cells), clumping (reflecting potential LPS contamination) and/or clouding of the media (indicative of bacterial/fungal contamination). All tissue culture was carried out in sterile conditions with mycoplasma contamination evaluated for. Experiments were carried out between passage 4 and passage 30. Significant deviation in cytokine production in control conditions from the established, expected range (1000-2000pg/mL TNF α in response to LPS 100ng/mL), or decreased percentage viability on routine passage

(<95%) led to discarding of the cells and re-instatement of the line from a frozen aliquot

2.2.2 Differentiation of Mono Mac 6 Cells

MM6 may be further differentiated via incubation with various ligands to induce distinct cellular phenotypes and responses to stimuli^{285,286}. These reagents aim to transform the relatively immature MM6²⁸⁷ into cells with characteristics that resemble mature monocytes or macrophages²⁸⁸.

Three different methods of differentiation were selected from the previous published literature: incubation with phorbol 12-myristate 13-acetate (PMA, Sigma, 10ng/mL)²⁸⁵, M-CSF, (Sigma, 20ng/mL) or $1\alpha, 25$ dihydroxycholecalciferol (VD3, dihydroxyvitamin D₃, calcitriol, Sigma, 10ng/mL)²⁸⁸. MM6 were cultured with these reagents in T75 flasks (25mL) seeded at 2×10^5 for either 48 or 72hours, scraped to ensure collection of newly adherent cells, washed, re-suspended in media alone to a density of 2×10^6 and plated in 96-well plates at 1×10^5 cells/well (50 μ L media) prior to stimulation. Viability (2.1.6) after co-incubation did not vary between reagents (exceeding 95%).

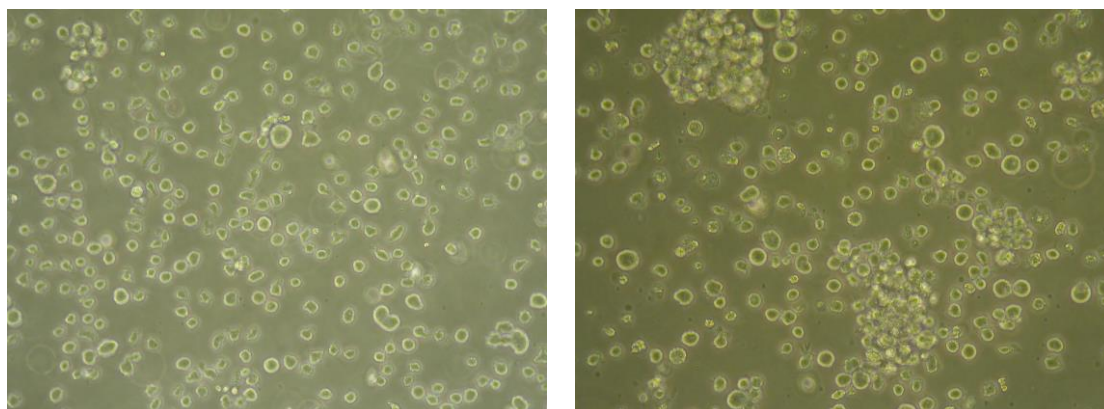


Figure f2.1. Photographs of MM6 taken via simple light microscopy (20X). **Left Panel:** 'undifferentiated' MM6 incubated in standard cell culture media alone. **Right Panel:** MM6 following incubation with PMA 10ng/mL for 48hrs. VD3 (10ng/mL) treated cells additionally demonstrated increased clustering (to a lesser degree) without clear morphological alteration.

2.3 Assays

2.3.1 Single-Analyte Enzyme Linked Immunosorbent Assay

The concentration of the cytokines TNF- α , IL-6 and IL-1 β and PGE₂ in cell culture supernatants and patient plasma was measured via enzyme-linked immunosorbent assay (ELISA). Pre-validated kits employing the 'sandwich' principle of analyte-specific capture and biotinylated detection antibodies were obtained from R&D systems (USA, DuoSet system) for the evaluation of TNF- α and from eBioscience

(USA, Ready-SET-Go! system) for IL-6 and IL-1 β , and conducted on half-volume (50 μ L) 96 well Corning CoStar high-binding, clear flat bottom polystyrene plates. Light absorbance of the streptavidin-horse radish peroxidase (HRP) catalysed breakdown of 3,3',5,5'-tetramethylbenzidine (TMB) was measured at 450nm against a reference wavelength of 595nm on a Tecan $\text{\textcircled{R}}$ GENios TM microplate spectrofluorometer and sample values interpolated from a standard curve of known antigen concentration on a plate by plate basis (Figure f2.2). Supernatants and plasma samples were thoroughly thawed and diluted in reagent diluent (PBS containing 5% bovine serum albumin) prior to addition to ensure working concentrations in the centre of the standard curve (1:2 MM6, 1:10 MDM, 1:10 whole blood) and the HRP-TMB reaction stopped via the addition of 1M sulphuric acid.

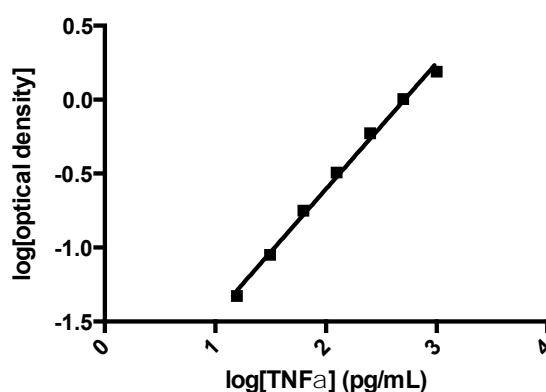


Figure f2.2. Example linearized standard curve generated during TNF α ELISA. A solution of known concentration (1ng/mL) of TNF α was made up and serial dilutions plated in duplicate. The logged average absorbance (optical density) of these wells (minus the mean zero standard) was then plotted against the log concentration. Linear regression was employed to draw a line of best fit and extrapolate unknown sample values. A minimum goodness of fit (R^2) was considered >0.98 .

PGE $_2$ concentration in biological fluids was determined using a R&D Systems Parameter TM Kit (Abingdon, Oxford). This assay relies on the forward sequential competitive binding technique whereby PGE $_2$ in a sample competes with HRP-labelled PGE $_2$ for a limited number of binding sites on a mouse monoclonal antibody. As sample is added to the pre-coated wells first, absorbance intensity is inversely proportional to the concentration of PGE $_2$ in the sample. As above, unknown concentrations was determined via comparison to known concentrations.

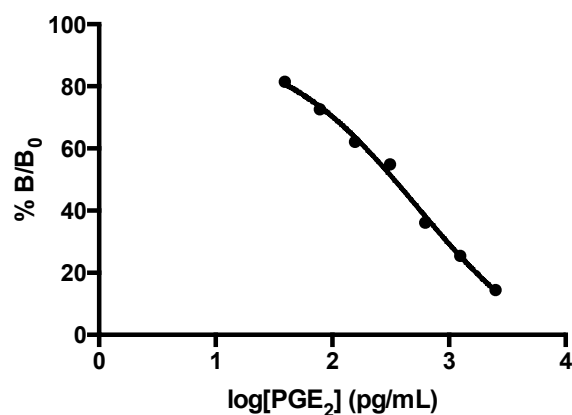


Figure f2.3. Example standard curve generated during PGE₂ ELISA. A solution of known concentration (2.5ng/mL) of PGE₂ was made up and serial dilutions plated in duplicate. The mean reading for each standard, control and sample was calculated and the average non-specific binding optical density subtracted. The % B/B₀ was calculated by dividing mean readings for samples or standards (B) by the zero standard (B₀, 0pg/mL) and multiplying by 100. This value was then plotted against the log PGE₂ concentration and four parameter logistic (4PL) regression employed to draw a curve of best fit and extrapolate unknown sample values. A minimum goodness of fit (R²) was considered >0.98.

2.3.2 Multiplex Cytokine Array

Concurrent evaluation of 10 cytokines known to be involved with the inflammatory response and immune regulation was undertaken via a high-sensitivity electrochemiluminescence assay on select plasma samples and supernatants from LPS-stimulated whole blood (2.3.3) derived from healthy volunteers undergoing IV endotoxemia (2.5). Briefly, after defrosting, samples were diluted 1:40 in RPMI, prior to placement on a Meso Scale Discovery (MSD®) V-PLEX™ Proinflammatory Panel 1 Kit coated with anti-human antibodies for IFN-γ, IL-10, IL-12p70, IL-13, IL-1β, IL-2, IL-4, IL-6, IL-8, and TNF-α, and incubated according to manufacturer instructions. After the addition of detection antibodies specific for the same analytes, plates were read using a MSD® QuickPlex SQ 120 imager (Institute of Child Health, UCL) to determine individual cytokine concentrations.

2.3.3 Whole Blood Cytokine Secretion

Unless otherwise stated, heparinized whole blood (Grenier Bio-One Vacuette® 9ml LH) was diluted 1:5 in RPMI (Gibco) in 15ml Falcon™ conical centrifuge tubes (Fisher Scientific, Pennsylvania, USA) and stimulated for 6hrs (37°C, 250rpm) with 1ng/ml LPS (*Salmonella abortus equi* S-form [TLRgrade™], Enzo Life Sciences®), as per Kox and co-workers²²⁰. Additional reagents were added as determined by

experimental conditions. After incubation samples were centrifuged (2000g/10mins/20°C) and supernatant stored at -80°C prior to analysis.

2.3.4 Mass Spectroscopic Assessment of Plasma Lipid Concentrations

Assessment of 64 known lipid mediator precursors, lipid mediators and their metabolites (Table t2.1) in plasma was undertaken by electrospray ionisation, liquid chromatography mass spectrometry (ESI/LC-MS) by Ambiotis (Toulouse, France). Plasma (EDTA BD Vactainer®) was centrifuged (2000g/10mins/20°C) and 3mL aliquots immediately made in glass vials pre-filled with 100mM butylated hydroxytoluene (BHT). These were stored at -80°C prior to analysis.

1	6-keto-PGF1a	33	RvD5
2	TxB2	34	RvD6
3	PGF2alpha	35	AT-RvD1
4	PGE2	36	PD1
5	PGD2	37	10S,17S-diHDHA (PDx)
6	8-iso-PGF2a	38	AT-PD1
7	PGE3	39	Maresin 1
8	11 HDHA	40	7S,14S-diHDHA
9	13 HDHA	41	RvE1
10	10 HDHA	42	RvE2
11	17 HDHA	43	RvE3
12	14 HDHA	44	LXA4
13	7 HDHA	45	LXB4
14	4 HDHA	46	5S,15S-diHETE
15	12 HETE	47	AT-LXA4
16	20 HETE	48	LTB4
17	5 HETE	49	20-OH-LTB4
18	8 HETE	50	tetranor-PGEM
19	11 HETE	51	tetranor-PGAM
20	15 HETE	52	2,3-dinor-8-iso-PGF2a
21	8 HEPE	53	2,3-Dinor-TxB2
22	11 HEPE	54	2,3-dinor-6-keto-PGF1a
23	15 HEPE	55	11-dehydro-2,3-dinor-TxB2
24	5 OXOETE	56	11-dehydro-TxB2
25	18 HEPE	57	15-keto-PGE2
26	5 HEPE	58	13,14-Dihydro-15-keto-PGE2
27	12 HEPE	59	13,14-Dihydro-15-keto-PGA2
28	9 HODE	60	15-deoxy-delta-12,14-prostaglandin J2
29	13 HODE	61	<i>5,6-EET</i>
30	RvD1	62	<i>8,9-EET</i>
31	RvD2	63	<i>11,12-EET</i>
32	RvD3	64	<i>14,15-EET</i>

Table t2.1: Lipid mediator precursor, lipid mediator and metabolite concentrations determined by Ambiotis (France) using ESI/LC-MS. Cytochrome 450 derived lipids (italicised) were deemed more technically challenging to measure and hence to be quantified only if deemed feasible after quality control.

2.4 Flow Cytometry

2.4.1 Sample Collection

Isolated leukocyte cell populations from whole blood (all constituents, PBMC) or cell culture (MDM, MM6) were, where appropriate, depleted of erythrocyte contamination by ACK lysis buffer and re-suspended in flow cytometry buffer (FACS buffer: 5% FBS in PBS), counted, and aliquots of between $0.2-1 \times 10^6$ cells/condition made in flow cytometry tubes or 'v' shaped plates. If ACK lysis buffer were employed cells were washed twice with flow cytometry wash buffer (FACS wash: FACS buffer with 2mM EDTA).

2.4.2 Antibodies

Working dilutions of each antibody were either established previously by the host laboratory by titration and assessment of expression of cell surface markers on circulating leukocytes or taken from the manufacturers literature. The list of antibodies used, including information on the fluorescent conjugate, clone, manufacturer, concentration employed, and whether an intra [I] or extra-cellular target [E] are given in Table t2.2. All antibodies employed were directly conjugated. Panels from this set were designed dependent upon experimental question with reference to spectral overlap and relative expression on target populations. FluoroFinder® was employed to aid panel setup and verification.

2.4.3 Cell Surface Staining

In order to reduce nonspecific binding cells were first incubated in Human TruStain FcX (Biolegend) (15mins, 4°C) to block Fc receptors. Cells were then centrifuged (500g/3mins/4°C), washed in FACS wash, re-spun and the pellet re-suspended in the volume of FACS buffer required to render the end volume of the cell/buffer/antibody mix 100µl. Selected antibodies targeted at cell surface antigens were then added as a pre-created cocktail ('master mix') and kept at 4°C for 30 minutes in the dark. After incubation, 50µL wash buffer was added to the mixture before centrifugation (500g/3mins/4°C). Cells were washed twice more by adding 100µL wash buffer and repeating the spin. If intra-cellular staining was not planned cells were re-suspended in 150µL FACS Buffer and 150µL fixative (0.1% paraformaldehyde in PBS) added prior to transfer to a 1mL microtube for analysis.

Target	Fluorochrome	Clone	Manufacturer	Stock (µg/ml)	V (µl/sample)
CD11b [E]	PerCP-Cy5	ICRF44	Biologend	NS	1.25µl
CD14 [E]	AF700	MSE2	BDPharm	500µg/ml	2.5µl
CD16 [E]	APC	3G8	Biologend	150µg/ml	1µl
CD16 [E]	PE/Cy7	3G8	Biologend	200µg/ml	1.25µl
CD33 [E]	PE	WM53	Serotec	1mg/ml	5µl
EP2 [I]	PE	Poly	Abcam	100µg/ml	3µl
EP4 [I]	APC	Poly	Abcam	100µg/ml	3µl
HLA-DR [E]	APC-H7	G46-6	BDPharm	NS	2.5µl
HLA-DR [E]	BV421	L243	Biologend	25µg/ml	1.25µl
CD3 [E]	FITC	HIT3a	Biologend	200µg/ml	1.25µl
CD19 [E]	FITC	HIB19	Biologend	400µg/ml	1.25µl
CD20 [E]	FITC	2H7	Biologend	100µg/ml	1.25µl
CD56 [E]	FITC	MEM-188	Biologend	400µg/ml	1.25µl
CD66b	FITC	G10F5	Biologend	300µg/ml	1.25µl
CD88 [E]	PE/Cy7	S5/1	Biologend	200µg/ml	2.5µl

Table t2.2: Antibodies employed. Target designates the cellular protein to which the antibody binds. [E]/[C] denotes whether the antibodies target epitope is located extra-cellularly ([E]) or intracellularly ([C], thus requiring permeabilisation). The fluorochromes give the labels a recognisable property for flow cytometry. The clones refer to the B cell clone from which these monoclonal antibodies have been obtained. It is advisable to use only one clone throughout to avoid any differences in binding affinity and eventually output signal.

2.4.4 Permeabilisation and Intra-Cellular Staining

To achieve intra-cellular staining, cells washed of excess extra-cellular antibodies were permeabilised via re-suspension in BD Bioscience Cytotfix/Cytoperm® solution (100µL) for 20mins at 4°C. Cells were subsequently washed twice with, and then re-suspended in BD Perm/Wash® solution, and fluorochrome-conjugated antibodies targeted at intra-cellular epitopes added to create a total volume of 100µL. This mixture was incubated for a further 30mins (4°C/dark). Cells were subsequently washed twice prior to re-suspension in FACS buffer, transfer to a microtube and made up to a volume of 300µL.

2.4.5 Compensation and Isotype Control

Where necessary, compensation controls were established for each fluorochrome used in the antibody mix to control for spectral overlap. BD CompBeads® were employed as they bind to all antibodies with uniform efficiency. Compensation controls were prepared by mixing 60µL of positive beads, 60µL of negative beads and the appropriate volume of antibody. The mixture was incubated for 30mins (4°C/dark). Stained beads were washed three times with 1mL of FACS wash with centrifugation at 800g (5mins/4°C). Following the final wash step, supernatant was discarded and the stained bead pellet re-suspended in 300µL PBS and transferred to a FACS tube. Compensation between fluorochromes was calculated automatically by the BD FACS Diva software. A maximum tolerance of 30% spectral overlap was allowed between fluorochromes.

Isotype controls are employed to help identify and discount non-specific binding (NSB). Here either the fraction-antigen binding (Fab) portion of a fluorochrome tagged antibody binds to a low affinity, non-specific target on the cell surface (or intracellular target if membrane integrity is compromised) or the fraction crystallisable (Fc) portion binds to Fc-receptors (FcR) expressed on certain cell types. Without appropriate controls this cell may be falsely identified as positive for the surface marker associated with the fluorochrome, being indistinguishable from one expressing the target epitope and specifically bound by the antibody-fluorochrome conjugate. Isotype controls are ideally antibodies from the same species and clone, with the same heavy chain, light chain, fluorochrome and F:P ratio (fluorescent molecules per antibody) as the antibody-conjugate to be employed but targeting a protein not found on the cell surface. Cells may then be incubated with this isotype and those binding it (inevitably non-specifically) may then be excluded from analysis leaving true-positives only.

Unfortunately several problems exist with the control: availability of isotype controls for each selected antibody-fluorochrome conjugate, cross-reactivity to a similar epitope on a different antigen, cost, requirement for additional cells from often limited samples etc. These are discussed in full here²⁸⁹, and have led to the adoption of alternate strategies to combat NSB. These include: titration of reagents to ensure high signal in bright populations while reducing spread in negative populations, employment of Fc-block, ensuring high and consistent cell viability (reducing 'stickiness' associated with necrosis/apoptosis) and use of FMOs to determine positivity. Given the complexity of the panels employed and these valid concerns a

strategy of pre-experimental optimisation, viability confirmation and Fc-block was selected as opposed to use of individual isotype controls.

2.4.6 Data Gathering and Analysis

Samples were analysed using the LSR Fortessa™ flow cytometer (BD Bioscience, USA) either on the same day as staining if permeabilisation had been performed or within 48hours of fixation if not.

Data were analysed using FlowJo software (FlowJo v7.6.1, Tree Star Inc., USA). Cell populations were identified using dot plots based on size (from forward scatter, FSC), granularity (from side scatter, SSC) and fluorescence. A gating strategy was adapted from previous data obtained by the host laboratory to identify individual cell populations. Cells positive for a marker were identified by performing fluorescence-minus-one (FMO) controls, made up of cells stained using an antibody mixture containing the entire panel except for one. Populations are labelled according to relative fluorescence intensity, indicated by superscript text i.e. x^{hi} or $x^{+/++}$ for high expression and x^{lo} or x^{-} for low or no expression.

Identified populations are given as a percentage of the total cell number (linear scale, mean \pm SD) and as absolute cell numbers (logarithmic scale, median \pm interquartile range). Differences between time points, differentiation and stimulation conditions are assessed where possible by paired Student's *t* tests for the percentage of total cells, or by Wilcoxon matched pairs tests for absolute cell numbers. Median fluorescence intensity (MFI) is given in arbitrary units (logarithmic scale, median \pm interquartile range) as this value varies between cytometers and used settings.

2.5 Intravenous Endotoxemia

2.5.1 Participants, Inclusion and Exclusion Criteria

Healthy, non-smoking male volunteers aged between 18 to 50 were advertised for via word of mouth and internal UCL email. After providing informed written consent potential participants were invited to participate in a 'health screen' to minimise risk from undergoing intravenous (IV) endotoxemia. Screening consisted of a full medical history, clinical examination of the cardiovascular and respiratory systems, routine clinical observations (blood pressure, pulse oximetry, heart rate, [Carescape V100, Dinamap, GE Healthcare] respiratory rate, temperature [ThermScan Pro 4000, Braun Welch Allyn]), 12-lead electrocardiogram (ECG) and core hematology and biochemistry investigations (full blood count, urea and electrolytes, liver function tests, C-reactive protein, bone profile, magnesium: all The Doctor's Laboratory, London, UK). Exclusion criteria included acute or chronic illness, regular prescribed medication use or current over-the counter NSAID use, abnormal physical examination, electrocardiogram or blood results (deemed to increase the likelihood of adverse events), drug or alcohol misuse, and recent vaccination or transfusion within 1 month. Abnormal results were reported to the participant's general practitioner. Individuals were paid £200 for completion of all elements of the study protocol.

2.5.2 Elicitation of Systemic Inflammation

Clinical Centre Reference Endotoxin (CCRE, E.coli O:113 EC-6) was made freely available by the National Institutes of Health (NIH), Bethesda, USA. CCRE is manufactured to Good Medicinal Practice standards and undergoes regular testing for safety, stability and efficacy by the NIH. It is supplied in single use sterile glass vials as a white lyophilised powder, each vials containing 10,000 endotoxin units (EU) (approximately 1 µg).

Participants were asked to refrain from food from midnight prior to intravenous (IV) endotoxin injection and alcohol and caffeine for the 24 hours before the start of the experiment. After confirmation of consent and health status participants were asked to lie on a bed, the head placed initially at 45° whilst two IV cannulae were inserted: a 20G in the dorsum of the non-dominant hand for endotoxin administration and an 18G in the opposite ante-cubital fossa. A 3-way tap was attached to this second cannula to allow fluid administration and repeated blood draw.

CCRE was re-constituted with 5ml sterile water for injection using full ANTT and agitated for a minimum of 1 hour to generate a 2000EU/ml / 200ng/ml solution. A dose of 2ng/kg was subsequently injected IV as a bolus over 2 minutes with a

subsequent flush of 10mls of 0.9% sodium chloride to elicit systemic inflammation. After administration of CCRE an infusion of Hartmann's Solution (Baxter™, UK) was commenced at a rate of 500ml/hour for two hours, then 166mls/hr for the following six hours to avoid dehydration and minimise the risk of endotoxin-associated cardiac arrhythmias.

All elements of the study were conducted in the UCL/University College London Hospital Clinical Research Facility (CRF). Two clinically qualified members of the research team were allocated to each procedure, one always attending the participant.

2.5.3 Clinical Monitoring and Sample Schedule

Participants were monitored for 8 hours post-injection. Clinical observations (as per health screen) were undertaken hourly. Participants were additionally asked to score symptom severity on visual analogue scales rating nausea, shivering, muscle ache, and headache with the option of adding additional symptoms.

Blood was drawn at baseline (cannula insertion, prior to CCRE injection), 1 hour, 1.5, 2, 3, 4, 6, and 8 hours post-injection. At each time point 5mLs of blood was drawn and discarded prior to sample collection to negate dead space. Mid-stream urine samples were collected at baseline, 2, 4 and 8 hours post-injection. At eight hours post-injection, following final sample draw and provided clinical observations were either trending towards or back at baseline, lines were removed and vital sign monitoring ceased (Figure f2.4).

Blood and urine were additionally collected at 24, 48, 72, 96 hours, day 7 and day 14 post-endotoxin injection. All samples were processed according to standard-operating procedures as detailed above. The full schedule of analysis is provided in Chapter 6.

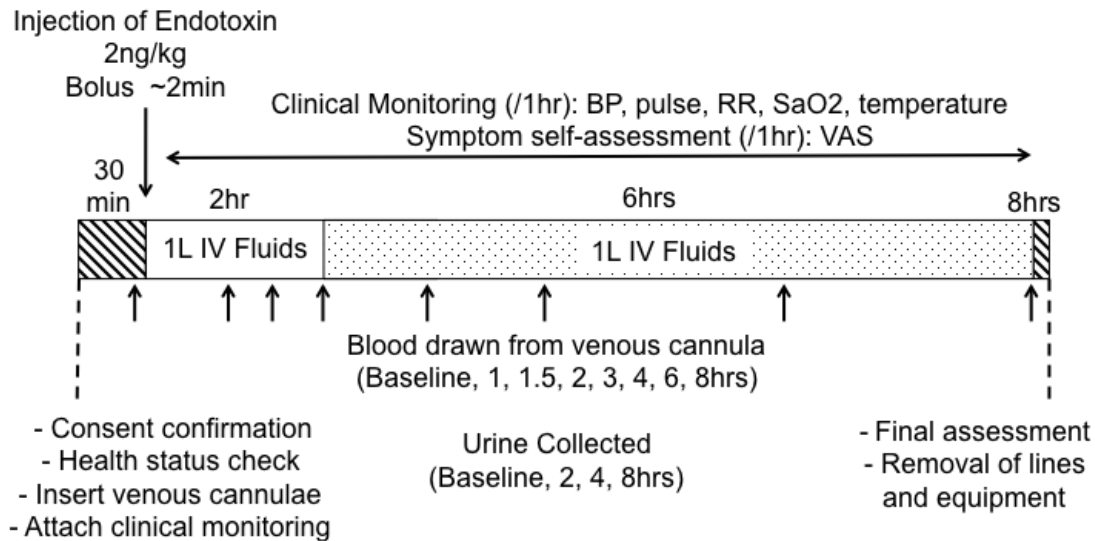


Figure f2.4: Schematic Representation of the IV Endotoxemia Protocol. Key procedures, interventions, monitoring and sampling time-points are illustrated.

2.6 Statistical analysis

2.6.1 Cell Culture and Ex Vivo Experiments

Various conventions for presenting n-values from cultured cell experiments exist. Unfortunately, no method is accepted and the majority of publications provide insufficient information to know how n-values have been calculated and what constitutes 'separate experiments'. Truly independent experiments may be considered those performed on separate days with fresh, newly reconstituted reagents²⁹⁰. When using primary cells it is desirable to perform experiments in separate donors, on separate days, with new drugs for each experiment. Where immortal cell lines, such as MM6, are employed which are clones and therefore cannot provide repeats with genetically different cells, it is important to perform experiments multiple times using different passages with newly generated reagents. However, practically this is not always possible and where donors and or/samples are limited, different techniques must be used to satisfy the scientific need for experimental repeats.

When undertaking the whole blood cytokine secretion assay blood was taken from 3-10 individuals (dependent upon experiment) selected by availability from a pool of 18 consenting healthy volunteers (72.2% male, mean age 32). Three separate tubes/individual/time point or condition were stimulated and supernatant from each of these assayed (ELISA) in duplicate, the mean of all values (6) taken as the overall reading. N was considered equivalent to the number of individual volunteers, enabling repeated-measures statistical analysis. This practice is in accordance with

previously published literature²⁹¹. Experiments involving the MM6 cell line were undertaken in 96 well plates. In general, each condition was replicated in four wells on two or three separate experimental days involving separate drugs and passage, cells being drawn from one on-going culture. Supernatants were assayed (ELISA) in duplicate or triplicate. Data points represent the mean value of technical repeats (8-12), n reflecting the number of biological (experimental) repeats. When plasma was employed figure legends indicate whether this was obtained from one or multiple individuals.

2.6.2 IV Endotoxemia

10 individuals were recruited. All physiological and haematological values were obtained from one reading/individual/time-point. Mean data from all 10 volunteers is presented. Cytokine profiles were obtained from the multiplex MSD array performed in duplicate, values for each individual representing the average of these. Data from bioassays represent either a) whole blood cytokine release: one tube/time-point or condition, each supernatant assayed (ELISA) in duplicate or b) MM6: plasma placed into four wells (96-well plate) with or without additional reagents, each supernatant assayed (ELISA) once. Individual values represent the average of ELISA values and n = 10 for all assays unless otherwise stated.

2.6.3 Software and Definitions

Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA). Differences were considered statistically significant at *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001, or non-significant (ns).

2.7 Ethical Approval

Ethical approval for the conduct of all studies on human volunteers was sought from the University College London Research Ethics Committee. Two separate applications were made: 'The effect of inflammation on local and systemic immune function', UCL Project ID 5060/001, covering the IV endotoxin model (Chapter 6) and 'LPS-stimulated whole blood cytokine release: comparison of different methodologies' UCL Project ID 4332/001, covering the whole blood cytokine secretion assay (Chapter 4). Both are attached as appendices to the thesis. Additional permission was gained from the UCL/UCLH Joint Research Office and the CRF Adoption Committee.

CHAPTER 3: Systematic Review

DO NON-STEROIDAL ANTI-INFLAMMATORY DRUGS ALTER PREDISPOSITION TO OR OUTCOME FROM ACUTE INFECTION? A SYSTEMATIC REVIEW OF THE CLINICAL LITERATURE

3.1 Introduction

3.2 Additional Methods

3.3 Results

3.4 Discussion

3.5 Summary

3.6 Appendix

The following literature review was conducted in conjunction with James Bott and Noam Roth, UCL Medical School Students, as part of an Acamedics Project supervised by Dr James Fullerton. JB and NR performed the literature search, reviewed the papers and extracted data. JNF conceived the review, analysed and interpreted the data and drafted the chapter.

3.1 INTRODUCTION

Non-steroidal anti-inflammatory drugs (NSAIDs) are among the most commonly administered medications globally, an estimated 30 million people using them every day^{292,293}. Exhibiting analgesic, anti-inflammatory and anti-pyretic properties clinically, they form part of the World Health Organisation's core list of Essential Medicines.

NSAIDs are a chemically heterogeneous group of compounds united by their mechanism of action²⁹⁴. As collective but variable inhibitors of both constitutively expressed cyclooxygenase (COX) 1 and inducible COX-2 they prevent the conversion of arachidonic acid to prostaglandin (PG) H₂ (via PGG₂): the first committed enzymatic step in the prostanoid synthetic pathway²⁹⁵. Their therapeutic (and side) effects predominantly derive from reduced production of the major downstream molecules PGD₂, PGE₂, PGF_{2α}, PGI₂/prostacyclin and thromboxane (TXA₂)^{296,297}. These bioactive lipid mediators play myriad physiological roles and are central in the inflammatory response, contributing directly or indirectly to hyperalgesia, vasodilation, oedema and fever^{151,298,299}. Less widely appreciated however are their effects on host defence.

Several eicosanoids, and in particular PGE₂, are now understood to exert distinct anti-inflammatory, immune regulatory and/or pro-resolution properties in addition to their more recognised roles^{189,300,301}. Recent studies have indicated that prostaglandins can impair innate and adaptive immune function in bacterial^{197,255,302,303}, viral³⁰⁴ and fungal³⁰⁵ infections, and elicit immunosuppression in discrete patient populations¹⁴². Indeed, certain pathogens exploit these mechanisms directly, *Pseudomonas aeruginosa*, *Cryptococcus spp.* and *Candida albicans* altering local lipid mediator concentrations at infected sites via direct release of eicosanoids, the enzymes required for their formation, or by stimulating host production, to protect themselves against elimination³⁰⁶⁻³⁰⁸. Correspondingly and as described in the Introduction, pre-clinical data has shown that NSAIDs enhance the effector functions of myeloid lineage cells both *in-vitro* and *in-vivo*, and that their administration repeatedly confers benefit in animal models of severe infection²⁶². The central mechanism appears to be ablation of the aforementioned immunosuppressive prostanoids^{255,309}.

Interest in immunoadjuvant therapy - where pharmaceutical agents are employed to restore or potentiate immune function with the aim of preventing infection and improving pathogen clearance – is growing³¹⁰. This strategy affords an antimicrobial chemotherapeutic approach that harnesses the host's innate defences whilst being

insusceptible to resistance. Given the pre-clinical evidence that NSAIDs and thus prostanoid blockade exerts an immunomodulatory effect, this systematic review seeks to summarize and broadly interrogate the clinical literature for such a signal. Benefit or harm from NSAID administration as either primary or adjunctive therapy in acute infection was sought, along with any suggestion that COX-inhibition alters susceptibility to, and severity of, infection. Sub-analyses focus on delineating whether particular demographic, pharmacological or infective factors determine either efficacy or risk of NSAID use in this context, with the aim of identifying future clinical and research opportunities.

3.2 ADDITIONAL METHODS

3.2.1 Systematic review

The systematic review was performed in line with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement³¹¹. Inclusion and exclusion criteria were established a priori with the objective of evaluating the clinical literature for any signal of harm or benefit associated with either pre-existing NSAID use during, or NSAID therapy commenced in response to, acute infection. Evidence of whether NSAID use for alternate clinical indications resulted in an altered incidence of acute infection was additionally sought. Pre-defined sub-group analyses investigated whether demographic (age), pharmacological (NSAID class, non-selective vs. COX-2 selective inhibition, dose, route or duration of therapy), co-prescription (acute mono-therapy vs. adjunctive prescription with established anti-microbial), or infective (pathogen, site of infection, severity) factors were linked with either efficacy or adverse outcomes from NSAID use in this setting.

3.2.2 Search strategy and inclusion criteria

A systematic review of articles using MEDLINE and MEDLINE In-Process and Other Non-Indexed Citations (1946 – November 2014) was performed. Databases were searched according to the strategy developed by the Cochrane Collaboration³¹². Additional studies were identified via independent manual trawl of the bibliographies of identified articles by two authors (JB and NR). The following key search terms were used “non-steroidal anti-inflammatory drug”, “NSAID”, “aceclofenac”, “acemetacin”, “aspirin”, “azapropazone”, “celecoxib”, “dexibuprofen”, “dexketoprofen”, “diclofenac”, “difunisal”, “etodolac”, “etoricoxib”, “fenoprofen”, “flurbiprofen”, “ibuprofen”, “indomethacin”, “ketoprofen”, “ketorolac”, “meclofenamic acid”, “mefenamic acid”, “meloxicam”, “namebutone”, “naproxen”, “nimeslide”, “oxaprozin”, “phenylbutazone”, “piroxicam”, “salicylic acid”, “sulindac”, “tenoxicam”, “tiaprofenic

acid”, and “tolmetin” and/or “infection”, “anti-microbial”, “sepsis”. All searches were limited to English language and “humans”.

Both observational and interventional (randomised and non-randomised) studies were included. No restriction was placed on patient age (neonate [<28 days], paediatric [28 days-17years], or adult [\geq 18years]), infective site, pathogen (bacteria, viral, fungal, protozoan) or drug (whether in current clinical use, dose, route or formulation). *In-vitro*, animal, and pre-clinical studies were excluded. Papers exploring the effect of NSAIDs on the clearance of chronic infection and where no acute infection was identified were also excluded, along with review articles not reporting original data.

3.2.3 Assessment of methodological quality

Articles meeting inclusion criteria after independent screening of title and abstract by 2 authors (JB and NR) were extracted and reviewed using predefined data fields. These included year of publication, geographic location, study design, patient demographics, details of associated pharmacological regimen, pathology and defined outcome measures. Disagreement between the 2 extracting authors was resolved by consensus. If this could not be reached a third author was deferred to for arbitration (JNF). To assess the risk of bias and determine methodological quality all studies underwent scoring using either the Jadad scoring system (for randomized and non-randomised controlled trials)³¹³ or the Newcastle-Ottawa Scoring system (NOS, for cohort and case-control studies)³¹⁴. Reduced risk of bias and/or acceptable methodological quality was defined by achievement of a Jadad score \geq 3 or a NOS of \geq 6 in line with previous systematic reviews^{315,316}. Papers were not excluded on the basis of score but analysed separately. Included studies were also inspected for author disclosures deemed to imply a conflict of interest, the source of research funding and, if stated, who acted as the guarantor of the article (responsible for data control and decision to publish).

3.2.4 Data interpretation

A pre-defined structured outcome matrix was utilized to classify the effect of NSAIDs on predisposition to, or outcome from, infection in studies meeting the inclusion criteria (Table t3.1). This non-validated construct was designed to be applicable across study types and to provide a broad un-weighted comparison of the effect of NSAIDs in disparate clinical contexts.

Outcomes were classified according to the original study authors determined effect – beneficial (positive), null or harmful (negative) - and whether this was subjective

(outcome category 1), objective (outcome category 2) or related to mortality (outcome category 3). In studies reporting multiple outcome measures a single category was attributed for further analysis (that affording the greatest certainty in effect – category 3>2>1).

Outcome category	Outcome definition
Positive 3	Survival benefit
Positive 2	Physiological/radiological/biochemical/microbiological/clinical benefit (e.g. reduced length of stay)
Positive 1	Change in clinically relevant symptoms (symptomatic benefit)
Null	No significant effect measured
Negative 1	Change in clinically relevant symptoms (symptomatic worsening)
Negative 2	Physiological/radiological/biochemical/microbiological/clinical harm (including new infective diagnoses)
Negative 3	Increased mortality

Table t.3.1. Structured outcome matrix employed to grade author-determined outcomes in reviewed studies

In order to verify the original author's conclusions, two authors (JB and NR) independently reviewed the reported effect direction, size and data interpretation. In addition, side effects of drug administration reported in the studies were recorded. In the event of disagreement regarding classification of a given study's outcome resolution was achieved as per study inclusion.

No meta-analysis was performed on the extracted data.

3.3 RESULTS

3.3.1 Article characteristics

The initial search strategy retrieved 330 studies and a further 20 were identified via bibliographic trawl (total 350). After removing duplicates (70), screening of titles and abstracts led to the exclusion of 157 papers (foreign language, *in-vitro*, animal, correspondence, comment or review articles). 27 papers were excluded as pre-clinical, mechanistic or pharmacological studies, post-hoc analyses of included studies or related to chronic infection (*Helicobacter pylori* and periodontitis). For 26 articles the full text could not be retrieved, leaving 70 studies for inclusion into the final review (see Section 3.6: Appendix). Study selection and exclusion is represented in Figure f3.1.

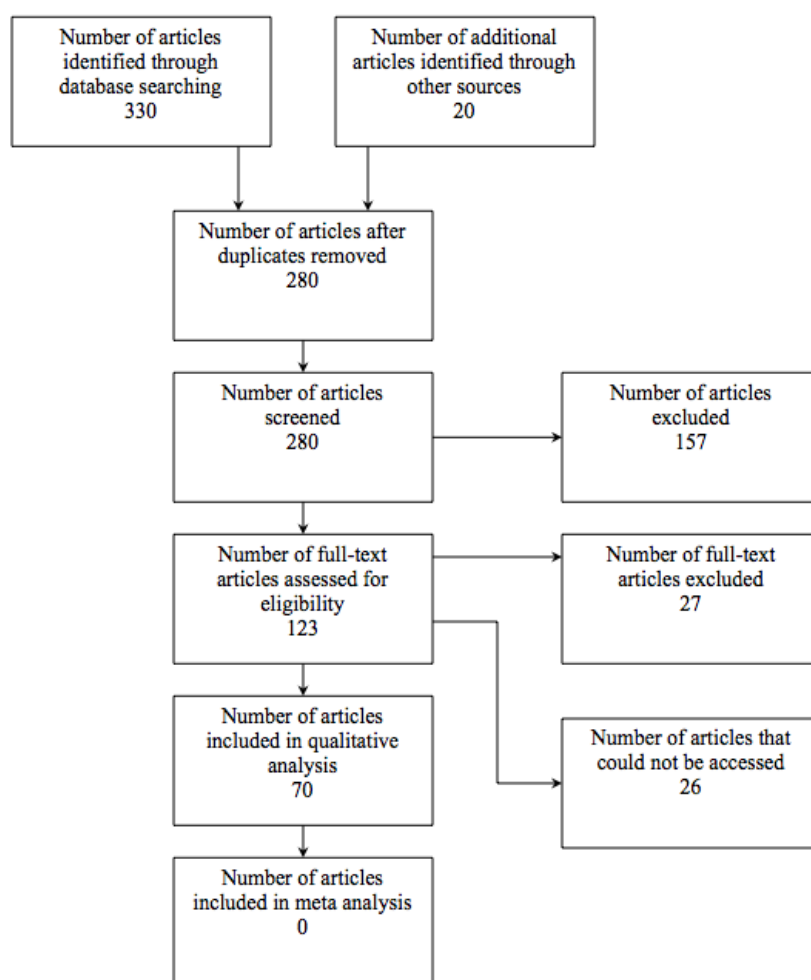


Figure f3.1. Flow chart illustrating article inclusion and exclusion.

The final group of 70 papers comprised 28 RCTs, 2 NRTs, 11 cohort studies (4 prospective, 7 retrospective), 15 case control studies, 5 case series and 9 case reports. Publication date ranged from 1960 to 2013 with the majority being published after 1980. Studies were predominantly (>75%) conducted in Europe and North America.

The majority of studies were small, 27/28 RCTs recruiting fewer than 500 patients (in all treatment arms combined) and 7/11 cohort studies included less than 1000 participants. The quality of included studies was variable. Employing a cut-off of ≥ 3 on the Jadad score for interventional studies only 12/28 RCT's and NRT's were deemed of adequate methodological quality. In contrast 25/26 cohort and case control studies achieved the pre-defined limit of NOS ≥ 6 . Risk of overt bias was explored by reviewing declared funding and conflict of interests. 11 of the RCTs were funded by pharmaceutical companies, in 4 they had provided funding to the principal investigator, and in one study controlled the data analysis and decision to publish.

Overall 25 studies (36%) reported mortality as the primary outcome (outcome category 3), 14 (20%) reported physiological, biochemical, radiological, microbiological or other objective clinical outcomes (outcome category 2) and 31 (44%) reported symptomatic alteration (outcome category 1).

Interventional studies demonstrated objective or subjective benefit from the primary or adjunctive use of NSAIDs in 76% (22) of papers, with a null effect (neither beneficial nor harmful) being reported in 24% (7). Only one RCT utilised mortality as the primary outcome measure²⁷⁶. No interventional study demonstrated harm from NSAID prescription in acute infection, as determined by their primary outcome measure (Figure f3.2, Panel A). The direction of effect of NSAIDs in RCTs deemed to be of adequate methodological quality (Jadad ≥ 3) was similar to those not achieving the pre-determined cut-off.

Observational studies were found to demonstrate a more diverse, polarized, range of outcomes, with clear clustering apparent (Figure f3.2, Panel B). Groups of reviewed papers separately indicated both benefit (including potential survival benefits from severe infection) and harm (association with increased predisposition to and severity of infection) from NSAID administration. Inevitably the vast majority of case reports and case series (12/14) reported negative outcomes from the use of NSAIDs during acute infection, this publication format being more commonly employed to describe novel or idiosyncratic adverse events.

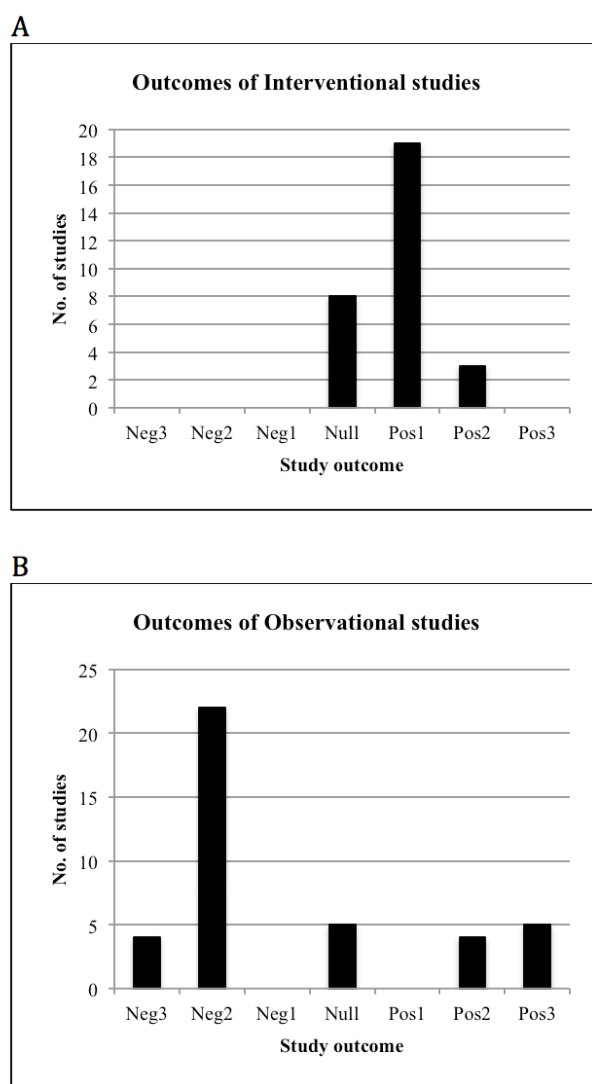


Figure f3.2. Outcomes reported in interventional (Panel A) and observational studies (Panel B), categorised according to a pre-defined outcome matrix (Table t3.1).

3.3.2 Pharmacological factors Agent, Dose, Route and Duration

Significant heterogeneity was demonstrated in the pharmaceutical agent, dose, route of administration and duration of therapy employed in the reviewed studies. Analysis, and hence comparability, was further impaired by missing data, the frequent use of multiple case definitions for inclusion and variable treatment regimens.

In total 23 different NSAIDs were employed across the manuscripts (Figure f3.3). Of these only 10 agents are currently licensed for use in the UK. 16 studies explored the

effect of 'NSAIDs' as a drug class as opposed to stating individual agents. Aspirin (12) and ibuprofen (11) were the most frequently investigated individual agents.

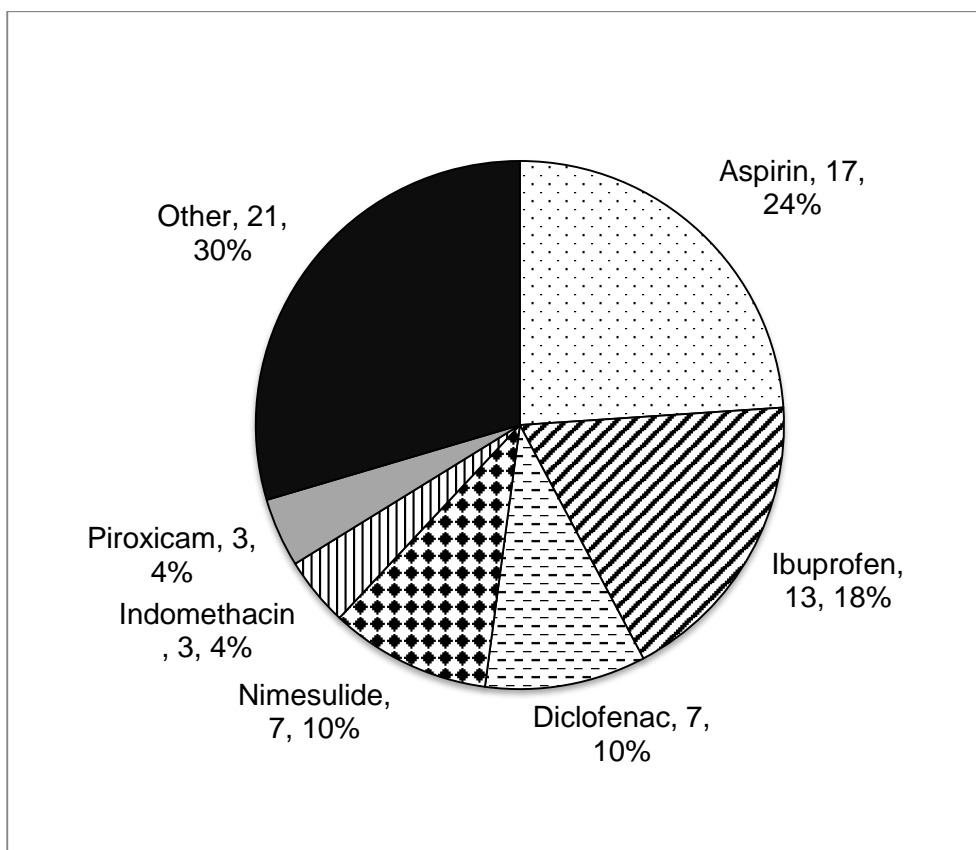


Figure f3.3. NSAID employed in included studies. A total of 23 different NSAIDs were investigated throughout the 70 studies. 'Other' includes 17 different agents investigated less than 3 times.

Divergence in treatment regimen and data omission can be exemplified by employing ibuprofen as an example. Ibuprofen was identified as the lone study drug in 11 papers (7 RCTs, 2 cohort, 1 case control, 1 case report). A dose was stated in only 9 manuscripts, with 2 of the 9 comparing multiple doses. 4 studies reported an absolute quantity, ranging from 400mg-1600mg/day administered as either a single dose or split into twice to four-times/daily divided dose regimens. The remaining studies, predominantly relating to paediatric or neonatal patients, employed a weight-based dose ranging from 5mg/kg twice/day to 10mg/kg four-times/day. Route of administration was described in 10/11 papers. Oral administration was the most frequently employed route of administration (8) followed by intravenous (IV, 2), with one study employing a mixed regimen of IV administration followed by per rectum. 9

studies reported duration of therapy with the majority of these (6) utilizing ibuprofen for between 1-3 days.

Given the number of agents employed and the variability between study protocols it is impossible to establish any meaningful relationship between either dose or duration of therapy and differences in outcome. No clear divergence in outcome between IV, oral or rectal administration of NSAIDs was apparent, although intramuscular (IM) administration of NSAIDs (in particular diclofenac) was linked to necrotizing fasciitis in 3 case reports/series³¹⁷⁻³¹⁹. In interventional studies the rationale for selection of individual elements of the treatment regimen (agent, dose, frequency of dosing etc.) was habitually neither stated, nor clearly related to either prior publications or *in-vivo*/pre-clinical work establishing efficacy. Therapeutic decisions must thus be assumed to have resulted from investigational team preference, local institutional policy or been determined by the clinical situation (patient age, renal function etc.).

NSAID Class and COX Selectivity

Dividing studies reporting single-agent NSAID use (45) according to the chemical class of the drug (acetic acids, fenamates, oxicams, propionic acids, pyrazolones, salicylic acids and sulphonamides, [see Rao et al.²⁹⁴]) revealed no discrete effects on outcome. Acetic acids were associated with negative outcomes more frequently than other groups, primarily due to the aforementioned reports of IM diclofenac use and separate case reports linking indomethacin with soft tissue infection³²⁰ and necrotizing enterocolitis³²¹. In contrast, sulphonamides (consisting of just nimesulide in reviewed papers) were associated with universally positive physiological (category 2) or symptomatic (category 3) outcomes in 4 RCTs³²²⁻³²⁵. Despite this, such small study numbers and the presence of multiple confounding factors render it impossible to draw meaningful conclusions as to chemical class effects.

The same single-drug studies were additionally subcategorised according to their COX 1/2 selectivity according to Warner et al³²⁶. The vast majority of NSAIDs employed in the reviewed manuscripts (19) were categorised into Group 1 (non-selective), with 2 NSAIDs in Group 2 (selective for COX-2: bromofenac and nimesulide), and 2 NSAIDs in Group 4 (weak inhibitors of both isoforms of COX: diflusal and niflumic acid). None were classified in Group 3 (strongly selective for COX-2). As above, heterogeneity in study populations and treatment protocols, in tandem with the paucity of agents in categories other than Group 1, render any comparison of relative

efficacy and hence the contribution of each isoenzyme to outcome of, or predisposition to, infection impossible.

Stand-Alone or Adjunctive Therapy

8 interventional studies tested the efficacy of NSAID prescription alone versus established anti-microbial therapy, an alternative analgesic/anti-pyretic or placebo in the management of acute infection. In the remaining 20 RCTs or NRTs NSAIDs were co-prescribed with standard therapy as an adjunct.

NSAIDs as single-agent therapy were found to be effective in symptom relief (principally pyrexia suppression) in upper or lower respiratory tract infections of presumed viral origin, however no conclusive evidence for attenuation of disease course was identified, reports describing conflicting evidence^{325,327}. Whilst one study reported ibuprofen as non-inferior to antibiotic (ciprofloxacin) for the management of urinary tract infection (UTI) this finding is tentative, arising from a pilot study designed to establish equipoise for a larger trial. Used adjunctively, all trials reported either a positive or null effect of NSAID addition to standard anti-infective therapy. Again, this primarily related to subjectively reported or objectively noted symptoms. Two RCT's reported improvement in either biochemical metrics when used in sepsis²⁷⁶, or muscle performance and mobility in geriatric patients hospitalized with infection-induced inflammation³²⁸.

3.3.3 Demographic factors

Age

50 papers reported the effect of NSAIDs in adult patients alone, 15 in the paediatric population, 3 in neonates, and in 2 studies inclusion criteria crossed the pre-defined age boundaries.

Systemic differences were evident between the manuscripts pertaining to different age categories. A greater proportion of papers in the paediatric literature explored NSAID's influence on the outcome of upper and lower respiratory tract infections (50%, 8/16) compared to the adult literature (excluding TB, 18%, 9/50), presumably related to relative incidence in the two different age groups. Additionally, the indication for therapy was more frequently to achieve relief from infective symptomology with outcome measures being aligned to this. Ibuprofen was employed in a greater proportion of reviewed neonatal/paediatric papers (43%, 6/14 papers using a single-agent) whilst aspirin use was described in only 1 paper reflecting concern over Reye's syndrome and absence of prescription for cardiovascular risk reduction. In total 4 of the reviewed paediatric and neonatal

papers were case reports. Of the remaining 16 that included individuals 18 years of age and under only 6 (37.5%) met the pre-defined cut-offs for methodological quality.

A clear dichotomy was evident in the paediatric/neonatal literature with regards the direction of NSAID's effect in the context of infection. On one hand COX-inhibition appears efficacious in reducing local and systemic symptomology in this age range. Multiple RCTs report a significant analgesic and antipyretic effect compared to placebo or alternate agents, along with focal infective symptom (e.g. cough or rhinorrhea) suppression³²⁹⁻³³⁵. Whilst some evidence of objective clinical improvement, such as reduced tonsillar hypertrophy³²⁴ and inflammatory indices (white cell count, C-reactive protein, IL-6, erythrocyte sedimentation rate^{325,336}) has been reported the significance of these findings is not clear. Equally no clear benefit of NSAID use in addition to an antibiotic compared to antibiotic alone was seen in two paediatric studies^{329,337}, although trends to reduced symptomology and quicker resolution were observed.

In contrast, several observational studies suggest an association between NSAID use and increased predisposition to bacterial infection. Whilst two case reports link NSAIDs with neonatal necrotizing enterocolitis (confounded by co-consumption of steroids and the co-existence of hyperprostaglandin E syndrome³²¹) and altered presentation (but not outcome) of bacterial meningitis³³⁸, the majority relate their prescription for a primary insult with subsequent severe soft-tissue infection, in particular bacterial superinfection following varicella zoster³³⁹⁻³⁴³.

3.3.4. Infective factors

Site

In the reviewed papers four focal infective sites were specifically addressed more than once: soft tissue (17), respiratory (16), lower gastro-intestinal (GI, 9), and urogenital (3). A further group of studies addressed clinical syndromes without discriminating between location of the primary insult (sepsis [9], acute lung injury [2]). Review of the data suggests NSAIDs exert divergent effects on the outcome or predisposition to infection in these separate sites (Figure f3.4).

Investigational studies have predominantly sought to test either the non-inferiority of treatment with NSAID alone compared to antibiotic, or the superiority of antimicrobial plus NSAID compared to antibiotic alone in the setting of upper respiratory (URTI) and urinary tract infections (UTI). With both sites COX-inhibition results in clear symptomatic benefit, ameliorating both systemic and local features subjectively. These effects relate to the analgesic and anti-inflammatory effect of NSAIDs and are

repeatedly demonstrated to be superior to placebo, non-inferior to antibiotics alone^{344,345}, equivalent or superior to alternative agents e.g. paracetamol^{331,334,346}, and to vary within class: certain NSAIDs being more efficacious^{333,334}. As described earlier, there is little evidence in the literature to support an assertion that NSAID administration leads to objective (outcome category 2 or 3) benefit over antibiotic administration alone or alternate analgesic/anti-inflammatory agents in URTI or UTI. There is no inference of an antimicrobial effect separate to their known properties.

Evidence of benefit in lower respiratory tract infections (LRTI) is less clear. A recent randomized placebo controlled trial of ibuprofen versus amoxicillin-clavulanic acid in uncomplicated acute bronchitis, found no significant reduction in symptoms by either agent compared to placebo and a greater adverse effect burden in the antibiotic arm³⁴⁷. In cases of community acquired pneumonia (CAP) two separate case control studies described either a significant independent association between acute NSAID prescription and the development of pleuropulmonary complications (37.5% NSAID vs. 7% no NSAID, OR 8.1; 95%CI 2.3-28)³⁴⁸ or, paradoxically, reduced need for intensive care treatment (5% vs. 24.4%) and a shorter hospital stay in those previously taking anti-platelet drugs (84% aspirin)³⁴⁹.

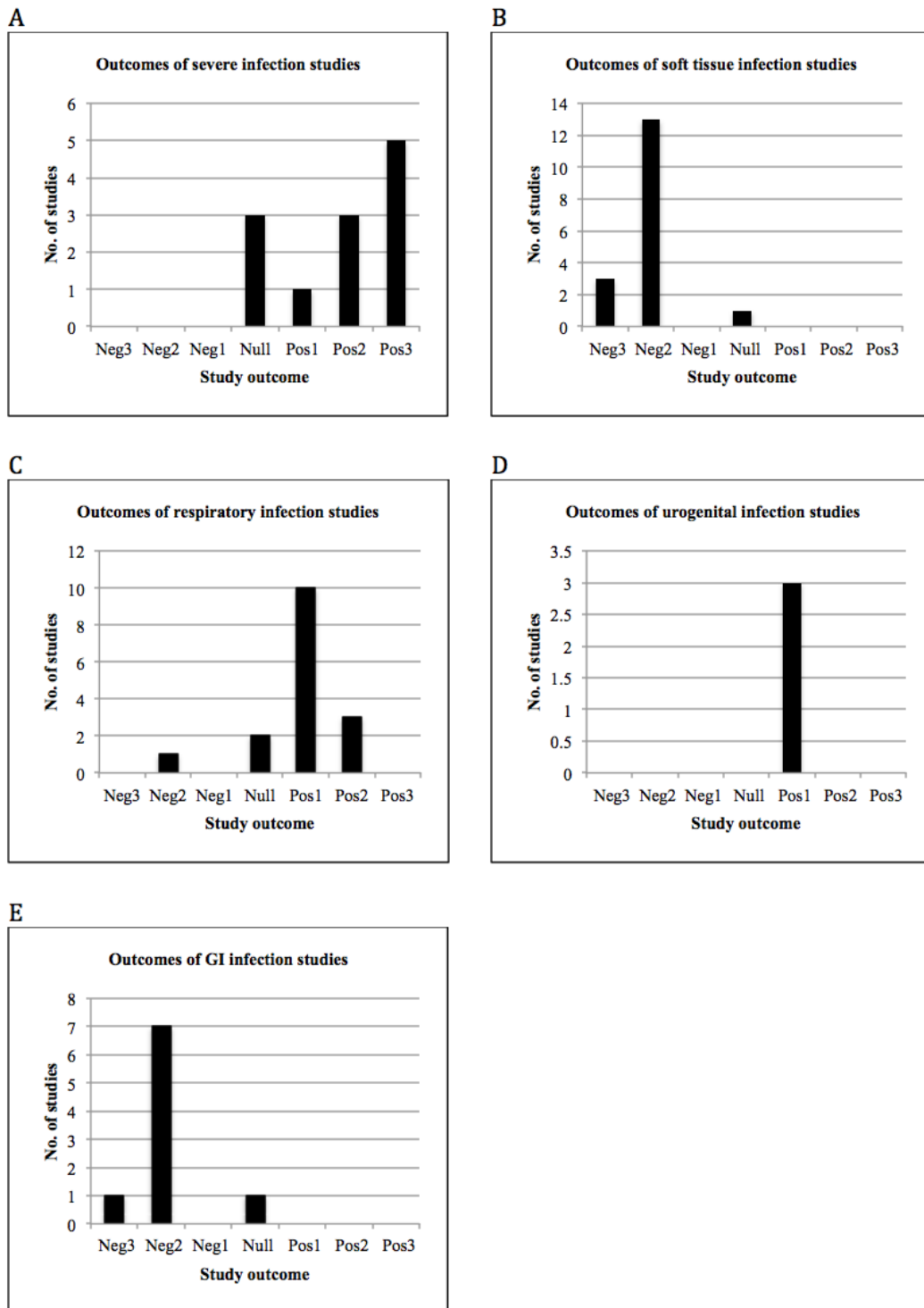


Figure f3.4 Comparative reported outcomes from NSAID administration during acute infection at different sites, including 'severe infection', as assessed by a pre-defined outcome matrix (Table 1). Included studies were grouped into severe infection (Panel A: sepsis, ICU, ALI), soft tissue infection (Panel B), respiratory (Panel C: URTI, LRTI including pneumonia), urogenital (Panel D), and gastro-intestinal (GI, Panel D).

Almost universally, reviewed papers describing soft tissue and lower GI pathology, relate NSAID administration to an increased incidence and/or severity of infection. As previously described, severe skin and soft-tissue infection (necrotizing cellulitis, necrotizing fasciitis, gangrenous myositis) is primarily associated with NSAID use for a primary insult, predominantly varicella zoster infection, but may include minor injury^{350,351} or surgery³²⁰. Case control studies estimate the additional risk of complications from NSAID use prior to or during paediatric varicella infection between 4.9 (rate ratio, 95% CI 2.1-11.4³⁵²) and 11.5 (odds ratio, 95% CI 1.4-96.9³⁴¹), with increased risk persisting in the adult zoster population but at a lower degree of magnitude (rate ratio 1.6, 95% CI 1.0-2.2³⁵²). One included single centre retrospective case series additionally suggested an increased risk of development of streptococcal toxic shock syndrome with acute NSAID administration³⁵³.

Of the reviewed papers six suggest an association with NSAID use and complications of diverticular disease. Two case-control studies report increased risk of pericolic abscess formation, generalized peritonitis following perforation, bleeding, or fistula formation in patients with diverticular disease taking NSAIDs^{354,355}, with a further indicating their use is related to an increased risk of recurrence in mild colonic diverticulitis managed conservatively³⁵⁶. Three cohort studies complement these, describing increased risk of symptomatic diverticular disease, specifically bleeding in NSAID users³⁵⁷⁻³⁵⁹. Despite this, paracetamol, an alternate analgesic, was seen to have a similar effect in one study³⁵⁸, and the finding of increased diverticulitis is neither universal nor pronounced (multivariable hazard ratio 1.25; 95% CI 1.05-1.47)³⁵⁹. Separately, one small single centre retrospective case-control study of 84 patients suggests an association between acute appendicitis and NSAID consumption in those over 50 years of age (OR 6.5, 95% 2.1-8.8) including a non-significant increased relative risk of death (OR 2.1, 95%CI 1.1-4.1)³⁶⁰, however this was not supported in a larger study³⁶¹.

Pathogen

The vast majority of included studies described either the prescription of NSAIDs in the context of bacterial or viral infection or their development during NSAID use. A much smaller number related explicitly to mycobacterial (1 tuberculosis, 1 leprosy), protozoan (1 malaria) or fungal (1 corneal ulcer) infection. Assessment for discrepancies in effect of NSAID on different pathogen groups was inherently conflated with infective site: viral infection being the predominant cause of respiratory tract infections, and bacteria being responsible for secondary soft-tissue and urinary

tract infections. Determination of gradated efficacy or harm in this context was felt disingenuous given the absence of clearly divergent results between categories.

Comparisons of specific sub-classes of pathogen (e.g. Gram-positive versus negative bacteria, aerobic versus anerobic) were rendered largely impossible due the paucity of studies specifying detailed microbiology. Case reports and series surrounding severe soft-tissue infection were the exception, frequently identifying Group A beta-haemolytic *Streptococcus* (GAS) as the cause of necrotizing fasciitis. Other bacteria implicated in these cases included *Streptococcus pyogenes*, *Streptococcus pneumoniae* and *Staphylococcus aureus*. Whilst it could be hypothesized that NSAIDs led to increased susceptibility to Gram-positive bacteria, the inclusion of studies advocating the efficacy of NSAIDs in reducing the incidence of Gram-positive infections in separate settings³⁶² and the comparable microbiological profile of patients with CAP taking NSAIDs or not³⁴⁸, suggests otherwise.

Severity

Infection severity was necessarily graded in a binary manner; included papers being split into those exploring the effect of NSAIDs on either sepsis or patients admitted to critical care, or not. 2 papers addressing severe pneumonia with septic complications were additionally reviewed. In total 14 manuscripts were classified as relevant to 'severe infection'.

Three RCT's explored the effect of NSAID administration on sepsis. One small trial (29 patients) designed to assess safety of IV ibuprofen administration in severe sepsis demonstrated a significant anti-pyretic effect but no corresponding haemodynamic, respiratory or metabolic alterations³⁶³. The second reported no significant effect of IV lornoxicam on any parameter, including biochemical and major pro-inflammatory cytokine concentrations compared to placebo³⁶⁴. Both studies indicated that short term NSAID administration was safe with no adverse GI, renal or hepatic sequelae.

The major interventional study to date in this population, randomising patients with sepsis and at least one organ failure to either placebo or IV ibuprofen (10mg/kg [max 800mg] every 6 hours for 8 doses), found significant reduction in urinary prostanoid metabolites with corresponding decreases in physiological variables (including oxygen consumption and lactic acidosis), but no statistically significant improvement in survival (37% NSAID vs. 40% placebo)²⁷⁶. This is supported by a small observational study in septic neonates where babies receiving ibuprofen for

treatment for patent ductus arteriosum were noted to have lower circulating C-reactive protein and IL-6 after day 4 compared to those not on NSAIDs³³⁶. No comment on outcome was made in this paper.

The null findings of RCTs contrast strongly with reviewed observational reports of NSAID use in severe infection. Prescription of anti-platelet regimes for cardiovascular, cerebrovascular or peripheral artery disease was associated with decreased risk of mortality in various subgroups of a mixed intensive care population (ORs 0.04-0.34, 93.5% of patients taking aspirin), even including those with high bleeding risk³⁶⁵. Patients with severe sepsis receiving anti-platelet agents for at least two days during their ICU stay (96.7% including aspirin) were reported to have a significantly reduced risk of in-patient mortality (In-ICU: OR 0.56, 95% CI 0.37-0.84, In-hospital: OR 0.57, 95% CI 0.39-0.83)³⁶⁶. This is supported by a large retrospective cohort study relating aspirin usage in the 24-hour period around the time of the systemic inflammatory response syndrome or sepsis recognition with reduced in-hospital death (10.9% aspirin vs. 17.2% propensity matched non-users, and 27.4% vs. 42.2% respectively)³⁶⁷. There was also evidence within the reviewed papers that this beneficial effect may extend to other NSAIDs, however this was abrogated if they were used in conjunction with aspirin³⁶⁸.

Observational data regarding a potentially preventative or protective role for COX-inhibiting drugs against severe infection was mixed. As previously discussed, one case-control study indicated that prior anti-platelet therapy (predominantly aspirin) may reduce the requirement for ICU admission and length of hospital stay in patients with CAP³⁴⁹, however pre-hospital aspirin therapy was not found to be independently associated with a reduction in either the development of sepsis, acute lung injury (ALI)/acute respiratory distress syndrome (ARDS) or in-hospital mortality in a separate prospective cohort study³⁶⁹. Despite concerns that acute and/or chronic NSAID use during evolving bacterial community-acquired infection may lead to the development of sepsis and septic shock raised in one article³⁴⁸ this was not borne out in a multi-centre case-control study³⁷⁰.

Two included studies investigated the effect of existing aspirin therapy on the development of ALI specifically, the most common cause of which is severe sepsis. A small, single-centre cohort study reported reduced incidence of ALI (12.7% vs. 28%; OR 0.37, 95% CI 0.16-0.84) in patients admitted to ICU with known risk factors (34% presenting with pneumonia, 27% meeting sepsis criteria) on anti-platelet therapy (99% aspirin), however there was no effect on ICU or in-hospital mortality³⁷¹. A

similar reduction in incidence of ALI was also noted in a multicenter international cohort study, however this did not reach significance on multivariate analysis and after adjusting for propensity to receive aspirin (Cochran-Mantel-Haenzel pooled OR 0.7; 95% CI 0.48-1.03)³⁷².

3.3.5 Adverse effects

No reviewed interventional study reported statistically significant increases in non-infective adverse effects with short term NSAID therapy. This includes typical NSAID adverse effects including increased risk of bleeding, GI ulceration and hypersensitivity reactions. Indeed, as described, benefit was witnessed from their use was observed even in groups at high-risk of bleeding in one epidemiological study³⁶⁵. Caution in their acute use should not be neglected however. One small trial of ibuprofen in sepsis noted a trend to reduced creatinine clearance³⁶³ whilst aspirin users experienced double the rate of renal impairment in propensity-matched non-users in a retrospective cohort study³⁶⁷.

Long-term usage of NSAIDs is commonly associated with both upper and lower GI side effects secondary to inhibition of 'protective' homeostatic PG production, and in the case of certain agents, direct toxicity. These may account for many of the complications, including perforation with resultant local abscess formation or generalized peritonitis, seen when employed in patients with known diverticular disease³⁵⁹.

3.4 DISCUSSION

A large published literature regarding the use of NSAIDs in clinical infection has accumulated. Whilst this variably describes the impact of NSAIDs on both the predisposition to, and the symptoms, severity and outcome from multiple infections, breadth has triumphed over depth. The articles included in this review demonstrate wide variance in experimental question, size, design and study population. Whilst partly a function of the expansive search and inclusion criteria employed, this likely reflects a fundamental lack of clear research aims and hence direction. Heterogeneity in case definitions and methodological quality, coupled to the frequent absence of either microbiological detail or a clear pharmacological strategy, render it challenging to draw substantive conclusions regarding the immunomodulatory potential of NSAIDs from this body of work.

3.4.1. Protopathic bias links NSAIDs to harm

The accumulated evidence does indicate that NSAIDs are clearly efficacious as analgesic, anti-pyretic and anti-inflammatory agents in the context of acute infection, superior to placebo and potentially alternate agents. This may be both a blessing (predominantly) and, paradoxically - as this review highlights - a curse.

Multiple epidemiological studies reported an association between NSAID administration and severe soft tissue infections, particularly GAS necrotizing fasciitis, and worsening of³⁵³ or increased severity³⁴⁸ of bacterial infection. The potential for a causal link between these events has been the subject of debate for around 30 years but is inherently confounded by the observational nature of studies and protopathic bias: where drugs are applied to treat symptoms that are actually early manifestations of the outcome of interest^{373,374}.

The apparent efficacy of NSAIDs in masking clinical indicators of disease severity (i.e. pyrexia, pain, local oedema and erythema) may delay illness recognition, presentation and appropriate intervention with resultant adverse outcomes: a feature shared with other analgesic agents^{343,375}. This may be exacerbated when the patient's ability to describe or qualify symptom evolution is impaired or confounded. Accordingly, this review found a preponderance of articles detailing the occurrence of secondary bacterial infection in the paediatric population or in the presence of a primary insult/existing diagnosis to which symptoms may be ascribed. To date, experimental animal models and prospective studies have failed to support the association between NSAIDs and invasive streptococcal disease (see Aronoff and Bloch for review³⁷⁶) or sepsis³⁷⁰, although concern lingers³⁴³. As such, whilst clinicians should have a high index of suspicion for infective complications/progression when prescribing NSAIDs acutely in this context, there is no clear clinical evidence of a negative immunomodulatory effect arising from COX-inhibition.

3.4.2. A potential role for NSAIDs in critical illness

A signal for the beneficial impact from NSAID administration, primarily aspirin, in sepsis was also evident: several observational studies over the past 5 years suggesting administration may reduce severity and improve outcomes when employed as an adjunctive treatment. Whilst these reports seem to stand in opposition to the major RCT in this area, which described significant improvement in biochemical markers but not mortality following ibuprofen administration in sepsis²⁷⁶, the debate may not be so clear-cut. The Ibuprofen in Sepsis Study was underpowered when seen in the context of modern day ICU trials, being designed to

detect a 35% reduction in mortality³⁷⁷. Further, in a post-hoc analysis of the data, patients admitted with hypothermic sepsis were found to have an exaggerated inflammatory response with significantly elevated serum cytokine and urinary PG metabolites. These individuals experienced a significant benefit from ibuprofen administration with 30 day mortality being reduced from 90% (18/20) to 54% (13/24)³⁷⁸. Given our increasingly nuanced view of sepsis and acceptance that a 'one-size fits all' therapeutic strategy is unlikely to be successful²⁹, this report offers hope despite its intrinsic methodological limitations.

Dysregulation of the inflammatory profile in terms of magnitude and duration is related to the occurrence of adverse outcomes in the critically ill, including prolonged hospital stay, acquisition of nosocomial infection and increased mortality⁴². Identification and subsequent stratification of ICU patients by associated humoral, cellular and functional biomarkers that delineate this population affords a rational approach to therapeutic decision making, allowing the administration of 'tailored' therapy^{379,380}. This strategy has already demonstrated potential when applied to the use of immunoadjuvant drugs³⁸¹. With recent work linking hypothermia at presentation with sepsis to clinically relevant immunosuppression³⁸², the tentative report of a mortality benefit from NSAID use in this population described above may reflect an inadvertent proof-of-principle study that prostaglandin blockade has a role in the immunostimulatory armamentarium alongside more established agents such as granulocyte-macrophage colony stimulating factor and interferon gamma^{381,383}. Indeed it has already shown promise in patients undergoing an alternate source of severe inflammation: major surgery^{210,211,384}.

How may NSAIDs exert a beneficial impact in this setting? The answer is likely multifactorial. Reduction of immunosuppressive prostanoid levels with consequent restoration of innate immune effector competence including phagocytosis and NADPH mediated killing^{199,203} would represent one central mechanism. NSAIDs proven ability to suppress fever could be another: a recent trial demonstrating superior outcomes in pyrexia septic patients cooled to normothermia³⁸⁵. Aspirin, for which there is the greatest evidence of benefit, may additionally augment antimicrobial function by increasing aspirin-triggered SPM release^{160,162} and via its antiplatelet effects (inhibition of TXA₂) mitigate microvascular thrombosis and subsequent organ failure³⁸⁶. Further, in high doses it may modulate NF-κB activation (via IκB kinase) and through acetylation of the endothelial nitric oxide (NO) synthase protein release NO, which reduces migration and infiltration of lymphocytes, regulates vascular tone and micro-vascular thrombi formation – all thought

pathogenic in ARDS (see for review^{377,387}). If confirmed, delineation of the key mechanistic pathway involved may allow refinement of therapeutic strategies, targeting more select pathways (e.g. the E-prostanoid 2 or 4 receptor, or microsomal PGE₂ synthase 1) to mitigate the risk of side effects.

3.4.3. Conclusions and limitations

Several limitations are evident with the review. The question asked was wide ranging and arguably un-focused, seeking to address the effect of any NSAID prescription on both predisposition to and outcome from all types and sites of infection. This was deliberate - the article being designed to primarily describe, and secondarily 'scope', the clinical literature on NSAIDs and infection for signals of either harm or benefit that may be evaluated in future more focused studies. Bias may have been introduced as only one database (MEDLINE and MEDLINE In-Process and Other Non-Indexed Citations) was searched, English language papers alone were eligible for inclusion, not all identified records were retrieved due to inability to gain institutional access and papers not identified through the initial search may have been missed on bibliographic trawl. Whilst this cannot be excluded it is unlikely that these restrictions would either exert systematic influence on the reviews conclusions or lead to the omission of whole themes. To further mitigate this, abstracts from papers where lack of institutional access prevented the full-text being obtained were separately evaluated to determine whether important or novel content was omitted. No clear new outcome patterns or themes were identified.

Heterogeneity within the study set and the breadth of the clinical question precluded performance of formal meta-analysis. The outcome matrix employed was non-validated, un-weighted and intended to provide an inclusive, summative guide to direction of effect. Consideration of discrete sub-classes of paper – particularly case reports and case-series – separately may have been beneficial, these studies exerting disproportionate influence on the overall analysis given the relative number of patients described. The majority of included studies were observational in nature and thus unable to establish causality. Given the multi-modal clinical effects of NSAIDs this renders determination of a mechanism of action even more challenging; recorded alterations in outcome (especially symptomatic) potentially being due to analgesic, anti-pyretic, anti-inflammatory, anti-platelet, anti-microbial effects or a combination of these discrete but overlapping properties. Exploration of existing clinical trial databases where patients have been randomised to NSAID and either placebo or alternate therapy in the absence of infectious disease (e.g. osteoarthritis)

for evidence of altered incidence, prevalence and/or severity of infection (as adverse events) may help ameliorate these concerns.

Despite these limitations, I believe the review highlights key deficiencies and areas of promise in the literature. NSAIDs are effective in reducing local and systemic symptoms of infection, however may obscure or delay the diagnosis of secondary infection or infective complications. There is little to no evidence of altered objective (e.g. biochemical markers or duration of illness) or mortality outcomes in non-severe infection of any aetiology when administered alone or as adjuvant to standard anti-microbial chemotherapy. Importantly, short-term NSAID administration in the context of acute infection appears safe, RCT's demonstrating no increase in rates of traditional side effects.

Observational evidence of clinical benefit in severe infections corresponds with animal-studies²⁶². To date there have been a paucity of high quality, appropriately designed and powered prospective clinical studies to either refute or support pre-clinical evidence of efficacy and/or harm from *in-vivo* lipid mediator manipulation in the context of infection. In particular establishment of both a clear mechanism of action (physiological/immunological/haematological, tissue/cell type, mediator, receptor) and pharmacological rationale has been absent (COX-isoform, timing, dose, route, duration of administration). Aspirin, the first NSAID, appears a promising agent and sepsis, arguably man's oldest ailment, the best target to validate this in. Clinical trials are already underway to test this hypothesis, investigating whether aspirin may prevent (ANTISEPSIS, Aspirin to Inhibit Sepsis, ACTRN12613000349741) or improve outcomes from sepsis (Aspirin for the Treatment of Sepsis, NCT01784159). Additionally, aspirin's ability to both avert the development of ARDS (LIPS-A, Lung Injury Prevention Study with Aspirin, NCT01504867) and treat it (STAR Trial, Aspirin as a Treatment for ARDS, NCT02326350) is being evaluated by Phase 2 and 3 trials³⁸⁷. Knowledge gleaned from these trials will hopefully validate the accumulated observational data, inform future patient stratification and facilitate refinement of therapeutic strategies.

3.5 SUMMARY

- The clinical literature describing NSAID use in the context of, or their effect on susceptibility to, acute infection demonstrates heterogeneity in demographic, pharmacological and infective dimensions.
- NSAID use in severe infection appears safe. RCT's do not demonstrate an increased rate of 'traditional' GI, renal, haematological and allergic side effects. Their use may however obscure new infective diagnoses or disease progression.
- Multiple observational studies report benefit from NSAID use, and in particular aspirin, in the context of critical illness (severe pneumonia, sepsis, acute lung injury). The mechanism of action underlying this effect is unknown and likely multi-factorial. Ablation of immunosuppressive COX-derived prostanoids represents one plausible explanation.
- Pre-clinical human studies to delineate biomarkers predictive of efficacy and define a pharmacologically rational approach to COX-inhibition in critical illness are required. These may inform future clinical trials of stratified immunomodulatory therapy.

3.6 APPENDIX: Articles meeting inclusion criteria, accessible online via institutional access. Key: *Adult:* age ≥18 years. *Paediatric:* 28 days – 17 years. *Neonate:* age <28 days. *Not specified:* Information not specified in the study. ‘–’: Information not relevant to the study. *n/a:* Not applicable. *X:* Information not accessible.

First Author	Journal	Reference	Observational/		Drug	Dose	Route	Duration	Infections	Pathogens	Type of pathogen	
			Interventional	n=							Outcome	
Desmeules R.	Can Med Assoc J	82:1219-22, 1960 Jun 11	Interventional	20	Phenylbutazone	400mg OD	PO	1-12 months	Tuberculosis	Mycobacterium tuberculosis	Mycobacterial	Null
Fraser Pk.	Lancet	1(7230):614-6, 1962 Mar 24	Interventional	600	Aspirin	332mg TDS	PO	Not specified	Upper respiratory tract infections	Not specified	Bacterial and Viral	Pos1
Doull JA.	Int J Lepr Mycobact Dis	35(2):128-39, 1967 Apr-Jun.	Interventional	400	Mefenamic acid	750mg	PO	24 weeks	Lepromatous leprosy	Not specified	Mycobacterial	Null
Nespoli L.	J Int Med Res	10(3):183-8, 1982.	Interventional	80	Fentiazac	100-200mg OD-BD	PR	7 days	Upper respiratory tract infection	-	Viral	Pos1
Pfandner K.	Arzneimittel-Forschung	34(1):77-9, 1984	Interventional	40	Nimesulide	200mg OD	PO	20 days	Urinary tract infection	Not specified	Bacterial	Pos1
Krige JEJ.	Lancet	1985; 2: 1432-3	Observational	1	Diflusal	Not specified	PO	1 day	Soft tissue infections	Group B streptococcus	Bacterial	Neg2
Brun-Buisson CJL.	BMJ	1985; 290: 1786	Observational	6	Aspirin, Diclofenac, Indomethacin, Phenylbutazone, Oxyphenbutazone	Not specified	PO	Not specified	Soft tissue infections	Group A streptococcus	Bacterial	Neg2
Weippl G.	Arzneimittel-Forschung	35(11):1724-7, 1985.	Interventional	120	Suprofen	50-200mg	PR	1 day	Not specified	Not specified	Bacterial and Viral	Null
Weippl G.	Arzneimittel-	35(11):1728-31,	Interventional	115	Suprofen	50-200mg	PO	2 days	Not specified	Not specified	Bacterial and	Pos1

Forschung 1985.											Viral	
Rimailho A.	J Infect Dis	155(1):143-6, 1987 Jan.	Observational	7	Aspirin, Diclofenac, Piroxicam, Niflumic acid	2g, 100mg, 20mg, 500mg	PO	Not specified	Soft tissue infections	Group A streptococcus	Bacterial	Neg2
Corder A	Br Med J Clin Res Ed.	295(6608):1238, 1987 Nov 14.	Observational	192	NSAIDs	n/a	PO	n/a	Diverticulitis	Not specified	Not specified	Neg2
Varsano IB.	Ann Otol Rhinol Laryngo	98(5 Pt 1):389-92, 1989 May	Interventional	81	Naproxen	7mg/kg TDS	PO	10 days	Acute otitis media	Not specified	Not specified	Null
Wilson RG.	Br J Surg	77(10):1103-4, 1990 Oct	Observational	92	NSAIDs	n/a	PO	4 weeks-5 years	Diverticulitis	Not specified	Not specified	Neg2
Bertin L.	J Ped	119(5):811-4, 1991 Nov	Interventional	231	Ibuprofen	10mg/kg TDS	PO	2 days	Upper respiratory tract infection	Not specified	Bacterial and Viral	Pos1
Chosidow O.	Arch Derm	127(12):1845-6, 1991 Dec	Observational	96	NSAIDs	n/a	PO	n/a	Soft tissue infections	Not specified	Not specified	Neg2
Haupt MT.	Crit Care Med	19(11):1339-1347. 1991 Nov.	Interventional	29	Ibuprofen	600mg OD, IV 800mg OD, IV 800mg TDS, PR	IV, PR	1 day	Septic shock	Not specified	Not specified	Pos1
Smith RJ.	South Med J	1991; 84: 785-7	Observational	1	Piroxicam	10mg x8	PO	1 day	Soft tissue infections	Group A streptococcus	Bacterial	Neg2
Campbell K.	Br J Surg	78(2):190-1, 1991 Feb.	Observational	50	NSAIDs	n/a	PO	n/a	Diverticulitis	Not specified	Not specified	Neg2
van Ammers PM.	S Afr Med J.	80(4):203-4, 1991 Aug 17.	Observational	3	Indomethacin, Flurbiprofen	100mg BD	PR	2-6 days	Soft tissue infections	Staph aureus, Strep pyogenes, klebsiella	Bacterial	Neg2

Campbell KL.	Br J Surg	79(9):967-8, 1992 Sep.	Observational	84	NSAIDs	n/a	PO	n/a	Acute appendicitis	Not specified	Not specified	Neg2
Lotti T.	Drugs	46 Suppl 1:144-6, 1993.	Interventional	120	Nimesulide	200mg OD	PO	9 days	Urinary tract infection	Not specified	Bacterial	Pos1
Gianiorio P.	Drugs	46 Suppl 1:204-7, 1993.	Interventional	40	Nimesulide	1.5mg/kg TDS	PO	3-7 days	Lower respiratory tract infection	Not specified	Viral	Pos2
Salzberg R.	Drugs	46 Suppl 1:208-11, 1993.	Interventional	100	Mefenamic acid, Nimesulide	5mg/kg	PO	3-10 days	Upper respiratory tract infection	Not specified	Viral	Pos1
Ugazio AG.	Drugs	46 Suppl 1:215-8, 1993	Interventional	100	Nimesulide	5mg/kg OD	PO	3-9 days	Upper respiratory tract infection	Not specified	Bacterial and Viral	Pos2
Barberi I.	Drugs	46 Suppl 1:219-21, 1993.	Interventional	70	Aspirin, Nimesulide	50mg BD, 350mg BD	PO	5 days	Upper and lower respiratory tract infections	Not specified	Not specified	Pos1
Hird B.	J Trauma	1994; 36:589-91	Observational	1	NSAIDs	Not specified	PO	Not specified	Soft tissue infections	Group A streptococcus	Bacterial	Neg2
Rowan JA.	Am J Obs & Gyne	173(1):241-2, 1995 Jul.	Observational	1	Diclofenac	75mg BD	PO	4 days	Soft tissue infections	Group A streptococcus	Bacterial	Neg2
Bernard GR.	N Engl J Med	1997; 336:912-918.	Interventional	455	Ibuprofen	10mg/kg QDS	IV	2 days	Sepsis	Not specified	Not specified	Pos2
Choo PW.	Anna Epidemiol	7(7):440-5, 1997 Oct.	Observational	89	Ibuprofen	n/a	n/a	n/a	Soft tissue infections	Not specified	Bacterial	Null
Evans JM.	Br J Surg	84(3):372-4,	Observational	138	NSAIDs	n/a	PO	n/a	Acute appendicitis	Not specified	Not specified	Null

1997 Mar												
Aldoori WH.	Arch Fam Med	1998 May-Jun;7(3):255-60	Observational	35615	NSAIDs	n/a	PO	n/a	Diverticulitis	Not specified	Not specified	Neg2
Zerr DM.	Pediatrics	103(4 Pt 1):783-90, 1999 Apr	Observational	19	Ibuprofen	n/a	PO	n/a	Soft tissue infections	Group A streptococcus, other Gram positives	Bacterial	Neg2
Ulukol B.	Eur J Clin Pharmacol	55(9):615-8, 1999 Nov.	Interventional	90	Ibuprofen, Nimesulide	2.5mg/kg BD, 10mg/kg TDS	PO	5 days	Upper respiratory tract infection	Not specified	Bacterial and Viral	Pos1
Passali D.	Clin Therapeut	23(9):1508-18, 2001 Sep	Interventional	241	Ketoprofen	160mg in 100mL BD	Gargled	7 days	Upper respiratory tract infection	Not specified	Not specified	Pos1
Frick S.	Clin Infect Dis	33(5):740-4, 2001 Sep 1.	Observational	2	Diclofenac, Tenoxicam	75mg OD	IM	1 day	Soft tissue infections	Strep pneumoniae	Bacterial	Neg3
Barnham MR.	Clin Microbiol Infect	8(3):174-81, 2002 Mar.	Observational	11	Ibuprofen, Aspirin, Diclofenac, Fenbufen	Not specified	PO	Not specified	Toxic shock	Strep pyogenes	Bacterial	Neg3
Vinh H.	Ped Infect Dis J	23(3):226-30, 2004 Mar	Interventional	80	Ibuprofen	10mg/kg QDS	PO	36 hours post-fever	Typhoid fever	Salmonella typhi	Bacterial	Pos1
Adler A.	Ped Cardiol	25(5):562-4, 2004 Sep-Oct	Observational	1	Aspirin	3mg/kg/day	PO	7 days	Infective endocarditis	Serratia marcescens	Bacterial	Pos2
Memis D.	Crit Care	8(6):R474-82, 2004 Dec.	Interventional	40	Lornoxicam	8mg BD	IV	3 days	Sepsis	Not specified	Bacterial	Null
Bachert C.	Clin Therapeut	27(7):993-1003, 2005 Jul.	Interventional	392	Aspirin	500mg OD, 1000mg OD	PO	1 day	Upper respiratory tract infection	Not specified	Viral	Pos1
Tamburini J.	J Infect	51(4):336-7, 2005 Nov	Observational	1	Ibuprofen	1200mg OD	PO	5 days	Meningitis	Neisseria meningitidis	Bacterial	Null

Goto M.	Int Med	46(15):1179-86, 2007	Interventional	174	Loxoprofen	60mg BD	PO	7 days	Upper respiratory tract infection	Not specified	Viral	Null
Sedlacek M.	Am J Kid Dis	49(3):401-8, 2007 Mar	Observational	872	Aspirin	n/a	PO	n/a	Bacteraemia	Staphylococcus	Bacterial	Pos2
Orlando A.	J Infect	54(3):e145-8, 2007 Mar	Observational	1	Ketorolac, Diclofenac	Not specified	IM	1 day	Soft tissue infections	E. Coli	Bacterial	Neg3
Souyri C.	Clin Exper Derm	33(3):249-55, 2008 May.	Observational	38	NSAIDs	n/a	n/a	n/a	Soft tissue infections	Group A strep, Staphylococcus, Pseudomonas aeruginosa, Proteus, Colibacillus	Bacterial	Neg2
Cetinkaya M.	Turk J Ped	50(4):386-90, 2008 Jul-Aug.	Observational	1	Indomethacin	1mg/kg	PO	18 days	Necrotizing enterocolitis	Klebsiella	Bacterial	Neg3
Mikaeloff Y.	Br J Clin Pharmacol	65(2):203-9, 2008 Feb	Observational	1067	NSAIDs	n/a	PO	n/a	Soft tissue infections	Not specified	Bacterial	Neg2
Dubos F.	Acta Dermatovenereologica	88(1):26-30, 2008.	Observational	159	NSAIDs	n/a	PO	n/a	Soft tissue infections	Not specified	Bacterial	Neg2
Legras A.	Crit Care	13(2):R43, 2009.	Observational	152	NSAIDs	n/a	n/a	6 days	Sepsis	Strep pneumoniae, Staph aureus, Strep pyogenes, E. coli	Bacterial	Null
Winning J.	Platelets	2009; 20(1):50-7	Observational	224	Aspirin	≤100mg OD	PO	>6 months	Pneumonia	Not specified	Not specified	Pos2
Kumar M.	Invest Ophthalmol	50(12):5601-8, 2009 Dec	Interventional	45	Aspirin	350mg BD	PO	30 days	Latent HSV-1	HSV-1	Viral	Null

Vis Sci

Winning J.	Crit Care Med	2010; 38(1):32-7	Observational	615	Aspirin	n/a	PO	n/a	Not specified	Not specified	Not specified	Pos3
Schechter BA.	Adv Ther	27(10):756-61, 2010 Oct.	Interventional	24	Bromfenac	0.09% solution, single eyedrop BD	Eyedrop	102 days max	Corneal ulcer	Not specified	Bacterial and Fungal	Pos1
Uri O.	J Plast Reconstr Aesthet Surg	63(1):e4-5, 2010 Jan.	Observational	1	Diclofenac	75mg OD	IM	5 days	Soft tissue infections	Staph aureus	Bacterial	Neg2
Bleidorn J.	BMC Medicine	26:8:30, 2010 May 26	Interventional	80	Ibuprofen	400mg TDS	PO	3 days	Urinary tract infection	E. coli	Bacterial	Pos1
Krudsood S.	Am J Trop Med Hyg	83(1):51-5, 2010 Jul	Interventional	60	Ibuprofen	400mg QDS	IV	3 days	Malaria	Plasmodium falciparum	Parasite	Pos1
Azuma A.	Pharmacology	85(1):41-7, 2010.	Interventional	170	Zaltoprofen	80mg OD, 160mg OD	PO	1 day	Upper respiratory tract infection	Not specified	Viral	Pos1
Beyer I.	BMC Musculoskeletal Disord	12:292, 2011.	Interventional	30	Piroxicam	10mg OD	PO	3 weeks max	-	-	-	Pos1
Voiriot G.	Chest	139(2):387-94, 2011 Feb.	Observational	90	NSAIDs	n/a	PO	5 days	Pneumonia	Strep pneumoniae, Legionella, Pseudomonas aeruginosa	Bacterial	Neg2
Strate LL.	Gastroenterology	140(5):1427-33, 2011 May.	Observational	47210	NSAIDs	325mg (1 to >6 doses/week)	PO	n/a	Diverticulitis	Not specified	Not specified	Neg2

Erlich JM.	Chest	2011; 139(2):289-95	Observational	161	Aspirin	n/a	PO	n/a	ALI	n/a	n/a	Pos2
O'Neal HR. Jr	Crit Care Med	2011; 39:1343- 1350	Observational	575	Aspirin	n/a	PO	n/a	Sepsis	-	-	Pos3
Kor DJ	Crit Care Medicine	2011; 39(11):2393- 400	Observational	3855	Aspirin	n/a	PO	n/a	ALI	n/a	n/a	Null
Azuma A.	Pharmacolog y	87(3-4):204-13, 2011.	Interventional	330	Zaltoprofen	160mg OD	PO	1 day	Upper respiratory tract infection	Not specified	Viral	Pos1
de Korte N.	Colorectal Disease	14(3):325-30, 2012 Mar	Observational	272	NSAIDs	n/a	PO	n/a	Diverticulitis	Not specified	Not specified	Neg2
Eisen DP.	Crit Care Medicine	40(6):1761-7, 2012 Jun	Observational	7945	Aspirin	150mg OD	PO	1 day	Sepsis	Not specified	Not specified	Pos3
Demirel G.	Early Hum Devel	88(4):195-6, 2012 Apr	Observational	121	Ibuprofen	10mg/kg OD, then 5mg/kg BD	PO	2 days	Sepsis	Not specified	Not specified	Pos2
Otto GP.	Platelets	2013; 24(6): 480-485	Observational	886	Aspirin	n/a	PO	n/a	Sepsis	Not specified	Bacterial	Pos3
Sossdorf M.	Crit care	2013; 8;17(1):402	Observational	979	NSAIDs, Aspirin	n/a	PO	n/a	Sepsis	Not specified	Not specified	Pos3
Llor C	BMJ	347:f5762. 2013 Oct	Interventional	416	Ibuprofen	600mg TDS	PO	10 days	Lower respiratory tract infection	Not specified	Viral	Null

CHAPTER 4: Stimulated Whole Blood

THE EFFECTS OF PROSTAGLANDIN E2 ON A CLINICALLY VALIDATED MEASURE OF IMMUNE DYSFUNCTION: WHOLE-BLOOD LPS-STIMULATED CYTOKINE SECRETION

4.1 Introduction

4.2 Additional Methods

4.3 Results

4.4 Discussion

4.5 Summary

4.6 Appendix

Publications:

Segre E and Fullerton JN. Stimulated Whole Blood Cytokine Release as a Biomarker of Immunosuppression in the Critically Ill: the Need for a Standardized Methodology.

Shock. *In Press*

4.1: INTRODUCTION

4.1.1 Monocyte deactivation

Monocyte deactivation is a key feature of CIID. Circulating monocytes extracted from CI patients characteristically display reduced production of pro-inflammatory cytokines in response to *ex-vivo* challenge^{138,388,389}, diminished phagocytosis^{390,391}, and loss of cell surface markers associated with antigen-presentation/T-cell stimulation including CD86 and HLA-DR^{392,393}. Deactivation has clear prognostic implications in this population. Reduced HLA-DR expression or cytokine production *ex-vivo*, either on ICU admission or which fails to return to normal values, is associated with increased rates of nosocomial infection^{134,137,393}, secondary sepsis³⁹⁴, and mortality^{136,138,395}. When combined with objective metrics of dysfunction in other leukocyte populations (PMN and T-cells) the predictive ability and clinical implications of these bioassays is further pronounced³⁹⁶.

Such data provide the rationale behind the undertaking of immunological monitoring and administration of immunoadjuvant therapy. This strategy dictates the stratification of CI patients into those with adequate or inadequate immune function, and the administration of tailored therapies to restore effector cell function in those with demonstrable immunoparalysis^{310,397-399}. Not only vital in identifying individuals as immune-compromised and likely to benefit from immunoadjuvant agents, bioassays of immune function may be used to titrate therapy against them, their normalisation being predictive of treatment efficacy and clinical benefit^{381,383}.

4.1.2 Immunorestorative therapy

To date, late-phase monocyte deactivation has been successfully reversed, at least partially, by both granulocyte-macrophage colony stimulating factor (GM-CSF)^{132,381,400-402} and interferon- γ (IFN- γ)^{131,383,403} in pre-clinical and small clinical trials. Significantly, rectification of HLA-DR expression and/or *ex vivo* cytokine production is paralleled by *in vivo* augmentation of pro-inflammatory TNF α and attenuation of anti-inflammatory IL-10 release^{137,383}. Further, restoration of immune competence via GM-CSF appears to be linked with improved clinical outcomes including reduced incidence of nosocomial infection¹³⁷, significant reduction in length of ICU and hospital admission, decreased time on mechanical ventilation, and improved acute physiology and chronic health evaluation (APACHE) II score³⁸¹.

As discussed in previous chapters, PGE₂ has long been known to suppress multiple monocyte and macrophage functions - including pro-inflammatory cytokine secretion - principally through EP2 and/or EP4-mediated increase in intra-cellular

cAMP^{207,404,405}. Given the ability of NSAIDs to improve survival in animal models of sepsis and their observed efficacy in critically ill patients, it is plausible that PGE₂ may contribute to clinically relevant monocyte deactivation. If this were true its ablation or antagonism would afford a further, potentially complimentary immunoadjuvant therapy to the afore-described agents.

4.1.3 Whole blood LPS-stimulated TNF α secretion

Ex vivo whole blood (WB) LPS-stimulated TNF α secretion has emerged as a key bioassay of immune competence. Thought to principally reflect monocyte function (being the primary cellular source of TNF α ⁴⁰⁶), a reduction in pro-inflammatory cytokine release has been demonstrated to be predictive of adverse clinical outcomes in the paediatric and adult CI population experiencing systemic inflammation as a result of either sterile or infective insults^{131,137,138,407,408}. Responsive to immunoadjuvant agents both *in vivo* and *ex vivo* with restoration of TNF α secretion being associated with improved outcomes, it represents an ideal functional assay with which to test a potential contribution of PGE₂ to clinically relevant monocyte deactivation.

4.1.4 Chapter aims

- Determine whether PGE₂ can induce monocyte deactivation in a clinically validated bioassay of immune-competence: whole blood (WB) LPS-stimulated TNF α secretion
- Deduce the pharmacological mechanism of any PGE₂-mediated effect and, if demonstrated, prove the efficacy of its antagonism determining whether this is independent of known immunoadjuvant therapies (GM-CSF and IFN- γ)
- Generate a key bioassay of PGE₂-mediated monocyte deactivation for use in the IV endotoxemia model of systemic inflammation through appropriate characterisation

4.2 ADDITIONAL METHODS

4.2.1 Whole blood LPS-stimulated cytokine secretion: variation in the assay

Venous blood was obtained from healthy volunteers and stimulated as per Chapter 2 Methods (2.1.1 and 2.3.3), supernatant being analysed for cytokines and PGE₂ by ELISA (2.3.1 and 2.3.2). The effect of several variables on the assay were evaluated including:

- Time-course of TNF α and PGE₂ release (30min, 1hr, 2, 4, 6, 8, 24hrs)
- Anti-coagulant (EDTA, Na Citrate, LH)
- LPS type (species [*Escherichia coli* 0111:B4 or 055:B5, *Salmonella abortus equi* S-form, [TLRgrade™], Enzo Life Science, *Salmonella Minnesota* (R-form, Serotype:R595 , Hycult®)], rough vs. smooth) and dose (1pg/mL-100ng/mL)
- Incubation conditions (dilution, temperature, shaking)
- Contribution of biological and technical variance

4.2.2 PGE₂-mediated modulation of whole blood LPS-stimulated cytokine secretion

The core WB assay described in 2.3.3 was employed to evaluate

- Dose-response curve of PGE₂
- Shift in the dose-response curve elicited by selective EP-receptor:
 - Agonists: Butaprost (EP2), CAY10598 (EP4)
 - Antagonists: PF-04418948 (EP2), MF498 (EP4)
- NSAID-modulation of TNF α release (Indomethacin)
- The contribution of cAMP to observed alterations via:
 - Forskolin: direct activator of adenylyl cyclase
 - Rolipram: selective phosphodiesterase 4 inhibitor
- The effect of established immunorestorative agents: GM-CSF and IFN- γ

All reagents were obtained from Cayman Chemical (MI, USA) unless otherwise stated and re-constituted in DMSO such that the eventual assay concentration was <0.01%.

4.3 RESULTS

4.3.1 Characterisation of the WB LPS-stimulated cytokine release model

A review of the literature on utilisation of *ex vivo* cytokine secretion as a measure of immune competence indicated that authors had employed multiple methodological variations in the assay. The effect of each of these was assessed to elucidate their comparative impact on supernatant TNF α concentration and variability (Figure f4.1).

Stimulation with LPS (1ng/mL) from any of four bacterial sources (as 4.2.1) elicited high levels of TNF α release (Panel A). Whilst statistically different (matched one-way ANOVA with Greenhouse-Geisser correction, $p < 0.0001$), the rough (R) LPS derived from *Salmonella Minnesota* being more potent, the biological relevance of this is unclear. TNF α was released in response to concentrations of *Salmonella Abortus Equi* (SAE) as low as 100pg/mL, with only a 10-fold increase – 1ng/mL – eliciting a maximal response, no further increase being seen even at 100ng/mL (Panel B). Cytokine release in the presence of alternate anticoagulants was observed to be significantly different, being lower when EDTA was employed compared to LH or Na Cit (Panel C: paired t-test, 2-tailed, both < 0.001). Whilst there was a trend towards lower TNF α release in Na Cit compared to LH this did not reach significance (paired t-test, 2-tailed, $p = 0.08$).

Supernatant TNF α concentration was observed to peak between 6 and 24hours, and showed no significant difference from 4-24hours (Panel D: matched one-way ANOVA with Greenhouse-Geisser correction, $p = 0.09$). Incubation at 37°C was required for cytokine release, samples being left at room temperature (20°C) failing to release significant quantities (Panel E). Whilst a lower mean concentration of TNF α was determined in shaken samples (S) than not-shaken (NS), which did not reach significance, they were less variable (S: 4934pg/mL SD 455pg/mL, NS: 5911pg/mL SD 783pg/mL; paired t-test, 2-tailed, $p = 0.12$). Previous authors have employed dilution of WB with cell culture media to aid ease of technical performance and to enhance supernatant yield. As expected, with an increased ratio of media to WB, the TNF α concentration per unit supernatant fell from a mean of 7267pg/mL (no diluent, 0) to 2107pg/mL (10:1) (Panel E). Variability however fell with increasing dilution (undiluted and 1:1 diluted samples, SD 4158pg/mL and 4161pg/mL respectively, 1:5 SD 3491pg/mL and 1:10 1925pg/mL).

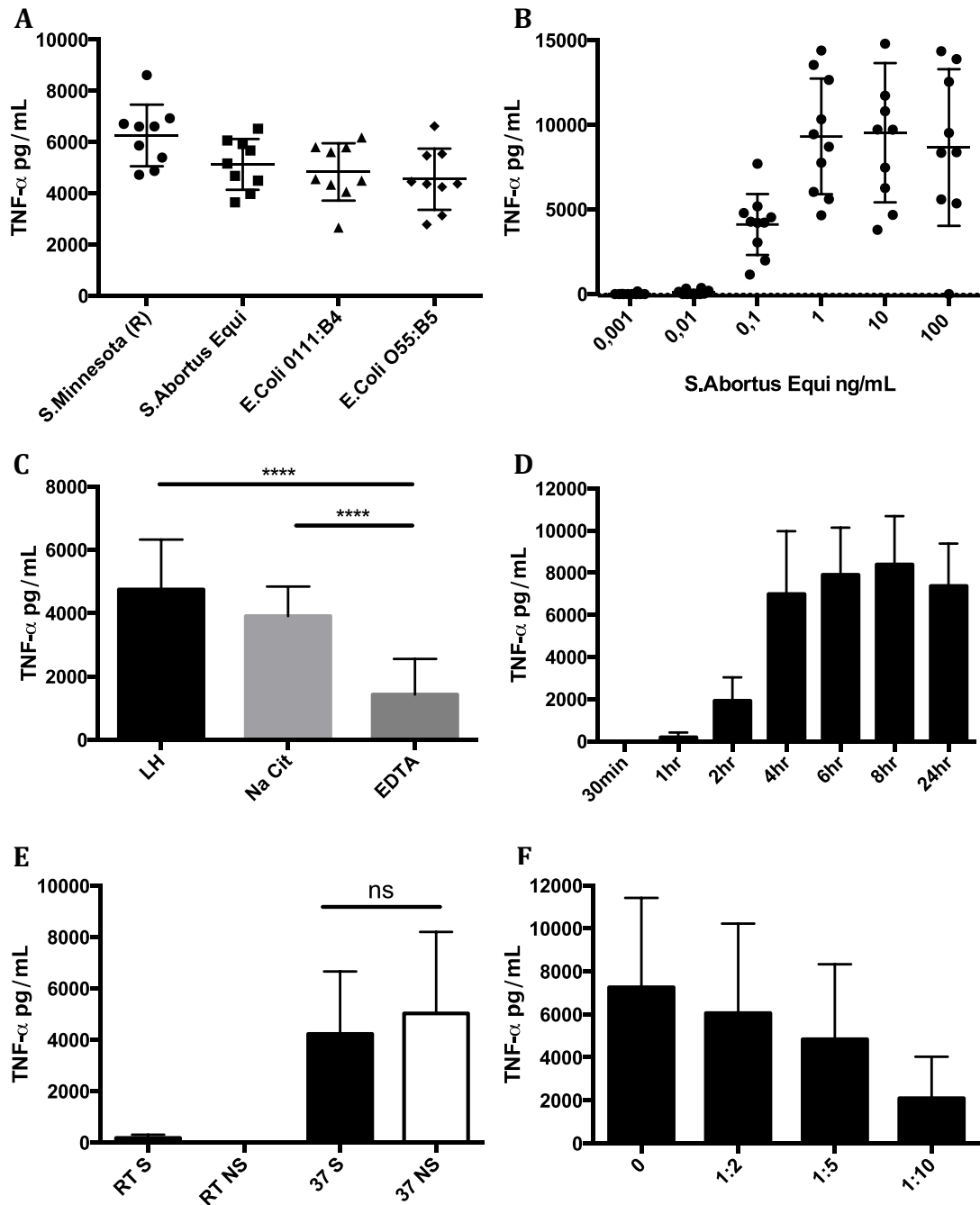
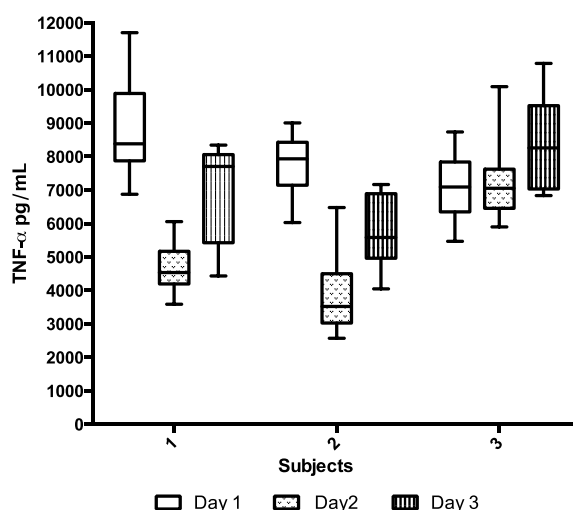


Figure f4.1. Methodological characterisation of the ex-vivo cytokine release assay. WB (1ml) from 10 HV (in triplicate) was stimulated with **A**: LPS (1ng/ml) from 4 different bacteria. TNF α from un-stimulated cells was undetectable **B**: Different doses of one type of LPS (SA, 1pg/mL – 100ng/mL) **C**: Having been anti-coagulated with either LH, Na Cit or EDTA (1ng/mL, SA) **D**: For different lengths of time prior to supernatant removal (SA, 1ng/mL, 30min-24h) **E**: In alternate incubation conditions either room temperature [RT, 20°C] or 37°C, whilst being shaken (S) or not shaken (NS), and **F**: Diluted in RPMI to different degrees (no diluent [0] to 1 part WB 9 parts RPMI [1:10]). N=10, all data in mean +/- SD.

Biological and technical variance in the assay was explored by undertaking ten technical repeats from three volunteers on three consecutive days using 1ng/mL SAE, 5:1 dilution, 37°C 250rpm, LH anticoagulant with supernatant aspiration at 6hours. As Figure f4.2 highlights, significant differences (all 2-way matched ANOVA, $p < 0.001$) between subjects, within subjects over time, and interaction between the two variables was observed. The latter indicates that individual subjects responses did not vary systematically with time, but likely via chance. Technical variance was demonstrable by examination of assay repeats (Table t4.1, Figure f4.2). Large standard deviations between identical samples ($n=10$ /subject/day) are observed, either reflecting genuine biological variability between WB drawn and handled identically or, more likely, cumulative variability from the conduct of the assay (e.g. length of incubation, dilution), sample storage (e.g. speed of freezing) and measurement error in recording TNF α .



Subject	Day 1		Day 2		Day 3	
	Mean	SD	Mean	SD	Mean	SD
1	8833.079	1577.628	4662.134	726.415	6992.939	1485.885
2	7815.977	894.5548	3822.005	1158.577	5739.586	1053.781
3	7085.192	981.8833	7259.572	1226.343	8441.676	1444

Figure f4.2 and Table f4.1: Intra-individual variation in LPS-stimulated whole blood TNF α release and PGE $_2$ -mediated suppression. Ten repeats of the WB assay (1mL WB, 5:1, 6hrs, SA 1ng/mL) from three individuals (1 male, 2 female) were performed on three consecutive days. The central line represents the mean, the upper and lower whiskers the maximum and minimum values, $n=10$ /individual/day. 2-way matched-sample ANOVA indicates significant differences between subjects ($P < 0.0001$, 14.26% variability), over time ($P < 0.0001$, 32.22% variability), and additionally interaction between the two ($P < 0.0001$, 19.47%).

4.3.2 PGE₂ as a mechanism of monocyte deactivation

WB was stimulated as per 2.3.3 to evaluate the effect of PGE₂ on cytokine secretion. 10ng/mL PGE₂ was observed to significantly suppress the release of both TNF α and IL-6 (multiple un-paired t-tests, equal SD assumed, Holm-Sidak correction for multiple comparisons) however it was not pan-inhibitory, eliciting non-significant increases in IL-1 β and IL-8 (Figure f4.3, Left Panel). Minimal IL-10 secretion was seen in response to LPS and this was not altered by the addition of PGE₂. PGE₂ inhibited LPS-stimulated TNF α release in a dose dependent manner, with an IC₅₀ of 317pg/mL (95% CI 105 – 959pg/mL) (Figure f4.3, Right Panel).

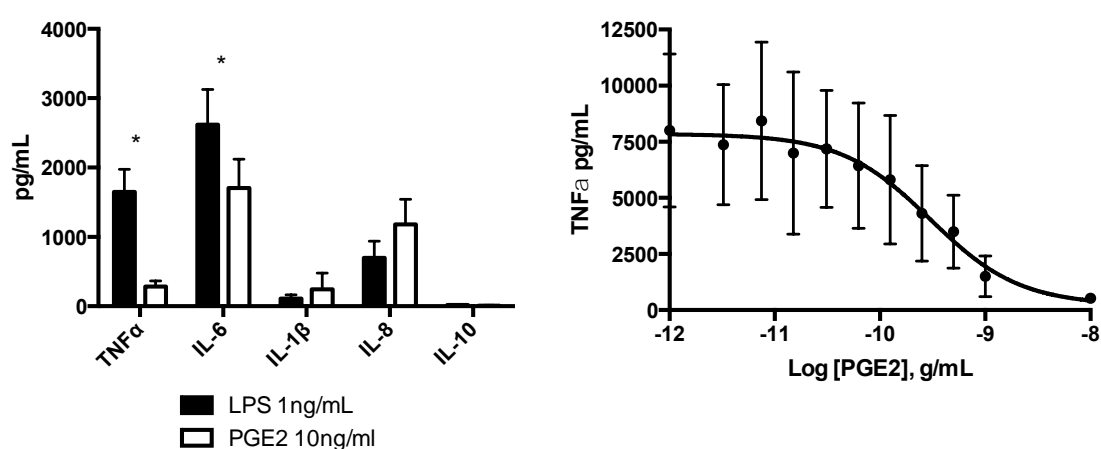


Figure f4.3: LPS-stimulated whole blood cytokine release and modulation by PGE₂. **Left Panel:** WB (1ml, 5:1 dilution, n=5 HV, 3 technical repeats/volunteer) was co-incubated with PGE₂ (10ng/mL) for 15mins prior to stimulation with LPS (SAE, 1ng/ml), supernatants being removed at 6hrs. Cytokine concentrations were determined by MSD V-Plex. Multiple un-paired T-test with Holm Sidak correction, * indicates significance. **Right Panel:** WB (n = 3 HV, 3 technical repeats/volunteer, mean +/- SD) was co-incubated with PGE₂ (1pg/mL – 10ng/ml) prior to stimulation as above. WB was pre-treated with indomethacin 10 μ M to prevent any exogenous prostanoid production. IC₅₀ was determined from a four-parameter dose-response curve.

A PGE₂-(G_s-coupled) EP4-cAMP axis appears to be the principal mechanism underlying TNF α suppression / monocyte deactivation. MF498 (1 μ M, a selective EP4 antagonist, Ki 0.7nM) causes a significant right-shift in the PGE₂ dose-response curve increasing the IC₅₀ 100-fold (PGE₂ IC₅₀ 200pM 95% CI 46–872pM, PGE₂-MF498 21nM 95% CI 1.5nM-305nM) whilst PF-04418948 (1 μ M, a selective EP2 antagonist, Ki 16nM) has no significant effect (IC₅₀ 380pM 95% CI 128pM-1.1nM) (Figure f4.4, Right Panel). In comparison, addition of the selective EP4 agonist CAY10598 mimicked the effect of PGE₂ at near equimolar concentrations (IC₅₀ 1nM

vs 1.7nM respectively), whilst the selective EP2 agonist butaprost did so only at ~50 times higher concentrations (IC50 73nM, Figure f4.4, Left Panel), reflecting their stated Ki of 1.2 and 73nM respectively.

The importance of the secondary messenger cAMP to the action of PGE₂ was emphasised by the potentiation of its effect in the WB assay by the addition of 10µM rolipram (RoP), which prevents catabolism of cAMP (Figure f4.4, Bottom Panel). Forskolin (100µM), a direct activator of adenylyl cyclase, mimicked the effect of PGE₂: an action that was again enhanced by RoP. Interestingly, the addition of RoP to LPS-stimulated WB led to an independent decrease in TNFα release, without the addition of an exogenous G_s-coupled receptor agonist or adenylyl cyclase activator. This implies the presence of an endogenous stimulator of cAMP production.

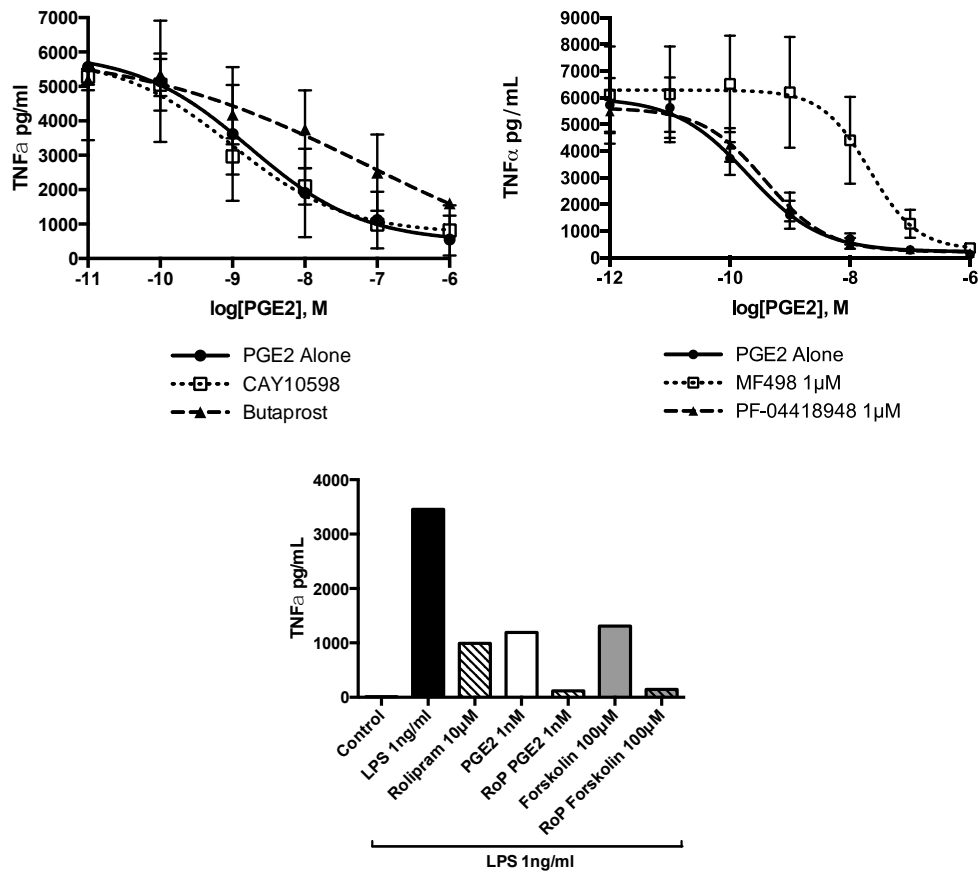


Figure f4.4: Pharmacological assessment of the receptor through which PGE $_2$ suppresses LPS-stimulated whole blood TNF α release. **Left Panel:** WB (n=3 HV, 3 technical repeats/volunteer) was incubated with 10pM-1 μ M PGE $_2$, butaprost (selective EP2 receptor agonist) or CAY10598 (selective EP4 receptor agonist) prior to stimulation with LPS. **Right Panel:** WB (n=3 HV, 3 technical repeats/volunteer) was co-incubated with PF-04418948 (1 μ M, selective EP2 receptor antagonist) or MF498 (1 μ M, selective EP4 receptor antagonist) prior to the addition of PGE $_2$ (1pM-1 μ M) and stimulation with LPS. IC $_{50}$ was determined from a four-parameter dose-response curve. Data points are mean \pm SD. **Bottom Panel:** WB (n=1 HV, 3 technical repeats) was incubated with PGE $_2$ 1nM, rolipram (RoP) 10 μ M, forskolin 100 μ M or combinations of the above and stimulated with LPS. All three agents suppressed TNF α concentrations in resultant supernatants, RoP further significantly potentiating the action of PGE $_2$ and forskolin. Data points are mean of 3 technical repeats, assayed (ELISA) in duplicate.

The generation and release of *ex vivo* generated PGE $_2$ by stimulated WB was tested for via ELISA (Figure f4.5). PGE $_2$ accumulated in the assay, first appearing around 1h and increasing up to 24h (666pg/mL, Top Panel). Co-incubation of LPS-stimulated WB with 1 μ M MF498 alone (or alternate selective EP4 antagonists, data not shown) resulted in increased TNF α release compared to LPS-stimulated blood

alone (Left Panel). This was replicated by the addition of the non-selective COX inhibitor indomethacin (10 μ M) to the assay, indicating that the effect of endogenous PGE₂ may be abrogated via either antagonism or prevention of TLR4-stimulated formation. To demonstrate that MF498 was not acting on COX, PGE₂ concentrations were determined in the same samples. Whilst indomethacin led to a complete suppression of PGE₂ formation, MF498 had no effect on this process.

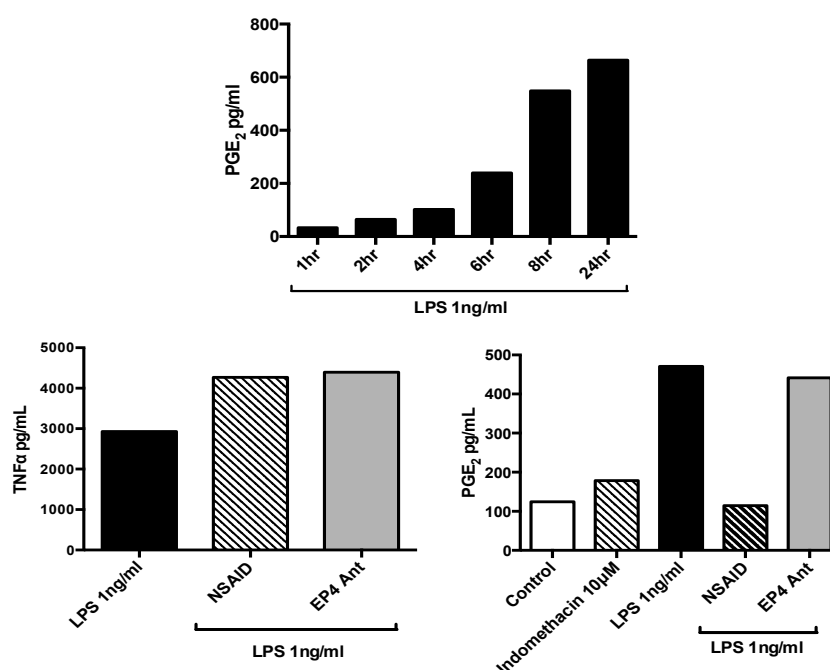


Figure f4.5: Endogenously generated PGE₂, induced by LPS, acts via the EP4 receptor to suppress TNF α secretion. **Top Panel:** LPS-stimulated WB was assayed for the presence of PGE₂ at time-points ranging from 1h-24h via ELISA (n=1 HV, 2 technical replicates, assayed (ELISA) in duplicate). Columns represent mean values. **Left Panel:** Both NSAID (indomethacin 10 μ M) and EP4 antagonist (MF498 1 μ M) addition to LPS-stimulated WB leads to enhanced TNF α accumulation (columns represent mean values of n=1 2 technical replicates, assayed (ELISA) in duplicate). **Right Panel:** Indomethacin, but not MF498, significantly suppresses PGE₂ generation *ex-vivo* (columns as per Left Panel).

4.3.3 Specificity and restoration of PGE₂ immunosuppressive effect

At physiologically relevant concentrations PGI₂, in addition to PGE₂ suppresses *ex vivo* TNF α secretion in the LPS-stimulated WB model. Carbaprostacyclin, a stable analog of PGI₂, was found to inhibit TNF α release with an IC₅₀ of 590pM (95% CI 444-785pM) in contrast to 427pM (95% CI 307-592pM) when directly compared to PGE₂. Carbocyclic thromboxane A₂, a stable analog of TXA₂, was found to have the nearest properties possessing an IC₅₀ of 184nM (95% CI 5.7nM to 5.8 μ M) (Figure f4.6).

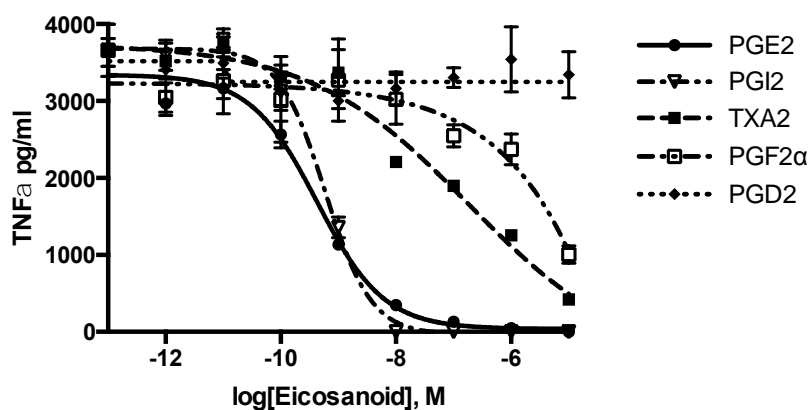


Figure f4.6: Comparative ability of alternate COX-derived prostanoids to elicit immunosuppression in the ex-vivo WB assay. Dose-dependent inhibition of LPS-stimulated (1ng/ml) TNF α release by PGD₂, PGF_{2 α} , PGI₂ (carbaprostacyclin, a stable analog of PGI₂) and TXA₂ (carbocyclic thromboxane A₂, a stable analog of TXA₂). WB was pre-treated with indomethacin 10 μ M to prevent exogenous prostanoid production. Data points represent the average of n=3 HV (2 technical repeats/volunteer, each assayed in duplicate [ELISA]), whiskers display SD. IC₅₀'s were determined via four-parameter dose-response curves.

IFN- γ and GM-CSF represent recognised immunoadjuvant therapies, capable of restoring ex vivo cytokine secretion in immunocompromised CI patients. As such their ability to reverse PGE₂-mediated cytokine secretion was assessed. Both agents were found to increase mean TNF α concentration (LPS 5482pg/mL, IFN- γ 8826pg/mL, GM-CSF 8666pg/mL) in assayed supernatants after LPS-stimulation but did not induce TNF α release when administered independently (Figure f4.7, Left Panel). They were found to 'reverse' PGE₂ 1ng/ml-mediated suppression of TNF α release to baseline (LPS 1ng/mL alone), however the co-administration of an EP4-receptor antagonist (MF498 1 μ M) was found to further increase observed TNF α concentrations (Figure f4.7, Right Panel). This indicates that IFN- γ and GM-CSF act via pathways independent of the PGE₂-EP4-cAMP axis and that they may represent complementary immunorestorative therapeutic strategies.

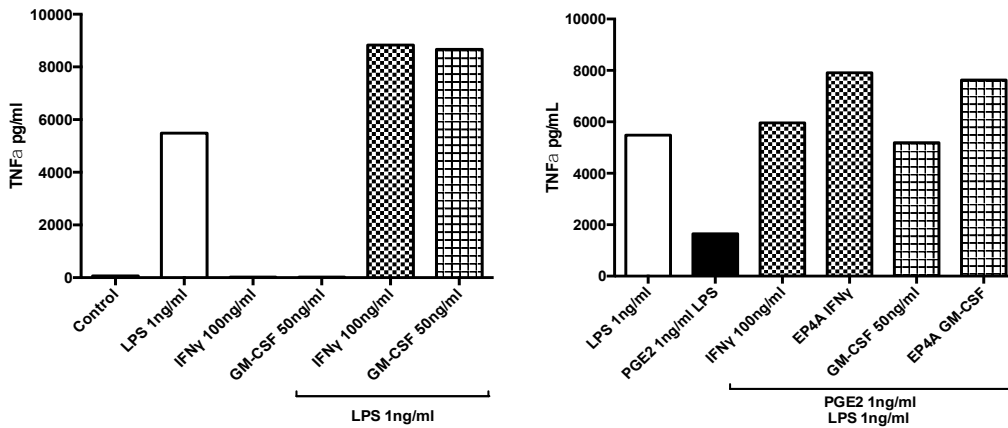


Figure f4.7: Comparison of established immunostimulatory agents to EP4 receptor antagonism in restoring LPS-stimulated WB TNF α secretion. **Left Panel:** WB was co-incubated IFN γ (100ng/ml, chess-board pattern) or GM-CSF (50ng/ml, cross-hatch pattern) and either stimulated with LPS-of not. Both agents led to significantly greater TNF α release ($p < 0.001$). **Right Panel:** WB was co-incubated with PGE $_2$ (1ng/ml) in the presence or absence of IFN γ (100ng/ml, chess-board pattern) or GM-CSF (50ng/ml, cross-hatch pattern) with the addition an EP4 receptor antagonist (MF498 1 μ M) where indicated. LPS 1ng/ml was used to stimulate the blood and supernatants were removed after 6hrs. WB was pre-treated with indomethacin 10 μ M to prevent any exogenous prostanoid production in both experiments. Bars represent the mean of $n=1$ HV, with 2 technical repeats/condition, each assayed (ELISA) 4 times.

4.4: DISCUSSION

PGE $_2$ is capable of inducing monocyte deactivation at physiological-relevant concentrations with an IC $_{50}$ ~300pg/mL, as determined by *ex vivo* WB LPS-stimulated TNF α release. The EP4 receptor appears central to this process, selective EP4 agonists and antagonists either mimicking the effect of PGE $_2$ or abrogating it. Like the EP2 receptor, EP4 is G $_s$ -coupled, cognate-binding serving to increase the intracellular concentration of cAMP⁴⁰⁹: a mechanism that has been repeatedly associated with the immunosuppressive action of PGE $_2$ *in vivo* and *in vitro*^{199,250,410}. Supportive evidence that this pathway is responsible for the reduction in observed TNF α release was provided through the replication of PGE $_2$'s action by forskolin, a cell permeable diterpenoid that directly activates adenylyl cyclase⁴¹¹, and it's augmentation by rolipram, a selective phosphodiesterase 4 inhibitor that reduces the catabolism of biosynthesised cAMP^{412,413}. LPS-stimulated but not un-stimulated WB was additionally observed to release PGE $_2$, acting as an autocrine/paracrine immunomodulator. To prevent endogenously generated eicosanoids impairing interpretation of the effect of exogenously added PGE $_2$ or alternate

immunomodulatory agents pre-treatment with COX-inhibitors (indomethacin 10 μ M) was instituted.

Preliminary evidence that reversal of PGE₂-mediated monocyte deactivation could represent a complimentary immunorestorative strategy to those already under investigation was obtained. Both GM-CSF and IFN- γ , and NSAIDs and EP4 antagonists were observed to independently increase TNF α release in the WB model, the latter seemingly due to ablation or blockade of the aforementioned endogenously released PGE₂. Whilst GM-CSF and IFN- γ , appeared to 'antagonise' the effects of PGE₂ - restoring TNF α concentration to baseline (LPS alone) when exogenous PGE₂ was added - the addition of an EP4 receptor antagonist led to a further increase in TNF α release. This indicates that the mechanism through which GM-CSF and IFN- γ work is separate to the PGE₂-EP4-cAMP axis described above. As such, 'anti-PGE₂' therapy may act synergistically with these established immunostimulatory agents, affording greater clinical gain.

Of note, PGE₂ was not the only COX-derived prostanoid to suppress WB TNF α release at physiological concentrations, PGI₂ possessing a similar IC₅₀. This is not unexpected: the IP receptor also being G_s-coupled and therefore potentially exerting this action via a common cAMP-protein kinase A axis. PGI₂ is now thought to play a regulatory or anti-inflammatory role in several disease states (which may be beneficial or detrimental), primarily through modulation of dendritic cell (DC) function (see⁴¹⁴ for review). Equally, previous authors have described how synthetic analogs of PGI₂ inhibit macrophage and monocyte-derived DC pro-inflammatory cytokine release, and thus the data described here may represent an extension of these findings^{415,416}. Care must be taken however in translating such results to the *in vivo* setting, artificially long-lasting analogs (in this case carbaprostacyclin) potentially being able to generate alterations in cellular function their more fleeting biological cousins cannot.

Additionally, PGE₂-induced monocyte deactivation may not however be absolute. 10ng/mL PGE₂ was observed to significantly reduce TNF α and IL-6 release but non-significantly increase IL-8 and IL-1 β . Whilst we and others have focused on TNF α due to its central place in the inflammatory cascade and the proven link between alteration in LPS-stimulated release and clinically meaningful outcomes, it is clear that PGE₂ may not universally suppress monocyte function, but phenotypically bias it. This may necessitate a more nuanced view than one of PGE₂ being entirely detrimental in the context of CI.

4.4.1 Assay selection and variability

Several different techniques for undertaking *ex vivo* WB LPS-stimulation have been reported in the literature. These variables were found to have differing degrees of impact on the assay and a core technique similar to that used by Kox et al.²²⁰ was eventually selected based on maximisation of TNF α release, minimisation of inter-replicate variability and retention of sensitivity to immunosuppressants. This involved using SAE 1ng/mL to stimulate 1ml of WB anticoagulated with lithium heparin and diluted with 4mls RPMI, which was then incubated at 37°C at 250rpm for 6hours.

Despite optimisation, significant technical and biological variability with the assay remained. Consequently, when employed as a marker of immune competence multiple technical replicates and internal controls should be employed. Many of the findings reported within this chapter, including evidence supporting cAMP involvement in PGE₂-mediated TNF α suppression and comparison of GM-CSF and IFN γ with EP4 antagonists was undertaken in blood obtained from a single individual. Despite technical replication this should be viewed as early proof-of-principle data and conclusions tempered appropriately.

4.5: SUMMARY

- PGE₂, at pathophysiological concentrations, induces suppression of whole blood *ex vivo* TNF α release commensurate with that seen in CIIID.
- The central mechanism for this effect appears to be a PGE₂-EP4 receptor-cAMP axis, although a contribution from the EP2 receptor cannot be refuted.
- Initial data suggests PGE₂ ablation or antagonism may provide a complimentary therapeutic strategy to that afforded by the established immunoadjuvant agents GM-CSF and IFN- γ : their co-administration synergistically increasing monocyte cytokine secretion. This requires further validation.
- Key variables influencing performance of the *ex vivo* WB LPS-stimulated cytokine release assay include dilution of blood, anticoagulant, incubation temperature and duration and concentration of LPS. An optimal method has been elucidated.

4.6: APPENDIX

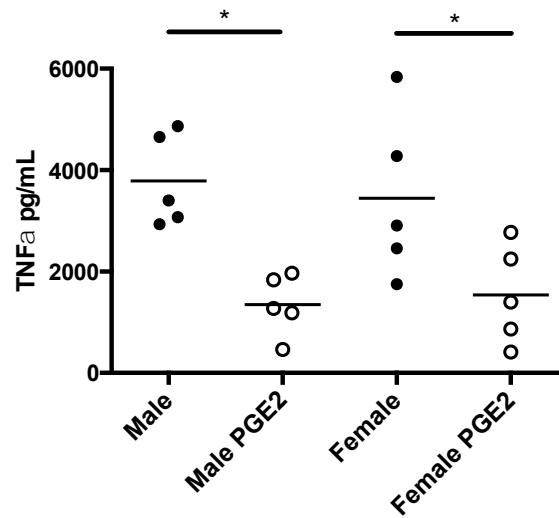


Figure f4.8: Sex differences in WB-LPS stimulated TNF α release. Whole blood (1ml) from 5 male and 5 female HV was stimulated with LPS (1ng/mL) in the presence or absence of PGE₂. In both groups PGE₂ elicited significant suppression of TNF α release (male: $p=0.0126$, female $p=0.0121$, both 2-tailed paired t-test), however there was no significant difference in either TNF α release between sexes or in the degree of PGE₂-mediated immunosuppression. Data points represent individual participants, mean of 3 technical replicates (tubes) each assayed (ELISA) in duplicate.

CHAPTER 5: Mono Mac 6

DERIVATION OF A FUNCTIONAL BIOASSAY TO DETECT PROSTAGLANDIN E2 IN CLINICAL SAMPLES

5.1 Introduction

5.2 Additional Methods

5.3 Results

5.4 Discussion

5.5 Summary

5.6 Appendix

5.1: INTRODUCTION

5.1.1 PGE₂ in health and disease

Predominantly acting as autocrine or paracrine signalling molecules, prostaglandins are evanescent. PGE₂ is rapidly cleared from the blood, only 3% of IV-administered tritium-labelled PGE₂ remaining after 1.5 minutes⁴¹⁷, the primary route of elimination being via 15-hydroxy-prostaglandin dehydrogenase (15H-PG DH) to 15-keto-13,14-dihydro-PGE₂ in the lungs⁴¹⁸. The concentration of PGE₂ in healthy individual's plasma is around 2-10pg/ml when measured via mass spectrometry⁴¹⁹, other methods providing considerably higher estimates (radioimmunoassay: 5-350pg/ml^{420,421}, ELISA: 150-850pg/ml⁴²²⁻⁴²⁴). This discrepancy may be due to formation of prostaglandins by leucocytes and platelets during isolation of the plasma, and potentially by non-enzymatic cyclization of polyunsaturated fatty acids⁴²⁵. The concentration of the principle metabolites of PGE₂, the 15-keto-13,14-dihydro-PGE₂ derivatives, have repeatedly been reported to be cumulatively around 30-50pg/mL⁴²⁵⁻⁴²⁷. These may represent a superior index of PGE₂'s entry into peripheral blood being formed principally outside this compartment.

In disease, specifically that with an inflammatory component, PGE₂ concentrations may be much higher - elevated levels being identified locally at wounds/infected sites⁴²⁸⁻⁴³⁰, their draining lymphatics⁴³¹ and in the circulation of patients with CI⁴³². During severe sepsis and septic shock, serum concentrations in the range of 500-1500pg/mL have been reported when measured via ELISA, 15-fold higher than in healthy controls⁴³³. Using electrospray ionisation liquid chromatography mass spectrometry (ESI/LC-MS/MS) we recently described a near 6-fold increase in mean plasma PGE₂ concentration in patients with acute decompensation of liver cirrhosis (90pg/mL) compared to healthy volunteers (15pg/mL)¹⁴², a condition associated with a similar immunosuppressive burden to CI^{434,435}. Local levels may escalate considerably higher, human burn blister exudates containing 2ng/mL^{429,430}, cantharidin (a sterile acantholytic) elicited blisters 5ng/mL⁴³⁶ and heat-killed *E.coli* inoculation provoking concentrations ~15ng/mL⁴³¹ in the efferent lymphatics. These rises may not be benign, but instead have both local^{437,438} and systemic functional consequences²¹² on host defence. Increasing immune effector cell sensitivity to PGE₂ may further compound the suppressive effect of an elevated concentration. This appears to be the result of altered receptor expression or humoral factors such as bacterial muramyl dipeptide and steroid hormones, with shifts in functional assay PGE₂ IC₅₀ from the low μ M to the low nM range being reported^{212,439-441}.

Animal studies suggest that tissue resident macrophages (splenic and peritoneal in particular^{442,443}) may be the source of circulating PGE₂ and further, that its *in vivo* ablation is immunostimulatory⁴⁴⁴. Studies of patients with thermal⁴⁴⁵ or traumatic injury^{210,446} and healthy volunteers injected with endotoxin²¹² suggest that circulating monocytes may also contribute to elevated plasma PGE₂ concentrations: prostanoid release increasing by up to 400% following *ex vivo* stimulation compared to that seen in healthy controls. A final contributing factor appears to be down-regulation of the pathways associated with PGE₂ catabolism, both 15H-PG DH and carbonyl reductase (a secondary, minor route of elimination) being significantly down regulated (up to 26-fold) at the transcriptional and post-transcriptional level in experimental models of sepsis^{447,448}.

5.1.2 Derivation of a PGE₂ bioassay

As described in Chapter 4, PGE₂ mimics the immunosuppression witnessed clinically in an established measure of monocyte deactivation - WB-LPS stimulated TNF α release - adding plausibility to the pre-clinical and observational clinical data implicating it as a contributing factor to CIID. Clearly however, not all CI patients will have a dysregulated inflammatory response, and of those that do, not all would develop immunosuppressive concentrations of PGE₂. Stratified medicine represents 'the potential to use biomarkers for identifying patients that are more likely to benefit... in response to a given therapy'⁴⁴⁹. If clinically proven, there is a need for rapid, meaningful methods to determine the presence and functional impact of PGE₂ in CI patients, stratifying them by immune competence into those that would benefit from anti-PGE₂ therapy and those it may do more harm than good⁴⁵⁰.

Mass spectrometry represents the gold standard for quantification of eicosanoids in biological fluids^{451,452}. Whilst unquestionably the most sensitive and specific technique it has disadvantages with regards to speed of sample processing, cost, complexity, the requirement of specialised training in its use, and availability - particularly in resource poor settings. In contrast, cell-based bioassays, already the mainstay of CIID detection (monocyte HLA-DR expression, *ex vivo* cytokine secretion) are quick, cheap, easy to perform, require minimal training and generate rapidly interpretable results which may inform clinical decision making. Importantly, a bioassay would not merely demonstrate the presence of PGE₂ or its receptor binding, but indicate its bioavailability and functional/downstream effects, linking an assessment of 'quantity' with its mechanistic importance in the observed pathophysiology. Predictive information regarding the result of PGE₂ blockade may also be gained.

The host laboratory have previously demonstrated the value of one such approach, placing plasma from cirrhotic patients on primary monocyte-derived macrophages (MDM) derived from healthy volunteers (HV) and using LPS-stimulated TNF α production as a read-out for the presence of PGE $_2$, a measure of its immunosuppressive impact and the effect of its reversal with selective EP-receptor antagonists¹⁴². Variants of this technique have also been employed by other investigators to determine the presence or absence of immunosuppressive humoral mediators in CI⁴⁵³. In preparatory work for this PhD during my Academic Clinical Fellowship, I placed sequential plasma samples (25% v/v) derived from nine patients admitted to UCLH with moderate-severe CAP (see 5.2.1) on HV MDM obtained via the technique described in 2.1.2. As illustrated in Figure f5.1, plasma from these patients was observed to reduce MDM TNF α release compared to HV plasma alone ($P < 0.001$), individuals' samples eliciting peak immunosuppression at different time-points (majority at 36hrs post-admission) and demonstrating divergent degrees of 'recovery' by day 5/discharge (6/9). This immunosuppressive effect was partially reversed by pre-incubation with AH6809 (an EP1-3/DP1 antagonist) at study recruitment, 36hrs and 5days/discharge, reaching significance at 0hrs (Figure f5.2), implying the presence of bioavailable, immunosuppressive PGE $_2$ in these individuals.

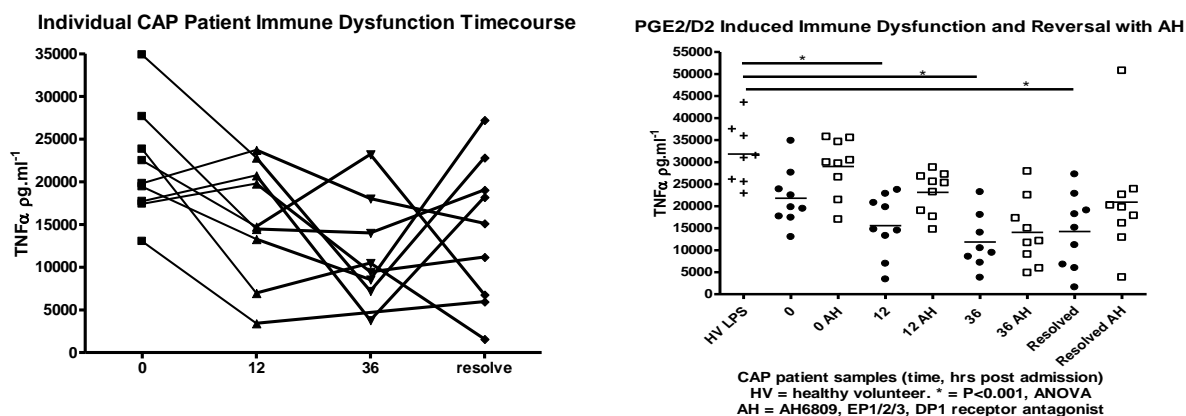


Figure f5.1: CAP patient plasma exerts a differential suppression of MDM cytokine production dependent on time post-admission. **Left Panel:** MDM were co-incubated with 25% v/v patient plasma (n=9) for 30mins prior to stimulation with LPS 100ng/mL. Data points represent the average of two technical (ELISA) repeats. Samples were obtained at study recruitment (0hr), 12hrs, 36hrs and at day 5 post-recruitment/discharge (resolved), whichever came earlier. Supernatants were removed 24hrs post-stimulation. **Right Panel:** MDM were pre-treated with or without 50 μ M AH6809 (a PGE $_2$ EP1-3/D $_2$ antagonist) for 30mins prior to plasma addition. Data points represent individual HV or patient samples (n=9, average of two technical (ELISA) repeats)

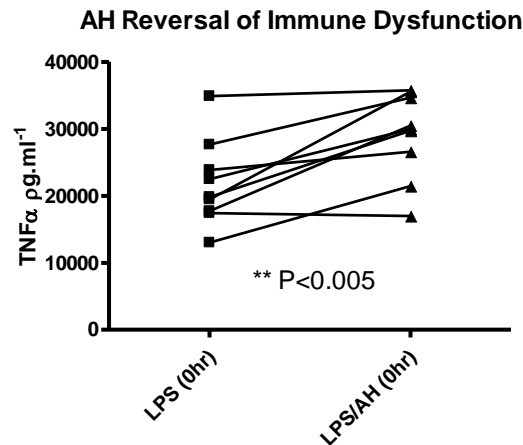


Figure f5.2: Individual (paired-sample) reversal of immune dysfunction with EP-receptor antagonism. MDM were co-incubated with 25% v/v patient plasma (n=9) for 30mins with or without 50 μ M AH6809 (a PGE₂ EP1-3/D₂ antagonist) prior to stimulation with LPS 100ng/ml. Data points represent individual patient values, each being the mean of two ELISA repeats. Stimulants were removed 24hrs post-stimulation. Paired t-test, ** = p<0.005.

5.1.3 Mono Mac 6

Despite the ability of the described bioassay, the use of primary cells to determine a PGE₂-mediated contribution to CIID has clear limitations. In my hands the method employed to yield MDM (2.1.2, derived from Smith et al.²⁸³) demonstrated low purity on flow cytometric analysis (MDM comprising only 25-50% of the stimulated population with a high proportion of T-lymphocytes) and both a poor and variable yield from a given quantity of blood. As such large (200mL) volumes are required, experimental planning is rendered challenging and the observed effect cannot be ascribed to a single cell population. Further concerns include both inter- and intra-individual variability regarding LPS-stimulated TNF α release, the lengthy lead-in time to generate 'testable' cells and the inability to 'up-scale' or render the assay standard – particularly relevant in the context of multi-site analysis.

The use of a cell line obviates many of these concerns, offering reliability, consistency and cell 'purity' at no clinical cost. Most importantly it should afford a significantly higher signal to noise ratio. Multiple human myeloid cell lines exist: THP-1, U937, Mono Mac 1 and Mono Mac 6 (MM6), each displaying unique characteristics secondary to their degree of development/maturity and genetic alterations that gave rise to their malignant potential. Of these, MM6 is considered to most closely resemble mature human monocytes by morphological, cytochemical and immunological criteria²⁸⁴ (see section 2.2 for further discussion).

After initial trial experimentation with U937 cells in which I found neither consistent response to LPS with regards to TNF α release, nor modulation of the elicited cytokine profile by PGE₂ (data not shown), I elected to efficacy of the MM6 cell line in this role. Relevant to my proposed usage these cells display alternate patterns of differentiation in response to their culture environment²⁸⁵, a functional AA-pathway with up-regulation of COX-2 in response to LPS⁴⁵⁴, PGE₂ generation in response to TLR-2 or 4 receptor stimulation in a dose-dependent fashion⁴⁵⁵, and most importantly, both eicosanoid-mediated regulation of agonist-induced TNF α release⁴⁵⁶ and features of monocyte deactivation in response to LPS^{457,458}. Already proposed for use in assays to determine pyrogen levels in biological samples²⁸⁸ and predict adjuvant safety *in vivo*⁴⁵⁹, it was hypothesised that MM6 would be suitable to detect the presence or absence of immunomodulatory PGE₂ in biological samples⁴⁶⁰.

5.1.4 Chapter Aims

- Develop a rapid, cheap and scalable bioassay to delineate the contribution of PGE₂ within a biological fluid to immune suppression
- Describe the characteristics of and pathways involved in this assay including principal receptors, intra-cellular messengers, specificity and sensitivity to alternate humoral mediators
- Compare the performance of the MM6 bioassay with that of primary MDMs

5.2: ADDITIONAL METHODS

5.2.1 Patient Samples

Samples were kindly donated by Dr Steve Harris and Prof Mervyn Singer Principal Investigators of the Sepsis Physiological and Organisational Timing: In Depth [(SPOT)id] study funded by the Wellcome Trust (Project: 088613/Z/09/Z) and the Intensive Care National Audit and Research Centre (ICNARC). All samples arose from University College London Hospital. Patients admitted to hospital with moderate-severe community acquired pneumonia (CAP) (Confusion, Urea, Blood Pressure, Respiratory Rate [CURB]-65 score⁴⁶¹ ≥ 2 , with clinical and radiological evidence of pneumonia) were recruited. After consent, venous blood was taken by trained research nurses at set time-points post-recruitment (0hrs [admission], 12hrs [day 1], 36hrs [day 2-3], 72hrs [day 5], and at discharge/10 days – whichever is later) in EDTA-sprayed Vacutainers® (BD), centrifuged (2000g/10mins/20°C), and resultant plasma aliquoted into appropriately labeled cryostorage tubes. Samples were stored by the host laboratory at -80°C until analysis. Institutional and ethical approval was granted for (SPOT)id by the University College London NHS Trust R&D office and the South East London Research Ethics Commission (ref: 10/H0807/78) respectively.

5.2.2 MDM Stimulation

Healthy volunteer MDM were isolated and plated in 96-well plates as per 2.1.2 or 2.1.3 where stated and incubated overnight (37°C/5% CO₂). The following day cells were treated sequentially with (dependent on experiment):

- i) PGE₂ receptor antagonist: AH6809 (EP1-3/DP1 antagonist), PF-04418948 (EP2), MF498 (EP4)
- ii) PGE₂ OR 25% v/v healthy volunteer or patient plasma
- iii) Lipopolysaccharide (LPS; *Salmonella abortus equi* S-form, [TLRgrade™], Enzo Life Science, 100ng/mL)

PGE₂ and its receptor antagonists were obtained from Cayman Chemical, reconstituted in DMSO to form stock solutions, and working concentrations made in appropriate culture media. 15 minutes was allowed between each addition step to allow receptor binding/activation of downstream cascades and facilitate reagent addition to requisite wells, providing time for consistent concentration equalisation. The validity of this protocol was tested via varying the time from reagent addition to LPS stimulation (see Figure f5.7, Left Panel). After addition of LPS cells were

incubated overnight (37°C/5% CO₂) and supernatants removed after either 6hrs (MDM via 2.1.3) or 24hrs (MDM via 2.1.2), and stored at -80°C prior to analysis.

5.2.3 MM6 Stimulation

MM6 were differentiated as per 2.2.2, washed, plated in 96-well plates at 1x10⁵ cells/well in 50µL media and incubated for 1hr (37°C/5% CO₂) prior to reagent addition or stimulation. Reagents were added in a standardised order as 5.2.2 unless otherwise stated to characterise:

- Effect of alternative differentiation protocols (2.2.2) on TNFα release and sensitivity to PGE₂
- Response to gram positive and negative stimuli (LPS; *Salmonella abortus equi* S-form, [TLRgrade™], Enzo Life Science, peptidoglycan (Sigma))
- Time-course of TNFα and PGE₂ release (30min, 1hr, 2, 4, 6, 8, 24hrs)
- Impact of healthy volunteer plasma (anti-coagulated with EDTA, Na Citrate, LH) or serum on cytokine release (25-100% v/v), including any sex differences
- Dose-response curve of PGE₂
- Shift in the dose-response curve elicited by selective EP-receptor:
 - Agonists: Butaprost (EP2), CAY10598 (EP4)
 - Antagonists:
 - EP2: PF-04418948, TG4-155 (Aobious, MA, USA), AH 6809
 - EP4: MF498, L161,982, ONO-AE3-208 (a kind gift from ONO Pharmaceuticals, Osaka, Japan), CJ-023,423
- The contribution of cAMP to observed alterations via:
 - Forskolin: direct adenylyl cyclase activator
 - Rolipram (RP): selective phosphodiesterase 4 inhibitor
- The effect of known immunosuppressant agents: IL-10 and dexamethasone
- The effect of established immunorestorative agents: GM-CSF and IFN-γ

Order of addition and intervals between steps was as 5.2.2. After LPS addition cells were incubated (37°C/5% CO₂) for 6hrs (unless stated) prior to supernatant aspiration and storage at -80°C. All reagents were obtained from Cayman Chemical (MI, USA) unless stated and re-constituted in DMSO such that the eventual assay concentration was <0.01%.

5.3: RESULTS

5.3.1 Differentiation Protocols

Either undifferentiated (not passaged for 48hrs) or differentiated MM6 (PMA [10ng/ml], M-CSF [20ng/ml] or $1\alpha, 25$ dihydroxycholecalciferol [VD3, 10ng/ml] for 48 or 72hrs) were analysed by flow cytometry. VD3 differentiation led to a significant increase in CD14 expression by 48hours ($p < 0.001$, Figure f5.3). In contrast co-incubation with alternative differentiation stimuli did not result in altered CD14 expression from baseline. PMA exposure for either 48 or 72 hours was associated with the development of a more macrophage-like phenotype, MM6 displaying reduced CD33, CD11b and CD11c (data not shown). CD16 and HLA-DR were unaffected by any of the three differentiation stimuli.

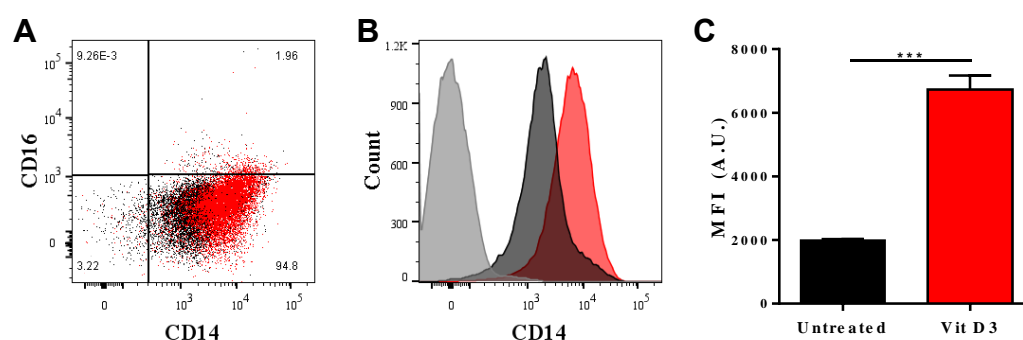


Figure f5.3: Differentiation with VD3 leads to increased CD14 expression on MM6. MM6 were co-incubated with VD3 (10ng/ml) for 48hr, or left un-differentiated prior to flow cytometric analysis. **Panel A:** No significant alteration in CD16 expression was observed, however CD14 significantly increased as demonstrated by typical dot-plot, histogram (**Panel B:** light grey = isotype control; dark grey = un-differentiated MM6; red = VD3 48hrs) and graphically (**Panel C:** mean of 3 separate experiments (discrete passage, drugs and day)). Un-paired t-test, $p < 0.001$).

Undifferentiated and differentiated MM6, displayed different response characteristics to LPS stimulation. Cells cultured with VD3 for 48hrs (VD3 MM6) demonstrated heightened sensitivity to LPS (achieving maximal TNF α production at 1ng/ml) and increased TNF α release (>1000pg/ml) compared to alternate differentiation protocols (Figure f5.4, Left Panel). This pattern was unchanged at 72hours. Minimal TNF α release was observed in response to peptidoglycan even at 1 μ g/ml (data not shown). MM6 additionally appeared to display sensitivity to PGE $_2$, co-incubation leading to reduced TNF α secretion, in line with primary MDM. This was additionally enhanced by VD3 (Figure f5.4, Right Panel).

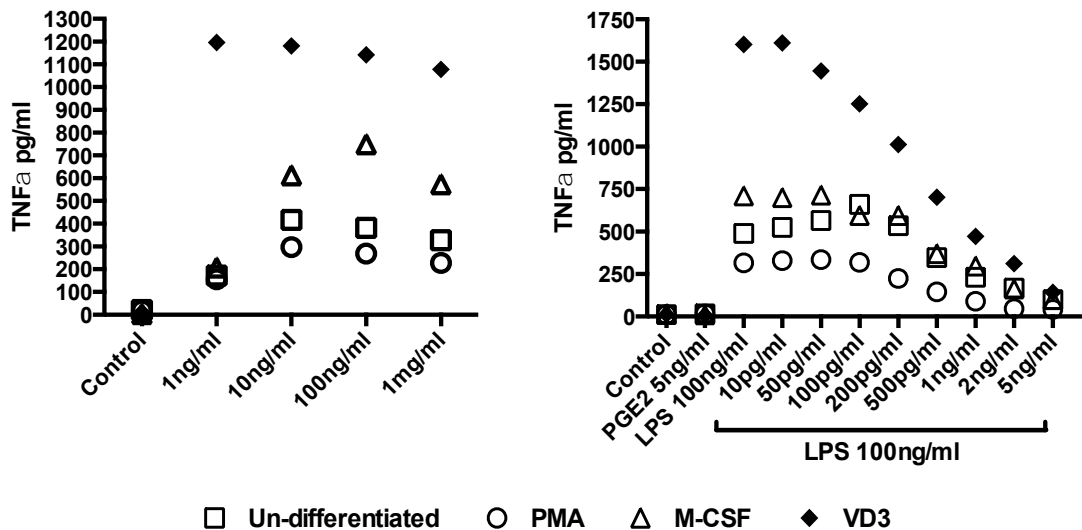


Figure f5.4: LPS-stimulated TNF α release is modified by differentiation stimuli. **Left Panel:** MM6 were co-cultured with either PMA, M-CSF, VD3 or left un-differentiated for 48hrs. Cells were subsequently stimulated with LPS of varying concentrations (1ng-1mg/ml) in 96well plates (1×10^5 cells/well) for 6hrs prior to supernatant removal. **Right Panel:** MM6 treated as above and stimulated with LPS 100ng/mL were co-incubated with increasing concentrations of PGE₂ (10pg/mL–5ng/mL). Individual data points represent the mean of 4 individual 96 wells, each assayed (ELISA) in duplicate. All stimuli were applied once to the same passage of cells.

5.3.2 Technical characterisation

PGE₂ (1ng/mL) caused significant suppression of MM6 IL-6 release in addition to TNF α , but not IL-1 β an alternate pro-inflammatory cytokine (Figure f5.5). Minimal IL-10, the archetypal anti-inflammatory cytokine, release was observed in response to LPS, a response un-altered by PGE₂. Significant decreases in monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein-1 β (MIP-1 β) were also elicited by PGE₂ but neither IL-8 nor MIP-1 α release was suppressed (data not shown)

VD3 MM6 TNF α release peaked between 2 and 6 hours post-stimulation with LPS, declining thereafter. Minimal PGE₂ release was observed over this early time-period (<50pg/mL). Instead the PGE₂ concentration reached its zenith at 24hr (130pg/mL), potentially reflecting accumulation secondary to the absence of *in vitro* catabolism.

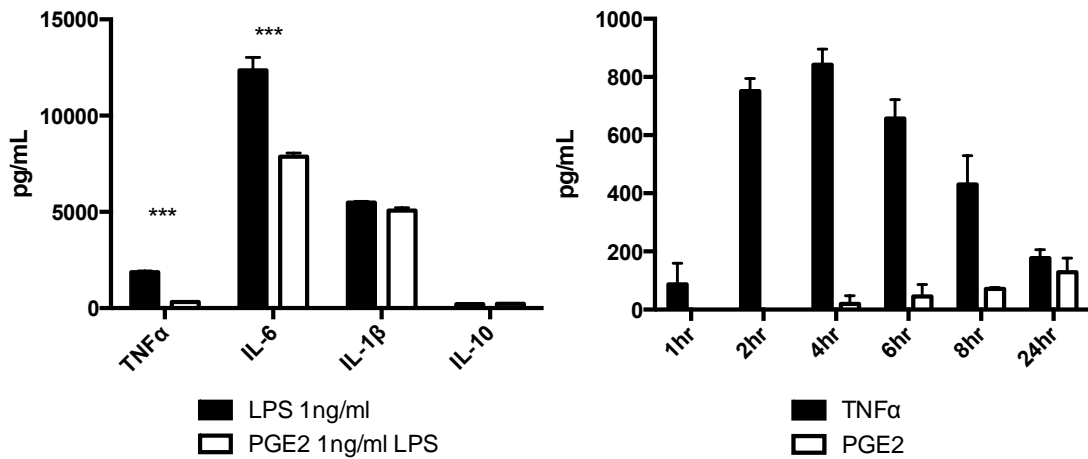


Figure f5.5: LPS-stimulated release of classic pro and anti-inflammatory cytokines and PGE₂. **Left Panel:** VD3 MM6 were stimulated with LPS (100ng/mL) in 96well plates (1x10⁵ cells/well, 8 wells/condition) and supernatants aspirated after 6hrs. Cytokine concentration was determined by LifeTechnologies Luminex® bead-based flow cytometric assay. Columns represent the mean value with SD of 8 wells treated on the same day. **Right Panel:** Supernatant was aspirated at variable time-points post-LPS stimulation and assayed for TNFα and PGE₂ via ELISA. Columns represent the average with SD of 2 discrete (passage/reagents/day) experiments, each condition being replicated in 4 wells.

Anticoagulants were found to exert an independent effect on VD3 LPS-stimulated MM6 TNFα release. Cells bathed in culture media which had been placed in an EDTA BD Vacutainer® were found to release significantly less TNFα than those exposed to untreated media, LH or Na Cit (Figure f5.6).

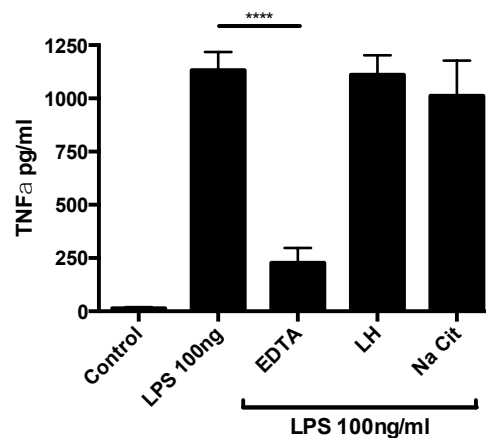


Figure f5.6: Effect of anti-coagulant on TNFα release. VD3 MM6 were treated with media that had been placed into BD Vacutainers® anticoagulated with lithium heparin (LH), EDTA or sodium citrate (Na Cit), prior to stimulation with LPS (100ng/mL). Columns represent the mean with SD of 2 discrete (passage/reagents/day) experiments, 4 wells/condition/day, each assayed (ELISA) in duplicate, each well equating to n = 1 (8). Un-paired t-test, *** = P<0.001.

A PGE₂-mediated signal was resilient to variations in cell culture protocol and sample handling. Treatment of VD3 MM6 with PGE₂ (1ng/ml) up to 1 hour prior to LPS-stimulation led to no appreciable loss of TNF α suppression, however between 1 to 2 hours attenuation was seen. Addition of 25% v/v HVP to the culture led to no appreciable increase in PGE₂ catalysis or loss of bioavailability (Figure f5.7, left panel). PGE₂ was additionally noted to be able to suppress TNF α release after LPS-stimulation. The ability of PGE₂ to induce monocyte deactivation was not affected by one freeze-thaw (-80°C) cycle indicating the validity of storing plasma for later analysis (Figure f5.7, right panel).

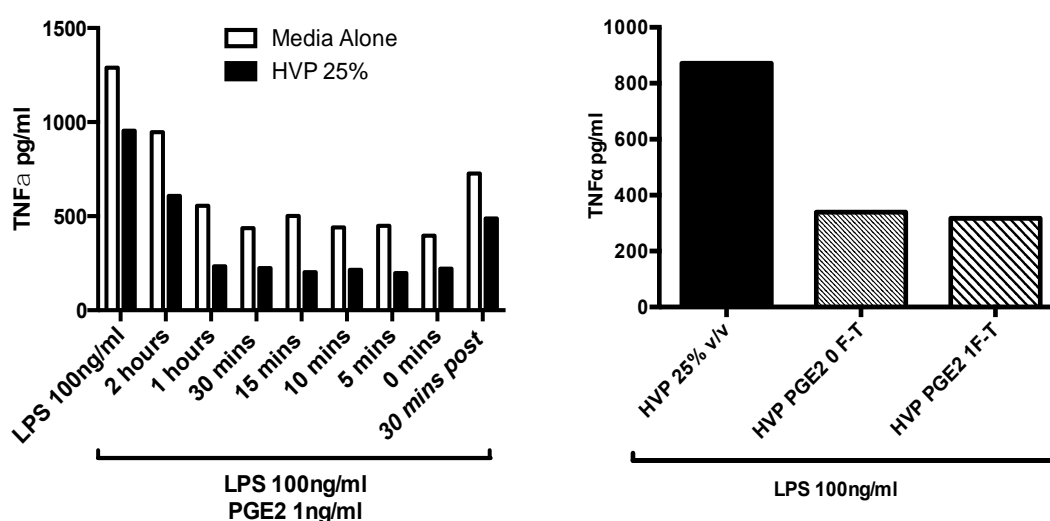


Figure f5.7: Variations in assay protocol and sample handling. **Left Panel:** PGE₂ (1ng/mL) was added in either media alone or 25% v/v HVP to VD3 MM6 at variable time-points prior to or following stimulation with LPS (100ng/mL). **Right Panel:** HVP alone or HVP spiked with 1ng/mL PGE₂ was either added to VD3 MM6 immediately or after undergoing one freeze-thaw cycle (F-T, -80°C) prior to stimulation. Columns represent the mean of 4 wells, each assayed (ELISA) in duplicate

5.3.3 Detection of PGE₂ in plasma

VD3 MM6 displayed a dose-dependent reduction in TNF α release when bathed in increasing proportions of LH anti-coagulated HVP during LPS stimulation (Figure f5.8, left panel). 2-way ANOVA found both HVP and PGE₂ (1ng/mL) to cause a significant decrease in TNF α release ($p < 0.0001$), although HVP accounted for a larger percentage of the total variation (67.8% vs 16.7%). As the percentage of HVP increased, a significant reduction in the PGE₂-mediated suppression of TNF α release was additionally observed (interaction $p < 0.0001$): PGE₂ 1ng/mL reducing TNF α release by a mean value of 54.6% in media alone, but only 29.6% at 75% v/v HVP. In 25% v/v HVP, PGE₂-mediated reduction from LPS-alone was preserved at 56.5%.

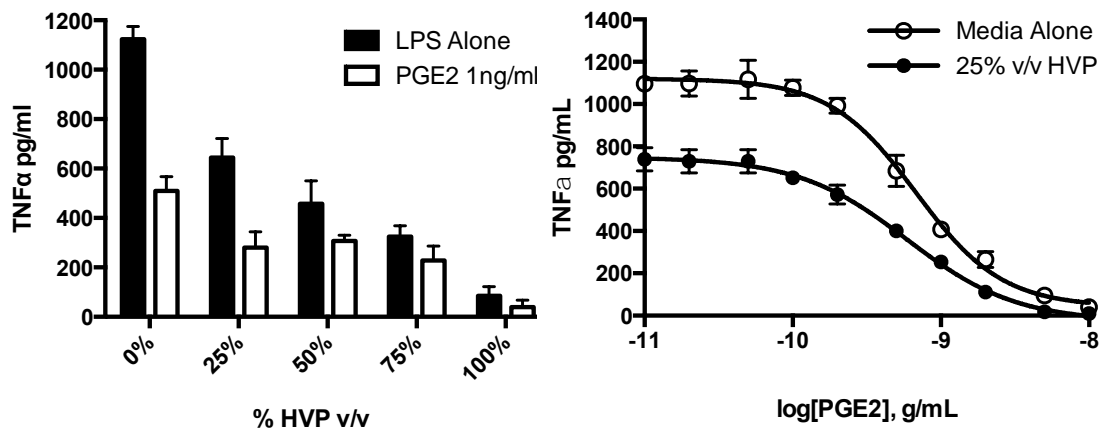


Figure f5.8: HVP independently suppresses LPS-stimulated TNF α release and impairs PGE $_2$'s action on MM6 in a dose-dependent manner. **Left Panel:** VD3-differentiated MM6 (1×10^5 cells/well) were incubated with increasing amounts of healthy volunteer plasma (LH anticoagulant, 0%-100% v/v) for 15mins prior to and during stimulation with 100ng/ml LPS. Supernatants were removed at 6hrs. **Right Panel:** As above, VD3 MM6 were incubated with either 0 or 25% v/v HVP and spiked with increasing concentrations of PGE $_2$ (10pg-10ng/mL). Bars and data points represent the average of 4 biological repeats with SD. Columns or data points represent the mean with SD of 2 discrete (passage/reagents/day) experiments, each condition being replicated in 4 wells/day and assayed (ELISA) in duplicate ($n = 8$). HVP was from the same individual throughout.

VD3 MM6 treated with either media or 25% LH HVP containing known concentrations of PGE $_2$ demonstrated no significant difference in IC $_{50}$, being 680pg/mL (95% CI 603-767pg/mL) and 590pg/mL (95% CI 503-692pg/mL) respectively (Figure f5.8, right panel).

Female plasma was found to have a marginally, yet significantly, greater suppressive effect than male plasma ($n=5$ /sex, mean \pm SEM 1004pg/mL \pm 28.28 vs. 1139pg/mL \pm 48.06 TNF α , $p = 0.041$, un-paired t-test), however the effect of PGE $_2$ was consistent, 1ng/mL eliciting a 65.3% and 63.8% reduction respectively (Figure f5.9).

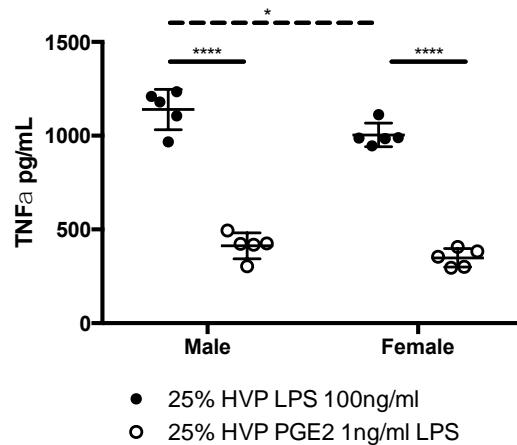


Figure f5.9: Differences in male and female HVP on the MM6 bioassay. VD3 MM6 were incubated with 25% v/v male (n=5) or female (n=5) HVP in the presence or absence of 1ng/ml PGE₂ and stimulated with 100ng/ml LPS. Data-points represent the mean of 4 wells treated with the same individuals plasma, assayed (ELISA) in duplicate. Line and whiskers indicate group mean and SD. Top: Dashed bar shows intra-sex difference in TNFα response p<0.05. Solid bars, intra-sex LPS vs. LPS + PGE₂ 1ng/mL differences p<0.0001 (both t-test).

5.3.4 Mechanism of PGE₂-mediated immunomodulation

PGE₂-mediated suppression of VD3 MM6 TNFα release appears to be through the EP4 receptor. Co-incubation with any of four EP4-selective antagonists (ONO-AE3-208 [K_i 1.3nM], MF498 [K_i 0.7nM], CJ-023,423 [K_i 13nM], L161,982 [K_i 24nM]; K_i for other EP/prostanoid receptors >2μM) prior to PGE₂ addition and LPS-stimulation led to a significant right-shift of the dose response curve (PGE₂ alone: 2.4nM [95% CI 1.7-3.4nM]. L161,982 was the least efficient, shifting the IC₅₀ to 160nM (95% CI 70-363nM), whilst MF498 was the most effective antagonist (4.9μM, 95% CI 934nM-26μM) (Figure f5.10, top panel). This effect was dose dependent, a greater right-shift being elicited with all antagonists as the dose was increased from 10nM to 1μM. None of the EP4 antagonists independently altered TNFα release (see Appendix f5.14).

In contrast, selective EP2 antagonists (TG4-155 [K_i 2.4nM] and PF-04418948 [IC₅₀ 16nM], both >1000-fold less active at EP4) and the non-selective EP1-4, DP1 antagonist AH6809, did not elicit any shift in the PGE₂ dose-response curve (Figure f5.10, middle panel). An EP4-mediated effect was further supported by the ability of CAY10598 (a selective EP4 receptor agonist, K_i 1.2nM, IC₅₀ 2nM, 95% CI 740pM-5.8nM) but not butaprost (a selective EP2 agonist, K_i 73nM) to suppress TNFα release from LPS-stimulated VD3 MM6 (figure f5.10, bottom panel).

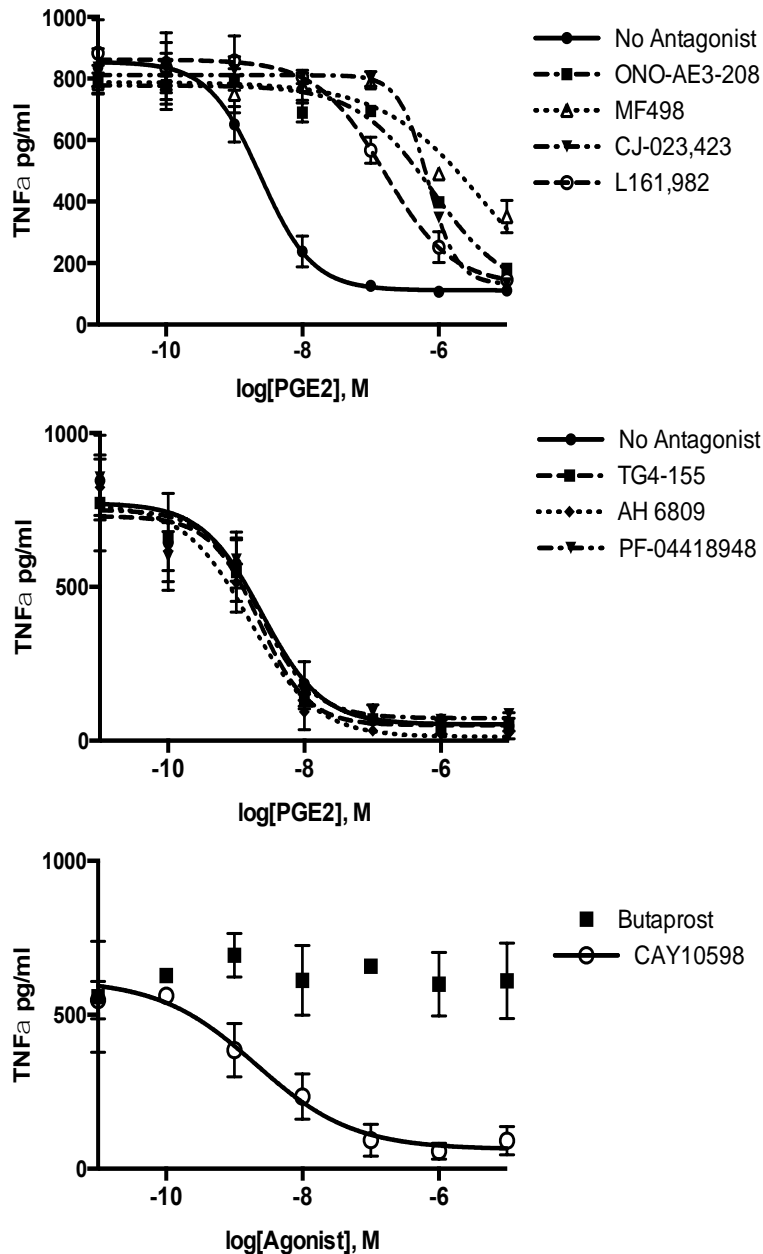


Figure 15.10: PGE₂ suppresses TNFα release in MM6 via the EP4 receptor. **Top Panel:** VD3 MM6 were co-incubated with varying concentrations of PGE₂ (10pM-10μM) in media alone or in the presence of one of four selective EP4 receptor antagonists (ONO-AE3-208, MF498, CJ-023,423, L161,982; all 1μM) and stimulated with LPS (100ng/mL) for 6hours. **Middle Panel:** As per Top Panel however EP4 receptor antagonists were substituted for three EP2 receptor antagonists (TG4-155 and PF-04418948; 1μM, AH6809; 50μM) **Bottom Panel:** VD3 MM6 were incubated for 15minutes with either butaprost (EP2 agonist) or CAY10598 (EP4 agonist) at concentrations ranging from 10pM-10μM prior to LPS stimulation. Supernatants were aspirated after 6hr. Data points represent the mean with SD of 3 discrete (passage/reagents/day) experiments, each condition being replicated in 4 wells/day and assayed (ELISA) in duplicate (n = 12)

EP4 is a G_s -coupled receptor, which upon ligand binding causes a rise in the intracellular second-messenger cAMP. Further confirmation of this pathway as the underlying mechanism behind PGE₂-mediated TNF α suppression / monocyte deactivation was sought via pre-treatment of VD3 MM6 with rolipram (RP, a selective phosphodiesterase 4 inhibitor) and forskolin (an independent activator of adenylyl cyclase). RP was found to increase the degree of TNF α suppression observed at any given concentration of PGE₂ (Figure f5.11, panel A), presumably via inhibition of the catalysis of EP4-generated cAMP. Correspondingly, forskolin was shown to mimic PGE₂'s effect, an action also potentiated by co-incubation with RP (panel B). Further, salbutamol (a β_2 -adrenoreceptor partial agonist; an alternate G_s -coupled receptor) was found to suppress TNF α release with an IC₅₀ of 20nM (95% CI 14-30nM, panel C). This was un-affected by co-incubation with an EP4-receptor antagonist.

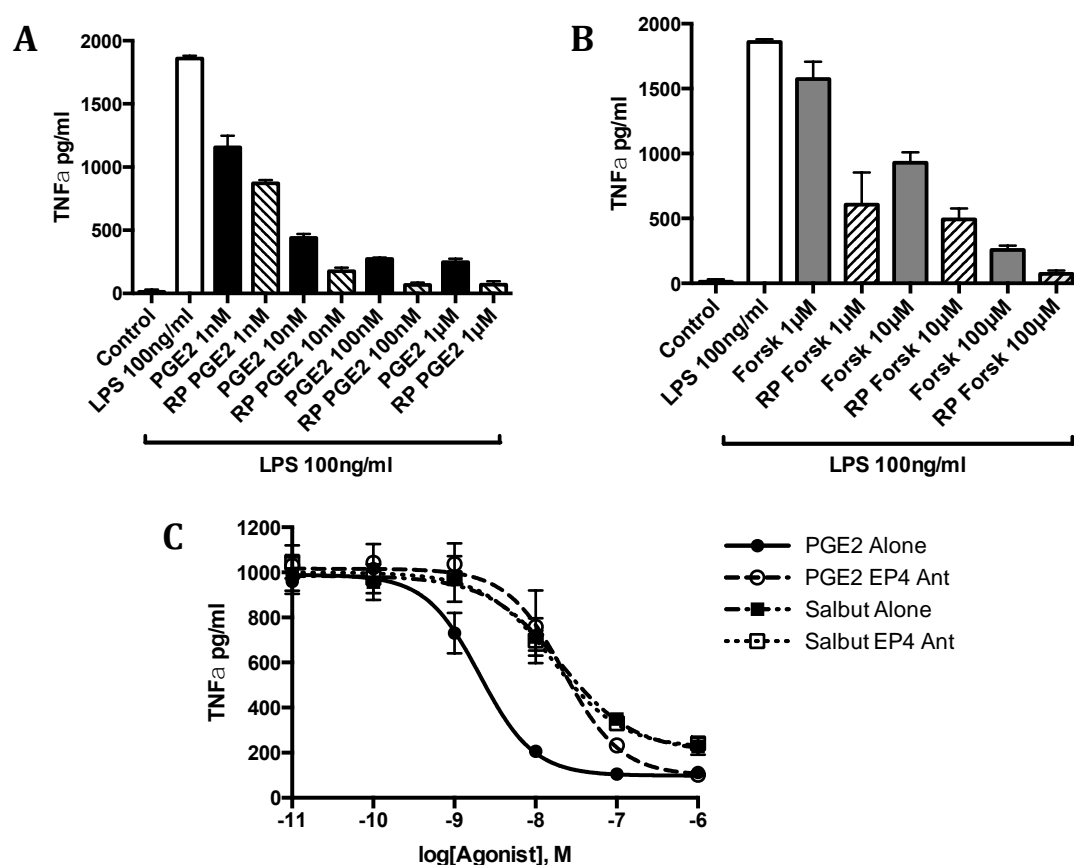


Figure f5.11: Increased intra-cellular cAMP is responsible for TNF α suppression in MM6. **Panel A:** Rolipram (RP, 10 μ M) potentiates the 'immunosuppressive' effect of PGE₂ on VD3 MM6 via preventing the catalysis of cAMP. **B:** Forskolin, an adenylyl cyclase activator, mimics the effect of PGE₂, an action potentiated by RP. **C:** Salbutamol increases intra-cellular cAMP via the β_2 -adrenoreceptor, inducing immunosuppression in an EP4-independent manner. Columns and data-points represent the mean with SD of 4 wells, each assayed (ELISA) in duplicate, each well equating to n = 1.

5.3.5 Bioassay specificity

In preliminary investigations, the VD3 MM6 bioassay displayed minimal alternation in TNF α release in response to either alternate COX-derived prostanoids (PGD₂, PGF_{2 α} , PGI₂ and TXA₂) or classic immunosuppressants (IL-10 and dexamethasone) at physiologically relevant or pharmacologically achievable doses (Figure f5.12). The IC₅₀ of PGE₂ was 1.9nM (95% CI 1.1-3.2nM), equivalent to 3ng/ml. IC₅₀s of alternate prostanoids could not be determined. Whilst PGE₂ 1ng/ml induced suppression of TNF α release by MM6, no apparent reduction was seen in response to either 10ng/ml IL-10 or 1 μ M dexamethasone. In contrast, WB *ex vivo* cytokine secretion was clearly suppressed even by concentrations 10-fold lower than these.

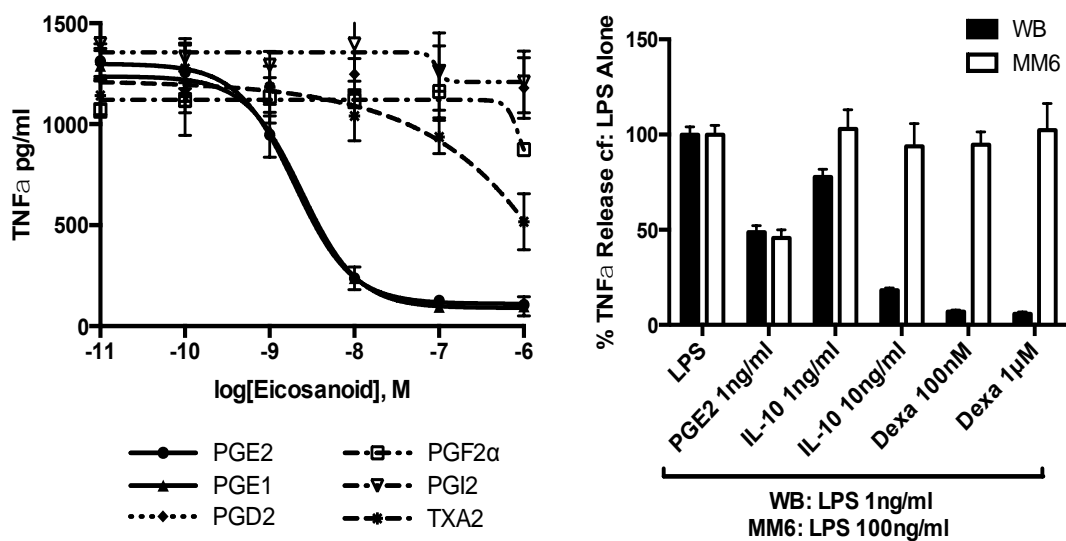


Figure f5.12: *Specificity of the VD3 MM6 assay.* **Left Panel:** Dose-dependent inhibition of LPS-stimulated (100ng/ml) VD3 MM6 TNF α release by COX-derived prostanoids PGD₂, PGF_{2 α} , PGI₂ (carbaprostacyclin, a stable analog of PGI₂) and TXA₂ (carbocyclic thromboxane A₂, a stable analog of TXA₂). Data points represent the mean and SD of 4 wells, each assayed (ELISA) in duplicate, each well equating to n = 1. **Right Panel:** MM6 or WB LPS-stimulated TNF α release, expressed as % of LPS alone, following pre-treatment with PGE₂, IL-10 or dexamethasone. Columns represent the mean with SD of 4 wells (MM6) or 3 volunteers (WB), n = 1 well or volunteer.

5.3.6 Comparability to primary MDM

Importantly, VD3 MM6 appear to display similar responses characteristics to primary HV-derived MDM (via protocol 2.1.3) with regards to PGE₂. MDM PGE₂-mediated TNF α suppression is seemingly mediated principally via the EP4-receptor, MF498 (EP4 antagonist) but not PF-04418948 (EP2 antagonist) significantly abrogating PGE₂'s effect (Figure f5.13, Left Panel, PGE₂ IC₅₀: 4.5nM [95% CI 3.0 - 6.6nM, with MF498 1 μ M: 72nM [95% CI 47 - 111nM). Both cells types also display near

equivalence in sensitivity to PGE₂, MDM having an IC₅₀ as above, MM6 - when TNF α release is normalized for comparison – an IC₅₀ of 7.7nM (95% CI 4.2 – 14.1nM) indicating they may act as accurate substitutes for primary cells (Figure f5.13, right panel). Of note, MM6 release appreciably less TNF α than MDM (~30 fold) and whilst HVP reduces release from MM6, it enhances that from MDM.

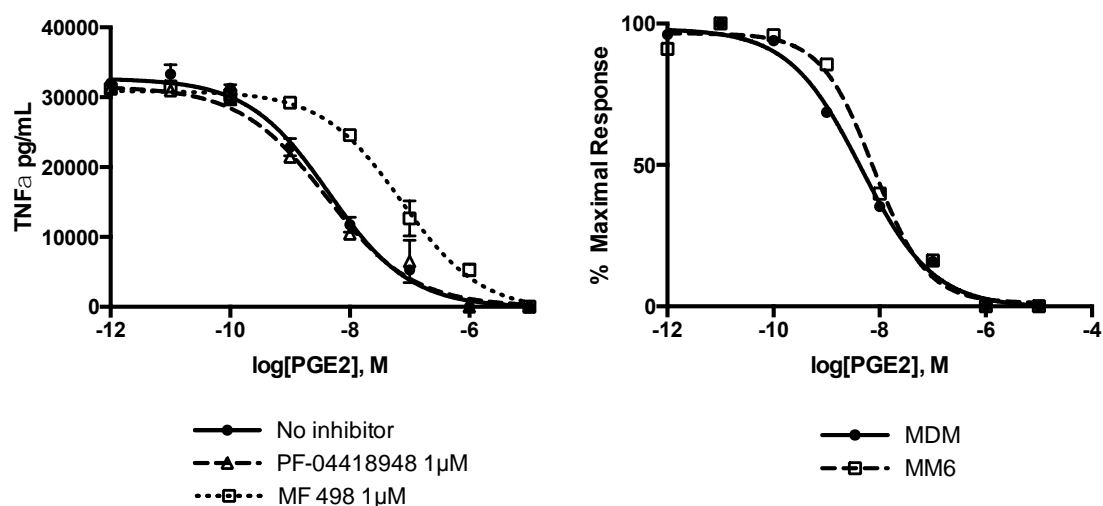


Figure f5.13: PGE₂ suppresses TNF α release in MDM via the same receptor (EP4) and with a similar IC₅₀ to MM6. **Left Panel:** MDM were co-incubated with varying concentrations of PGE₂ (10pM-10μM) in media alone or in the presence of either a selective EP4 receptor antagonist (MF498 1μM) or EP2 receptor antagonist (PF-04418948 1μM) prior to stimulation with LPS (100ng/mL). **Right Panel:** Dose response curves (four parameter variable slope) were calculated for MDM and MM6 in response to PGE₂, normalised for TNF α release (LPS alone = 100%). Data-points represent the mean with SD of 8 wells (MM6) or 4 wells (MDM, cells being derived from 1 volunteer), n = 1 well or volunteer.

5.4: DISCUSSION

MM6 display many favourable characteristics to support their use as a bioassay for the presence and functional relevance of PGE₂. Following incubation with VD3 for 48hours they demonstrate exquisite sensitivity to LPS, rapidly (within 2-4hours) generating a consistent quantity of TNF α across passages. PGE₂ elicits a clear, reproducible, dose-dependent suppression of TNF α , with a threshold of ~100pg/ml and IC₅₀ 590pg/mL (95% CI 503-692pg/mL) when incubated with 25% v/v plasma. This effect appears to be mediated by the EP4 receptor alone and is secondary to elevated intracellular cAMP. Preliminary experiments indicate the assay to be specific, demonstrating little or no response to either alternate COX-derived prostanoids or classic anti-inflammatory mediators (IL-10, glucocorticoids), the

contributory role of PGE₂ to monocyte deactivation in a given biological sample being confirmed via reversal with selective EP4 antagonists (e.g. MF498 1µM). To date VD3 MM6 have demonstrated similar qualitative (IC50) if not quantitative (TNFα production) responses to LPS and PGE₂ as primary MDM, making them an appropriate substitute, although this finding requires replication in MDM derived from a greater number of donors. Further, the PGE₂-mediated effect appears robust to variations in protocol and sample handling. As with the WB assay, alternate cytokines including IL-1β and IL-10 do not display PGE₂-mediated suppression, however the heterogeneous modulation of cytokine biosynthesis by cAMP has long been recognised⁴⁰⁴.

In line with previous publications VD3 was observed to increase MM6 CD14 expression and TNFα release in response to LPS^{288,462}. As a known co-receptor for TLR4 this is perhaps not surprising, although it's activation traditionally requires the presence of LPS binding protein⁴⁶³. PMA incubation was associated with a shift to a more macrophage-like phenotype with increased clumping, adherence (associated with an increase in CD11b and CD11c), and loss of CD33. This was not accompanied by increased TNFα release in response to LPS or peptidoglycan. M-CSF surprisingly had no effect on the MM6, inducing no phenotypic or functional alteration.

Of concern, plasma taken from healthy volunteers suppressed MM6 LPS-stimulated TNFα release in a dose-dependent manner. This effect is the opposite of that seen in primary MDM and could only be partially overcome by employing higher concentrations of LPS (data not shown). Further, higher concentrations of plasma reduced the ability of exogenous PGE₂ to suppress MM6 TNFα release. This may reflect altered bioavailability (e.g. binding by albumin) or increased rates of catabolism. The latter seems less likely however, PGE₂ being added to MM6 in media alone or in the presence of HVP at increasing times before LPS stimulation demonstrating loss of efficacy at comparable rates. This finding accords with previous data demonstrating that the half-life of PGE₂ in culture is in the order of hours, not seconds as *in vivo*⁴⁶⁴.

At 25% v/v plasma PGE₂ was demonstrated to retain it's potency, displaying equivalent dose-response characteristics to addition in media alone, and was consequently selected as the preferred technique. Use of 25% v/v as opposed to 100% v/v plasma reduces the sensitivity of the assay four-fold, the effective IC50 consequently being in the order of 2.5ng/mL. Whilst in the physiological range of

PGE₂ in exudates or efferent lymphatics draining an inflammatory site such concentrations are unlikely to be achieved in plasma. Further work is required to assess means of ameliorating the independent 'immunosuppressive' effect of plasma on MM6 TNF α release (e.g. protein denaturation) or of enhancing the lipid mediator signal in a non-cytotoxic manner (e.g. acetone/chloroform extraction and re-suspension)⁴⁶⁵.

In accordance with previous publications, and as seen with the *ex vivo* WB cytokine release assay, EDTA was determined to exert an independent effect, reducing TNF α release⁴⁶⁶⁻⁴⁷⁰. Lithium heparin, which unlike EDTA and sodium citrate, does not rely on a calcium-chelating mechanism to prevent coagulation exerted the least interference with the bioassay. No attempt at either dilution of EDTA or calcium replacement was attempted to determine whether this could reverse or reduce the observed effect on LPS-stimulated TNF α release. Given that the SPOT(id) patient samples were obtained in EDTA-containing tubes this represents a not insignificant oversight as correction for assay interference would have facilitated their analysis and potentially established clinical proof-of-principle for the MM6 PGE₂ bioassay.

As discussed, the inflammatory profile of patients is now known to determine outcome; individuals exhibiting a sustained, exaggerated response being more likely to experience adverse outcomes⁴². Identification of these individuals is key, facilitating the administration of a growing arsenal of immunorestorative agents. Those patients whose plasma exerts a proportionally greater immunosuppressive effect on MM6 (as per the MDM in Figure f5.1) may represent this 'at risk' cohort. Reversal of observed monocyte deactivation (reduced cytokine secretion) *in vitro* by EP4 antagonism would indicate the presence and mechanistic contribution of PGE₂ and individuals could, hypothetically, be stratified to receive 'anti-PGE₂' therapy (e.g. NSAIDs, EP receptor antagonists). The MM6 bioassay represents one attempt to provide a rapid, cheap, reproducible assay to both demonstrate a role for PGE₂ in CIID, and potentially, facilitate such clinical decision-making in the future. Whilst demonstrating multiple advantageous characteristics its utility remains to be proven.

5.5: SUMMARY

- MM6 display alternate differentiation pathways in response to VD3 and PMA. VD3 differentiation significantly increases CD14 expression, sensitivity to LPS, and subsequent TNF α release
- PGE₂ causes dose-dependent suppression of MM6 TNF α release at physiologically relevant concentrations (IC50 ~600pg/mL), seemingly via an EP4 mediated increase in intracellular cAMP. Preliminary experiments indicate neither alternate COX-derived prostanoids, nor classic negatively immunomodulatory agents (IL-10, glucocorticoids), replicate this effect.
- MM6 display equivalent qualitative (IC50) if not quantitative (TNF α production) responses to LPS and PGE₂ as primary MDM, potentially making them an appropriate substitute. This requires further verification.
- Healthy volunteer plasma reduces MM6 LPS-stimulated TNF α release in a dose-dependent manner and, above a threshold of 25% v/v, reduces the potency of PGE₂. EDTA independently interferes with cytokine release.
- The MM6 bioassay may represent a novel tool to quantify the proportional contribution of PGE₂ to monocyte deactivation in biological samples and to provide an estimate of bioactive PGE₂ concentrations.

5.6: APPENDIX

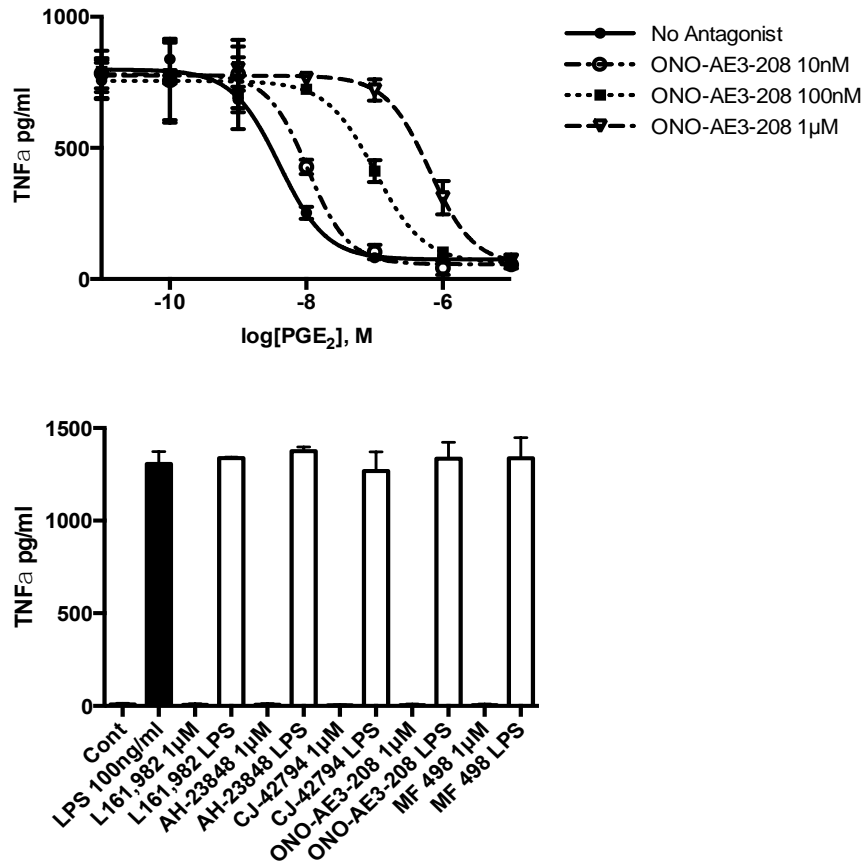


Figure f5.14: *EP4* receptors antagonist characterisation. **Top Panel:** VD3 MM6 were co-incubated with varying concentrations of PGE₂ (10pM-10μM) in media alone or in the presence of one of four selective EP4 receptor antagonists (ONO-AE3-208, MF498, CJ-023,423, L161,982) at different concentrations (10nM-1μM) prior to stimulation with LPS (100ng/mL). The ability of EP4 receptors antagonists to reverse PGE₂'s effect was concentration dependent in all cases. ONO-AE3-208 is shown as an exemplar **Bottom Panel:** EP4 antagonists were not found to independently increase TNFα release. All data points represent mean +/- SD.

CHAPTER 6: Intravenous Endotoxemia

DETECTION OF AN IMMUNOMODULATORY PROSTAGLANDIN E2 SIGNAL IN A HUMAN MODEL OF SYSTEMIC INFLAMMATION: INTRAVENOUS ENDOTOXEMIA

6.1 Introduction

6.2 Additional Methods

6.3 Results

6.4 Discussion

6.5 Summary

6.6 Appendix

6.1 INTRODUCTION

Investigation of the inflammatory response and its component pathways in the clinical population is challenging. Demographic (age, sex, ethnicity), temporal (time of onset, duration of exposure), pathological (type of initiating stimuli, severity of insult, co-morbidity burden) and therapeutic (drugs, location/level of care) variance impedes access to and interrogation of key biological mechanisms. The traditional solution has been to use animal models. Despite affording multiple advantages, their fundamental similarity to human pathophysiology^{471,472} and the relevance of their output, has come under increasing scrutiny⁴⁷³⁻⁴⁷⁵. An alternative is to employ reductionist human models.

Developed in the late 1960's, intravenous (IV) endotoxin administration to man offers a platform through which to discover, delineate and potentially drug inflammatory pathways⁴⁷⁶⁻⁴⁷⁹. National Institutes for Health Clinical Center Reference Endotoxin (CCRE, *Escherichia coli* O:113:H10:K negative, used synonymously with LPS below) is a potent TLR4 agonist that triggers the inflammatory cascade in a dose-dependent manner. Injected as a bolus of 2-4ng/kg, it reliably and reproducibly generates vascular, haematological, endocrine, immunological and organ-specific functional effects that parallel, to varying degrees, those seen in the early stages of a 'sepsis-like' state. The associated short-lived, moderate, systemic inflammatory response permits controlled investigation of single components of the highly complex host-pathogen interaction that develops during bacterial infection. Crucially, the human endotoxin model appears to recapitulate many of the features of CIID facilitating exploration of both its mechanistic drivers and therapeutic strategies to rectify it.

6.1.1 IV endotoxin as a model of CIID

At a transcriptomic level, *in vivo* LPS administration has been repeatedly demonstrated to cause similar patterns of up and down regulation of inflammatory networks to pathophysiological states including trauma, burn and sepsis, potentially reflecting a common human response to systemic stress^{42,471}. Functionally, exposure to endotoxin appears to have multi-modal immunological consequences. Repeated administration of IV endotoxin results in a dramatic reduction in response to this key bacterial ligand mirroring monocyte deactivation following sepsis, trauma or burn. Indeed, after 5 consecutive daily challenges *in vivo* cytokine responses were observed to be 95-99% lower than after first exposure⁴⁸⁰. Kox and co-workers demonstrated that this effect, as in the CI population, was neither short term, nor relied on repeated dosing. Administration of a second dose of IV endotoxin (2ng/kg) 2 weeks after a first challenge (also 2ng/kg) was still associated with a significantly

attenuated response: key pro- and anti-inflammatory cytokines plasma concentrations being 10-46% lower²²⁰. This may reflect a period of immune suppression arising from the on-going release of endogenous mediators, or reflect functional alterations in cytokine-releasing cells as a result of endotoxin tolerance. Significantly, this 'immunoparalysis' appears amenable to therapeutic intervention, the same authors demonstrating the efficacy of IFN- γ and potentially GM-CSF as immunorestorative therapies in the model – either preventing or ameliorating the loss of cytokine response to the second challenge³⁸³.

Despite the fact that LPS may be a ligand for TLR4, endotoxin appears to induce anergy to multiple TLR ligands including those that signal solely through a MyD88-independent pathway, WB taken from volunteers 3-8hr after IV endotoxin being hyporesponsive to TLR2, TLR3, TLR5 and TLR7 signalling, reflecting extensive cross-tolerance⁶¹. Nor does endotoxin appear to affect myeloid lineage cells alone, lymphocytes too appear deactivated post-experimental exposure, demonstrating reduced responses to phytohaemagglutinin (PHA) and IL-2 release *ex vivo*⁴⁸¹. In short, human endotoxemia appears to offer a unique, valid, manipulable window into CIID.

6.1.2 IV endotoxin administration triggers lipid mediator release

IV endotoxin administration to humans has previously been shown to elicit lipid mediator release and been used to investigate the contribution of COX to the inflammatory response. This suggests it additionally represents a suitable model to study the systemic immune consequences of PGE₂.

Bolus doses of endotoxin lead to a significant increase in urinary concentrations of both 6-keto PGF_{1 α} (the principal metabolite of PGI₂) and 11-dehydro thromboxane B₂ (the principal metabolite of TXA₂) in a dose-dependent manner (4ng/mL > 2ng/mL), indicating increased systemic production. COX2 appears to be the isoform primarily responsible for biosynthesis, ibuprofen (a non-selective COX1/2 inhibitor) or celecoxib (COX2 selective), but not low-dose aspirin (81mg), ablating this rise and also significantly attenuating the constitutional and febrile response⁴⁸². Ibuprofen, and hence prostanoid suppression, additionally appears to alter the human metabolic and neuroendocrine response to LPS⁴⁸³⁻⁴⁸⁵. Whilst not measured to date it can only be assumed that elevated PGE₂ forms part of this response – hypothalamic PGE₂ release believed to be one of the core mechanisms underlying the human pyrexial response⁴⁸⁶.

Importantly, there is evidence that NSAIDs may alter immune competence in this model. Rodrick and colleagues noted that whilst administration of 800mg of ibuprofen orally 90min prior to and again at the time of endotoxin injection did not modify subsequent cellular kinetics, it did restore otherwise suppressed mitogenic PBMC responses to PHA to normal and increase IL-2 production *ex vivo*⁴⁸¹. IL-1 and TNF α production from adherent PBMC (monocyte/macrophage) was not re-established however potentially indicating discrete mechanisms of deactivation. These findings complement their earlier demonstration that bolus endotoxin administration results in augmented macrophage PGE₂ release and increased lymphocyte sensitivity to PGE₂, mimicking the changes seen in PBMC obtained from burn patients, and which may be rescued by COX inhibition *in vivo* or *ex vivo*²¹².

Evidence for the inflammation-induced release of SPM in humans is less convincing. Recent work found only minute concentrations of select mediators in the plasma (maresins and PD1) and none of others (RvD1 and RvE1), with no evidence of their formation in response to a low dose endotoxin challenge (0.6ng/kg) even when volunteers had been taking omega-3 supplementation¹⁷⁸. In contrast others report key roles for these molecules, especially those derived from the administration of drugs including aspirin⁴⁸⁷ and statins⁴⁸⁸ in select settings.

6.1.3 Chapter aims

- Describe the profile of plasma COX-derived prostanoids in response to endotoxin for the first time in man, comparing these to known cytokines and clinical markers
- Validate the IV endotoxin administration as a model of CIID using established measures of immune dysfunction
- Determine the contribution of PGE₂ to observed immune defects using the LPS-stimulated WB cytokine release and MM6 bioassay as metrics
- Implicate PGE₂ blockade as a viable therapeutic strategy to restore immune competence in the critically ill

6.2 ADDITIONAL METHODS

6.2.1 Monocyte HLA-DR (mHLA-DR) determination; QuantiBrite™ System

Samples of peripheral blood were obtained in EDTA-coated BD Vacutainers®. 50µL of WB was stained with 20µL of QuantiBrite HLA-DR/Monocyte mixture (anti-HLA-DR PE [clone L243]/anti-monocyte PerCP-Cy5.5 [CD14, clone MφP9], Becton Dickinson [BD], San Jose, CA) at 4°C for 30min-1hr in the dark. Samples were then lysed with 450µL BD FACS Lysing solution (1x) and kept in the dark at 4°C until analysis.

Live cells were first gated out using forward (size) and side (granularity) scatter characteristics. Monocytes were identified on the basis of CD14 expression and mHLA-DR expression was measured on their surface (mono-parametric histogram) as geometric mean fluorescence intensity (MFI) related to the entire population. These results were transformed into number of antibodies (AB) per cell (C) (AB/C) using calibrated PE-QuantiBrite™ beads (BD) and GraphPad Prism software. QuantiBrite™ Beads were run in advance of every participant. This protocol represents the current gold standard for mHLA-DR determination and has been extensively validated^{489,490}.

6.2.2 Neutrophil CD88 (complement component 5a receptor) expression

Blood was obtained as above and processed as per 2.4. A standardised gating strategy was employed to identify neutrophils using flow cytometry (adapted from ^{491,492}). Live cells were first gated using forward and side scatter characteristics (>50k), with subsequent doublet exclusion undertaken via forward scatter height/area. Lymphocyte and natural killer cell lineage marker (CD3, CD19, CD56) and HLA-DR negative cells were further examined for CD16 expression. CD16+ cells with high granularity (side scatter) were identified as neutrophils and cell surface CD88 (PE-Cy7) expression described as MFI (see Figure 6.16, Appendix). All antibodies employed are described in 2.4.3.

6.2.3 Identification of persistent lymphopenia

Persistent lymphopenia during CI has recently been identified as predictive of adverse clinical outcomes including nosocomial acquisition and mortality. Variable definitions have been applied including absolute lymphocyte count $<1.2 \times 10^3/\mu\text{L}$ (severe lymphopenia $<0.6 \times 10^3$)^{382,493}, $<1.0 \times 10^3/\mu\text{L}$ ⁴⁹⁴ and $<0.5 \times 10^3/\mu\text{L}$ ⁴⁹⁵. Haematological analysis (full blood count) of participant samples was performed via University College London Hospital (Sysmex XE-2100, Kobe, Japan. The Doctor's Laboratory, London) and the presence of lymphopenia evaluated against all published cut-off's.

6.3 RESULTS

6.3.1. Characterisation of the inflammatory profile

Injection of 2ng/kg EC-6 endotoxin induced a reproducible systemic inflammatory response in all volunteers. Symptoms, including nausea, headache, muscle ache and shivering (rigors), peaked between 1.5-2hr after administration before gradually abating (Figure f6.13, Appendix). All participants described either no symptomology at 8h or only minimal residual effects, none requiring further monitoring. Vital signs followed a similar yet temporally distinct pattern, the majority reaching their point of greatest divergence from baseline between 3-4hr (Figure f6.14, Appendix). Consistent increases in respiratory rate (RR), pulse and tympanic temperature were observed. No significant alteration in systolic blood pressure was seen. Arterial oxygen saturations exhibited a clinically insignificant late decrease with a nadir at 6hr around 97%, potentially reflecting increased capillary permeability and hence lung-water and/or pulmonary cellular exudate. 90% of participants achieved systemic inflammatory response syndrome (SIRS) criteria, the majority meeting all 3 of RR >20bpm, pulse \geq 90 and temperature \geq 38°C. A total white blood cell count (WBC, leukocytes) \geq 12x10⁹ cells/L – the final SIRS criteria - was witnessed in 7 participants, WBC habitually peaking 8hr post-injection (Figure f6.14, Appendix). The final individual achieved a pulse of 91bpm and RR of 20 however did not exceed this value.

Individual leukocyte populations displayed discrete kinetics in response to endotoxin administration (see Figure f6.1). Neutrophil (PMN) numbers were observed to rise rapidly and immediately, reaching a peak 5-6 fold higher than baseline at 8hr in 90% of participants, returning to normal by 48-72hr. This was mimicked by C-reactive protein (CRP), a hepatically-secreted acute phase reactant employed clinically to monitor inflammation. CRP rose significantly by 8hr, peaked at 24hr (mean 29.9mg/L) and was present at elevated concentrations to day 7. In contrast, striking early monocytopenia and lymphopenia was observed, these populations reaching their nadir by 2hr and 4hr and recovering to normal concentrations by 8hr and 24-48hr respectively. The location and fate of these cells during this period is unclear, as is whether repopulation of the blood compartment is by the same or newly released cells.

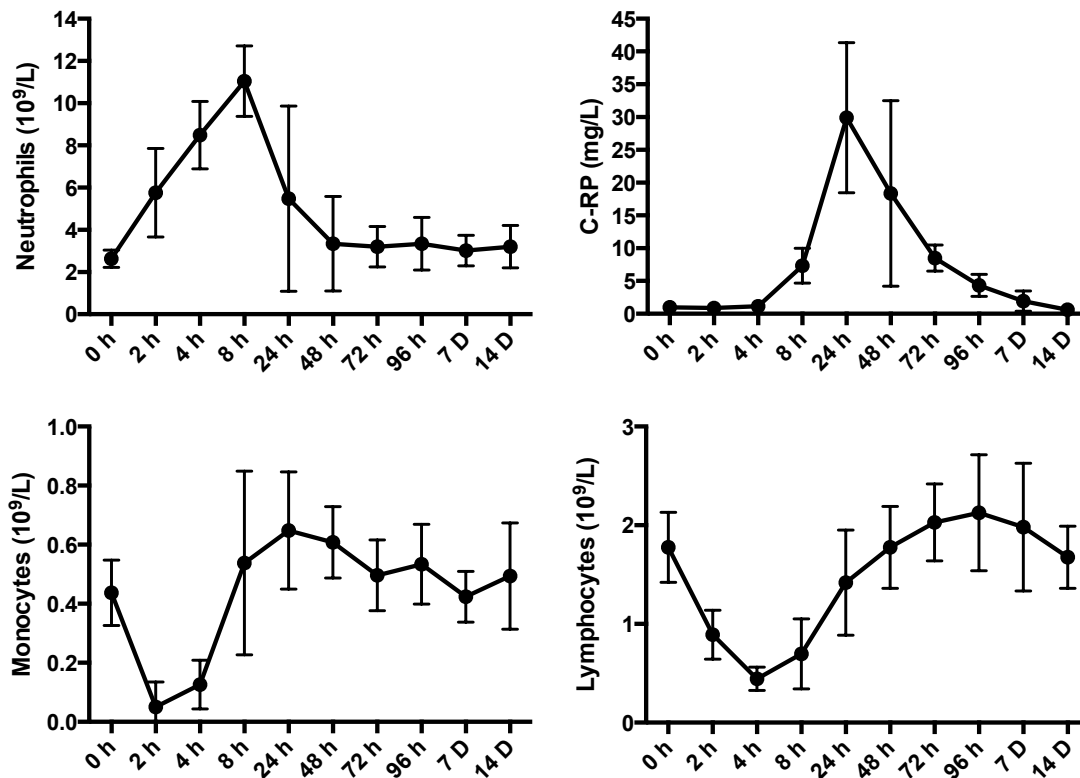


Figure f6.1. Leukocyte and C-reactive protein (CRP) kinetics post-IV endotoxin injection. Individual leukocyte populations and CRP were determined by The Doctor's Laboratory between baseline and 14days, via UCLH. EC-6 administration resulted in a clear neutrophilia in all participants, peaking at 8hr, and acute phase response, typified by CRP, that reached its zenith by 24hrs. In contrast, the immediate post-endotoxin period was characterised by monocytopenia and lymphopenia, which had returned to baseline by 8hr and 24-48hr respectively. Data-points represent mean and SD of n=10 volunteers.

Plasma cytokine concentrations displayed complimentary and contrasting profiles. Significant quantities of the classic pro-inflammatory cytokines TNF α , IL-6, IL-8, but not IL-1 β , were recorded in plasma. Natural killer and lymphocyte released IFN- γ and the anti-inflammatory cytokine IL-10 were also found but at substantially lower concentrations (Figure f6.2). TNF α was the first to be observed, rising by 1hr, peaking at 1.5hr (mean 495pg/mL) and gradually returning to baseline by 8hr. IL-8 and IL-6 displayed similarly broad-based increases but rose and peaked 30minutes later (775pg/mL and 389pg/mL respectively). IL-10 and IFN γ sequentially reached their zeniths at 3 and 4hrs respectively both with mean concentrations of 54pg/mL. Individual cytokine time courses are displayed in Figure 6.15 (Appendix).

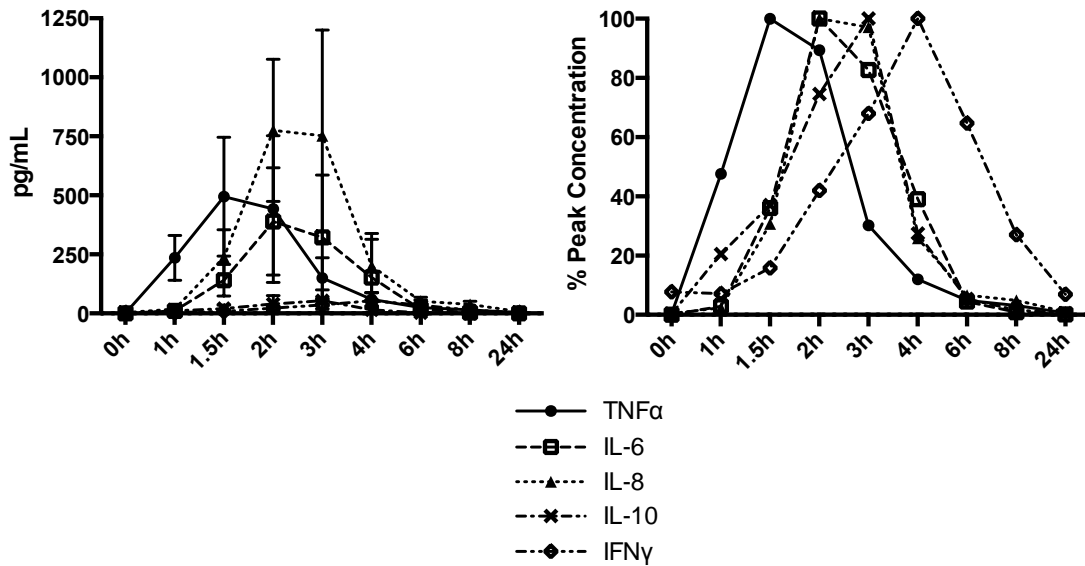


Figure f6.2. *Plasma cytokine concentration.* Plasma was assayed for cytokine concentrations via the MSD V-Plex system. **Left Panel:** Individual cytokines are displayed as mean absolute concentration (pg/mL, n=10 volunteers) with SD. **Right Panel:** Cytokines are expressed as % individual peak concentration (normalised) allowing appreciation of their discrete yet overlapping time-courses. Data-points represent the mean of n=10 volunteers.

6.3.2 Prostaglandin E_2 and EP receptor expression

Mass spectrometric analysis of participants' plasma (n=5, as per 2.3.4) revealed increased circulating concentrations of COX-derived prostanoids in response to IV endotoxin administration (Figure f6.3). PGE_2 , $PGF_{2\alpha}$ and TXA_2 were all significantly elevated, mean PGE_2 peaking at 3hours 7.8x higher than baseline values (10pg/mL compared to 1.3pg/mL). TXA_2 demonstrated the greatest alteration, increasing 23 fold from 8.2pg/mL to 191pg/mL on average. No clear pattern was observed in either PGD_2 or the PGI_2 metabolite 6keto- $PGF_{1\alpha}$ concentration following systemic inflammation, indicating discrete alterations in PG synthase enzymes downstream of COX or altered binding, catalysis and/or elimination of these molecules. Alterations in the concentration of alternate AA, EPA, DHA and linoleic acid derived lipid mediators, mediated by COX, LOX, cytochrome 450 (CYP450) and non-enzymatic oxidization (NEO) are displayed in Figures f6.17-f6.22 (Appendix).

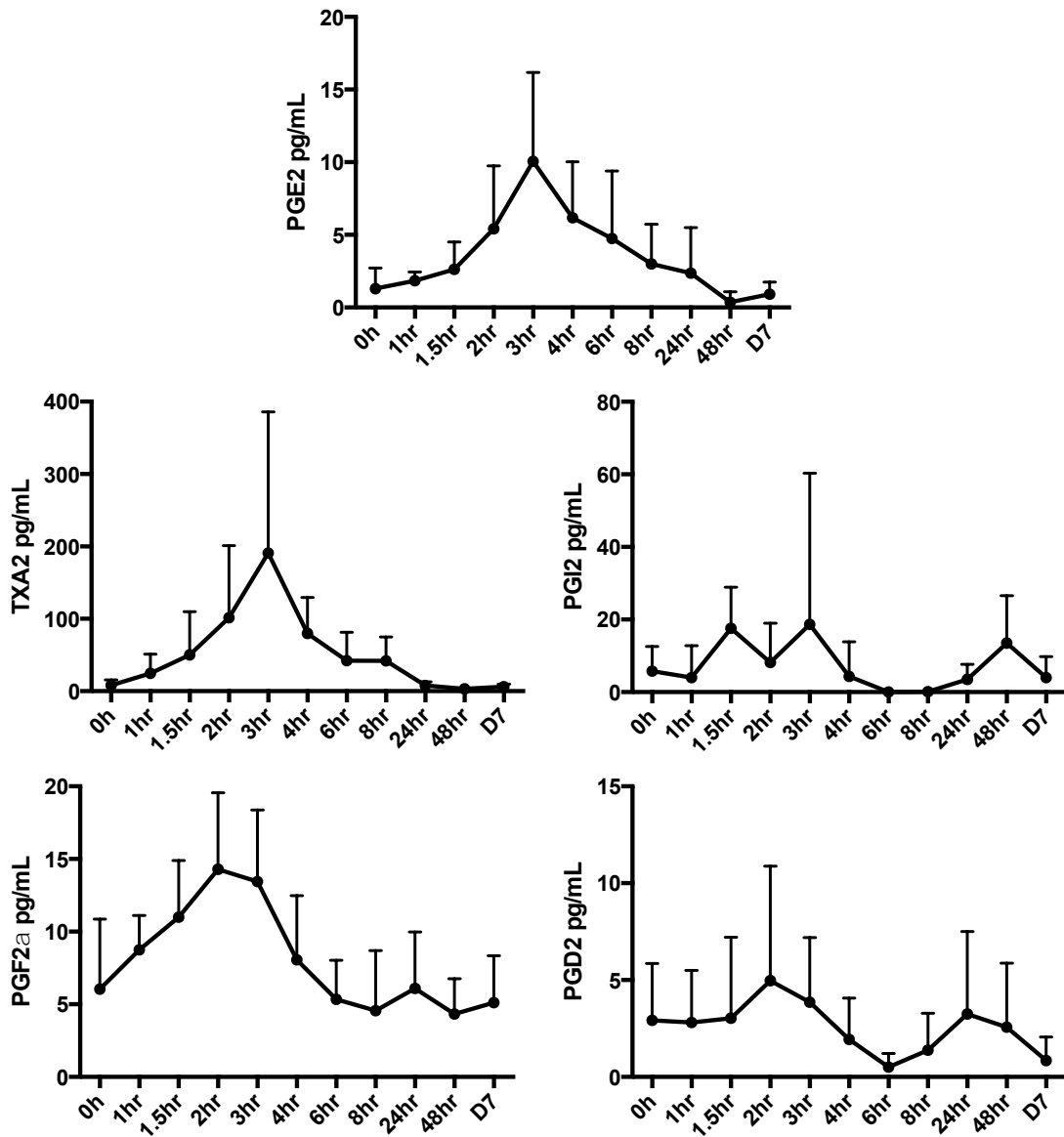


Figure f6.3. IV-endotoxin induced plasma COX-derived prostanoid concentration. Plasma was assayed for prostanoid concentration by electrospray ionisation, liquid chromatography mass spectrometry (ESI/LC-MS) by Ambiotis (Toulouse, France). Panels display individual COX-derived prostanoids as stated on the y-axis. Data-points are the mean and SD of n = 5 participants. PGI₂ is represented by its primary metabolite 6-keto-PGF_{1α}.

The potential contribution of elevated PGE₂ to immune dysfunction was tested via the *ex vivo* LPS-stimulated WB (Chapter 4) and the MM6 bioassays (Chapter 5) elucidated previously. Addition of either an EP2 (PF-04418948) or EP4 receptor antagonist (MF498) to LPS-stimulated WB from individuals 4, 24, 48 or 7days after IV-endotoxin injection did not lead to significant increases in *ex vivo* TNF α release compared to baseline ($p= 0.95$, repeated measures 2-way ANOVA). In comparison, significant variation did occur over time. WB at 4hr released significantly less

(0pg/mL), and, that taken at 24 and 48hr, but not day 7, secreted significantly more TNF α than at baseline ($p < 0.0001$ and $p < 0.001$ respectively, repeated measures 2-way ANOVA with Dunnett's multiple comparisons test) (Figure f6.4)

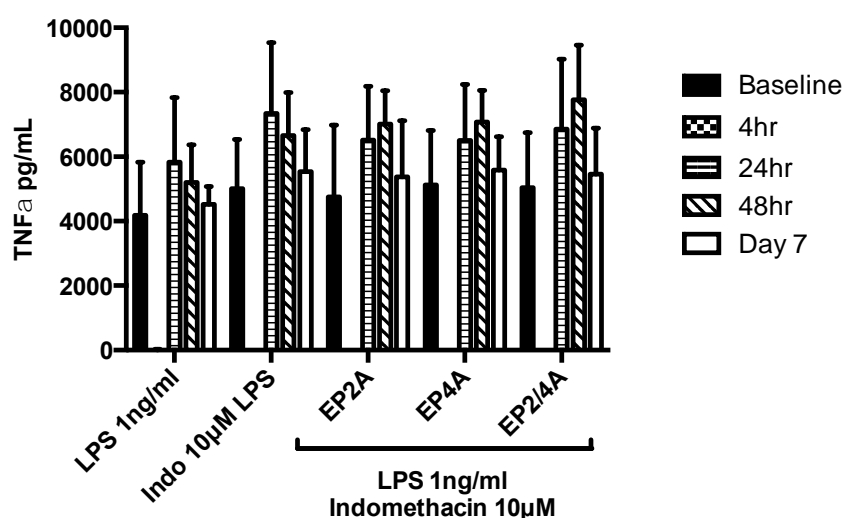


Figure f6.4. Addition of *E*-Prostanoid (EP) receptor 2 and 4 antagonists does not alter ex-vivo WB TNF α release. WB was taken at baseline, 4hr, 24hr, 48hr and 7 days after administration of IV endotoxin and stimulated with 1ng/mL LPS in the presence or absence of an EP2 (PF-04418948 1 μ M) or EP4 receptor (MF498 1 μ M) antagonist. Indomethacin 10 μ M was employed to prevent ex vivo prostanoid release. Bars represent the mean and SD of $n = 5$ participants (1 tube/condition/time-point/volunteer, TNF α assayed [ELISA] in duplicate).

No evidence of an immunosuppressive mediator in the plasma was detected by the MM6 bioassay post-endotoxin administration. Addition of 25% v/v plasma taken at time-points between 2h to 72h after injection failed to suppress MM6 TNF α release below baseline (linear regression of mean TNF α values, $n=7$; R^2 0.149, no significant deviation from zero, $p=0.3455$. Figure f6.5, Left Panel). Nor was there any indication of an immunosuppressive role for PGE $_2$: 2 way ANOVA comparing the effects of time and EP4 antagonism on TNF α release finding no significant effect of MF498 addition ($p=0.07$). Indeed, in direct opposition to the hypothesised effect of PGE $_2$ in the CI, antagonism of PGE $_2$ -EP4 binding in the assay appeared to non-significantly reduce TNF α release at the majority of time-points (Figure f6.5, right panel).

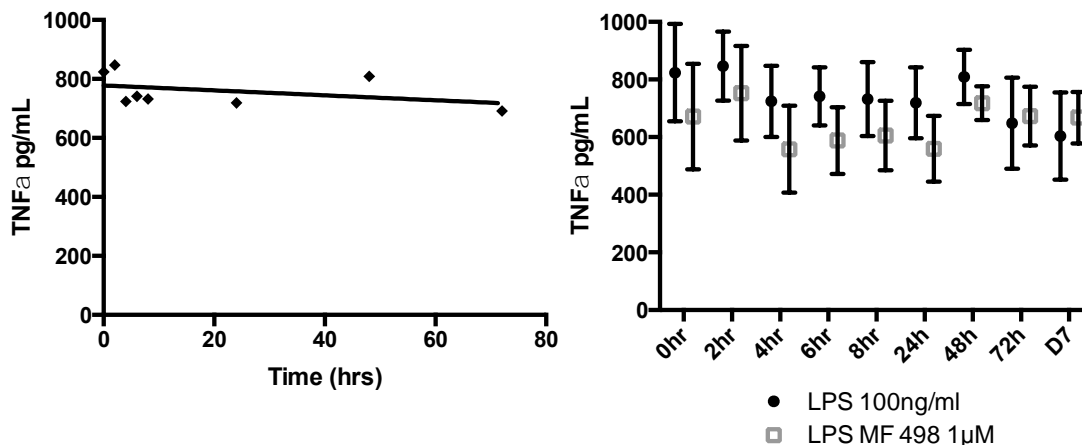


Figure f6.5. Plasma acquired post-IV endotoxemia is not immunosuppressive. **Left Panel:** VD3 MM6 (as per Chapter 5, 4 wells) were incubated with 25% v/v plasma (LH) acquired at set time-points following IV endotoxin administration and stimulated with 100ng/mL LPS. Linear regression was employed to create a line of best fit through the mean of acquired values (baseline to 72h, R^2 0.149). Data-points represent the average of 4 wells/volunteer, assayed (ELISA) in duplicate of $n = 7$ volunteers. **Right Panel:** MM6 were stimulated in the presence (grey boxes) or absence (black circles) of an EP4 receptor antagonist (MF498 1 μ M). Data-points represent the mean + SD of 7 participants (baseline to day 7, as above).

One potential explanation for how such low concentrations of PGE₂ (<50pg/mL) could contribute to circulating monocyte deactivation was alteration in EP-receptor expression and hence sensitivity to a given concentration of PGE₂. WB obtained at different set time points after endotoxin administration however demonstrated no significant alteration in dose-response to PGE₂ with IC₅₀ at baseline being estimated between 148 pg/mL-1ng/mL, at 24h 230pg/mL-5.4ng/mL, 48h 202pg/mL-2.1ng/mL and 265pg/mL-8ng/mL at 7days (Figure f6.6). Further, a change in the dose-response to neither butaprost (EP2 agonist) nor CAY10598 (EP4 agonist) was witnessed at the same time-points. These findings were corroborated by flow cytometric analysis of classical (CD14+) monocyte EP2 and EP4 expression, which found no sustained alteration in either receptor (Figure f6.7). Of note and as already described, whilst no change in EP-receptor expression could be pharmacologically detected, systematic alteration in TNF α release by stimulated WB at different times post-endotoxin was repeatedly witnessed (up shift of the dose-response curve).

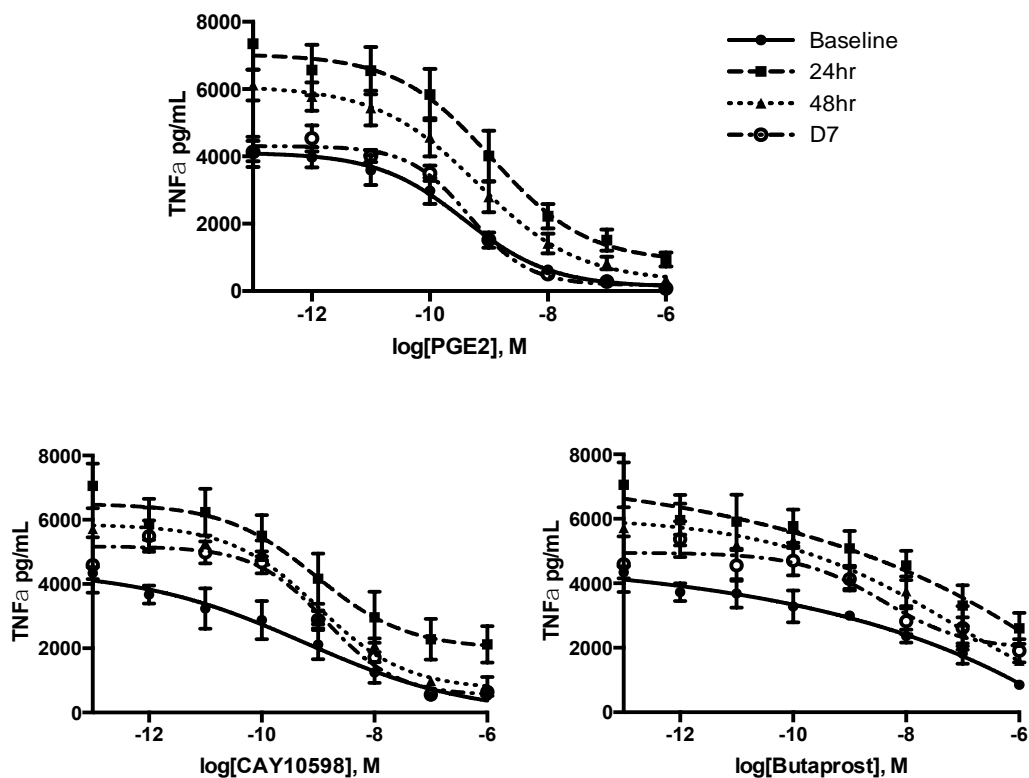


Figure f6.6. Pharmacological evidence for alteration in monocyte *E*-Prostanoid (EP) receptor 2 and 4 expression. WB was taken at baseline, 24hr, 48hr and 7 days after administration of IV endotoxin and stimulated with 1ng/mL LPS in the presence of increasing concentrations ($1 \times M$) of PGE₂, butaprost (EP2) or CAY10598 (EP4). Indomethacin 10 μ M was employed to prevent *ex vivo* prostanoid release. Data-points represent the mean and SEM of n=3 volunteers (1 tube/condition/time-point/volunteer, assayed (ELISA) induplicate). Dose-response curves and IC₅₀ derived by a variable slope four-parameter logistic curve.

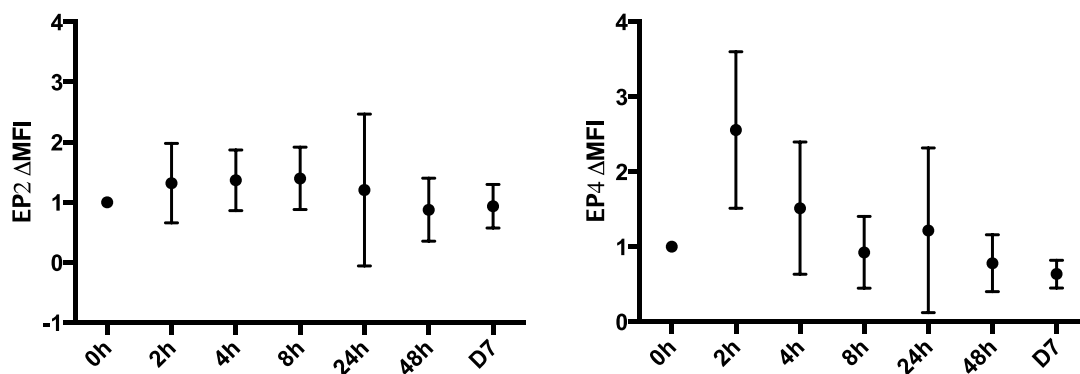


Figure f6.7. Flow cytometric assessment of classical monocyte (CD14+) EP2 and 4 expression. WB was lysed and leukocytes stained to identify the CD14+ monocyte sub-population and EP2 and EP4-receptors. Geometric mean fluorescence from 4 participants is expressed as mean +/- SD fold-change in relation to individual baseline values.

6.3.3 IV endotoxin: A model of immunosuppression?

Despite the assertions of previous authors, the observed alterations in *ex vivo* WB cytokine secretion appear inconsistent with 2ng/kg IV endotoxin representing a model of sustained immune dysfunction. Whilst TNF α release by LPS-stimulated blood was dramatically suppressed in the hours immediately following IV endotoxin administration it was rapidly restored to baseline levels (Figure f6.8). Indeed, at 24hours and 48hours post-injection blood appeared 'primed' to a secondary challenge, significantly increased 'supra-normal' levels of TNF α being recorded in response to the same dose of LPS. Repeated measure 2 way ANOVA showed a significant overall difference in TNF α release at different time points, with Dunnett's multiple comparison test highlighting 2h, 4h and 8h as significantly lower than baseline (all <0.001) and 24h and 48h significantly higher (both >0.0001). Day 7 was non-significantly different from baseline. Whilst addition of an NSAID (10 μ M indomethacin) to the WB assay led to increased TNF α release through the prevention of autocrine/paracrine PGE₂ signalling (see Chapter 4), it neither restored early TNF α secretion, nor led to significant proportional enhancement at 24 or 48h compared to baseline ($p > 0.05$). This implies no enduring alteration in monocyte COX activity results from IV endotoxin administration

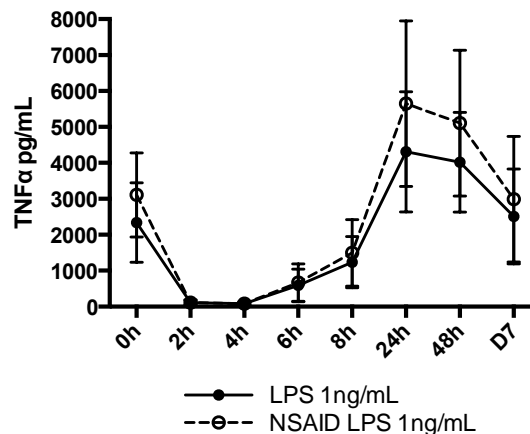


Figure f6.8. *Suppression of ex vivo LPS-stimulated WB TNF α release is not sustained.* WB was taken at baseline, 2hr, 4hr, 8hr, 24hr, 48hr and 7 days after administration of IV endotoxin and stimulated with 1ng/mL LPS in the presence (open circles, dashed lines) or absence (filled circles, solid line) of indomethacin 10 μ M. Data points represent mean and SD of n=10 participants (1 tube/condition/time-point/volunteer, assayed (ELISA) in duplicate).

To determine whether reduction in LPS-stimulated WB *ex vivo* cytokine release could purely be attributed to alterations in cell number TNF α was expressed as a fraction of monocyte number (pg/1000 monocytes, Figure f6.9, left panel). This correction did

not alter the relationship between time and observed TNF α concentrations: suppression in cytokine release still being seen at 4hr ($P < 0.0001$) and 8hr but at a lower level of significance ($p < 0.05$, repeated measure 2 way ANOVA with Dunnett's multiple comparison test), with statistically significant elevated release at 24 and 24hrs (both $p > 0.001$). *In vivo* administration of endotoxin does therefore appear to induce, albeit temporarily, a reduction in *ex vivo* monocyte response to secondary challenge (tolerance) or decreased functional capacity. The source of circulating cytokines may not however be monocytes in the blood compartment – peak plasma TNF α concentration corresponding with the nadir of blood monocyte numbers (Figure F6.X, right panel).

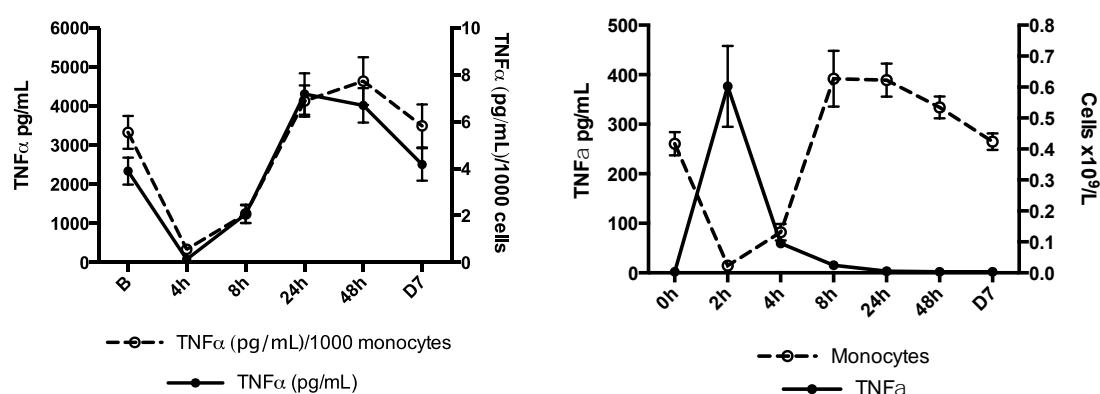


Figure f6.9. Alteration of *ex vivo* LPS-stimulated WB TNF α release is not secondary to alterations in cell number alone. **Left Panel:** TNF α concentrations derived from stimulated WB taken at 4hr, 8hr, 24hr, 48hr and 7 days after administration of IV endotoxin are presented as absolute values (left y axis, solid lines, black circles) and as a function of monocyte number determined from matched samples (right axis, pg/1000 monocytes, dashed line, open circles). Data points are mean and SD of $n=10$ participants (1 tube/condition/time-point/volunteer, assayed (ELISA) in duplicate, 1 full blood count/volunteer/time-point). **Right Panel:** Plasma TNF α concentration (solid line, black circles, left axis, pg/mL) in relation to temporally matched monocyte number (dashed line, open circles, right axis, cells $\times 10^9/L$). Data points represent mean and SD of $n=10$ participants

To further characterise bolus IV endotoxin administration as a model of CIID three alternate established measures of immune dysfunction exploring separate cell populations were employed. Monocyte HLA-DR expression (mHLA-DR, $n=3$) was found to mimic *ex vivo* cytokine secretion, demonstrating an initial mean reduction to 87% (SD 6.9%) of baseline 4hr post-injection of endotoxin, before rebounding to reach supra-normal levels at 24hrs (117%, SD 10.6%; Figure f6.10). Expression was observed to subsequently decrease at 72hrs, being sustained at day 7. This is of unclear physiological significance.

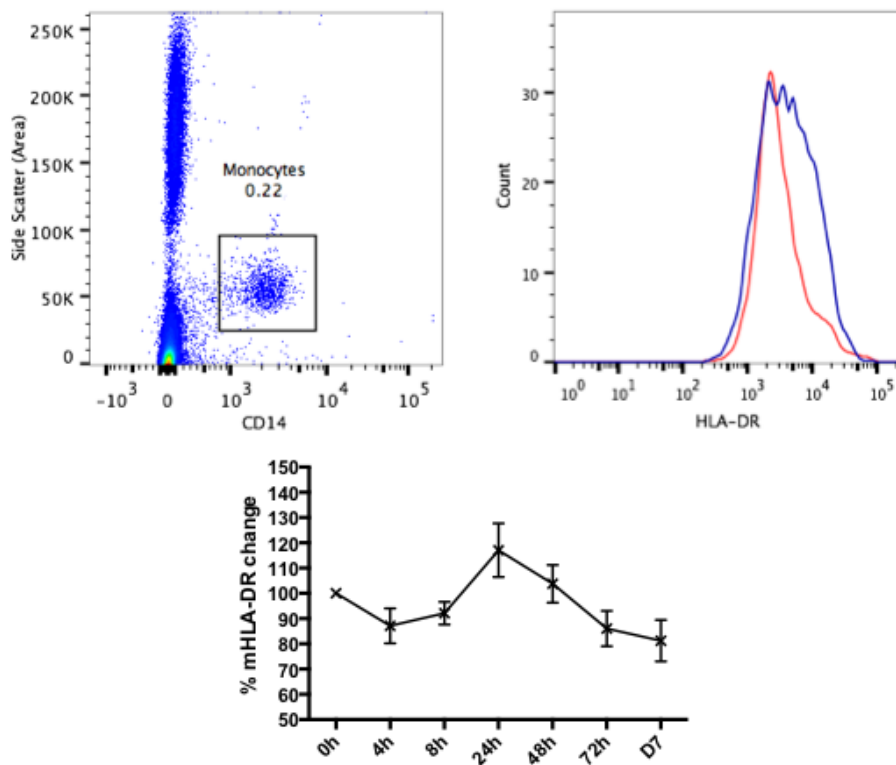


Figure f6.10. Monocyte HLA-DR (mHLA-DR) expression post-IV endotoxin administration. **Top Left Panel:** Monocytes were identified by their CD14+ characteristics as per 6.2.1 **Top Right Panel:** Example illustration of mHLA-DR expression, red (baseline) and blue (24hr). Data is displayed as a histogram, from which geometric mean fluorescence intensity (GMFI) was determined. **Bottom Panel:** GMFI were transformed into number of antibodies (AB) per cell (C) (AB/C) using calibrated PE-Quantibrite™ beads (BD) and GraphPad Prism software, and expressed as percentage change relative to baseline expressed over time. Data points represent mean and SD of n=3 participants.

A decrease in neutrophil CD88 expression reflects increased circulating concentrations of the anaphylotoxin activated complement component 5 (C5a), a known contributor to immune dysfunction. Mean fluorescence intensity was observed to initially fall to 66.6% (SD 12.9%) of baseline by 4hr suggesting compromised neutrophil function, but again mirrored *ex vivo* cytokine secretion, recovering to 81% (SD 17.9%) by 24h and 110% (SD 33.7%) by 48hr (Figure f6.11).

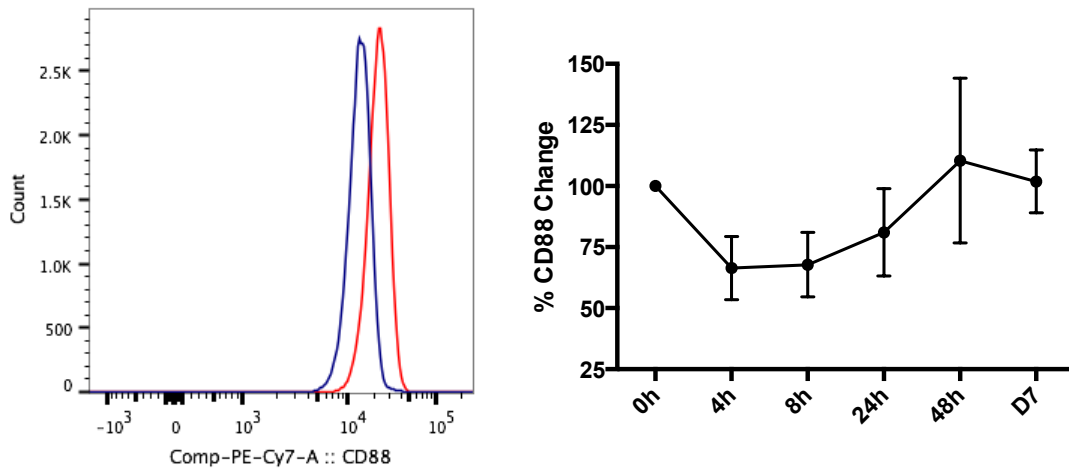


Figure f6.11. Neutrophil CD88 (complement C5a receptor) receptor expression. **Left Panel:** Neutrophils were identified as per the gating strategy stated in 6.2.2 and illustrated in Figure f6.16. Example illustration of CD88 expression red (baseline) and blue (24hr). Data is displayed as a histogram, from which geometric mean fluorescence intensity (GMFI) was determined. **Right Panel:** Mean GMFI with SD are expressed as percentage change relative to baseline expressed over time (n=3 participants).

Finally, the occurrence of sustained lymphopenia was tested for using the multiple definitions employed in the literature to date (Figure f6.12). Whilst the point mean absolute lymphocyte cell count for all participants (n=10) met the upper two cut-offs ($<1.2 \times 10^3/\mu\text{L}$ ^{382,493} and $<1.0 \times 10^3/\mu\text{L}$ ⁴⁹⁴) at 2 and 8hr, all 3 were only met at 4hr ($<0.5 \times 10^3/\mu\text{L}$ ⁴⁹⁵) – the lymphocyte nadir. By 24 hours the absolute lymphocyte count had risen to $1.42 \times 10^3/\mu\text{L}$ (SD 0.53) and at 48hr returned to baseline levels ($1.78 \times 10^3/\mu\text{L}$, SD 0.41). The absolute count was noted to peak at day 4 ($2.13 \times 10^3/\mu\text{L}$, SD 0.59).

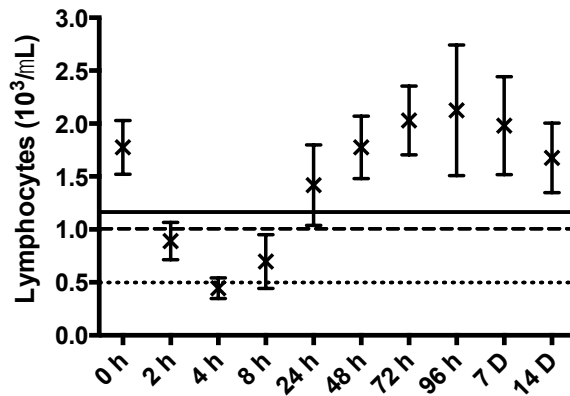


Figure f6.12. Absolute lymphocyte count as a marker of immune compromise. Lymphocyte count was performed via University College London Hospital (Sysmex XE-2100, Kobe, Japan. The Doctor's Laboratory, London). Published cut-offs used to define lymphopenia predictive of immune compromise are illustrated by horizontal lines. Solid: Drewry et al. $<1.2 \times 10^{3382,493}$, dashed: Heffernan et al. $<1.0 \times 10^3/\mu\text{L}^{494}$, dotted: Inoue et al. $<0.5 \times 10^3/\mu\text{L}^{495}$. Data-points represent mean and SD of $n=10$ volunteers (1 full blood count/volunteer/time-point).

6.4 DISCUSSION

Contrary to expectations bolus challenge with 2ng/kg IV CCRE did not institute a phase of sustained immunoparalysis. Three separate metrics related to three discrete cell populations (monocyte HLA-DR expression, neutrophil CD88 expression and absolute lymphocyte count), all known to predict clinically relevant immune dysfunction individually and cumulatively³⁹⁶, showed short-term (<24hour) loss of competence with restoration by 24-48hours after endotoxin injection. Mimicking WB *ex vivo* LPS-stimulated cytokine secretion they all additionally showed an apparent 'rebound' or 'priming' effect, achieving supra-normal values in the days following the initial challenge. Whilst noted by previous authors, to the best of our knowledge, this phenomenon has neither been explored nor described in such detail previously⁴⁸¹.

As anticipated, endotoxin challenge did however elicit a consistent systemic inflammatory response, 90% of participants achieving SIRS criteria. Subjectively reported symptoms, vital signs, cellular kinetics and both pro and anti-inflammatory cytokines followed predictable courses, comparable to those described by previous authors^{479,496}. Against these established parameters we have, for the first time, described the concentration and profile of prostanoids in plasma. TXA_2 , $\text{PGF}_2\alpha$ and PGE_2 all showed significant elevation from baseline, the latter increasing nearly 8-fold by 3hours before returning to baseline by 24-48 hours. As stated, the variance in

profiles demonstrated by each individual lipid mediator, in particular the lack of appreciable increase by PGD₂ and PGI₂, may reflect either distinct regulation by synthases downstream of COX, altered binding, catalysis or elimination *in vivo*. The lack of appreciable concentrations of 6-keto PGF_{1α} (PGI₂-metabolite) is surprising given previous descriptions of its appreciable increase in urine concentration following endotoxin administration⁴⁸². One explanation may lie in isolated augmentation of renal synthesis.

No evidence of an immunosuppressive PGE₂ signal was detected in either the WB or MM6 bioassay. This is not surprising. Firstly, as noted, monocyte deactivation, as reflected by *ex vivo* WB cytokine secretion was only found at the 2, 4 and 8 hour time points. At this early stage neither addition of indomethacin to the assay, nor EP2, EP4 antagonists or both, led to restoration in responsiveness. At the originally predicted target time points of immunosuppression (24 hours – day 7) there was no monocyte reactivation to restore. Secondly, if accurate, the concentrations of PGE₂ identified in the plasma (maximum ~10pg/mL) were insufficient to elicit significant suppression in cytokine release in either assay having IC₅₀ in the order of 300pg/mL (WB) and 600pg/mL (MM6). In the CI patient population this situation may be altered however, previous authors finding that cellular (lymphocyte) sensitivity to PGE₂ was increased ~100-fold, being greater in those who had experienced more severe initial inflammatory insults²¹². No evidence of such a shift in monocyte sensitivity to PGE₂ was elicited in the current set of experiments however, both pharmacological (IC₅₀ of PGE₂, butaprost or CAY10598) and flow cytometric (EP2 and EP4 receptor expression) methodologies indicating no significant temporal change.

6.4.1 Rejection of the hypothesis?

IV administration of endotoxin to humans clearly has multiple benefits – elimination of inter-species translational barriers, increased signal to noise ratio compared to clinical subjects etc. – but an equal number of limitations. Whilst accurately mimicking the clinical phenotype of ‘sepsis-like’ states this is only achieved qualitatively, not quantitatively or temporally. Administration of a single TLR ligand directly into the bloodstream that is cleared within 15 minutes of injection clearly cannot replicate the complexity of bacterial invasion and persistence at a tissue site, nor stimulate an equivalent response from the host. It is already known that mimicry of every pathophysiological characteristic feature of disease is not achieved⁴⁹⁷ and ‘any expectation that the model fully replicates the clinical condition of severe, localized or systemic gram-negative infection is un-warranted’⁴⁷⁸. Indeed, the lack of

requirement for clinical intervention for the phenotypic consequences of endotoxin administration to resolve stands testament to its divergence from disease states.

By ethical necessity only a relatively modest inflammatory response may be elicited. 2ng/kg was selected for this experimental set having previously been shown to induce both short and long-term *in vivo* immunoparalysis^{220,480}. This may however have been insufficient to generate immunosuppressive concentrations of PGE₂. McAdam and co-workers demonstrated that 4ng/kg CCRE provoked urinary TXA₂ and PGI₂ metabolite concentrations 3-4 fold higher than 2ng/kg, following a similar time-course to that described here⁴⁸². In other words prostanoid release appears to be initiating stimuli dose-dependent in a non-directly proportional manner, larger insults triggering still greater eicosanoid biosynthesis. In animal models where PGE₂ ablation has successfully ameliorated risk of secondary infection mortality from the initial challenge was 30-50%¹⁹⁴. In hypothermic septic patients with excessive prostanoid generation who derived benefit from ibuprofen administration mortality was 90%³⁷⁸. Clearly PGE₂ was never predicted to be a 'universal immunosuppressant', instead contributing to loss of immune competence only in select CI individuals with a dysregulated, excessive inflammatory response⁴². Taken in context with the aforementioned data, failure to identify a PGE₂ signal in the current study does not appear extraordinary, but potentially a limitation of the technique.

A final confounding factor may be the differences between *in vivo* and *ex vivo* immune responses and their relationship between each other. Whilst *ex vivo* measures of immune competence have been shown to rapidly return to normal following exposure to endotoxin, these may not mirror the *in vivo* situation where aberrations persist²²⁰. Whilst anti-PGE₂ strategies appear ineffective *ex vivo* in this body of work they may actually have exerted benefit if employed *in vivo*, replicating the success of IFN- γ ³⁸³. Indeed, there is clinical evidence to suggest this may be the case arising from patients pre-treated with NSAIDs undergoing major surgery^{211,384}. Therefore, whilst not supported here, it seems premature to reject the possibility of PGE₂-mediated immune dysfunction and the potential utility of PGE₂ ablative or blocking therapy in stratified patients.

6.5 SUMMARY

- IV endotoxin (2ng/kg) reliably induces COX-derived prostanoids release, TXA₂, PGF_{2α} and PGE₂ being found at concentrations 3 to 23x baseline. Peak concentrations are around 3hours post-injection
- Whilst endotoxin exposure elicits temporary reductions in whole blood *ex vivo* cytokine release in response to LPS, monocyte HLA-DR expression, neutrophil CD88 expression and absolute lymphocyte count this effect is only short-lived, all metrics returning to normal or exceeding baseline values by 24-48hours.
- Prevention of PGE₂ production via NSAID addition or antagonism of it's binding to the EP2 and EP4 receptors failed to restore LPS-stimulated WB cytokine release during this period. No signal of PGE₂-mediated immunosuppression was detected by the MM6 bioassay, 25% v/v plasma failing to reduce TNFα release at any time point
- IV endotoxin led to neither alteration in monocyte EP2 or EP4 receptor expression, nor pharmacologically determined sensitivity to PGE₂.
- Whilst a valuable tool to explore resolving systemic inflammation, human IV endotoxemia may be unable to recapitulate the immunological features of critical illness

6.6 APPENDIX

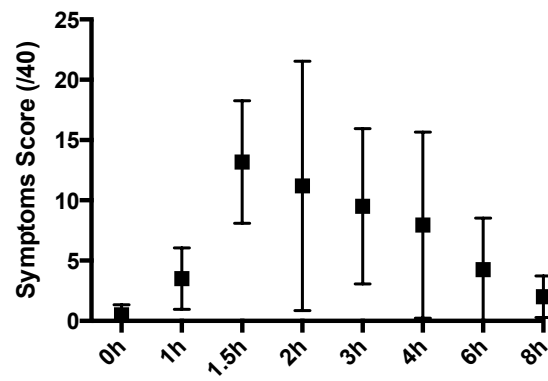


Figure f6.13. *Time-course of cumulative symptom scores.* Participants (n=10) were asked to score their experience on 4 visual analog scales (max 10/scale) describing headache, muscle ache, nausea and shivering at sample collection time-points. Data-points represent mean and SD.

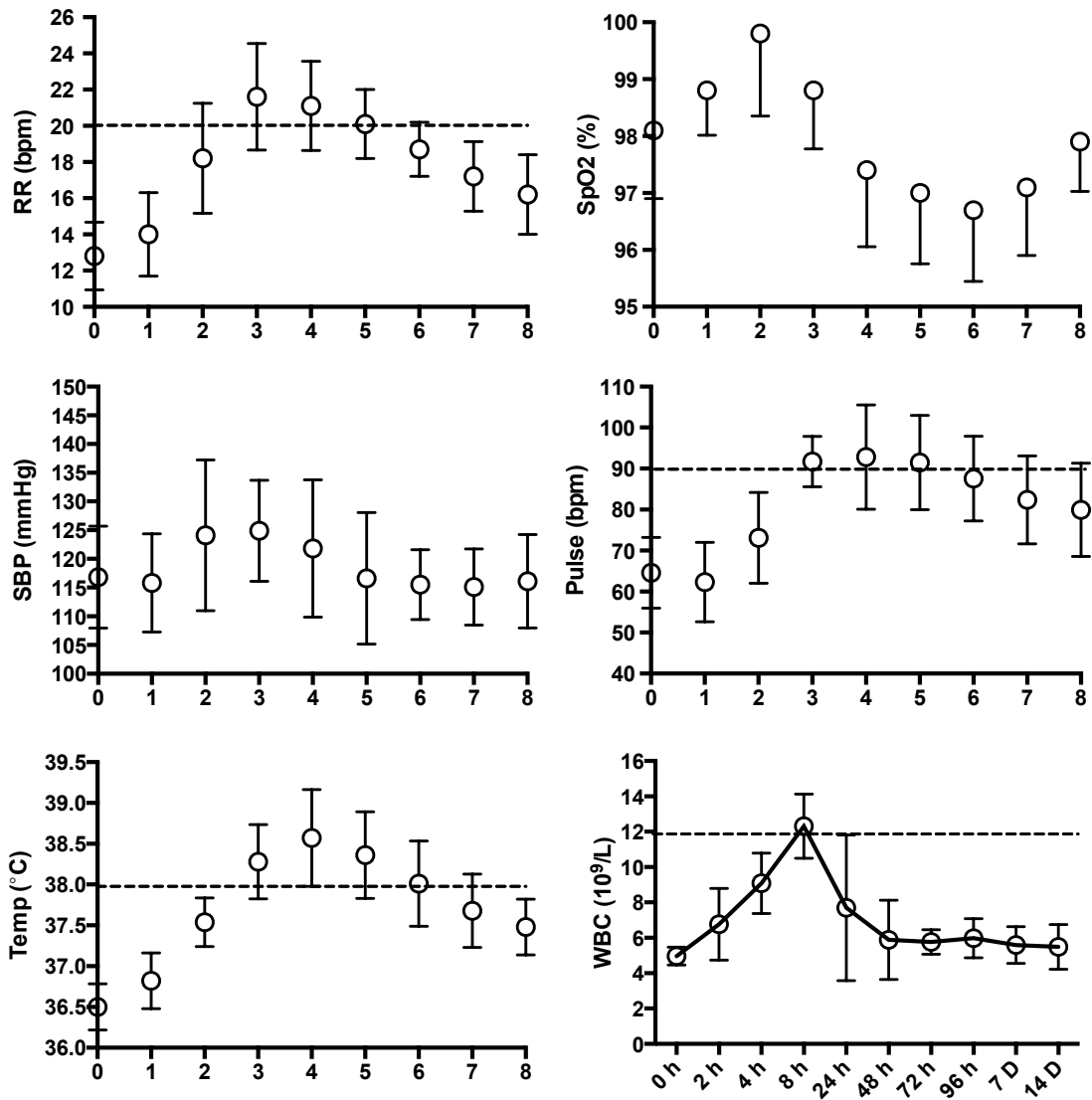


Figure f6.14. Sequential clinical observations and total white blood cell count (WBC) in relation to SIRS definition. Respiratory rate (RR, breaths per minute), arterial oxygen saturation (SpO₂, %), systolic blood pressure (SBP, mmHg), pulse rate (beats per minute), and temperature (°C) were recorded hourly post-endotoxin injection for 8 hours. Total WBC (10⁹cells/L) was taken at baseline (0h), 2h, 4h, 8h, 24h, daily till 96hr, at day 7 and 14. Where appropriate cut-off's used to define SIRS have been inserted (dashed lines): RR>20bpm, pulse >90bpm, temperature >38°C, WBC >12x10⁹/L. 9 out of 10 participants met SIRS criteria (2 or more of the above). Data-points represent mean and SD.

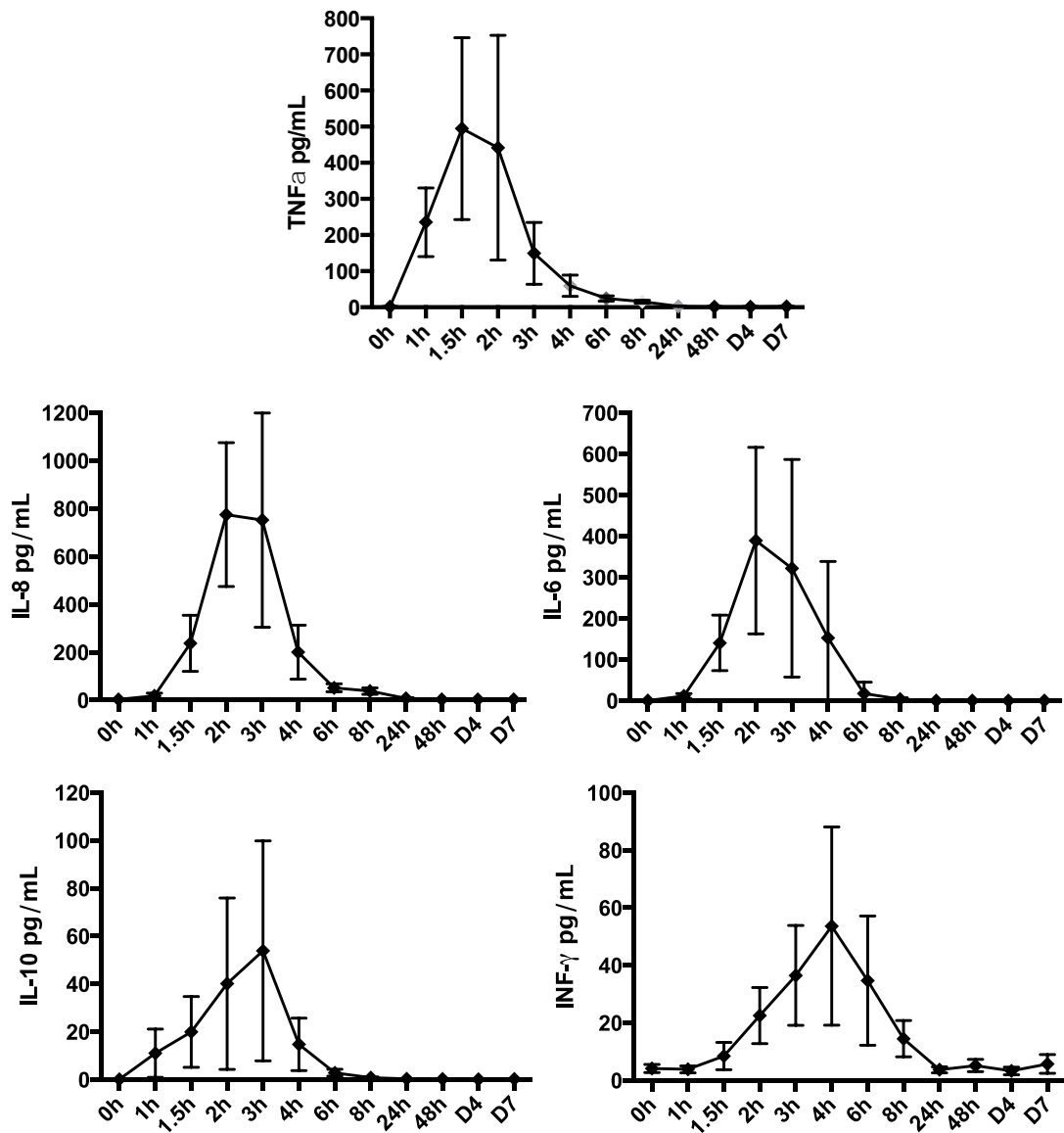


Figure f6.15. Individual plasma cytokine concentration. Plasma was assayed for cytokine concentrations via the MSD V-Plex system. Data-points represent mean and SD of 10 participants (0hr-24hr) and 5 from (48hr-D14).

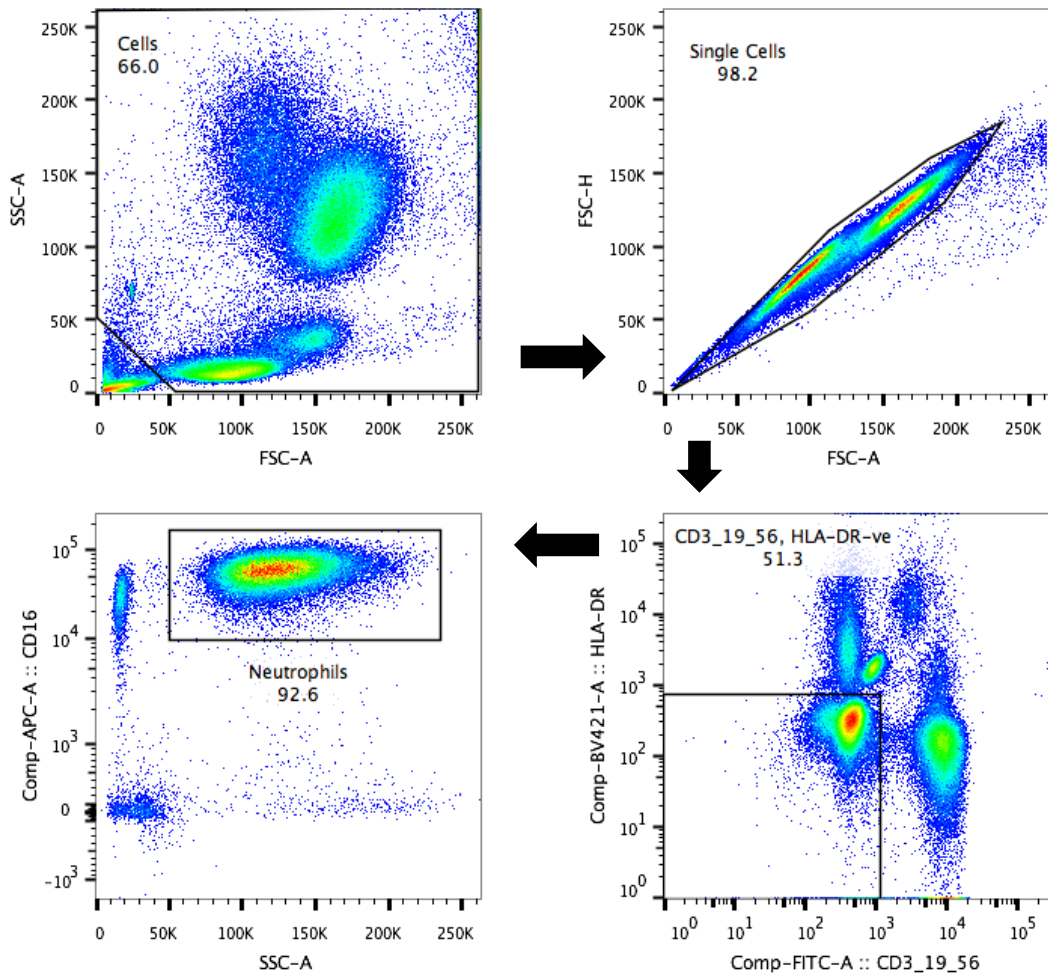


Figure f6.16. Gating strategy to identify neutrophils for surface CD88 (C5a receptor expression). Live cells were first gated using forward and side scatter characteristics (>50k), with subsequent doublet exclusion undertaken via forward scatter height/area. Lymphocyte and natural killer cell lineage marker (CD3, CD19, CD56) and HLA-DR negative cells were further examined for CD16 expression. CD16+ cells with high granularity (side scatter) were identified as neutrophils. Representative pseudocolour dot plots shown (FlowJo, TreeStar). Arrows indicate sequential gating-windows.

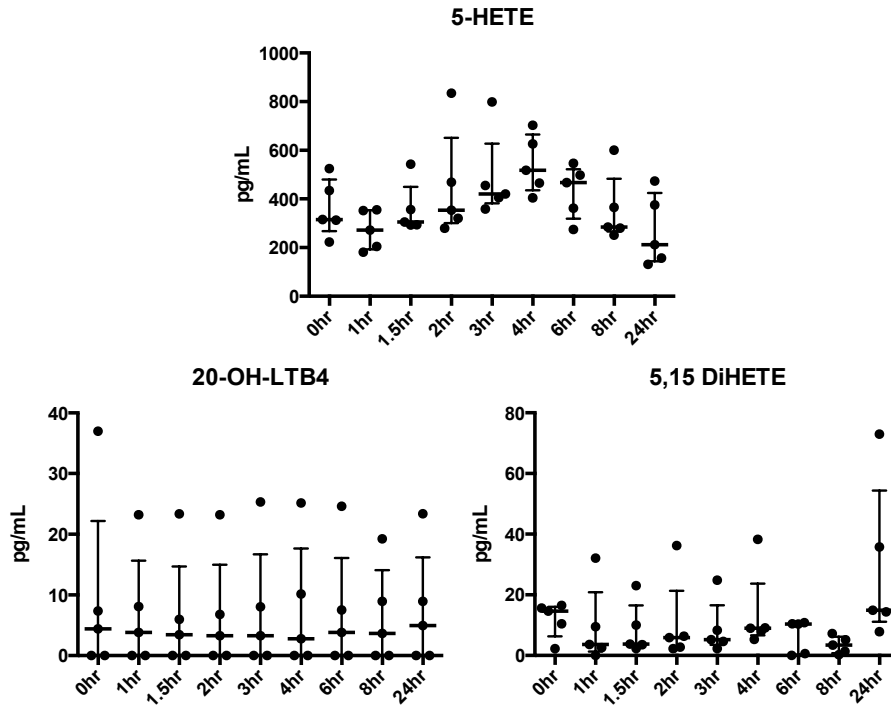


Figure f6.17. IV-endotoxin induced alterations in the concentration of 5-LOX mediated, arachidonic acid derived, lipid mediators. Panels display individual values, median and IQR of 5 participants.

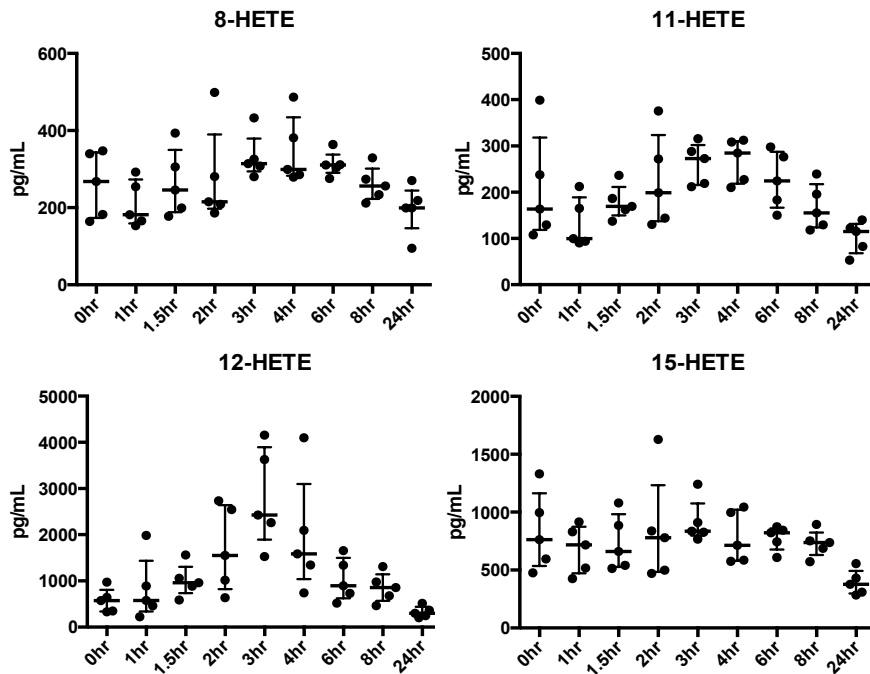


Figure f6.18. IV-endotoxin induced alterations in the concentration of 12-LOX, 15-LOX and NEO-mediated, arachidonic acid derived, lipid mediators. Panels display individual values, median and IQR of 5 participants.

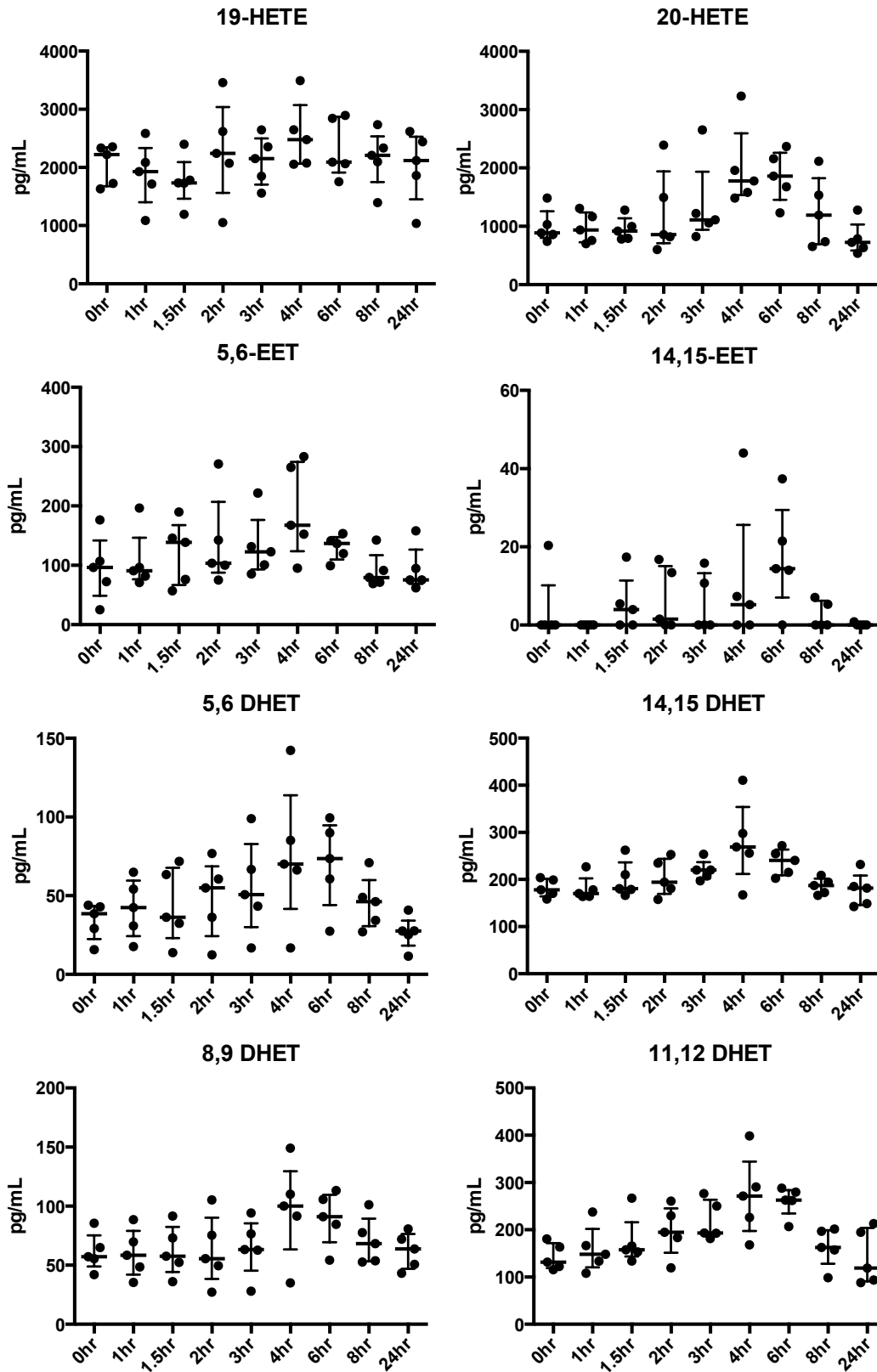


Figure f6.19. IV-endotoxin induced alterations in the concentration of CYP450-mediated, arachidonic acid derived, lipid mediators. Panels display individual values, median and IQR of 5 participants.

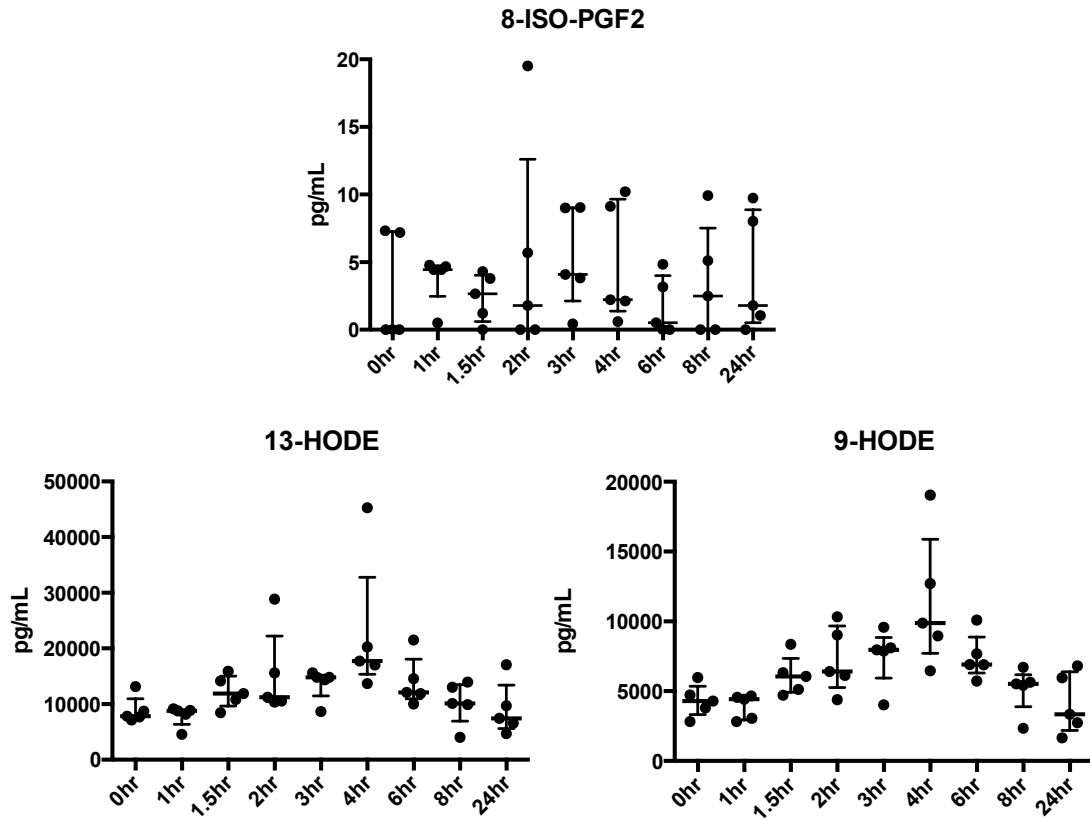


Figure f6.20. IV-endotoxin induced alterations in the concentration of isoprostanes and linoleic acid derived, lipid mediators. The latter may be generated by COX, 15-LOX, CYP450 and NEO. Panels display individual values, median and IQR of 5 participants.

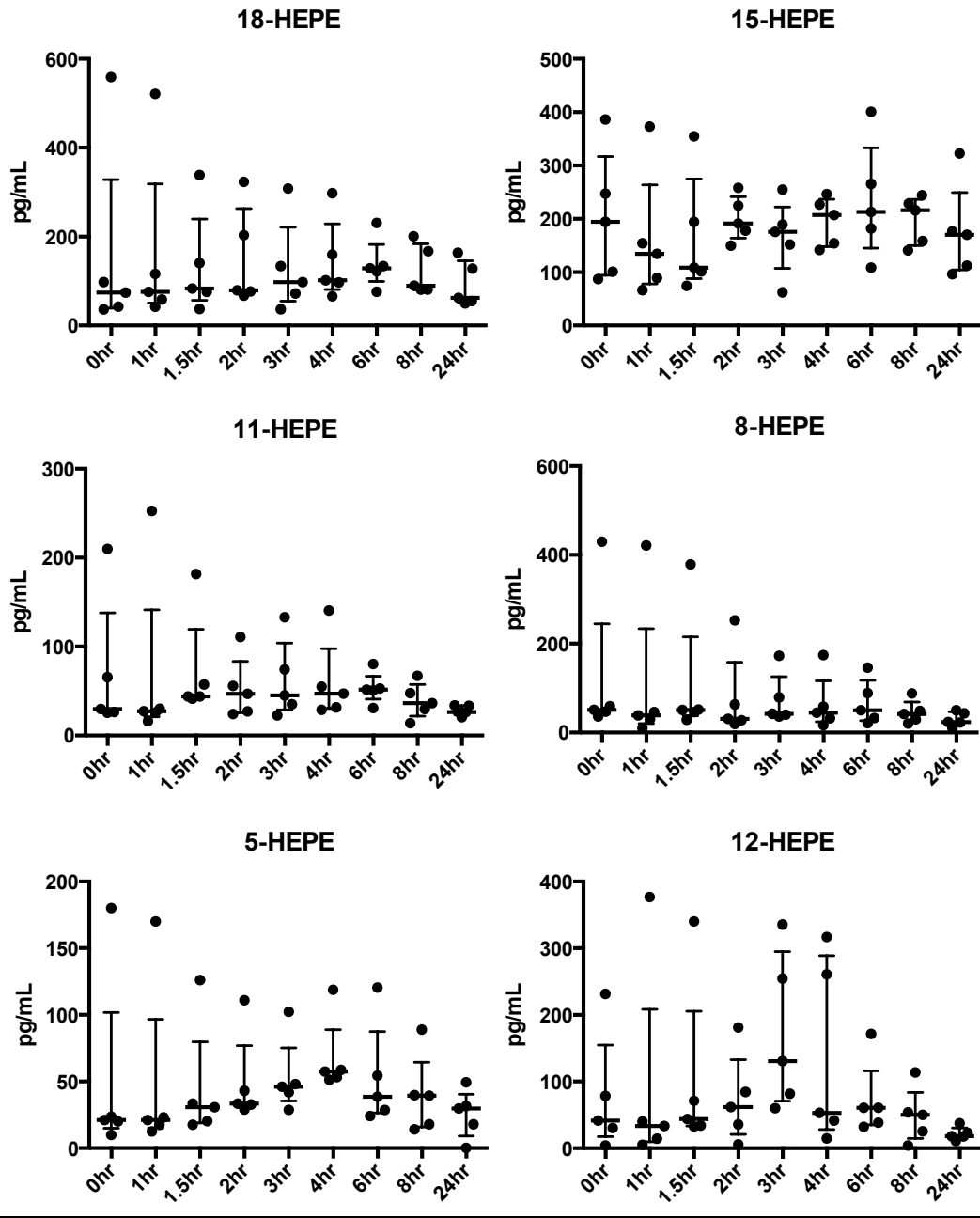


Figure f6.21. IV-endotoxin induced alterations in the concentration of COX, 15-LOX and NEO mediated, EPA derived lipid mediators. Panels display individual values, median and IQR of 5 participants.

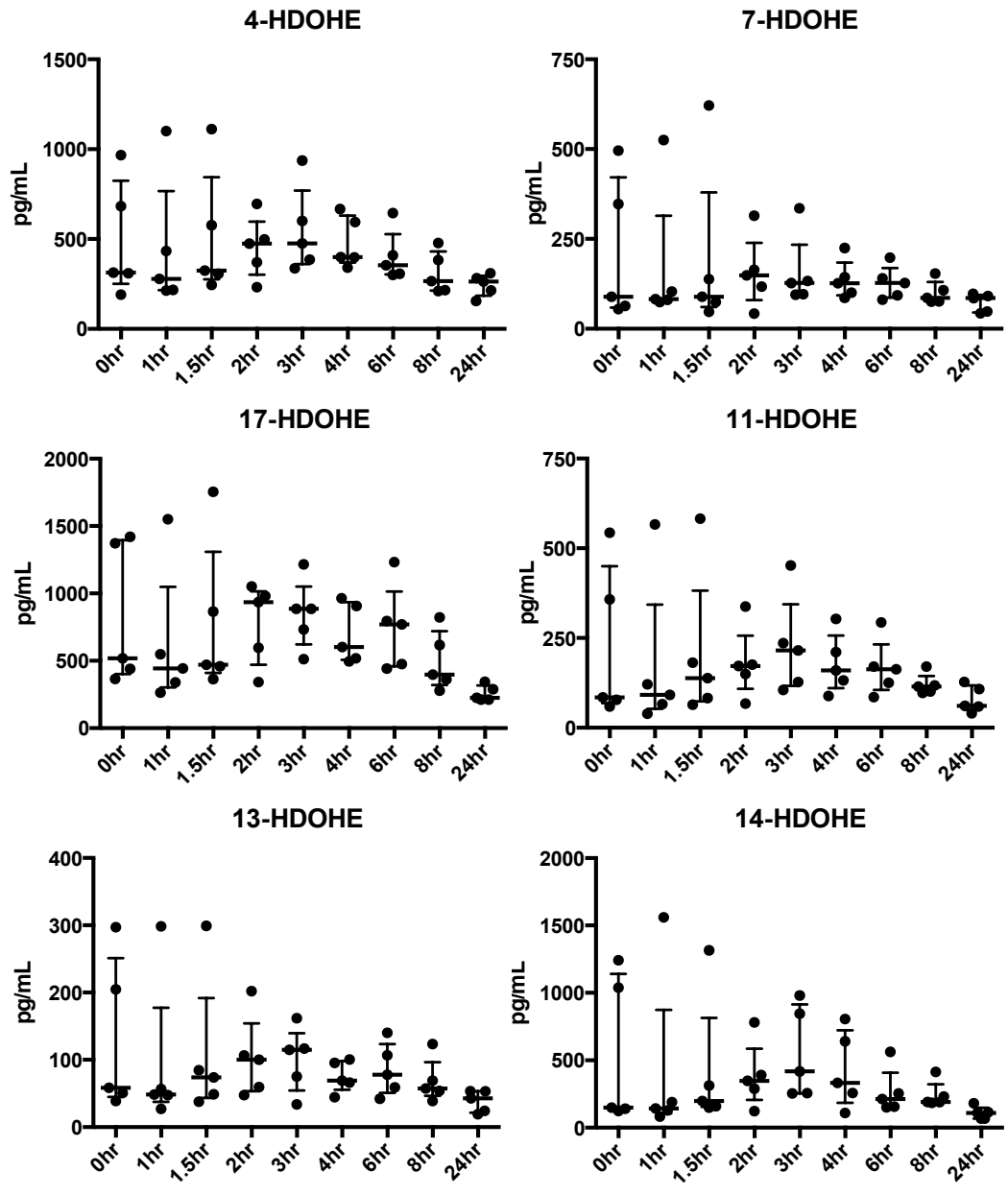


Figure f6.22. IV-endotoxin induced alterations in the concentration of NEO mediated, DHA derived lipid mediators. Panels display individual values, median and IQR of 5 participants.

CHAPTER 7: General Discussion

This thesis has sought to test the hypothesis that PGE₂ mechanistically contributes to CIID via systematic literature review, *in vitro/ex vivo* experiments and an *in vivo* pre-clinical human study. Whilst producing data that supports the assertion, neither acceptance nor rejection of the central premise is possible. There remains a compelling argument that PGE₂, a lipid mediator capable of suppressing multiple aspects of immune effector cell function, plays a pathological role in the context of severe inflammation. Failure to establish this paradigm using the methods employed in this body of work does not constitute failure of the concept, but instead reflects their technical limitations. As Kalinski elegantly states, PGE₂ has the ‘paradoxical status of a pro-inflammatory factor with immunosuppressive activity’³⁰¹. Whilst demonstration of the former is readily achievable, delineating a pathologic contribution from the regulatory capacity of PGE₂ *in vivo* is eminently more challenging.

7.1 A question of degree

Immunosuppression and consequent adverse outcomes is not a universal phenomenon in every CI patient⁹⁹. Instead it appears linked to dysregulation of the inflammatory profile in size and duration in response to an initial severe insult⁴²: mild, resolving inflammation being associated with a qualitatively and quantifiably different cellular and humoral reaction to widespread, non-resolving inflammation, as seen in sepsis⁴⁹⁸. Experimentally in man such a severe inflammatory is impossible to replicate. The closest approximation available - the IV bolus endotoxin model of systemic inflammation (Chapter 6) – whilst transiently replicating many features of CI, phenotypically appears to represent the former self-limited inflammatory response, and can potentially only provide limited insight into the latter^{479,497}. The lack of sustained immune paralysis (at least as determined by *ex vivo* metrics²²⁰) and functional effects of PGE₂ observed in this body of work must be interpreted in this light.

Correspondingly, whilst review of the clinical literature (Chapter 3) provides consistent epidemiological evidence that COX inhibition (and hence PG ablation) may be beneficial following severe infective insults, it appears to afford none in minor infection. This is not unexpected. From a teleological perspective it seems inconceivable that PGE₂ - a mediator which regulates multiple central aspects of the inflammatory response including vasodilation and cellular migration locally⁴⁹⁹⁻⁵⁰¹, and pyrexia centrally⁴⁸⁶ – would be immunopathological under ‘normal’ inflammatory conditions, as routinely elicited by invasive pathogens, and consequently what

benefits NSAIDs would provide in this setting. The same is not true in severe inflammatory states.

In humans there is evidence that PG biosynthesis is related to the size of the inflammatory insult, increasing disproportionately to the initiating stimuli^{210,482}. Larger, persistent challenges may thus be associated with greater prostanoid release from tissue-associated and circulating cells²¹². Increased PGE₂ production may be compounded by local failure of autocrine/paracrine signalled eicosanoid class switching¹⁴⁷, or downregulation of orthodox clearance pathways including 15-hydroxyprostaglandin dehydrogenase (15-HPGD) and carbonyl reductase, both locally and in the lungs^{448,502,503}. Additional biochemical alterations associated with CI may further contribute to PGE₂ becoming pathogenic, in particular reduced serum albumin, a protein that both binds and catabolises PGE₂ under homeostatic conditions to reduce its bioavailability^{142,504,505}. Accentuating these effects are inflammation-induced alterations in E-prostanoid receptor distribution and/or density on key circulating leucocyte populations that render effector cells 'vulnerable' to their PGE₂-rich milieu^{217,506 212}. It is likely that the dynamic interaction of these factors contributing to PGE₂-mediated immune paralysis accounts for the observation that neither all CI patients, nor every individual with an elevated circulating PGE₂ concentration, demonstrates immunosuppression: multiple simultaneous aberrations being required for the effect to be seen clinically^{142,378}.

Mimicry of this combination of effects by IV endotoxemia was either not achieved or highly unlikely. Whilst the chosen dose elicited SIRS near universally (90%) and generated a classic cytokine response in-line with previous authors⁴⁷⁶, peak plasma prostanoid concentrations were orders of magnitude lower than those reported in critical illness⁴³³. A higher dose (4ng/kg) would have elicited greater PGE₂ synthesis⁴⁸², but would have however come at a symptomatic cost⁵⁰⁷. Whether this would have been sufficient to generate sustained immune paralysis is unclear. It is known that IV endotoxemia does not replicate alterations in microvascular leak seen in critical illness states and hence the fluctuations in serum albumin required to 'expose' PGE₂-driven immune paralysis may have failed to occur^{508,509}. Whilst only monocytes were explored we also demonstrated no alteration in EP receptor expression either pharmacologically or via flow cytometry. Alteration in synthetic and catalytic enzyme transcription was not evaluated.

In short, whilst the endotoxin model, as used, provides a key platform on which to explore the (self-resolving) inflammatory response in man, the initiating insult

employed may be insufficiently severe to generate the severity of inflammation required for the development of immunopathology. Detection and elaboration of an excessive PGE₂-mediated regulatory signal in this system was thus impossible.

7.2 *Alternative approaches*

Regardless of the difficulties posed a translational approach should not be neglected however, and variations in the techniques employed may yet yield benefit. As stated, an increased dose of endotoxin might afford greater replication of the immunosuppressive phenotype. Even if not, the discrepancy in PGE₂ release between the two doses (2 and 4ng/kg) may allow relative inference of an excessive regulatory role. A further intriguing proposition would be replication of murine 'two-hit' models of systemic inflammation and subsequent infective challenge in humans¹⁹⁰. Here, IV endotoxemia would be used to elicit the first hit, replicating trauma, burn or sepsis, and a second challenge would be provided by either inhaled⁵¹⁰ or intradermal endotoxin⁵¹¹, mimicking the two most common sites of nosocomial infection. This approach would allow exploration of the relevance of proven sustained *in vivo* immune tolerance and/or dysfunction^{220,512}, and crucially ascertain the impact of inflammation in one compartment (blood) on function in another (pulmonary, skin)⁷⁶. Finally, direct IV infusion of PGE₂ into healthy volunteers has previously been undertaken, and may permit focused investigation of the functional consequences of this molecule and the benefit of associated immunorestorative therapies *in vivo*⁵¹³.

Whilst exogenous PGE₂ was observed to recapitulate the functional deficits observed during critical illness in a validated measure of immune compromise (Chapter 4), this was only achieved at plasma concentrations recorded in sepsis⁴³³. If pre-clinical experimental medicines are wholly unable to replicate the clinical phenotype then patient-derived samples will be required to delineate a role for PGE₂, despite the attendant increase in signal-to-noise ratio. Whilst patients with sepsis are the obvious choice - given previous suggestions of the efficacy of PGE₂ ablative strategies³⁷⁸ – an alternative would be to use a more homogenous, controlled population. An immunosuppressant role for PGE₂ has also been alluded to in patients undergoing emergency major surgery²¹¹. Elective orthopaedic procedures, with their relative uniformity of inflammatory insult, routine multi-dimensional assessment of baseline functional status (to which immunological could be added) and range of clinical outcomes, may offer the ideal patient cohort for this purpose⁵¹⁴. Given the complex range of factors that likely determine the immunosuppressive potency of PGE₂ aside from concentration (albumin, target cell EP receptor expression etc.) it is hoped that,

after further development, the MM6 bioassay will provide a superior read-out of this effect than standard physico-chemical methods alone (Chapter 5).

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Appendices: UCL Research Ethics Council Applications

Reference: 5060_001 – Intravenous endotoxemia

Reference: 4332_001 – Stimulated human whole blood

IMPORTANT: ALL FIELDS MUST BE COMPLETED. THE FORM SHOULD BE COMPLETED IN PLAIN ENGLISH UNDERSTANDABLE TO LAY COMMITTEE MEMBERS.

SEE NOTES IN STATUS BAR FOR ADVICE ON COMPLETING EACH FIELD. YOU SHOULD READ THE ETHICS APPLICATION GUIDELINES AND HAVE THEM AVAILABLE AS YOU COMPLETE THIS FORM.

APPLICATION FORM

SECTION A APPLICATION DETAILS

A1	Project Title: The effect of inflammation on local and systemic immune function	
	Date of Submission: 23/09/2013	Proposed Start Date: 01/12/2013
	UCL Ethics Project ID Number: 5060/001	Proposed End Date: 31/05/2015
	If this is an application for classroom research as distinct from independent study courses, please provide the following additional details:	
	Course Title: N/A	Course Number: N/A

A2	Principal Researcher	
	<i>Please note that a student – undergraduate, postgraduate or research postgraduate cannot be the Principal Researcher for Ethics purposes.</i>	
	Full Name: Professor Derek. W Gilroy	Position Held: Professor of Experimental Immunology and Wellcome Trust Senior Research Fellow.
	Address: Centre for Clinical Pharmacology, Division of Medicine Rayne Building 5 University Street LONDON WC1E6JF	Email: [REDACTED]
		[REDACTED]
		[REDACTED]
	Declaration To be Signed by the Principal Researcher	
	<ul style="list-style-type: none"> ▪ I have met with and advised the student on the ethical aspects of this project design (<i>applicable only if the Principal Researcher is not also the Applicant</i>). ▪ I understand that it is a UCL requirement for both students & staff researchers to undergo Disclosure and Barring Service (DBS) Checks when working in controlled or regulated activity with children, young people or vulnerable adults. The required DBS Check Disclosure Number(s) is: N/A ▪ I have obtained approval from the UCL Data Protection Officer stating that the research project is compliant with the Data Protection Act 1998. My Data Protection Registration Number is: Z6364106/2013/09/08 ▪ I am satisfied that the research complies with current professional, departmental and university guidelines including UCL's Risk Assessment Procedures and insurance arrangements. ▪ I undertake to complete and submit the 'Continuing Review Approval Form' on an annual basis to the UCL Research Ethics Committee. ▪ I will ensure that changes in approved research protocols are reported promptly and are not initiated without approval by the UCL Research Ethics Committee, except when necessary to eliminate apparent immediate hazards to the participant. ▪ I will ensure that all adverse or unforeseen problems arising from the research project are reported in a timely fashion to the UCL Research Ethics Committee. ▪ I will undertake to provide notification when the study is complete and if it fails to start or is abandoned. 	

SIGNATURE:

DATE:

A3	Applicant(s) Details (if Applicant is not the Principal Researcher e.g. student details):	
	Full Name: Dr James Fullerton	
	Position Held: Wellcome Trust Research Training Fellow (PhD Student)	
	Address: Clinical Pharmacology Rayne Building 5 University Street WC1E 6JF	Email: [REDACTED]
		[REDACTED]
		[REDACTED]
	Full Name:	
	Position Held:	
	Address:	Email:
		Telephone:
		Fax:

A4	<p>Sponsor/ Other Organisations Involved and Funding</p> <p>a) Sponsor: <input checked="" type="checkbox"/> UCL <input type="checkbox"/> Other institution If your project is sponsored by an institution other than UCL please provide details:</p> <p>b) Other Organisations: If your study involves another organisation, please provide details. <i>Evidence that the relevant authority has given permission should be attached or confirmation provided that this will be available upon request.</i> University College London Hospital (via use of the Clinical Research Facility). R&D approval has been sought via the Joint Research Office and is available upon request.</p> <p>c) Funding: What are the sources of funding for this study and will the study result in financial payment or payment in kind to the department or College? <i>If study is funded solely by UCL this should be stated, the section should not be left blank.</i> Wellcome Trust Research Training Fellowship and Pfizer 'Anti-infectives Specialist Programme for Young Researchers' (ASPYRe Award, both awarded to Dr James Fullerton. The latter is a one-off stand-alone award to support investigator-initiated research. It is provided with no restrictions, requirements or obligations (other than interim and final reports, and due acknowledgement on scientific output). No Pfizer products will be employed in the study.</p>
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A5	<p>Signature of Head of Department or Chair of the Departmental Ethics Committee (This must not be the same signature as the Principal Researcher)</p> <p>I have discussed this project with the principal researcher who is suitably qualified to carry out this research and I approve it. The project is registered with the UCL Data Protection Officer, a formal signed risk assessment form has been completed, and appropriate insurance arrangements are in place. <i>Links to details of UCL's policies on data protection, risk assessment, and insurance arrangements can be found at: http://ethics.grad.ucl.ac.uk/procedures.php</i></p> <p>UCL is required by law to ensure that researchers undergo a Disclosure and Barring Service (DBS) Check if their research project puts them in a position of trust with children under 18 or vulnerable adults.</p> <p>*HEAD OF DEPARTMENT TO DELETE BELOW AS APPLICABLE*</p> <p>I am satisfied that checks: (1) have been satisfactorily completed (2) have been initiated (3) are not required</p> <p>If checks are not required please clarify why below.</p>
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Chair's Action Recommended: Yes No

A recommendation for Chair's action can be based only on the criteria of minimal risk as defined in the Terms of Reference of the UCL Research Ethics Committee.

PRINT NAME: Prof Raymond MacAllister

SIGNATURE:

DATE: 23/09/13

SECTION B

DETAILS OF THE PROJECT

B1

Please provide a brief summary of the project in simple prose outlining the intended value of the project, giving necessary scientific background (*max 500 words*).

Nearly 30% of intensive care (ICU) patients develop at least one hospital-acquired infection (HAI), a burden 6-times greater than that on standard wards. HAI in this setting confers a 2-3 fold increased risk of in-patient mortality and is estimated to cost the UK up to £1billion/year. In particular critically ill patients are vulnerable to secondary infection with less virulent, multi-drug resistant, opportunistic organisms and to re-activation of latent pathogens. Aside from known iatrogenic (medically caused) and physical risk factors (e.g. sedation, in-dwelling lines), a failure of the body's defence system is now recognized as the key causative factor - a phenomenon that may be described as critical illness-induced immune dysfunction (CIID).

Inflammation is an evolutionarily ancient, highly conserved process, designed to protect us against foreign bodies or agents. Triggered by a multiple different events such as infection (by bacteria, viruses etc.), tissue injury (trauma, burns, major surgery) or chemicals (e.g. alcohol causing pancreatitis) it is a feature of nearly all critical illness. Recent data has found that patients on ICU who have adverse outcomes (HAI, prolonged hospitalization, in-patient death) experience a dysregulated inflammatory response both in magnitude and duration. Predominance and prolongation of regulatory/anti-inflammatory processes and mediators (signaling chemicals) appear to contribute directly to CIID, however the relative contribution and time-course of different molecules is not known. In work already performed by our group looking at the blood of patients with pneumonia, we have identified both an excess of one inflammation regulating chemical - prostaglandin E2 - and an absence of another - aspirin-triggered lipoxin - as potential key drivers of immunosuppression and HAI susceptibility.

The proposed project seeks to establish, characterize and interrogate, for the first time, a robust clinically-relevant 'two-hit' human healthy volunteer model of systemic inflammation (mirroring sepsis, trauma, burn injury or major surgery) coupled with a biologically valid secondary infective challenge (mimicking HAI). We believe this model will allow us to compile a compendium of information that will provide the most complete description of CIID to date. In particular it will uniquely allow exploration of inflammation-induced alterations in defence function over time and in different body compartments - the blood and skin.

Current systems for exploring CIID are inadequate, relying on increasingly repudiated animal models or observational human data. In contrast this model will provide a controlled, safe, adaptable platform upon which to accurately dissect the cause of CIID in humans. The study aims to identify viable pharmacological opportunities that both reduce susceptibility to HAI in the ICU population and work synergistically with antibiotics to promote the clearance of infection. Additionally, biomarkers potentially predictive of HAI risk (reflecting immune impairment) may be identified. Sub-classification of the critically ill population using measures of immune function to monitor disease progress and individualize treatment regimens, dictating what drugs should be given and when, have recently been shown to be effective. We propose, the therapeutic challenge of two immunosuppressive pathways in humans using commonly used drugs to mechanistically test the contribution of prostaglandin E2 and aspirin-triggered lipoxin.

B2

Briefly characterise in simple prose the research protocol, type of procedure and/or research methodology (e.g. observational, survey research, experimental). Give details of any samples or measurements to be taken (max 500 words).

2 studies are proposed. Both involve the same participant population, methodology, procedures and measurements. The first will examine the nature and time-course of inflammation-induced immune (defence) dysfunction, the second will employ two drugs (ibuprofen and aspirin) to mechanistically explore the contribution of two inflammation-regulating chemicals (prostaglandin E2 and aspirin-triggered lipoxin) to observed defects. Participants will be randomly allocated to two groups of pre-determined size in both studies, reflecting time-point of secondary immune measurement (Study 1) or drug allocation (Study 2). The model will combine two previously employed safe, validated, standardised techniques (endotoxemia and skin-window formation) to achieve our objectives.

Healthy male volunteers aged 18 to 50 will be recruited. Enrollment will involve undergoing a medical screening assessment to ensure participant safety. Exclusion criteria include acute or chronic illness, regular prescribed medication use, abnormal physical examination, electrocardiogram or blood results, drug or alcohol misuse, and recent vaccination or transfusion within 3months. Each participant may be enrolled in one of the studies only once. All phases of the study will be conducted in the Clinical Research Facility (CRF), UCL/UCLH. Completion of the full protocol will involve 7 visits to the CRF and a total 20hours of participant's time over 3weeks.

Immune function will be assessed before (baseline) and after (experimental) systemic inflammation (repeated-measures design). Samples will be taken from two body compartments: blood (systemic) and skin (local). In Study 1 local immune function will be assessed either on day 3 or day 7 post-inflammation (determining timecourse). In Study 2 this will occur on day 3 however participants will have consumed either aspirin or ibuprofen during the intervening period (determining mediator effect). Systemic assessment will be constant throughout (blood sampling on day 1, 3, 7, 14 post-inflammation)

Intravenous injection of endotoxin (National Institute of Health, E.coli O:113 EC-6 2ng/kg), a sterile bacterial product, will be used to induce inflammation. Participants will be monitored (standard observation set) by a doctor for 10 hours post-injection, blood being drawn from an in-dwelling venous line (to avoid repeated stab) every 1-2 hours (re: systemic immune assessment). A self-assessed symptom score will be undertaken bi-hourly. A fluid drip will be given to counter physiological changes.

Local immune assessment will be a (gram-positive) sterile bacterial challenge to suction-blister induced 'skin windows' (streptokinase and heat-killed S.aureus labeled with a dye, replicating peripheral venous access or wound infection). Blisters will be formed using pre-established protocols using aseptic technique. A specially developed 'exudate-collecting chamber' (ECC, see PIL) will hold participant-derived (autologous) serum, containing the reagents, over the 'windows'. Samples will be aspirated at 2 time-points within 12 hours, to assess the kinetics of the immune response. Participants will be ambulant in-between.

Both blood and local samples will contain two key elements. Plasma/exudate (acellular) will be used to test concentrations of chemicals/mediators. Cellular material will be employed to look at changes in gene expression caused by inflammation. Crucially the ECC will allow functional analysis of the inflammatory and immune response in a biologically valid, 'real-life' setting (in-vivo).

Attach any questionnaires, psychological tests, etc. (a standardised questionnaire does not need to be attached, but please provide the name and details of the questionnaire together with a published reference to its prior usage).

B3

Where will the study take place (please provide name of institution/department)?

If the study is to be carried out overseas, what steps have been taken to secure research and ethical permission in the study country? Is the research compliant with Data Protection legislation in the country concerned or is it compliant with the UK Data Protection Act 1998?

University College London (UCL)/University College London Hospital (UCLH) Clinical Research Facility (CRF)

B4	<p>Have collaborating departments whose resources will be needed been informed and agreed to participate? <i>Attach any relevant correspondence.</i></p> <p>N/A</p>
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B5	<p>How will the results be disseminated, including communication of results with research participants?</p> <p>Participants will be invited to return 2 days after the screening visit to be informed of the results of their medical screening. Any abnormal examination or test results will prompt exclusion from the study. If none are present enrollment will be confirmed and baseline immune assessment performed. Abnormalities will also, with the participant's permission, be communicated to their general practitioner to determine the need for further investigation/medical intervention.</p> <p>Results will be disseminated via traditional scientific channels (peer-reviewed journal publications, conference presentation) and described on the Gilroy Group's open-access website. Participants will be offered the opportunity to receive copies of research output via email.</p>
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B6	<p>Please outline any ethical issues that might arise from the proposed study and how they are be addressed. <i>Please note that all research projects have some ethical considerations so do not leave this section blank.</i></p> <p>Scientific importance: This proposed study provides a significant advance on current research methodology providing a construct upon which the cellular and signaling processes underlying CIID may be explored and potential therapeutic interventions identified and tested. The proposal has been independently assessed and approved by the Wellcome Trust's Research Training Fellowship Committee and Pfizer's Anti-Infective Scientific Advisory Committee.</p> <p>Recruitment, consent and payment: Participants will be recruited by generic, un-selected internal UCL email. The Chief Investigator (CI) will assess capacity prior to recruitment and no vulnerable adults will be used in the study. Potential participants will be provided with a PIL at least 3 days in advance of the screening visit providing time to reflect on the protocol and desire to participate. The opportunity to ask questions/seek clarification will be provided. Written consent will be taken at the screening visit after verbal confirmation of understanding, and re-affirmed prior to each interventional procedure, participants being free to withdraw without penalty at any time.</p> <p>Organization and Management: All procedures will be performed at the University College London Hospital (UCLH)/University College London (UCL) National Institute of Health Research (NIHR) Clinical Research Facility (CRF), a 20-bed facility situated in a wing of UCLH. This facility represents a purpose-built environment for investigators to conduct pre-clinical research studies as safely, effectively and efficiently as possible. Specialized staff (nursing, pharmacy) for medication/treatment administration and equipment required to monitor/evaluate their effects (blood pressure, oxygen saturations etc.) are present along with dedicated laboratory facilities for sample processing. UCLH's A&E, acute medical, surgical and intensive care facilities provide clinical support in the event of adverse reaction or un-expected events during the protocol.</p> <p>Effect on Participants: The protocol involves undertaking procedures on healthy volunteers, affords them no health benefit and may potentially cause harm. The steps taken to minimize this are detailed in D4. The techniques/procedures involved are well established with known safety profiles, the novelty of the research residing in their combination.</p> <p>Sample Storage and Data Protection: Participant data will be quasi-anonymised with allocation and use of a study number throughout. Only the CI and PI will have access to this code. No personal details will be retained aside from age, confirmation of normal health status (results from screening visit) and contact details (email address). All information will be recorded in a password-protected UCL-database. Only the research team and regulatory bodies (UCL R&D, UCL Research Ethics Committee) will have access to this database. Paper records will be kept in locked cabinets in the Rayne Institute, UCL. On completion of the study information linking participants to samples will be destroyed.</p> <p>All samples will be labeled with the study number and stored for a maximum of 10 years in locked freezers</p>
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within the Rayne Institute, UCL (or suitable location with a Human Tissue Act License). Participants will be made fully aware of the tests that will be performed on their samples, including the retention of cellular material and that the results of the study are intended for public display as posters, presentation, and/or as a journal article. These will contain no identifiable information. Any future research on stored samples will require Research Ethics Committee approval.

Funding and Conflict of Interest: All researchers taking part on the study can confirm they have no conflicts of interest.

SECTION C

DETAILS OF PARTICIPANTS

C1

Participants to be studied

C1a. Number of volunteers:	35
Upper age limit:	50
Lower age limit:	18

C1b. Please justify the age range and sample size:

Calculations independently performed by Mr Paul Bassett (Statistician, UCL Joint Research Office)

Overall: Study 1: 12 participants are required. Study 2: 16 participants are required

With an estimated 'drop-out'/non-completion rate of 15-20% it is estimated that 15 and 20 participants will have to be recruited to each study respectively - 35 in total.

Study 1 (Primary end-point): The sample size was calculated on the primary outcome measure, alteration in ex-vivo whole blood stimulated TNF- α production and selected to obtain a precise estimate of TNF- α concentration at day 3 (first group) or day 7 (second group). TNF- α will be expressed as the percentage reduction from baseline, with levels at day 3 estimated to be 50% of those at baseline. Based on previous published data[20] and preliminary work in the Gilroy group, the standard deviation of the percentage reduction is estimated to be 15%. In order to obtain an estimate of the mean percentage reduction that is correct to within 12% of the true population value using a 95% confidence level, it is calculated that 6 subjects are required. As each group will be considered separately a total of 12 subjects are needed. It is anticipated that not all participants enrolled will complete the full study protocol (drop-out/excluded). Recruitment will continue until target numbers are reached. With an estimated 15-20% drop-out/exclusion rate a maximum of 15 participants will need to be enrolled. The 12% cut-off was selected as sufficient to achieve a scientifically meaningful answer to the question whilst not involving unnecessary recruitment. Alternative cut-offs and numbers of subjects required to achieve these are listed at the bottom of this section.

Study 2 (Secondary end-point): The sample size was based on a comparison in the percentage increase in whole blood stimulated ex-vivo TNF- α production from inflammation-induced nadir towards baseline in patients plasma treated or not with cyclooxygenase inhibitor/prostaglandin antagonists. Based on previous studies[20] and group data, the standard deviation of the percentage reduction is estimated to be 15%. A difference in the percentage reduction between groups of 25% would be considered to be a clinically important difference. Using a 5% significance level and 90% power, it is calculated that 8 subjects per group, 16 in total, are required for the study. As in study 1 if a 15-20% drop-out/exclusion rate is allowed for it is expected that a maximum number of 20 participants will have to be enrolled to achieve this target.

Addendum: Alternative Study 1 'cut-off' values: 20% in each direction of the true value would need 2 subjects; 18% in each direction of the true value would need 3 subjects; 16% in each direction of the true value would need 4 subjects; 14% in each direction of the true value would need 5 subjects; 12% in each direction of the true value would need 6 subjects; 10% in each direction of the true value would need 9 subjects; 8% in each direction of the true value would need 14 subjects; 6% in each direction of the true value would need 24 subjects

C2	<p>If you are using data or information held by a third party, please explain how you will obtain this. You should confirm that the information has been obtained in accordance with the UK Data Protection Act 1998.</p> <p>N/A</p>
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C3	<p>Will the research include children or vulnerable adults such as individuals with mental health problems or with learning disabilities, the elderly, prisoners or young offenders? <input type="checkbox"/> Yes <input checked="" type="checkbox"/> No</p> <p>How will you ensure that participants in these groups are competent to give consent to take part in this study? <i>If you have relevant correspondence, please attach it.</i></p>
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C4	<p>Will payment or any other incentive, such as gift service or free services, be made to any research participant?</p> <p><input checked="" type="checkbox"/> Yes <input type="checkbox"/> No</p> <p>If yes, please specify the level of payment to be made and/or the source of the funds/gift/free service to be used.</p> <p>Max £200/participant (see below). Funding is derived from the Dr James Fullerton's Wellcome Trust Research Training Fellowship.</p> <p>Please justify the payment/other incentive you intend to offer.</p> <p>£200 will be allocated to each volunteer for the study, payable upon completion of all procedures (final visit, day 14 post-endotoxemia). This is considered suitable to compensate for the mild discomfort associated with interventional procedures, travel expenses, and the inconvenience of lifestyle modification. The study is expected to take around 20 hours of participants time and reflects a rate of £10/hour. Partial completion will lead to incremental payment so as not to compel/coerce participants to complete all aspects of the protocol should they wish to withdraw. £50 will be paid for baseline immune assessment, £100 for undergoing endotoxin injection and monitoring.</p>
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C5	<p>Recruitment</p> <p>(i) Describe how potential participants will be identified:</p> <p>Self-identification. An advertising email (attached) will be sent to UCL staff and students describing the study in brief and stating eligibility criteria.</p> <p>(ii) Describe how potential participants will be approached:</p> <p>Contact details of study investigators are included in the advertising email and will be used by prospective recruits to contact the investigators if they consider themselves eligible and are interested in participating.</p> <p>(iii) Describe how participants will be recruited:</p> <p>Once contact with the investigators is made and initial eligibility ascertained, a Participant Information Leaflet will be distributed and the opportunity to discuss the study in detail (phone, email and/or meeting) provided (min period 3 days) before consent is sought. All aspects of the study including design, timelines, potential side effects, required commitment, and the right not to participate and to withdraw at any time without penalty will be fully explained.</p>
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Attach recruitment emails/adverts/webpages. A data protection disclaimer should be included in the text of such literature.

C6	<p>Will the participants participate on a fully voluntary basis? <input checked="" type="checkbox"/> Yes <input type="checkbox"/> No</p> <p>Will UCL students be involved as participants in the research project? <input checked="" type="checkbox"/> Yes <input type="checkbox"/> No</p> <p><i>If yes, care must be taken to ensure that they are recruited in such a way that they do not feel any obligation to a teacher or member of staff to participate.</i></p> <p>Please state how you will bring to the attention of the participants their right to withdraw from the study without penalty?</p> <p>The right to withdraw will be emphasised verbally in advance of, and during, the consenting process, as well as in writing. A copy of the signed consent form will be provided to participants. The right to withdraw is also explicitly stated in written form in the Participant Information Leaflet.</p>
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C7	<p>CONSENT</p> <p>Please describe the process you will use when seeking and obtaining consent.</p> <p>Potential participants who reply to the email advertisement will have their initial eligibility confirmed. If eligible they will be provided with a Participant Information Leaflet and given a minimum period of 3 days to decide whether they wish to enrol. During this period they will be free to contact the investigators to clarify any elements of the study. Timelines, visits to UCL, requirement of the participant to comply with the study protocol, as well as potential for harm will be discussed. Voluntary entry and withdrawal without penalty will be reaffirmed. If happy to proceed consent will be taken by the applicant prior to any study intervention, understanding being confirmed verbally prior to written consent being sought.</p> <p><i>A copy of the participant information sheet and consent form must be attached to this application. For your convenience proformas are provided in C10 below. These should be filled in and modified as necessary.</i></p> <p>In cases where it is not proposed to obtain the participants informed consent, please explain why below.</p> <p>N/A</p>
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C8	<p>Will any form of deception be used that raises ethical issues? If so, please explain.</p> <p>No</p>
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C9	<p>Will you provide a full debriefing at the end of the data collection phase? <input type="checkbox"/> Yes <input checked="" type="checkbox"/> No</p> <p>If 'No', please explain why below.</p> <p>Participants will be offered the opportunity to have the final scientific output forwarded to them along with a lay summary. Initial data is not likely to be of interest to most of our volunteers in this study.</p>
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C10	<p>Information Sheets And Consent Forms</p> <p>A poorly written Information Sheet(s) and Consent Form(s) that lack clarity and simplicity frequently delay ethics approval of research projects. The wording and content of the Information Sheet and Consent Form must be appropriate to the age and educational level of the research participants and clearly state in simple non-technical language what the participant is agreeing to. Use the active voice e.g. "we will book" rather than "bookings will be made". Refer to participants as "you" and yourself as "I" or "we". An appropriate translation of the Forms should be provided where the first language of the participants is not English. If you have different participant groups you should provide Information Sheets and Consent Forms as appropriate (e.g. one for children and one for parents/guardians) using the templates below. Where children are of a reading age, a written Information Sheet should be provided. When participants cannot read or the use of forms would be inappropriate, a description of the verbal information to be provided should be given. Please ensure that you trial the forms on an age-appropriate person before you submit your application.</p>
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Both the Participant Information Leaflet and Consent Forms are provided as separate documents

SECTION D DETAILS OF RISKS AND BENEFITS TO THE RESEARCHER AND THE RESEARCHED

D1	<p>Have UCL's Risk Assessment Procedures been followed? <input checked="" type="checkbox"/> Yes <input type="checkbox"/> No</p> <p>If No, please explain.</p>
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D2	<p>Does UCL's insurer need to be notified about your project before insurance cover can be provided? <input type="checkbox"/> Yes <input checked="" type="checkbox"/> No</p> <p><i>The insurance for all UCL studies is provided by a commercial insurer. For the majority of studies the cover is automatic. However, for a minority of studies, in certain categories, the insurer requires prior notification of the project before cover can be provided.</i></p> <p>If Yes, please provide confirmation that the appropriate insurance cover has been agreed. <i>Please attach your UCL insurance registration form and any related correspondence.</i></p>
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D3 Please state briefly any precautions being taken to protect the health and safety of researchers and others associated with the project (as distinct from the research participants).

Interventional procedures (blister formation, venepuncture, venous cannula insertion) will only be performed by appropriately trained, clinically qualified and experienced investigators. Investigators have gained experience using the blister equipment in Prof. Arne Akbar's Lab (Division of Infection and Immunity).

All study procedures will take place in the Clinical Research Facility, a purpose built and designed facility. Investigators performing tasks which risk exposure to bodily fluids will have appropriate immunization records. UCL Occupational Health will provide support in the un-likely event of needle-stick etc. There is also rapid access to the accident and emergency department at University College London Hospital if a health problem becomes manifest during the study

D4 Will these participants participate in any activities that may be potentially stressful or harmful in connection with this research? Yes No

If **Yes**, please describe the nature of the risk or stress and how you will minimise and monitor it.

Medical Screening: Medical screening may detect previously un-diagnosed conditions. It may also flag up false positives (where the test indicates an abnormality however there is no underlying medical problem), causing un-necessary concern. To maximize safety and minimize the risk of false-positives only tests felt to directly impact on risk of undergoing the study will be performed. Any abnormalities discovered will either be discussed directly with the subjects General Practitioner after gaining consent, or provided to the participant so they may discuss them with their GP. The GP will determine the need for further investigation or medical care not a member of the investigative team.

Interventional Procedures

1) Skin window formation: Blister formation requires participants to remain relatively still for around 1-1.5hours and is associated with a mild tingling/tugging sensation but is not painful. Subjects are free to read, listen to music etc. during this procedure and will have an investigator with them throughout, monitoring the process. De-roofing is associated with mild discomfort. There is a very small risk of infection. This will be minimized by meticulous attention to aseptic technique throughout including skin cleansing with 70% isopropyl alcohol solution and the use of sterile, one-use razors to remove any hairs. The investigators have considerable experience in performing this process.

Blister formation can lead to temporary skin marking which usually clears after 4-6 weeks, but may persist for longer on darker skin. Unusually it may take several months to clear. Participants will be provided with images of the blister, skin windows and their healing over time in the Participant Information Leaflet to facilitate informed choice on their desire to undergo this procedure. Skin care advice will be offered.

2) Exudate Collecting Chamber (ECC): The ECC and associated accessories have been specifically designed and manufactured for this project. The ECC consists of a single-use silicone structure cast from a 3D-printed mould, which matches the formed skin-windows and is secured to the forearm via a 'bezel' and strap (see images in the Participant Information Leaflet). The silicone employed is NuSil MED 6015, supplied by Polymer Systems Technology Ltd. (UK), an optically clear elastomer that has been approved for medical use, including implantation. Dr Richard Day (Senior Lecturer, UCL Applied Biomedical Engineering Group) is responsible for manufacture. A transparent silicone has been employed so that any signs of spreading erythema (potentially signifying infection and/or adverse reaction) may be observed. The ECC will be sterilized prior to application and participants asked to report any adverse symptoms to the investigators whilst wearing the device. The investigative team will be contactable at all times throughout this procedure. The ECC strap exerts a pressure similar to that of a watch strap and the whole device will be protected by a comfortable dressing.

Autologous serum will be employed in the wells of the ECC. Subjects will be exposed to streptokinase twice as a gram-positive stimulant. The risk of sensitization is felt to be very low due to dose and site (200units in contact with dermal capillaries). Previous severe reactions have been associated with use of streptokinase as a thrombolytic (dose 1.5million units, intravenous).

3) Endotoxaemia: Intravenous (IV) injection of endotoxin is a safe, accepted and well-characterized model, which has previously been performed at UCL. The endotoxin used will be supplied by the National Institute of Health, USA (EC-6, E.Coli O:113) where its use, dose and safety profile has been validated and verified. EC-6 is manufactured in accordance with GMP procedures. It will be stored and re-constituted by trained Investigational Pharmacists in the Clinical Research Facility, and the dose adjusted to participant weight (2ng/kg) to ensure a consistent, equivalent response between participants.

ET at the dose specified elicits flu-like symptoms including high temperature, shivering/chills, headache, back pain, nausea and muscle ache that resolve within 4-6 hours. Physiological changes include increased heart rate and core temperature (1-4C). Blood pressure (BP) may fall but infrequently to clinically significant levels. Features of systemic inflammation generally subside by 6-8 hours. The ET itself is undetectable in the blood within 30-60minutes.

Safety will be ensured by several measures: Prior medical screening; Location: the UCL/UCLH Clinical Research Facility (CRF) is specifically designed to conduct such pre-clinical research with appropriate monitoring and resuscitation facilities. The CRF is attached to UCLH, a large teaching-hospital; Staff: a medically qualified investigator will be present throughout, supported by research nurses as required; Dose of Endotoxin: 2ng/kg is at the lower end of the range known to elicit systemic inflammation, and half the upper-limit; Monitoring: standard clinical monitoring will be employed (as stated) to ensure no significant physiological deterioration takes place; Fluids: Intravenous fluids (a drip) will be given over the first 6 hours post-endotoxin injection to counter-act endotoxin-induced physiological alterations; Time: 10 hours of monitoring will be performed in the CRF with subjects asked not to leave the building. This exceeds the expected duration of endotoxin-induced physiological effects. If at the end of this monitoring period clinical observations remain deranged this will be extended. In the highly un-likely event of significant physiological disturbance independent clinical assessment at UCLH will be sought. All participants will have 24-hour telephone access to the CI if they have concerns or become un-well post-procedure.

4) Blood Sampling: Venous blood samples will be taken throughout the study as detailed in the protocol. In total, venepuncture will take place 8 times. An experienced practitioner maintaining aseptic technique throughout will minimize the risk of infection and/or bruising. During endotoxaemia a venous cannula will be placed in the anti-cubital fossa (front of elbow) and blood drawn from this to avoid repeated stabs. All participants will be made fully aware of the investigations that will be performed on the samples.

Cyclooxygenase suppression: In Study 2 aspirin or ibuprofen will be employed to manipulate the downstream products of cyclooxygenase and lipoxygenase – prostaglandins and lipoxins. Participants will start taking their allocated study drug the evening after undergoing endotoxaemia, and continue till the evening of day 2 (the day before Investigative Immune Assessment). This will comprise 7 doses of ibuprofen 400mg, taken three-times a day or 3 doses of aspirin 75mg taken once a day. These drugs are extremely safe when administered on such short-term basis, having a minimal side-effect profile. They are consequently available over-the-counter without prescription. Participants describing previous allergy, adverse reaction or intolerance or these or related drugs or clinical features that suggest high-risk of experiencing side effects will be excluded. Participants will also be asked to contact the investigative team or seek medical assistance if they experience any adverse events whilst taking the drugs. Both ibuprofen and aspirin will be supplied by the UCLH Pharmacy who will issue dosing and safety advice.

D5

Will group or individual interviews/questionnaires raise any topics or issues that might be sensitive, embarrassing or upsetting for participants?

If **Yes**, please explain how you will deal with this.

No

D6	<p>Please describe any expected benefits to the participant.</p> <p>No benefits for participants is expected. Previously un-diagnosed medical conditions may be detected by screening investigations/examination.</p>
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D7	<p>Specify whether the following procedures are involved:</p> <p>Any invasive procedure(s) <input type="checkbox"/> Yes <input checked="" type="checkbox"/> No</p> <p>Physical contact <input checked="" type="checkbox"/> Yes <input type="checkbox"/> No</p> <p>Any procedure(s) that may cause mental distress <input type="checkbox"/> Yes <input checked="" type="checkbox"/> No</p> <p>Please state briefly any precautions being taken to protect the health and safety of the research participants.</p> <p>Standard hand hygiene procedures and aseptic non-touch technique will be employed throughout. The option of a chaperone being present during physical examination will be provided</p>
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D8	<p>Does the research involve the use of drugs? <input checked="" type="checkbox"/> Yes <input type="checkbox"/> No</p> <p>If Yes, please name the drug/product and its intended use in the research and then refer to Appendix I</p> <p>EC-6 Clinical Reference Centre Endotoxin (CCRE), National Institute of Health (USA). 2ng/kg, IV, bolus)</p> <p>Ibuprofen (400mg, TDS, PO, total 7doses)</p> <p>Aspirin (75mg, OD, PO, total 3 doses)</p> <p>Does the project involve the use of genetically modified materials? <input type="checkbox"/> Yes <input checked="" type="checkbox"/> No</p> <p>If Yes, has approval from the Genetic Modification Safety Committee been obtained for work? <input type="checkbox"/> Yes <input type="checkbox"/> No</p> <p>If Yes, please quote the Genetic Modification Reference Number:</p>
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D9	<p>Will any non-ionising radiation be used on the research participant(s)? <input type="checkbox"/> Yes <input checked="" type="checkbox"/> No</p> <p>If Yes, please refer to Appendix II.</p>
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CHECKLIST

Please submit either 12 copies (1 original + 11 double sided photocopies) of your completed application form for full committee review or 3 copies (1 original + 2 double sided copies) for chair's action, together with the appropriate supporting documentation from the list below to the UCL Research Ethics Committee Administrator. You should also submit your application form electronically to the Administrator at: ethics@ucl.ac.uk

Documents to be Attached to Application Form (if applicable)	Ticked if attached	Tick if not relevant
Section B: Details of the Project		
• Questionnaire(s) / Psychological Tests	<input checked="" type="checkbox"/>	<input type="checkbox"/>
• Relevant correspondence relating to involvement of collaborating department/s and agreed participation in the research.	<input type="checkbox"/>	<input checked="" type="checkbox"/>
Section C: Details of Participants		
• Parental/guardian consent form for research involving participants under 18	<input type="checkbox"/>	<input checked="" type="checkbox"/>
• Participant/s information sheet	<input checked="" type="checkbox"/>	<input type="checkbox"/>
• Participant/s consent form/s	<input checked="" type="checkbox"/>	<input type="checkbox"/>
• Advertisement	<input checked="" type="checkbox"/>	<input type="checkbox"/>
Section D: Details of Risks and Benefits to the Researcher and the Researched		
• Insurance registration form and related correspondence	<input type="checkbox"/>	<input checked="" type="checkbox"/>
Appendix I: Research Involving the Use of Drugs		
• Relevant correspondence relating to agreed arrangements for dispensing with the pharmacy	<input checked="" type="checkbox"/>	<input type="checkbox"/>
• Written confirmation from the manufacturer that the drug/substance has been manufactured to GMP	<input checked="" type="checkbox"/>	<input type="checkbox"/>
• Proposed volunteer contract	<input type="checkbox"/>	<input type="checkbox"/>
• Full declaration of financial or direct interest	<input checked="" type="checkbox"/>	<input type="checkbox"/>
• Copies of certificates: CTA etc...	<input type="checkbox"/>	<input type="checkbox"/>
Appendix II: Use of Non-Ionising Radiation		

Please note that correspondence regarding the application will normally be sent to the Principal Researcher and copied to other named individuals.

IMPORTANT: ALL FIELDS MUST BE COMPLETED. THE FORM SHOULD BE COMPLETED IN PLAIN ENGLISH UNDERSTANDABLE TO LAY COMMITTEE MEMBERS.

SEE NOTES IN STATUS BAR FOR ADVICE ON COMPLETING EACH FIELD. YOU SHOULD READ THE ETHICS APPLICATION GUIDELINES AND HAVE THEM AVAILABLE AS YOU COMPLETE THIS FORM.

APPLICATION FORM

SECTION A APPLICATION DETAILS

A1	Project Title: LPS-Stimulated Whole Blood Cytokine Release: Comparison of Different Methodologies	
	Date of Submission: 19/08/2015	Proposed Start Date: 1/09/2015
	UCL Ethics Project ID Number: 4332/001	Proposed End Date: 29/02/2016
	If this is an application for classroom research as distinct from independent study courses, please provide the following additional details:	
	Course Title: N/A	Course Number: N/A

A2	Principal Researcher <i>Please note that a student – undergraduate, postgraduate or research postgraduate cannot be the Principal Researcher for Ethics purposes.</i>	
	Full Name: Professor Derek. W Gilroy	Position Held: Professor of Experimental Immunology and Wellcome Trust Senior Research Fellow.
	Address: Centre for Clinical Pharmacology, Division of Medicine Rayne Building 5 University Street LONDON WC1E6JF	Email: [REDACTED]
		Telephone: [REDACTED]
		Fax: [REDACTED]
	Declaration To be Signed by the Principal Researcher	
	<ul style="list-style-type: none"> ▪ I have met with and advised the student on the ethical aspects of this project design (<i>applicable only if the Principal Researcher is not also the Applicant</i>). ▪ I understand that it is a UCL requirement for both students & staff researchers to undergo Disclosure and Barring Service (DBS) Checks when working in controlled or regulated activity with children, young people or vulnerable adults. The required DBS Check Disclosure Number(s) is: N/A ▪ I have obtained approval from the UCL Data Protection Officer stating that the research project is compliant with the Data Protection Act 1998. My Data Protection Registration Number is: Z6364106/2013/09/08 ▪ I am satisfied that the research complies with current professional, departmental and university guidelines including UCL's Risk Assessment Procedures and insurance arrangements. ▪ I undertake to complete and submit the 'Continuing Review Approval Form' on an annual basis to the UCL Research Ethics Committee. ▪ I will ensure that changes in approved research protocols are reported promptly and are not initiated without approval by the UCL Research Ethics Committee, except when necessary to eliminate apparent immediate hazards to the participant. ▪ I will ensure that all adverse or unforeseen problems arising from the research project are reported in a timely fashion to the UCL Research Ethics Committee. ▪ I will undertake to provide notification when the study is complete and if it fails to start or is abandoned. 	

SIGNATURE:

DATE: 19/08/2015

A3	Applicant(s) Details (if Applicant is not the Principal Researcher e.g. student details):	
	Full Name: Dr James Fullerton	
	Position Held: Wellcome Trust Research Training Fellow (PhD Student)	
	Address: Clinical Pharmacology Rayne Building 5 University Street WC1E 6JF	Email: [REDACTED]
		Telephone: [REDACTED]
		Fax: [REDACTED]
	Full Name:	
Position Held:		
Address:	Email:	
	Telephone:	
	Fax:	

A4	Sponsor/ Other Organisations Involved and Funding
	a) Sponsor: <input checked="" type="checkbox"/> UCL <input type="checkbox"/> Other institution If your project is sponsored by an institution other than UCL please provide details:
	b) Other Organisations: If your study involves another organisation, please provide details. Evidence that the relevant authority has given permission should be attached or confirmation provided that this will be available upon request.
	c) Funding: What are the sources of funding for this study and will the study result in financial payment or payment in kind to the department or College? If study is funded solely by UCL this should be stated, the section should not be left blank. Wellcome Trust Research Training Fellowship awarded to Dr James Fullerton

A5	Signature of Head of Department or Chair of the Departmental Ethics Committee <i>(This must not be the same signature as the Principal Researcher)</i>
	<p>I have discussed this project with the principal researcher who is suitably qualified to carry out this research and I approve it. The project is registered with the UCL Data Protection Officer, a formal signed risk assessment form has been completed, and appropriate insurance arrangements are in place. Links to details of UCL's policies on data protection, risk assessment, and insurance arrangements can be found at: http://ethics.grad.ucl.ac.uk/procedures.php</p> <p>UCL is required by law to ensure that researchers undergo a Disclosure and Barring Service (DBS) Check if their research project puts them in a position of trust with children under 18 or vulnerable adults.</p> <p>*HEAD OF DEPARTMENT TO DELETE BELOW AS APPLICABLE*</p> <p>I am satisfied that checks: (1) have been satisfactorily completed (2) have been initiated (3) are not required</p> <p>If checks are not required please clarify why below.</p> <p>Chair's Action Recommended: <input type="checkbox"/> Yes <input type="checkbox"/> No</p> <p><i>A recommendation for Chair's action can be based only on the criteria of minimal risk as defined in the Terms of Reference of the UCL Research Ethics Committee.</i></p>

PRINT NAME: Prof Raymond MacAllister

SIGNATURE:

DATE: 19/08/15

SECTION B

DETAILS OF THE PROJECT

B1

Please provide a brief summary of the project in simple prose outlining the intended value of the project, giving necessary scientific background (max 500 words).

Lipopolysaccharide (LPS, a bacterial product) stimulated whole blood tumour necrosis factor alpha (TNF- α , a pro-inflammatory mediator) release is a key marker of immune competence in humans. In septic patients and those suffering trauma or burn injury TNF- α release is reduced, correlating with adverse outcomes including acquisition of hospital-acquired infection and mortality. Despite this no standardised method of undertaking the assay has been described with varying types of LPS, concentrations, anti-coagulants etc. being employed, hindering comparability and reproducibility.

This study seeks to explore the effect of these variables on the LPS-stimulated whole blood cytokine release assay, highlighting their impact and suggesting an optimal method for implementation by the research and clinical community.

B2

Briefly characterise in simple prose the research protocol, type of procedure and/or research methodology (e.g. observational, survey research, experimental). Give details of any samples or measurements to be taken (max 500 words).

A pool of 15 healthy, non-smoking male and female volunteers aged 18 to 50 will be recruited. Exclusion criteria will be acute or chronic illness, regular prescribed medication use, and drug or alcohol misuse.

From the pool of recruited volunteers a number (1-10) will be asked to donate blood (between 10 to 30mL) either as a single event or as part of a multi-day sequence (maximum 3 consecutive days) dependent on experimental protocol. Whole blood will be stimulated ex-vivo with lipopolysaccharide (a bacterial product) and incubated for 30minutes to 24hours, centrifuged and the resultant supernatant stored at -80C. This will be assayed for pro-inflammatory cytokines (predominantly) tumour-necrosis factor-alpha at a later time point.

Blood will be collected using a 21 gauge butterfly needle using full aseptic non-touch technique and drawn into BD or Grenier BioOne vacutainers. No cellular material will be stored and samples pseudo-anonymised, all volunteers being allocated a study number known only to the research team.

Attach any questionnaires, psychological tests, etc. (a standardised questionnaire does not need to be attached, but please provide the name and details of the questionnaire together with a published reference to its prior usage).

B3

Where will the study take place (please provide name of institution/department)?

If the study is to be carried out overseas, what steps have been taken to secure research and ethical permission in the study country? Is the research compliant with Data Protection legislation in the country concerned or is it compliant with the UK Data Protection Act 1998?

All samples will be drawn, stored and processed at the Rayne Building, Division of Medicine, UCL.

B4

Have collaborating departments whose resources will be needed been informed and agreed to participate?

Attach any relevant correspondence.

N/A

B5 How will the results be disseminated, including communication of results with research participants?

Scientific findings will be disseminated by standard research methods (presentation at conferences, publication). All volunteers will be offered the opportunity to receive the collated results and to discuss the findings with the research team.

B6 Please outline any ethical issues that might arise from the proposed study and how they are be addressed. Please note that all research projects have some ethical considerations so do not leave this section blank.

Recruitment, consent and payment: Participants will be recruited by word of mouth. The research team will assess capacity prior to recruitment and no vulnerable adults will be used in the study. Written consent will be taken and verbal confirmation of understanding, re-affirmed prior to each interventional procedure, participants being free to withdraw without penalty at any time. Subjects will be offered no financial reimbursement for their participation.

Effect on Participants: The protocol involves venupuncture alone and as such no serious adverse events are anticipated. The maximum amount of blood taken per week/volunteer will be <100mls and as such does not represent a physiologically significant burden.

Sample Storage and Data Protection: Participant data will be quasi-anonymised with allocation and use of a study number throughout. Only the CI and PI will have access to this code. No personal details will be retained.

All samples will be labelled with the study and volunteer number and stored for a maximum of 1 year in locked freezers within the Rayne Institute, UCL. After this time they will be destroyed. Participants will be made fully aware of the tests that will performed on their samples. Any data disseminated will contain no identifiable information. Any future research on stored samples distinct from that described here will require separate Research Ethics Committee approval.

Funding and Conflict of Interest: All researchers taking part on the study can confirm they have no conflicts of interest.

SECTION C **DETAILS OF PARTICIPANTS**

C1 **Participants to be studied**

C1a. Number of volunteers:	15
Upper age limit:	50
Lower age limit:	18

C1b. Please justify the age range and sample size:

The age range was selected to include the normally working-age population, with an upper cut-off designed to exclude any age-related changes in physiology. The pool of 15 volunteers was deemed sufficient such that enough volunteers may be able to attend on any given day to meet experimental criteria.

C2 If you are using data or information held by a third party, please explain how you will obtain this. You should confirm that the information has been obtained in accordance with the UK Data Protection Act 1998.

N/A

C3	<p>Will the research include children or vulnerable adults such as individuals with mental health problems or with learning disabilities, the elderly, prisoners or young offenders? <input type="checkbox"/> Yes <input checked="" type="checkbox"/> No</p> <p>How will you ensure that participants in these groups are competent to give consent to take part in this study? <i>If you have relevant correspondence, please attach it.</i></p>
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C4	<p>Will payment or any other incentive, such as gift service or free services, be made to any research participant? <input type="checkbox"/> Yes <input checked="" type="checkbox"/> No</p> <p>If yes, please specify the level of payment to be made and/or the source of the funds/gift/free service to be used.</p> <p>Please justify the payment/other incentive you intend to offer.</p>
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C5	<p>Recruitment</p> <p>(i) Describe how potential participants will be identified:</p> <p>Researchers, staff and students working in the Division of Medicine will be offered the opportunity to join the pool of volunteers.</p> <p>(ii) Describe how potential participants will be approached:</p> <p>Individuals in the Rayne Building will be approached by the CI (JNF) and asked whether they wish to participate.</p> <p>(iii) Describe how participants will be recruited:</p> <p>All aspects of the study including design, required commitment, and the right not to participate and to withdraw at any time without penalty will be fully explained. If happy to join the volunteer pool individuals will be asked to sign a consent form.</p> <p><i>Attach recruitment emails/adverts/webpages. A data protection disclaimer should be included in the text of such literature.</i></p>
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C6	<p>Will the participants participate on a fully voluntary basis? <input checked="" type="checkbox"/> Yes <input type="checkbox"/> No</p> <p>Will UCL students be involved as participants in the research project? <input checked="" type="checkbox"/> Yes <input type="checkbox"/> No</p> <p><i>If yes, care must be taken to ensure that they are recruited in such a way that they do not feel any obligation to a teacher or member of staff to participate.</i></p> <p>Please state how you will bring to the attention of the participants their right to withdraw from the study without penalty?</p> <p>The right to withdraw will be emphasised verbally in advance of, and during, the consenting process, as well as in writing. A copy of the signed consent form will be provided to participants.</p>
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C7	<p>CONSENT</p> <p>Please describe the process you will use when seeking and obtaining consent.</p> <p>All aspects of the study including design, required commitment, and the right not to participate will be fully explained. The ability to voluntarily withdraw without penalty will be reaffirmed. If happy to proceed consent will be taken by the applicant prior to any study intervention, understanding being confirmed verbally prior to written consent being sought.</p> <p><i>A copy of the participant information sheet and consent form must be attached to this application. For your convenience proformas are provided in C10 below. These should be filled in and modified as necessary.</i></p> <p>In cases where it is not proposed to obtain the participants informed consent, please explain why below.</p> <p>N/A</p>
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C8	<p>Will any form of deception be used that raises ethical issues? If so, please explain.</p> <p>No</p>
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C9	<p>Will you provide a full debriefing at the end of the data collection phase? <input type="checkbox"/> Yes <input checked="" type="checkbox"/> No</p> <p>If 'No', please explain why below.</p> <p>Participants will be offered the opportunity to have the final scientific output forwarded to them along with a lay summary</p>
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C10	<p>Information Sheets And Consent Forms</p> <p>A poorly written Information Sheet(s) and Consent Form(s) that lack clarity and simplicity frequently delay ethics approval of research projects. The wording and content of the Information Sheet and Consent Form must be appropriate to the age and educational level of the research participants and clearly state in simple non-technical language what the participant is agreeing to. Use the active voice e.g. "we will book" rather than "bookings will be made". Refer to participants as "you" and yourself as "I" or "we". An appropriate translation of the Forms should be provided where the first language of the participants is not English. If you have different participant groups you should provide Information Sheets and Consent Forms as appropriate (e.g. one for children and one for parents/guardians) using the templates below. Where children are of a reading age, a written Information Sheet should be provided. When participants cannot read or the use of forms would be inappropriate, a description of the verbal information to be provided should be given. Please ensure that you trial the forms on an age-appropriate person before you submit your application.</p>
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Information Sheet for Participants in Research Studies

You will be given a copy of this information sheet.

Title of Project: **LPS-Stimulated Whole Blood Cytokine Release: Comparison of Different Methodologies**

This study has been approved by the UCL Research Ethics Committee (Project ID Number): **4332/001**

Name Professor Derek W Gilroy

Work Address Centre for Clinical Pharmacology, Rayne Building, 5 University Street

Contact Details 

Introduction

We are recruiting healthy, non-smoking volunteers aged 18-50 for this research project and we would like to invite you to participate. You should only participate if you want to; choosing not to take part will not disadvantage you in any way. Before you decide whether you want to take part, it is important for you to read the following information carefully and discuss it with others if you wish. It is important for you to understand why it is being done and what it will involve, so please take the time to read the following information

Why is this study being done?

Medical conditions associated with overwhelming inflammation such as sepsis, trauma and burn injury are known to reduce the body's ability to defend itself and leave patients susceptible to infections. One way of measuring immune competence (the strength of the body's defences) is to take blood from these individuals and 'challenge' it with a bacterial product (lipopolysaccharide) in a test tube. A poor reaction by the blood, releasing low levels of anti-infective chemicals (cytokines), is predictive of vulnerability to secondary infections and has previously been linked with increased risk of in-hospital death. Despite the importance of this test no standard means of performing it has been selected, different institutions using different volumes of blood, bacteria, length of time before it is interpreted etc. This study will investigate the importance of these different technical factors on the reaction of blood taken from healthy volunteers and attempt to determine an optimal, reproducible method to measure immune competence.

What does it involve for me?

We are looking to recruit 15 people like you who are prepared to give a small volume of blood on days when you are asked and you are free to donate. On each occasion we will take between 10-30mls of blood (around one egg cup full). On certain occasions we will ask, if you are able, to give blood on consecutive days (maximum 3). All blood taking will be performed by an experienced clinician using appropriate aseptic technique. It is anticipated that the study will be completed in a period of 1-2 months.

After being taken your blood will be challenged with bacterial product for a period ranging from 1 hour to 24 hours in a variety of conditions and its response (cytokine release) measured, before being discarded. No cells or DNA containing material will be retained.

Will this study help me?

This study will not benefit you directly. However, it will allow us to refine an important, simple test of immune function.

Could I come to any harm if I take part in this study?

There is a minimal risk of bruising and infection from taking blood. It may also cause slight discomfort. Aseptic technique will be used throughout to minimize the risk of infection.

Are there any factors that would exclude me from taking part in the study?

You will not be able to take part in this study if you have any acute or chronic medical conditions, or take regular prescribed or over-the-counter medication. You should not take part in this study if you are already involved in another study.

What are the arrangements for compensation?

No financial remuneration will be provided for taking part in this study.

Do I have to take part in this study?

You do not have to take part in this study if you do not want to. If you decide to take part you may withdraw at any time without giving a reason and without any penalty. If you decide, now or at a later date that you do not wish to participate in this research project, it is entirely your right.

Whom do I speak to if a problem arises?

If you have any complaint about the way in which this research project has been, or is being conducted, please, in the first instance discuss them with the researcher. If the problems are not resolved or you wish to comment in any other way, please contact Professor Derek Gilroy or Dr. James Fullerton. Centre for Clinical Pharmacology, The Rayne Institute (UCL), 5 University Street, London WC1E 6JJ.

Who is organising and funding the research?

The research is organised by the Centre for Clinical Pharmacology, UCL. The study is being funded by the Wellcome Trust.

How will confidentiality be protected?

No personal information aside from age, sex and health status will be collected or retained. You will be assigned a study number and all samples and records labelled with this. Only the investigators will have access to this code and the data-files. All information generated in the course of the study is confidential and will be kept in a locked room and on password-secured UCL computers.

What happens if anything goes wrong?

We believe that the study is very low risk. However, we carry insurance to make sure that if your health does suffer as a result of being in this study, then you will be compensated. In such a situation, you will not have to prove that the harm or injury which affects you is anyone's fault. If you are not happy with any proposed compensation, you may have to pursue your claim through legal action.

Finally: you do not have to join this study. You are free to decide not to be in this trial or to drop out at any time. If you decide not to be in the study, or drop out, there will be no penalty involved. This will not put at risk your ordinary medical care or benefits you are otherwise entitled.

Please discuss the information above with others if you wish or ask us if there is anything that is not clear or if you would like more information. It is up to you to decide whether to take part or not; choosing not to take part will not disadvantage you in any way. If you do decide to take part you are still free to withdraw at any time and without giving a reason. If you decide to take part, you will be given this information sheet to keep and be asked to sign a consent form. All data will be collected and stored in accordance with the Data Protection Act 1998.

Details of how to contact the researchers

You can contact

Professor Derek Gilroy on [REDACTED]

Dr. James Fullerton on [REDACTED]

Thank-you for taking your time to read this information leaflet. If you have any further questions please do not hesitate to contact the researchers using the above details.

Informed Consent Form for Participation in Research Studies

Please complete this form after you have listened to an explanation about the research.

Title of Project: **LPS-Stimulated Whole Blood Cytokine Release: Comparison of Different Methodologies**

This study has been approved by the UCL Research Ethics Committee (Project ID Number): **4332/001**

Thank you for your interest in taking part in this research. Before you agree to take part, the person organising the research must explain the project to you.

If you have any questions arising from the explanation already given to you, please ask the researcher before you to decide whether to join in. You will be given a copy of this Consent Form to keep and refer to at any time.

Participant's Statement

I

- have read the notes written above and understand what the study involves.
- understand that if I decide at any time that I no longer wish to take part in this project, I can notify the researchers involved and withdraw immediately.
- consent to the processing of my personal information for the purposes of this research study.
- understand that such information will be treated as strictly confidential and handled in accordance with the provisions of the Data Protection Act 1998.
- agree that the research project named above has been explained to me to my satisfaction and I agree to take part in this study.

Signed:

Date:

SECTION D DETAILS OF RISKS AND BENEFITS TO THE RESEARCHER AND THE RESEARCHED

D1

Have UCL's Risk Assessment Procedures been followed?

Yes No

If No, please explain.

D2	<p>Does UCL's insurer need to be notified about your project before insurance cover can be provided? <input type="checkbox"/> Yes <input checked="" type="checkbox"/> No</p> <p><i>The insurance for all UCL studies is provided by a commercial insurer. For the majority of studies the cover is automatic. However, for a minority of studies, in certain categories, the insurer requires prior notification of the project before cover can be provided.</i></p> <p>If Yes, please provide confirmation that the appropriate insurance cover has been agreed. <i>Please attach your UCL insurance registration form and any related correspondence.</i></p>
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D3	<p>Please state briefly any precautions being taken to protect the health and safety of researchers and others associated with the project (as distinct from the research participants).</p> <p>Venepuncture will only be performed by appropriately trained, clinically qualified investigators with complete vaccination records.</p>
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D4	<p>Will these participants participate in any activities that may be potentially stressful or harmful in connection with this research? <input type="checkbox"/> Yes <input checked="" type="checkbox"/> No</p> <p>If Yes, please describe the nature of the risk or stress and how you will minimise and monitor it.</p>
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D5	<p>Will group or individual interviews/questionnaires raise any topics or issues that might be sensitive, embarrassing or upsetting for participants?</p> <p>If Yes, please explain how you will deal with this.</p> <p>No</p>
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D6	<p>Please describe any expected benefits to the participant.</p> <p>No benefits for participants is expected.</p>
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D7	<p>Specify whether the following procedures are involved:</p> <p>Any invasive procedure(s) <input type="checkbox"/> Yes <input checked="" type="checkbox"/> No</p> <p>Physical contact <input checked="" type="checkbox"/> Yes <input type="checkbox"/> No</p> <p>Any procedure(s) that may cause mental distress <input type="checkbox"/> Yes <input checked="" type="checkbox"/> No</p> <p>Please state briefly any precautions being taken to protect the health and safety of the research participants.</p> <p>Standard hand hygiene procedures and aseptic non-touch technique will be employed throughout.</p>
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D8	<p>Does the research involve the use of drugs? <input type="checkbox"/> Yes <input checked="" type="checkbox"/> No</p> <p>If Yes, please name the drug/product and its intended use in the research and then refer to Appendix I</p> <p>Does the project involve the use of genetically modified materials? <input type="checkbox"/> Yes <input checked="" type="checkbox"/> No</p> <p>If Yes, has approval from the Genetic Modification Safety Committee been obtained for work? <input type="checkbox"/> Yes <input type="checkbox"/> No</p> <p>If Yes, please quote the Genetic Modification Reference Number:</p>
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D9	<p>Will any non-ionising radiation be used on the research participant(s)? <input type="checkbox"/> Yes <input checked="" type="checkbox"/> No</p> <p>If Yes, please refer to Appendix II.</p>
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CHECKLIST

Please submit either 12 copies (1 original + 11 double sided photocopies) of your completed application form for full committee review or 3 copies (1 original + 2 double sided copies) for chair's action, together with the appropriate supporting documentation from the list below to the UCL Research Ethics Committee Administrator. You should also submit your application form electronically to the Administrator at: ethics@ucl.ac.uk

Documents to be Attached to Application Form (if applicable)	Ticked if attached	Tick if not relevant
Section B: Details of the Project		
• Questionnaire(s) / Psychological Tests	<input type="checkbox"/>	<input checked="" type="checkbox"/>
• Relevant correspondence relating to involvement of collaborating department/s and agreed participation in the research.	<input type="checkbox"/>	<input checked="" type="checkbox"/>
Section C: Details of Participants		
• Parental/guardian consent form for research involving participants under 18	<input type="checkbox"/>	<input checked="" type="checkbox"/>
• Participant/s information sheet	<input checked="" type="checkbox"/>	<input type="checkbox"/>
• Participant/s consent form/s	<input checked="" type="checkbox"/>	<input type="checkbox"/>
• Advertisement	<input type="checkbox"/>	<input checked="" type="checkbox"/>
Section D: Details of Risks and Benefits to the Researcher and the Researched		
• Insurance registration form and related correspondence	<input type="checkbox"/>	<input checked="" type="checkbox"/>
Appendix I: Research Involving the Use of Drugs		
• Relevant correspondence relating to agreed arrangements for dispensing with the pharmacy	<input type="checkbox"/>	<input checked="" type="checkbox"/>
• Written confirmation from the manufacturer that the drug/substance has been manufactured to GMP	<input type="checkbox"/>	<input checked="" type="checkbox"/>
• Proposed volunteer contract	<input type="checkbox"/>	<input checked="" type="checkbox"/>
• Full declaration of financial or direct interest	<input type="checkbox"/>	<input checked="" type="checkbox"/>
• Copies of certificates: CTA etc...	<input type="checkbox"/>	<input checked="" type="checkbox"/>
Appendix II: Use of Non-Ionising Radiation		

Please note that correspondence regarding the application will normally be sent to the Principal Researcher and copied to other named individuals.