Eicosanoid and cytokine responses to bacterial infection

by

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Summary

Infectious diseases remain some of the most serious health threats facing the world. The immune system is equipped to initiate a rapid and specific response to foreign invaders of the body, with its ultimate aim being to protect an organism from injury and disease. Eicosanoids, including prostaglandins and leukotrienes, are a family of lipids that play key roles in inflammation including helping leukocytes fight infection. Cells of the innate immune system including tissue macrophages, neutrophils and sentinel dendritic cells are major contributors of local eicosanoids. In mammals an inflammatory insult will result in a cytokine cascade whereby tumour necrosis factor α (TNF-α) is released, followed by interleukin-1β (IL-1β) and then IL-6. Downstream of these cytokines, others are released that serve as potent chemoattractants to induce migration of neutrophils and macrophages to the site of infection.

It is known that exposure to varying bacterial components results in a different profile of lipids and cytokines, and by characterising mediator signals it may be possible to define biomarker fingerprints predictive for early bacterial infections. To analyse this, a combination of a targeted lipidomic approach and cytokine immunoassays were employed to identify neutrophil and macrophage responses to individual bacterial components and the whole organism.

Work in this thesis has identified potential markers of bacterial infection, such as 12-HETE, 14-HDOHE and TNF-α, which, along with future advances, could be used to develop novel strategies for clinicians, nurses and primary care staff to analyse patients suspected of bacterial infection at the bedside. Work here provides an insight into how the eicosanoid and cytokine storms are generated alongside each other to accompany classic inflammation during specific bacterial infection. The ability to distinguish between species of bacteria causing infection could prove invaluable, reducing the time taken to establish the cause of infection, ultimately leading to better patient outcomes.

Abbreviations

AA	Arachidonic acid
ACCP	American College of Chest Physicians
BSA	Bovine serum albumin
Ca^{2+}	Calcium
CFU	Colony forming units
$CHCl_3$	Chloroform
CHIPS	Chemotaxis inhibitory protein of staphylococci
COX	Cyclooxygenase
CpG	Cytidine-phosphate-guanosine
CRP	C-reactive protein
CysLTs	Cysteinyl-leukotrienes
DNA	Deoxyribonucleic acid
DIC	Differential interference contrast
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
Eap	Extracellular adhesion protein
ECACC	European collection of authenticated cell cultures
E. coli	*Escherichia coli*
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ESBLs	Extended Spectrum Beta-Lactamases
FCS	Foetal bovine serum
FLAP	5- lipoxygenase activating protein
fMLP	N-formylmethionyl-leucyl-phenylalanine
GC	Gas chromatography
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GPI	Glycosylphosphatidylinositol
H_2O	Water
HCl	Hydrogen chloride
HEPES	Hydroxyethyl piperazineethanesulfonic acid

HETEs	Hydroxyicosatetraenoic acid
HKLM	Heat-killed *Listeria monocytogenes*
HPETEs	Hydroperoxyeicosatetraenoic acids
HPLC	High-performance liquid chromatography
ICU	Intensive care unit
IL-1β	Interleukin-1beta
IL-6	Interleukin-6
IL-8	Interleukin-8
IsoPs	Isoprostanes
KCl	Potassium chloride
LBP	Lipopolysaccharide binding protein
LOX	Lipoxygenase
LPS	Lipopolysaccharide
LRR	Leucine-rich repeats
LTA	Lipoteichoic acid
LTs	Leukotrienes
LXs	Lipoxins
MALDI-TOF	Matrix assisted laser desorption/ionisation – time of flight
MeOH	Methanol
Mg	Magnesium
MRM	Multiple reaction-monitoring
MRSA	Methicillin-resistant *Staphylococcus aureus*
MS	Mass spectrometry
MyD88	Myeloid differentiation factor 88
NaCl	Sodium chloride
NaH_2PO_4	Monosodium phosphate
NCTC	National collection of type cultures
NETs	Neutrophil extracellular traps
NMEC	Neonatal meningitis *Escherichia coli*
NOD	Nucleotide-binding oligomerization domain
NLR	NOD-like receptor
OPD	O-phenylenediamine dihydrochloride

PAMP	Pathogen-associated molecular pattern
PBP	Penicillin-binding protein
PBS	Phosphate buffer saline
PBST	Phosphate buffer saline with tween
PCT	Procalcitonin
PGs	Prostaglandins
PLA_2	Phospholipase A_2
PMA	Phorbol 12-myristate 13-acetate
PMN	Polymorphonuclear neutrophils
PMNL	Polymorphonuclear leukocytes
PRRs	Pattern recognition receptors
PTG	Peptidoglycan
PUFAs	Polyunsaturated fatty acids
Q-PCR	Quantitative polymerase chain reactions
RIG	Retinoic acid-inducible gene
ROS	Reactive oxygen species
SCC	Staphylococcal cassette chromosome
SCCM	Society of Critical Care Medicine
SIRS	Systemic inflammatory response syndrome
SNAP	Synaptosome-associated protein
SNAREs	Soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors
Spp	Species
SSC	Surviving sepsis campaign
TLR	Toll like receptor
TNF-α	Tumor necrosis factor-alpha
UK	United Kingdom
UPEC	Urophathogenic *Escherichia coli*
UT	Untreated
UTIs	Urinary tract infections
UV	Ultraviolet
VAMP	Vesicle-associated membrane proteins
WBCs	White blood cells

Chapter 1

Introduction

1.1 The immune system

The immune system is an organisation of cells and molecules which have specialised roles in defending the host against infection (Delves & Roitt 2000). The human immune system develops in order to protect the host from opportunistic invasion of microorganisms such as bacteria, viruses, fungi and parasites. It works simultaneously to combat and destroy foreign pathogens entering the body, whilst also recognising and removing host cells that are damaged, senescent or cancerous. The ability of the immune system to be able to distinguish between self and non-self is crucial to its success and therefore the survival of the organism.

1.1.1 Innate immunity during infection

The immune system employs two branches: innate (natural) immunity and adaptive (acquired) immunity. Innate immunity is a non-specific cell-mediated response which essentially creates a rapid first line of defence against infection (Rittirsch et al. 2008). Adaptive immunity also possesses a cellular response alongside an antibody-mediated (humoral) factor that culminates in a highly specific, efficient response (Steinke & Borish 2006). These two components act together in the clearance of pathogens.

The innate immune system is intrinsic to the organism, and consists of all the immune defences that lack immunological memory (Delves & Roitt 2000). The innate immune system is able to target a broad spectrum of pathogenic infections as it does not require the production of antibodies. It is composed of a complex network of both cellular and molecular factors, which cooperate to counteract any invasion by pathogenic microorganisms. The major function of the innate immune system in vertebrates includes the recruitment of immune cells to the site of infection through the generation of chemical mediators; identification and removal of foreign invasion in organs, tissue, blood and lymph by specialised white blood cells, through phagocytosis and generation of neutrophil extracellular traps (NETs); activation of

the complement cascade to identify bacteria, activate cells and promote clearance of antibody complexes; activation of the adaptive immune system and acting as a chemical and physical barrier to infectious agents (chemical measures include clotting factors in blood and physical factors such as the skin) (Dunkelberger & Song 2009; Parham 2015) (Figure 1.1).

The cellular components of the innate immune system include the phagocytic cells neutrophils and macrophages, which will be discussed in this thesis. A molecular aspect of the innate immune response is called complement. This is a complex network of plasma and membrane-associated serum proteins, which work in a chain-like manner whereby each component interacts with the next in a cascade of proteolytic interactions to generate subsequently active molecules (Dunkelberger & Song 2009). At the end, this results in the accumulation of complement components in the membrane of targeted cells to generate pores that kill the cell by causing lysis. In a process called opsonisation, certain members of the complement cascade also work together with cellular components and mark targeted cells (with complement component 3bi (C3bi)) for removal by phagocytic cells. Other complement components generate a chemotactic gradient (of complement component 5a(C5a)), which recruits phagocytic cells to the vicinity (Dunkelberger & Song 2009). In all instances, the resultant cell debris is swept away by the phagocytic foot soldiers of the innate immune system, neutrophils and macrophages. In performing these functions, complement provides a vital first-line barrier to invading pathogens.

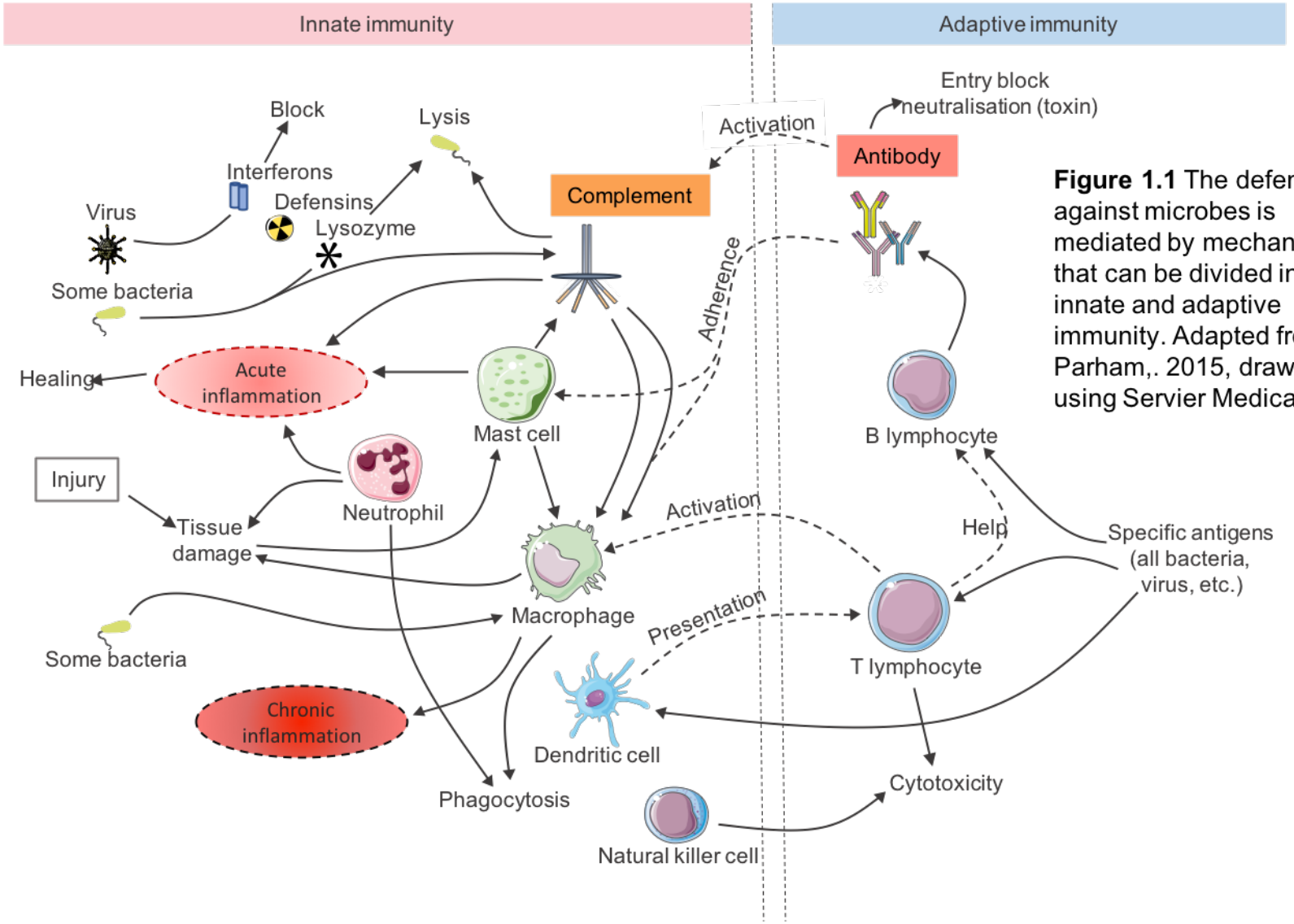


Figure 1.1 The defence against microbes is mediated by mechanisms that can be divided into innate and adaptive immunity. Adapted from Parham,. 2015, drawn using Servier Medical Art.

Inflammation is an essential host defence mechanism, whereby the innate immune system plays a pivotal role, as it mediates the initial response and protects the body against pathogenic microorganisms (Russell & Schwarze 2013). Resolution of inflammation is an active process, where the production of counter-regulatory molecules such as anti-inflammatory cytokines (for example, IL-4, IL-10 and IFN-alpha), resolvins, lipoxins and protectins attempt to restore an immunological equilibrium, rendering inflammation self-limiting under normal conditions (Cohen 2002; Russell & Schwarze 2013). In early infection circulating neutrophils and macrophages produce and respond to pro-inflammatory signals such as cytokines and lipid mediators, facilitating their migration to the site of inflammation and allowing them to phagocytose foreign pathogens (Strassheim et al. 2002; Lerman et al. 2014). Although these inflammatory mediators are important for the host defence, in uncontrolled conditions, damage to the host can result when there is an excessive and non-resolving inflammatory response (Lerman et al. 2014). This overzealous activity is demonstrated in sepsis for example, where homeostatic control declines during an inflammatory response to infection, leading to a pro-inflammatory, pro-coagulation state that can cause organ dysfunction and death (Russell & Schwarze 2013).

Systemic inflammation is commonly demonstrated amongst the elderly, infants, immunocompromised and critically ill patients (Pugin 2012). The concept of the systemic inflammatory response syndrome (SIRS) was conceived by Roger C. Bone in 1991 and was soon incorporated into the definition of sepsis by the American College of Chest Physicians/Society of Critical Care Medicine (ACCP/SCCM) consensus conference (Bone et al. 1992a; Bone et al. 1992c). The host response to injury is termed SIRS when there is an over-whelming disproportionate activation of the immune system (Pugin 2012). This response can be attributed to various underlying causes, besides that of infectious insult, including pancreatitis, ischemia, haemorrhagic shock and multiple trauma and tissue injury, thus, the generic term SIRS incorporates both sterile and non-sterile inflammation (Bone et al. 1992a; Stoppelkamp et al. 2015; Dellinger et al. 2013).

1.1.2 Infectious diseases

Infectious diseases are disorders caused by pathogenic organisms. Whilst this includes bacteria, viruses and many types of fungi, discussion here will focus specifically on bacteria. They remain some of the most serious health threats facing the world. Successfully combating infections caused by pathogenic organisms is essential for the host survival (Das 2011). The immune system is equipped to initiate a rapid and specific response to foreign invaders of the body, with its ultimate aim being to protect an organism from injury and disease (Tecchio et al. 2014).

The signs, symptoms and severity of the immune response vary depending on the organism causing the infection. The human body is equipped with an indigenous microbiota, which develops with the host from birth and is subject to complex interplay that depends on the host genome, nutrition and life-style (Nicholson et al. 2012).
Indigenous microbiota are also a source of protection from other invading bacteria. The complex host-microbe and microbe-microbe interactions that exist in the body have a beneficial role, including the human skin microbiota and the gut microflora. For example, the most common clinical isolate of cutaneous microbiota on the body, *Staphylococcus epidermidis* (*S. epidermidis*), comprises around 90% of the aerobic resident flora (Cogen et al. 2008). *S. epidermidis* promotes cutaneous defence through elicitation of host immune responses. Data suggest that *S. epidermidis* plays a protective role by influencing the innate immune response of keratinocytes through toll-like receptor signalling (Cogen et al. 2008). Also, the leading human pathogen *Staphylococcus aureus* (*S. aureus*) can result in a range of clinical diseases from skin infections to invasive life-threatening diseases such as sepsis (Cogen et al. 2008). However, despite the usual classification of *S. aureus* as a transient pathogen it may also be considered a normal component of the nasal microflora and is not synonymous with infection (Cogen et al. 2008). Like *S. epidermidis*, healthy individuals rarely contract invasive infections caused by *S. aureus* where they are in effect acting as commensals rather than pathogens.

However, the indigenous microbiota is also a potent source of opportunistic infections that arise when the mechanisms that normally confine the microbes to a particular site are disrupted (Cho & Blaser 2012). Anaerobic infections following bowel surgery, urinary tract infections, chronic respiratory tract infections, catheterisation, dental/gingival diseases, and skin conditions, such as surgical site infections and abscesses, come into this category. An overuse of antibiotics may disrupt the delicate balance of the cutaneous microflora leaving individuals susceptible to pathogens previously kept at bay by the existing resident and mutual microbiota.

1.1.2.1 Specific bacterial infections

1.1.2.1.1 Wounds

The skin is the body's largest organ and is the first defence barrier to external threat. The surface of the skin is not sterile and is populated with microorganisms, known as normal flora. Wound and skin infections represent an invasion of tissues by one or more species of microorganisms. The infection triggers the body's immune system, causing inflammation and tissue damage. Tissue injury is followed by the sequential infiltration of the wound by neutrophils and macrophages (Brancato & Albina 2011). Neutrophil abscess formation is a critical event in the innate immune response against invading pathogens and is required for wound resolution and bacterial clearance (Kim et al. 2011). Macrophages control the cellularity of wounds through their capacity to induce apoptosis and phagocytose a variety of wound cells. Macrophage-targeted cells include neutrophils during the inflammatory phase of repair and fibroblasts and endothelial cells during its resolution (Brancato & Albina 2011).

Many infections remain confined to small areas, such as scratches, and can be self-resolving. Other infections may persist and if untreated, increase in severity and spread further into the body. Wounds are breaks in the skin and tissue, which may be superficial but also include punctures, burns or through surgical intervention. Chronic wounds are a major cause of morbidity, affecting more than 1% of the UK population (Edwards &

Harding 2004). Chronic wounds tend to occur in individuals with an increased risk of bacterial invasion as a result of trauma or systemic disease such as diabetes mellitus and rheumatoid arthritis (Edwards & Harding 2004). Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the most common causes of wound infections in both hospital and community settings (Patel 2007). MRSA infections can range from minor infections of the skin to more serious chronic wound infections, contaminated implants, pneumonia and sepsis (Patel 2007). The emergence of methicillin resistance is due to the acquisition of a transferable DNA element called staphylococcal cassette chromosome *mec* (SCC*mec*), a cassette (type I-V) carrying the *mecA* gene, coding penicillin-binding protein (PBP) 2a (Ma et al. 2002; Cogen et al. 2008; Foster 2004). Through site-specific recombination, the DNA element integrates into the genome. Normally, β-lactam antibiotics bind to the PBPs in the cell wall, disrupt peptidoglycan layer synthesis and kill the bacterium (Cogen et al. 2008). However, β-lactam antibiotics cannot bind to PBP2a, allowing a bacterium containing the *mecA* gene to survive β-lactam killing (Foster 2004). *S. aureus* expresses many virulence factors (ability to cause disease; degree of pathogenicity) that contribute to evasion, which are both secreted, and cell-surface associated. These include the secretion of chemotaxis inhibitory protein of staphylococci (CHIPS) which binds to the formyl peptide receptor and C5a receptor on neutrophils, subsequently interfering with neutrophil chemotaxis (de Haas et al. 2004). Bacterial extracellular adhesion protein (Eap) is a novel anti-inflammatory factor that inhibits leukocyte recruitment by adhering to the intracellular adhesion molecule-1 on neutrophils (Indesmith et al. 2003).

1.1.2.1.2 Urinary tract infections, meningitis and pneumonia

Other common infections which induce inflammatory responses include urinary tract infections (UTIs), pneumonia and septicaemia. *Escherichia coli* (*E. coli*) represents a diverse organism that is normally a harmless commensal but only needs to acquire a combination of mobile genetic elements to become a highly adapted pathogen capable of causing a range of diseases, from gastroenteritis to infections of the urinary tract,

bloodstream and central nervous system. *E. coli* is the most common Gram-negative pathogen in patients of all ages and the most common cause of UTIs (Swami et al. 2012). Around 70-80% of health care-acquired infections are attributed to the use of indwelling catheters (Nicolle 2014). Urophathogenic *E. coli* (UPEC) can cause cystitis in the bladder and acute pyelonephritis in the kidneys (Croxen & Finlay 2010). The ability to ascend the urinary tract from the urethra to the bladder and kidneys reflects exceptional mechanisms for evading innate immunity and avoiding clearance by micturition (Croxen & Finlay 2010). There are several highly regulated virulence factors that contribute to this complex pathogenesis, including secretion of toxins, multiple pili, a polysaccharide capsule and biofilm formation (Croxen & Finlay 2010; Nicolle 2014). During infection, the resulting influx of phagocytes causes tissue damage, and UPEC attachment and invasion results in apoptosis and exfoliation of bladder cells (Croxen & Finlay 2010).

The most frequent cause of Gram-negative-associated meningitis in new-borns is caused by neonatal meningitis *E. coli* (NMEC) (Croxen & Finlay 2010). Fatality rates can reach up to 40% and survivors are often left with severe neurological defects. NMEC is a common inhabitant of the gastrointestinal tract, where the bacteria must enter the bloodstream and cross the blood-brain barrier into the central nervous system which can lead to meningeal inflammation and pleocytosis of the cerebrospinal fluid (Croxen & Finlay 2010). NMEC survival in the blood is crucial as progression of the disease is dependent on high bacteraemia ($>10^3$ colony-forming units per ml of blood). Virulence factors which help protect the bacteria from host immune defences include possession of an antiphagocytic capsule, invasion of macrophages and monocytes prevent apoptosis and chemokine release providing a niche for replication (Selvaraj & Prasadarao 2005; Sukumaran et al. 2004).

Klebsiella pneumoniae can be carried asymptomatically by individuals in their nose, throat and on their skin (Doorduijn et al. 2016). *Klebsiella pneumoniae* is recognised as an important opportunistic pathogen that frequently causes UTIs and pneumonia in immunocompromised individuals, neonate and the elderly (Struve & Krogfelt 2003). It is

characteristic that most *Klebsiella pneumoniae* infections are preceded by colonization of the patients' gastrointestinal tract, which is considered the main reservoir of transmission of the bacteria (Struve & Krogfelt 2003). One of the main virulence factors expressed by *Klebsiella pneumoniae* is a capsule that protects the pathogen against phagocytosis, antimicrobial peptides and complement-mediated lysis (Doorduijn et al. 2016). The acquisition of antibiotic resistant genes and intrinsic resistance to several classes of antibiotics limits the treatment options for infections caused by *Klebsiella pneumoniae* (Doorduijn et al. 2016). There is a current global spread of *Klebsiella Pneumoniae* strains producing Extended Spectrum Beta-Lactamases (ESBLs) and carbapenemases (Hawkey & Jones 2009). These enzymes inactivate β-lactams, a class of antibiotics that forms the basis of effective treatment for *Klebsiella pneumoniae*-infected patients (Doorduijn et al. 2016). As a result, the antimicrobial peptide colistin has been reintroduced to treat multi-drug-resistant *Klebsiella pneumoniae* infections. However, there have been reports of colistin-resistant *Klebsiella pneumoniae*, threatening the effectiveness of colistin as a last antibiotic for multi-drug resistant Klebsiella infections (Wang et al. 2016).

1.1.2.1.3 Sepsis

Sepsis arises when the body's response to an infection damages its own tissues and organs. It is a syndrome that comprises physiological, pathological and biological abnormalities induced by infection (Singer et al. 2016). The septic response is an extremely complex chain of events involving pro- and anti-inflammatory processes, humoral and cellular reactions and circulating abnormalities and represents infection in its worst case (Limongi et al. 2016). Last revised in 2001, the sepsis definition has been used for the past quarter of a century, sculpting the basis of research into sepsis and propelling the development of its clinical recognition and management (Levy et al. 2003; Gotts & Matthay 2016). However, substantial advances made within the pathobiology (changes in organ function, immunology, cell biology, circulation, morphology and

biochemistry), management and epidemiology of sepsis have called for a re-evaluation of definitions (Singer et al. 2016).

In February 2016, the European Society of Intensive Care Medicine and the SCCM published a new consensus definition of sepsis and related clinical criteria (Table 1.1). Sepsis is now defined as life-threatening organ dysfunction caused by a dysregulated host response to infection (Singer et al. 2016). In the United Kingdom (UK) there are an estimated 150,000 cases of sepsis each year, with a survival rate between 30-50%, resulting in 44,000 deaths; claiming more lives than breast, bowel and prostate cancer combined (Daniels 2009; Daniels 2011). In 2011, the Centre for Maternal and Child Enquiries (CMACE) found sepsis to be the commonest direct cause of maternal death in the UK (Cantwell et al. 2011). Alongside the mortality associated with sepsis also lies the social and economic impact. This life-threatening condition has a major bearing on healthcare resources and expenditure and fuels the need for early detection of infection.

The specific species of bacteria discussed throughout section 1.1.2.1 will be employed in this thesis to look for initial marker profiles of infection. They were chosen as they are highly pathogenic and represent some of the most common causes of bacterial infections. Early detection of infectious diseases plays a crucial role in all treatment and prevention strategies. Rapid and accurate identification of the underlying agent using diagnostic testing is essential to select the correct control measure.

1.1.2.2 Clinical diagnosis of specific infections

Bacterial infections can be challenging to diagnose, this is because clinical presentation of infections from different causative agents can be similar. For example, in certain instances it may be difficult to differentiate viral from bacterial infections. Inflammatory states, such as trauma, pancreatitis, transplant rejects and vasculitis, might also have clinical presentation similar to that for an infection (Simon et al. 2004). Treating viral

illnesses or non-infective causes of inflammation with antibiotics is not only ineffective, but also contributes to the development of resistance (WHO, 2000), increases costs and adding to the risk of toxicity and allergic reactions. Studies carried out by the World Health Organisation (WHO) found that for every 100 respiratory infections, only 20 required antibiotic treatment (WHO, 2000).

The most accurate way to diagnose bacterial infection is by culture (Simon et al. 2004). Laboratory methods for detecting pathogens from blood cultures or wounds require long incubation times that do not support rapid detection and fast decisions for selecting the most appropriate therapeutic interventions (Wolk et al. 2009). It is now accepted that early recognition and therefore appropriate intervention of therapy are key to preventing patient deterioration, which in severe cases may lead to death (Dellinger et al. 2013). The significant mortality and rising economic costs of critically ill patients are driving research towards gaining a greater understanding into basic immunity, in turn creating a more effective therapeutic approach (Osuchowski et al. 2007). Diagnostic biomolecular markers could simplify and accelerate diagnosis, verification and timely correct therapy for bacterial infections. Current diagnostic markers in used are discussed later in this chapter. One potential target for this is through the use of immune mediators secreted by innate immune cells.

1.1.3 White blood cells

White blood cells (WBCs) were first divided into subpopulations based on differences in their morphologies by Paul Ehrlich in 1898 (Figure 1.2) (Werber 1986). Ehrlich described one subgroup to appear to contain multiple clustered nuclei as opposed to a single nucleus. However, upon further examination, he observed the appearance of nuclear 'lobes', which remained connected by fine tethers and proceeded to call them 'cells with polymorphous nuclei' (Ehrlich 1898). This group of cells became the focus of much attention and were later renamed by the Russian scientist Ilya Mechnikov as

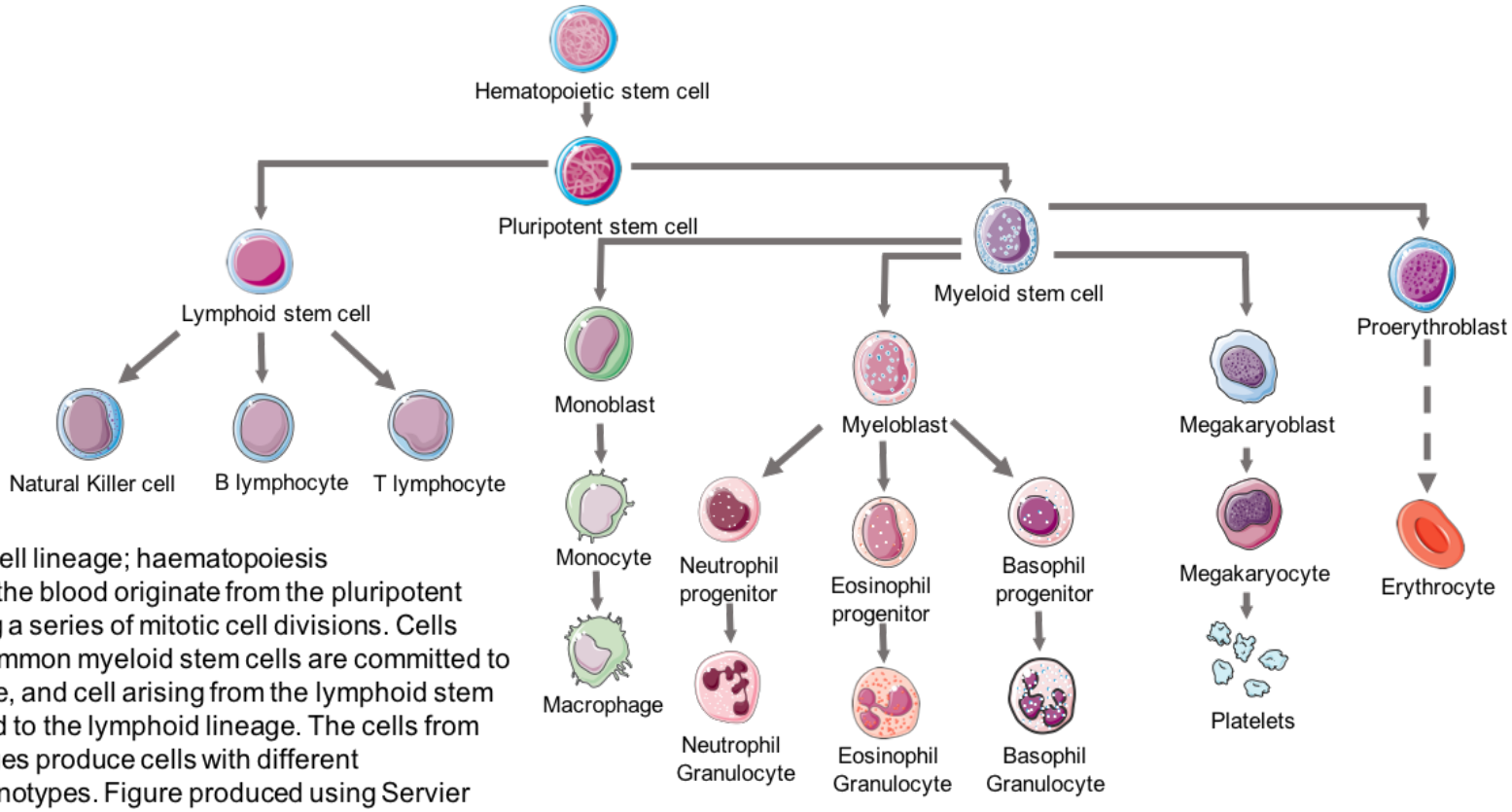


Figure 1.2 Blood cell lineage; haematopoiesis
All components of the blood originate from the pluripotent stem cell, following a series of mitotic cell divisions. Cells arising from the common myeloid stem cells are committed to the myeloid lineage, and cell arising from the lymphoid stem cells are committed to the lymphoid lineage. The cells from different cell lineages produce cells with different morphological phenotypes. Figure produced using Servier Medical Art.

'polymorphonuclear leukocytes' (PMNLs) in 1905. PMNLs is a term that is still used to describe this subpopulation of WBCs. Following pioneering studies by Mechnikov came the discovery of macrophages and the proposal of phagocytes and phagocytosis (Metchnikov 1939).

Although WBCs account for only 1% of blood, they are important components of the blood system and their impact is significant. Along with WBC, the peripheral blood consists of multiple cell types, including red blood cells (erythrocytes), platelets, as well as a liquid component known as plasma (Figure 1.3). All WBCs are derived from multipotent cells in the bone marrow (Figure 1.4), known as hematopoietic stem cells, and are stored in the blood and lymphatic tissues. Also called leukocytes, WBCs are cells of the immune system and are essential in the defence against infectious diseases and foreign invaders (Cavenagh 2007).

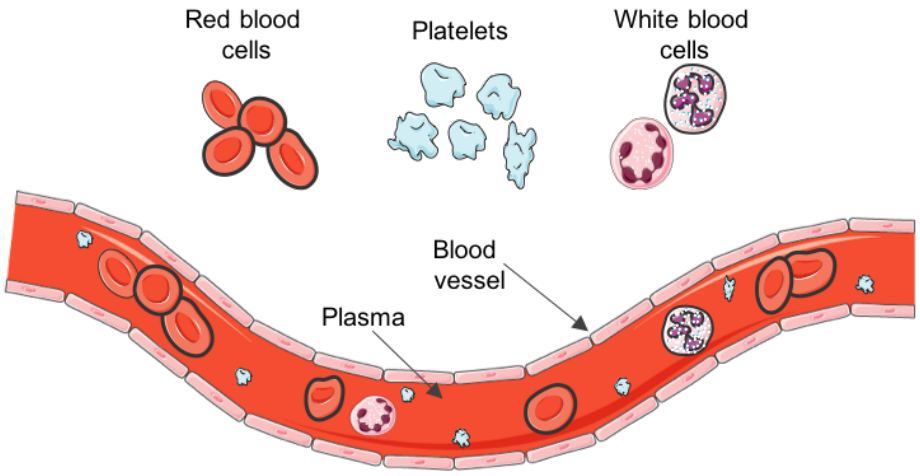


Figure 1.3 White blood cells are an important component of the blood system, which is also made up of red blood cells, platelets, and plasma.

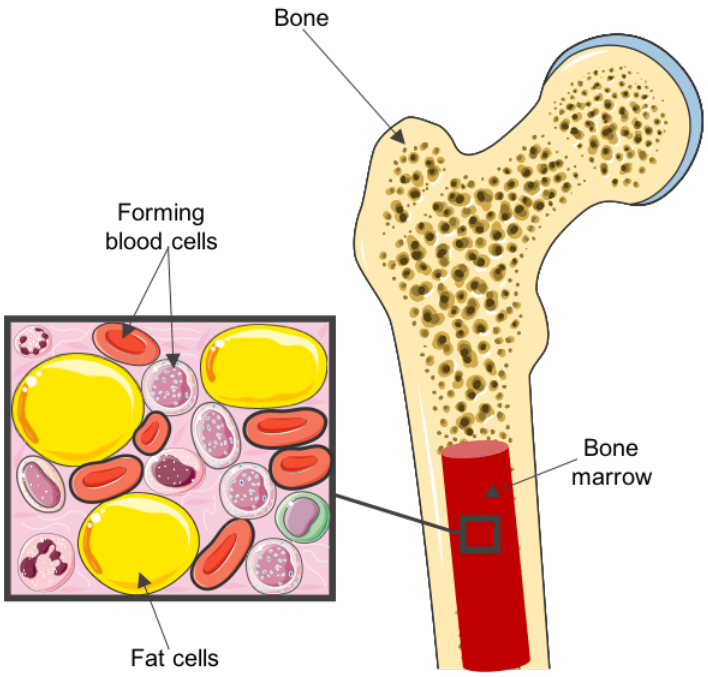


Figure 1.4 White blood cells are made inside the bone marrow and are stored in the blood and lymphatic tissues. As some white blood cells have a short lifespan of one to three days, the bone marrow is constantly generating them. Figure drawn using Servier Medical Art.

1.1.4 Phagocytes

Phagocytes (from the ancient Greek – *phagein*, meaning 'to devour' and *kytos*, meaning 'cell'), are a class of cells that help protect the host by engulfing any potentially dangerous cell debris and foreign microorganisms. Phagocytosis (from the ancient Greek *phagein*, *kytos* and *osis* meaning 'process'), is the mechanism by which, namely phagocytes, internalise foreign invaders of the body within compartments known as phagosomes and proceed to degrade them (Metchnikov 1905; 1939; Silva & Correia-Neves 2012). Other lower organisms also utilise phagocytosis mainly for acquisition of nutrients (Silva & Correia-Neves 2012).

Neutrophils and macrophages are leukocytes that are collectively known as phagocytic cells. Vertebrates have well established phagocytic cells, which are crucial to their innate immune response to invading pathogens. They also represent the two innate immune

cell types closely linked with the extreme inflammatory response characterising severe sepsis (Strassheim et al. 2002).

1.1.4.1 Neutrophils

Neutrophils, also known as polymorphonuclear neutrophils (PMN) due to their multi-shaped nuclei, are the most abundant cell type contributing approximately 40-80% of the WBCs in circulation (Stearns-Kurosawa et al. 2011). They are continuously generated in the bone marrow from the myeloid hematopoietic system (Kolaczkowska & Kubes 2013), and are produced at around 10^8 cells/minute (Prince et al. 2011). Neutrophils are short-lived, terminally differentiated cells with a circulating half-life of 6-8 hours, hence their rapid rate of production (Summers et al. 2010). They are also the first line of defence against infection during innate immunity, often being the first leukocytes to move towards inflammatory lesions to perform host defence mechanisms, including production of reactive oxygen species (ROS), phagocytosis and neutrophil extracellular traps (NETs) (Waksman et al. 1990; Clark et al. 2007). When activated by a microbial or inflammatory stimulus, such as various cytokines and growth factors as well as bacterial products, their longevity increases by several fold ensuring the presence of primed neutrophils at the site of inflammation (Summers et al. 2010; Kolaczkowska & Kubes 2013).

Although neutrophil production is constitutive during homeostasis, an increased neutrophil response is often essential for host survival (Huo et al. 2017). During SIRS and severe sepsis, for example, there can be up to a 10-fold increase in the production of these immune effector cells in the bone marrow, and their recruitment into the host circulation (Drifte et al. 2013).

1.1.4.2 Macrophages

Monocytes circulate in the blood, bone marrow and spleen (Geissmann et al. 2010). Under normal conditions circulating blood monocytes are short-lived, undergoing spontaneous apoptosis on a daily basis (Parihar et al. 2010). Upon stimulation, for example through infection, monocytes are rapidly recruited from the blood to the tissue where they differentiate into tissue macrophages (Daigneault et al. 2010). Once differentiated these long-lived cells (life-span ranging several days to over a week) have considerable plasticity, whereby the environmental cues they receive dictate their functional phenotype (Figure 1.5) (Daigneault et al. 2010; Leavy 2011). For example, when in a proinflammatory environment, macrophages are driven towards a classically activated M1 state, characterised by the production of pro-inflammatory cytokines (such as TNF-α, IL-1β, IL-6, IL-8) and are modulators of cellular behaviour. Pro-inflammatory cytokines can directly or indirectly regulate inflammatory reactions through adhesion molecules or other cytokines (Anderstam et al. 2012). When in the presence of specific regulatory/anti-inflammatory cytokines (including interleukin-4 (IL-4), interleukin-13 (IL-13)) they are termed alternatively activated M2 macrophages, which can be further subdivided to accommodate similarities and differences between IL-4 (M2a), immune complex + toll like receptor (TLR) ligands (M2b) and interleukin-10 (IL-10) (M2c) (Martinez & Gordon 2014; Genin et al. 2015). Macrophages not only contribute to innate immune responses but they also form the bridge into adaptive immunity via antigen processing (Daigneault et al. 2010).

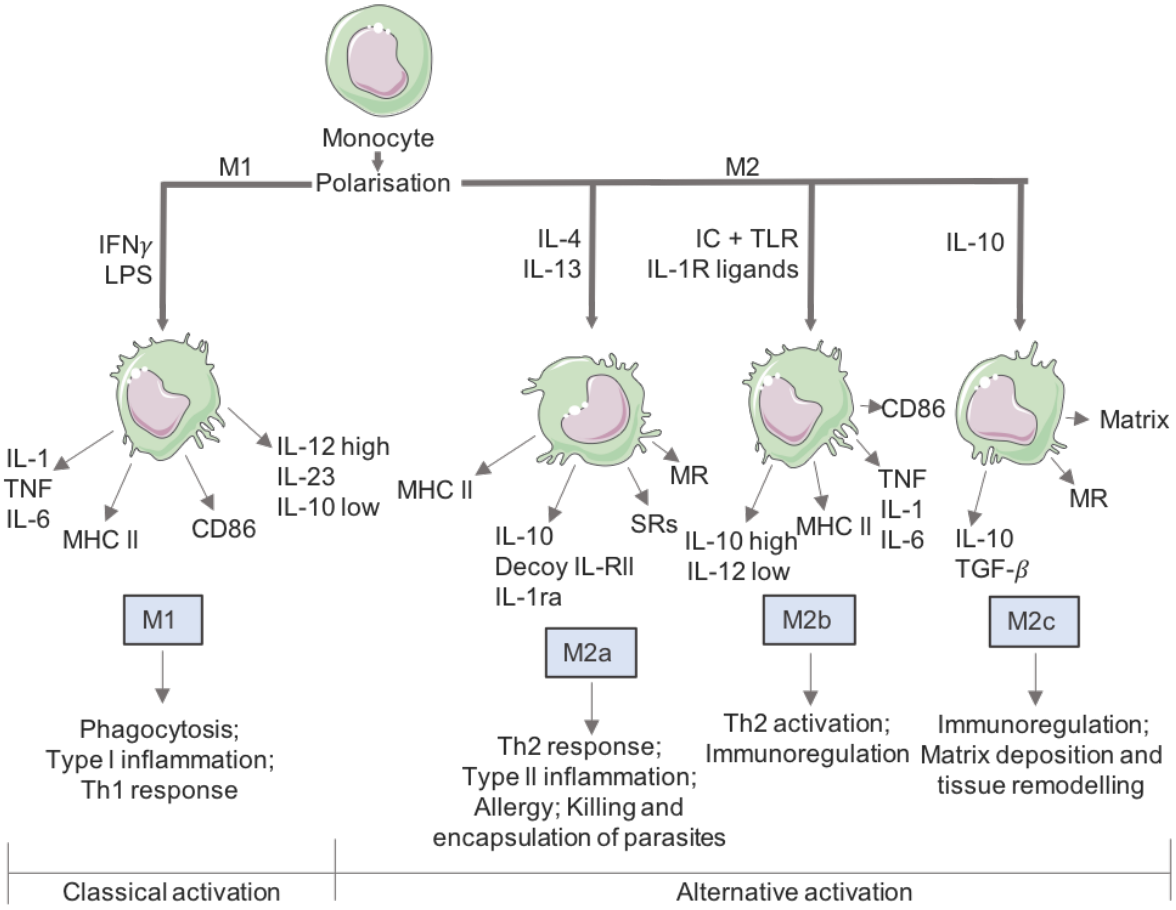


Figure 1.5 Macrophage polarisation. Micro-environment stimuli define macrophage polarisation via classical activation (M1) or alternative activation (M2). Pathogen-derived LPS alone or in combination with IFN-γ leads to classical activation of M1 macrophages, which improves microbicidal activity and secretion of pro-inflammatory mediators. Alternative macrophage activation could be subdivided into three subpopulations. M2a differentiation is promoted by IL-4 or IL-13, and this subpopulation is associated with Th2 response, allergy process, internalisation, and parasite killing. M2b is related to the presence of immune complexes (IC), TLR or IL-1R agonists, and promotion of immunoregulation. IL-10 secretion lead to differentiation into M2c, which also induces immunoregulation, tissue remodelling, and repair. SR, scavenger receptors; MR, mannose receptors. Figure adapted from Martinez and Gordon, 2014; drawn using Servier Medical Art.

1.1.4.3 Neutrophil and macrophage infiltration

Inflammatory leukocytes, including neutrophils and macrophages, are equipped with surface receptors including a specific family of pattern recognition receptors (PRRs) called toll-like receptors (TLRs) (Yang et al. 2014). These transmembrane receptors recognise pathogen-associated molecular patterns (PAMPs), which are macromolecular motives from microorganisms (Schulte et al. 2013). Examples of PAMPs include lipopolysaccharide (LPS), which is only present in Gram-negative bacteria and a TLR4 agonist, flagellin, which is the major component of the bacterial flagellar filament and a TLR5 agonist, and bacterial lipoproteins such as pam3CSK4 and FSL-1, which are TLR1/2 and TLR6/2 agonists, respectively (Table 1.1) (Schulte et al. 2013). Binding of the PAMPs to the cell surface TLRs initiates a signal transduction cascade, leading to the activation of nuclear factor-κB (NF-κB) and subsequent translocation into the nucleus inducing cytokine, chemokine, nitric oxide and lipid mediator synthesis (Clark et al. 2011; Schulte et al. 2013). In neutrophils and macrophages these mechanisms of defence, including the synthesis of a range of effector cytokines and chemokines, such as tumour necrosis factor (TNF) and interleukin-1, are major contributors to the pathophysiological change in infectious and inflammatory diseases including septic shock (Cohen 2002).

Neutrophils and macrophages also biosynthesise an array of lipid mediators of inflammation derived from fatty acids including arachidonic acid (AA), known as eicosanoids and also perform other host defence mechanisms including phagocytosis, generation of oxygen-derived free radicals and the release of proteolytic enzyme (St-Onge et al. 2007).

PAMPs are recognised by pattern recognition receptors. Bacterial proteins are *N*-formylated, and the formylated peptides activate chemoattractant formylated-peptide receptors (FPRs) (Zhang et al. 2010). These bacterial stimuli activate neutrophils through the formylated peptide receptor-1 (FPR-1) on their surface, subsequently mediating an

inflammatory response through the release of the immune modulators MMP-8 and IL-8 and phosphorylation of several MAP kinase enzymes (Calfee & Matthay 2010). The principle ligands for FPR1 are bacterial and mitochondrial formylated peptides, which are actively secreted by invading pathogens or passively released from apoptotic host cells after tissue injury (Dorward et al. 2015). As mentioned, TLRs respond to many PAMPs including the bacterial CpG DNA that stimulates TLR9. Mitochondria provide a link between internal and external triggers of the innate immune response, where mitochondrial DNA and bacterial DNA share many similar structural motifs (Calfee & Matthay 2010). The mitochondrial genome (mtDNA) contains the CpG DNA repeats and codes for formylated peptides because they have evolved from saprophytic bacteria to endosymbionts to organelles (Zhang et al. 2010; Taanman et al. 1999). The mitochondrial formyl peptides act as neutrophil chemoattractants and activators of related cell lines (Rabiet et al. 2005). Formylated peptides, such as the synthetic peptide N-formylmethionyl-leucyl-phenylalanin (fMLP, also known as fMLF), stimulates a bacterial challenge through G-protein-coupled receptors FPR-1 and FPRL-1, with high and low affinity, respectively (Zhang et al. 2010). Alternative neutrophil activating agents include LTB_4, PAF, C5a and IL-8.

Activation of neutrophils by G-protein-coupled receptors causes an increased intracellular calcium concentration ($[Ca^{2+}]_i$) (Hauser et al. 2000), heterologous and homologous G-protein-coupled receptor desensitisation (Tarlowe et al. 2003) and activates MAP kinases (MAPKs) (West et al. 2007). Mitochondrial damage associated molecular patterns from human myocytes induce human neutrophil $[Ca^{2+}]_i$ fluexes equal to 1 nM fMLP (Zhang et al. 2010). During infection, pathogens target and destroy host tissue with the synchronised released of both bacterial- and host-derived formylated peptides, therefore connecting FPR1 to both the infective and sterile inflammatory processes (Dorward et al. 2015).

Table 1.1 Toll-like receptors and their pathogen derived activators (Bauer 2008).

Pathogen-associated molecular pattern (PAMP)	Pattern recognition receptor (PRR)	Pathogen
Pam3CSK, FSL, Zymosan.	TLR1,2,6	Gram-positive bacteria (e.g *S. aureus*)
LPS, Lipid A	TLR4	Gram-negative bacteria (e.g. *E. coli*)
Flagellin	TLR5	Bacteria, Flagellum
dsRNS	TLR3	Virus
ssRNA	TLR7,8	Virus
CpG DNA	TLR9	Bacteria, DNA

1.2 Therapeutic agents

Antimicrobial resistance is defined as the ability of an organism to resist the action of an antimicrobial agent to which it was previously susceptible (Beceiro et al. 2013). Antibiotic resistance is an ever-increasing problem, and one of the greatest healthcare challenges currently faced, particularly within the intensive care unit (ICU) patient cohort (Fullerton et al. 2014). There are a diminishing number of antimicrobial agents that remain effective against common pathogens, both Gram-positive (primarily MRSA) and Gram-negative (*Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*) (Harbarth et al. 2003). The World Health Organization stated that, "Antimicrobial resistance...threatens the effective prevention and treatment of an ever-increasing range of infections caused by bacteria.... A post-antibiotic era—in which common infections and minor injuries can kill—far from being an apocalyptic fantasy, is instead a very real possibility for the 21st Century." (WHO 2014).

Antibiotics are available to effectively inhibit bacterial cell wall synthesis, protein synthesis and DNA replication, which include penicillins, tetracyclines and rifampicin (Neu 1992). The microbes that were once susceptible are becoming even more difficult to combat as treatment is compromised by the development of antibiotic-resistant strains of microbial pathogens (Mazel & Davies 2016). A variety of biochemical processes are involved to enable bacterial resistance to antibiotics, these include chromosomal mutations, inductive expression of latent chromosomal genes or acquisition of genetic material through transformation (exchange of DNA), transduction (bacteriophage), or conjugation by plasmids (extrachromosomal DNA) (Mazel & Davies 2016; Neu 1992).

Administration of antibiotics, often to a presumed pathogen, is the single most important life-saving measure in diseases such as bacterial meningitis and sepsis, subsequently followed by supportive care (Brouwer et al. 2010; Hutchins et al. 2014). It is well understood that rapid, appropriate antibiotic treatment is paramount and any delay can reduce survival (Garnacho-Montero et al. 2006; Ferrer et al. 2009). A range of studies carried out on infected and septic patients showed that inappropriate antimicrobial therapy was a constant predictor of poor outcomes (Harbarth et al. 2003; Garnacho-Montero et al. 2003). One retrospective review carried out between 1989 and 2004 of 2,700 Canadian patients admitted with septic shock found that 50% received effective antibiotic treatment within six hours of the onset of hypotension (Kumar et al. 2006). Patient survival rate has been associated with nearly a 12% reduction for each hour antibiotic administration was delayed, after the onset of shock (Kumar et al. 2006). A more recent analysis in 2014 from 18,000 patients admitted with septic shock or severe sepsis from 165 ICUs across Europe, USA and South America, found a correlation in steadily increasing hospital mortality as the delay in antibiotic administration increased (Ferrer et al. 2014).

The incidence of urinary tract infections (UTIs), skin and soft tissue infections, pneumonia and sepsis are amongst conditions increased due to the changing

epidemiology of bacterial infections (Robson & Daniels 2008). It is also due to an increasingly older population displaying multiple comorbidities, in turn leading to an increase in hospital-acquired infections (through the use of catheterisation and cannulation). Alongside this, is also antibiotic resistance, due to the over-prescribing of antibiotics or patient misuse (Dellinger et al. 2013; Greenhow et al. 2014).

From this, it is evident that there is an ever-increasing need for the development of assays that are rapid, sensitive and specific for the identification of bloodstream pathogens, which will in turn allow for timely, appropriate and accurate antimicrobial therapy (Choi et al. 2013).

1.3 Bacterial pathogens and innate immunity

The human body contains bacteria both inside and on the surface, especially on the skin and the mucous membranes (Kendall & Nicolaou 2013). Many of these bacteria are innocuous, several are beneficial, and some are even necessary. However, other bacteria, which are considered as pathogens, are able to colonise, invade and damage the host and thus cause illness. Pathogenicity describes the ability of an agent to cause disease, and the pathogenic bacteria possess several factors that enable them to enhance their virulence (i.e., the degree of pathogenicity) (Beceiro et al. 2013). It is common for most pathogens to make use of a combination of two properties to cause disease: i) toxicity, the degree to which a substance causes harm, and ii) invasiveness, the ability to penetrate into the host and spread (Madigan et al. 2009). The final consideration that forms the balance of an infectious disease process will depend on the virulence or pathogenicity of the microbe alongside the host status (in relation to risk factors such as immune status, age, diet and stress), which determines the host susceptibility to infection (Beceiro et al. 2013).

The human host and bacteria have co-evolved over millions of years, during which pathogenic bacteria have altered their virulence to adapt to host defence systems (Beceiro et al. 2013). This contrasts with the relatively recent evolution and spread of antimicrobial resistance, which has occurred mainly in the last 50 years since antibiotics were first used. However, despite these differences, timescales of evolution of these processes share some common characteristics, such as the ability to survive under adverse conditions (host defence systems and antimicrobial therapies), the transfer of virulence and resistance factors between species by gene transfer, and the fact that antibiotic resistance is often associated with infection and therefore is also related to virulence (Akira et al. 2006; Beceiro et al. 2013). Other characteristics that are common in both virulence and resistance include the direct involvement of porins (outer membrane protein channels, which allow diffusion of hydrophilic molecules) (Tsai et al. 2011), cell wall alterations (Moya et al. 2008), and two-component systems that activate or repress the expression of various genes (Yeung et al. 2011).

Opportunistic pathogens are not able to produce infection in healthy individuals as they lack the necessary mechanisms of toxicity and invasiveness that enable primary pathogens to overcome the host immune system (Beceiro et al. 2013). However, in some cases such as individuals who are immunocompromised, have indwelling catheters, contaminated implants, hip replacements, opportunistic pathogens can produce infection, which can be prevented mainly with use of antimicrobial therapies.

1.3.1 Toll-like receptors as pattern-recognition receptors

Vertebrates are constantly threatened by the invasion of microorganisms. The host has evolved systems of immune defence to eliminate infecting pathogens in the body. As previously stated, the initial innate immune system recognises signature molecules of pathogens via PRRs. To date, several classes of PRRs including, TLRs, retinoic acid-inducible gene (RIG)-l-like receptors and nucleotide-binding oligomerization domain

(NOD)-like receptor (NLRs) have been identified. PRRs possess common characteristics, which are i) recognition of microbial components known as PAMPs, ii) constitutive expression of PRRs in the host and detection of the pathogen regardless of the life-cycle stage, and iii) PRRs are germline-encoded, nonclonal, expressed on all cells of a given type and independent of immunological memory (Akira et al. 2006).

The study of innate immunity in mammals amplified after the discovery of Toll and the role of this protein in the innate immune defence of *Drosophila melanogaster* (Philpott & Girardin 2004). It was initially identified as a gene product essential for the embryonic development of the fly (Anderson et al. 1985), before later being shown to play a critical role in the antifungal response of flies (Lemaitre et al. 1996) and then Gram-positive bacterial infections (Lemaitre et al. 1997). The first discovery of Toll-related proteins in mammals was in 1998 (Rock et al. 1998) which was rapidly followed by the demonstration that the mammalian TLR4 was the long-sought after signalling receptor in a protein complex responsible for recognition of LPS and cellular responses leading to this event (Poltorak et al. 1998).

TLRs are transmembrane proteins with an extracellular domain which consists of leucine-rich repeats (LRR) and a cytoplasmic domain called the Toll/IL-1 receptor or TIR domain (Figure 1.6)(Kumar et al. 2009). Stimulation of the TLR ligand leads to the recruitment of adaptor proteins containing the TIR domain, such as myeloid differentiation factor 88 (MyD88), to the cytoplasmic portions of the TLRs through

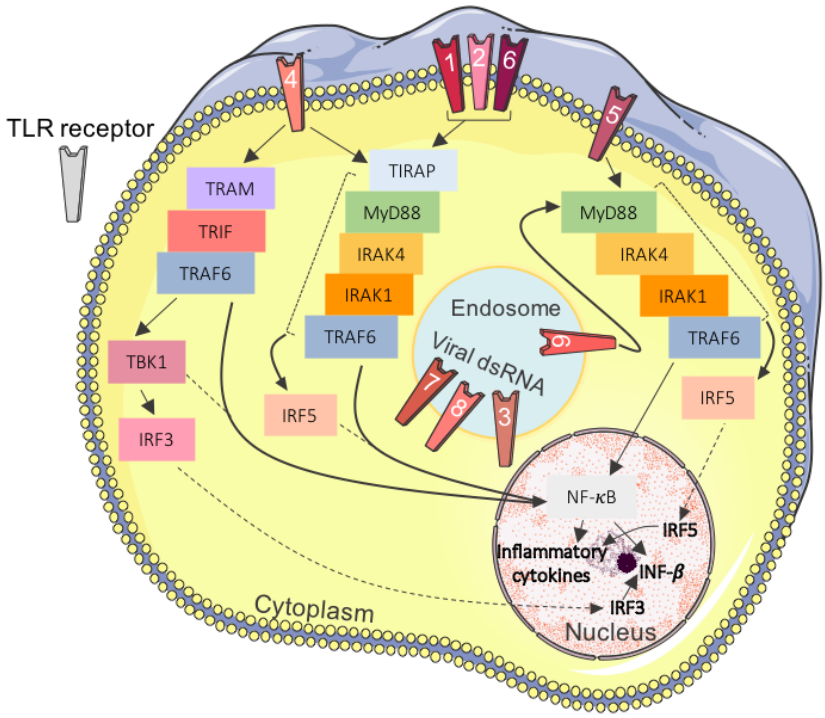


Figure 1.6 Schematic representation of bacterial toll-like receptor (TLR) signalling pathways. All TLRs apart from TLR3 (viral receptor) share the MyD88-dependant pathway that activates NF-κB and sequentially induces genes encoding inflammatory cytokines. (Pathway adapted from Merly and Smith., 2005; drawn using Servier Medical Art).

homophilic interaction of their TIR domains (Akira et al. 2006). This triggers a downstream signalling cascade and drives the expression of many genes, many of which are involved in the proinflammatory response (Philpott & Girardin 2004).

1.3.2 Bacterial recognition by TLRs

In January 2018, the world health organisation (WHO) reported the most common antibiotic-resistant bacteria were *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus* and *Streptococcus pneumoniae*, followed by *Salmonella* species (spp). Bacteria can be classified into two distinct groups according to the different staining characteristics of their cell walls, namely Gram-positive and -negative bacteria (Figure 1.7). The names come from the Danish physician Hans Christian Gram, who

developed the staining technique (Claus 1992). Gram staining involves three processes, staining the pathogen with a water-soluble dye called crystal violet followed by decolourisation and counterstaining usually with safranin. Due to differences in the thickness of the peptidoglycan layer in the cell membrane between Gram-positive and -negative bacteria, Gram-positive bacteria (with a thicker peptidoglycan layer) retain the crystal violet stain during the decolourisation process; which uses organic solvents such as ethanol or acetone as the decolouriser. While the Gram-negative bacteria lose the crystal violet stain and are instead stained red by the counterstain safranin in the final staining process. Since safranin is lighter than crystal violet, it does not disturb the purple colour in Gram-positive cells.

It is some of these unique cell wall components that act as PAMPs, which are recognised by individual TLRs and stimulate the immune cells. LPS, also known as endotoxin, is generally perceived as the most potent immunostimulant among these cell-wall components (Akira et al. 2006). The structure of LPS molecules vary in their phosphate patterns, numbers of acyl chains and fatty-acid composition within different bacteria (Akira et al. 2006). Lipid A is a lipid segment of LPS that is responsible for most of the pathogenic events associated with Gram-negative bacterial infections such as endotoxic shock. LPS from Gram-negative bacteria associates with LPS binding protein (LBP), present in the bloodstream, before subsequently binding to CD14, a glycosylphosphatidylinositol (GPI-linked protein expressed on the surface of phagocytes (Akira et al. 2006). LPS is then transferred to MD-2, which is associated with the extracellular portion of TLR4, followed by oligomerisation of TLR4 (Poltorak et al. 1998; Shimazu et al. 1999).

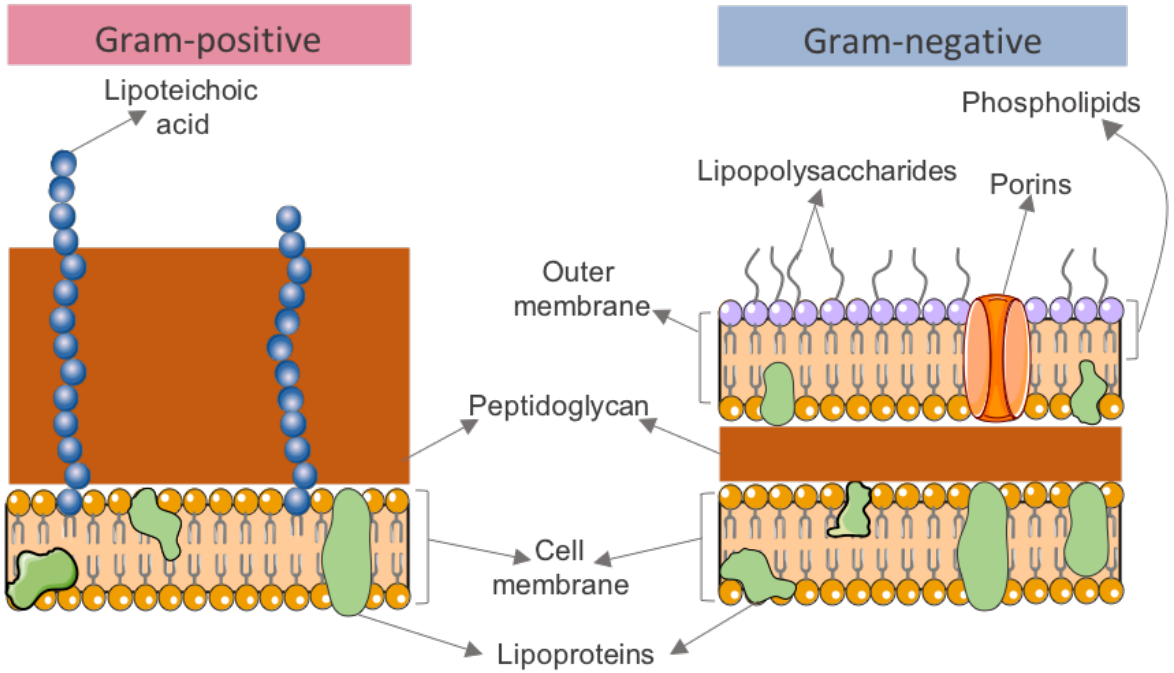


Figure 1.7 Schematic representation of bacterial Gram-positive and Gram-negative cell walls. Gram-positive bacteria (e.g. MRSA) have a thick layer of peptidoglycan. Lipoteichoic acids and lipoproteins are embedded in this cell wall. The cell wall of Gram-negative bacteria (e.g. *E. coli* and *Klebsiella*) is characterized by the presence of LPS. Figure adapted from Akira et al., 2006; drawn using Servier Medical Art.

Lipoproteins and peptidoglycan (PTG) are potent immunostimulants present in both Gram-positive and -negative bacteria. The Gram-positive bacterial cell wall contains components that also stimulate the innate immune system. These bacteria do not contain LPS, however, they do have lipoteichoic acid (LTA) which appears to function in a similar manner as an immune activator (Merly & Smith 2005). TLR2 plays a major role in Gram-positive bacterial detection and is involved in the recognition of microbial components including lipoproteins, peptidoglycan and LTA (Merly & Smith 2005). TLR2 appears to interact alongside TLR1 and TLR6 to form a collaboration, which discriminates between subtle changes in the lipid portions of the lipoproteins (Merly & Smith 2005; Akira et al. 2006).

Another potent activator of the innate immune response is flagellin, the major protein constituent of the bacteria flagella, the motility apparatus used by both Gram-positive and -negative microbial pathogens (Hayashi et al. 2001). TLR5 is the receptor responsible for the detection of flagellin and specifically recognises the constant domain D1, which is comparatively conserved amongst different species (Hayashi et al. 2001).

TLR9 regulates the recognition of the unmethylated 2'-deoxyribo (cytidine-phosphate-guanosine) (CpG) DNA motifs found in bacteria and viruses (Krieg 2002). The methylated CpG motif does not activate mammalian innate immune cells (Akira et al. 2006). CpG-DNA displays potent immunostimulatory activities including induction of inflammatory cytokines and Th1 immune responses (Akira et al. 2006). The particular DNA sequence that provokes an immune response varies between species (Merly & Smith 2005). As TLR9 resides in the endosome, the bacterial DNA must be transported to this intracellular compartment where the acidic and reducing conditions lead to the degradation of the double-stranded DNA into multiple single-stranded CpG-motif-containing regions that subsequently interact with TLR9 (Latz et al. 2004; Ahmad-Nejad et al. 2002).

1.4 Eicosanoids

The term 'eicosa' (meaning twenty in Greek) represents the number of carbon atoms in arachidonic acid (Cook 2005). Eicosanoids, including prostaglandins (PGs) and leukotrienes (LTs) are a family of lipid signalling molecules generated by all cutaneous cell types and contributing to homeostatic processes and inflammatory responses associated with injury, allergy, infection and other acute or chronic conditions (Harizi et al. 2008; Nicolaou 2013). The major precursor of these oxygenated compounds is the fatty acid derivative arachidonic acid (AA) (Cook 2005). Eicosanoids are primarily generated through an oxidative pathway from AA, but are also known to be produced through pathways from eicosapentaenoic and dihomo-γ-linolenic acids (Levin et al. 2002; Wada et al. 2007).

Innate immune cells including tissue macrophages, sentinel dendritic cells and neutrophils are major producers of local eicosanoids, exerting their effect in both an autocrine and paracrine fashion (Cook 2005; St-Onge et al. 2007). There is increasing evidence to suggest that eicosanoids collaborate with other signalling mediators, particularly cytokines and chemokines, playing a vital role in the modulation of physiological processes in both homeostatic and inflammatory conditions (Cook 2005; Stafford & Marnett 2008).

The biosynthesis of eicosanoids is dependent upon the availability of free AA (Figure 1.8) (Harizi et al. 2008). Tissue exposure to physiological and pathological stimuli, for example growth factors or cytokines, causes the release of AA from the phospholipid cell membrane, in a Ca^{2+}-dependant manner; catalysed by phospholipase A_2 (PLA_2) and it is subsequently converted into different eicosanoids (Harizi et al. 2008; St-Onge et al. 2007). There are three main pathways that enzymatically metabolise AA;

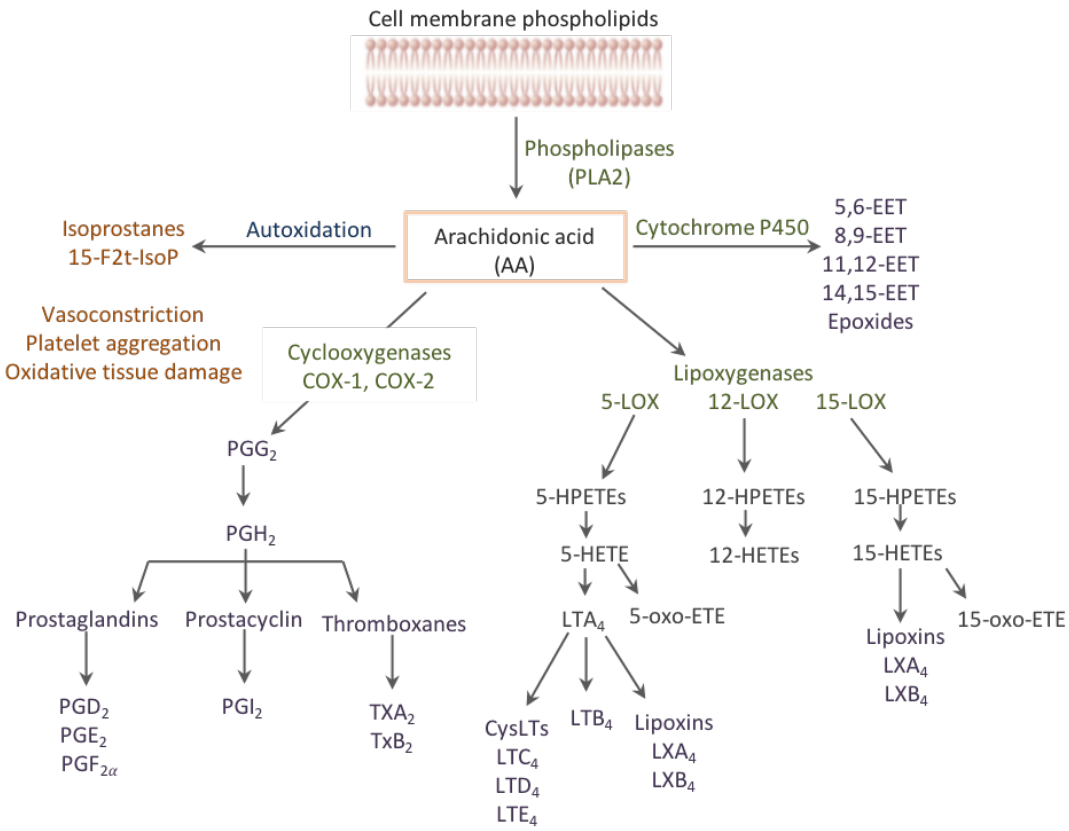


Figure 1.8. Summary of eicosanoid biosynthesis pathways from arachidonic acid (AA). In response to stimuli, such as cytokines, stress and hormones, AA is released from the phospholipid membrane by phospholipase enzymes (especially cytosolic phospholipase A_2, $cPLA_2$). The free AA is then converted into biologically active eicosanoids through cyclooxygenase (COX), lipoxygenase (LOX) and cytochrome P450 enzymatic pathways, and also non-enzymatic autoxidation. COX isozymes (COX-1 and COX-2) catalyse the formation of products collectively known as prostanoids. LOX enzymes (5-LOX, 12-LOX, 15-LOX) catalyse the formation of Leukotrienes, HETEs and Lipoxins. The cytochrome P450 epoxygenase pathway catalyses the formation of EETs and epoxins. Non-enzymatic autoxidation, leads to the production of isoprostanes. Diagram adapted and reproduced from Hammond and O'Donnell 2012

cyclooxygenase (COXs), lipoxygenase (LOXs) and cytochrome P450 epoxygenase (Dennis & Norris 2015).

Reactive oxygen species are generated under oxidative stress due to an imbalance between pro-oxidant and antioxidant states. Free-radical-induced peroxidation of AA, leads to the non-enzymatic formation of a family of prostaglandin-like compounds called isoprostanes (IsoPs) (Comporti et al. 2008). Once formed, they are cleaved by phospholipases and free IsoPs are released from tissues into circulation, where they are partially metabolised (Comporti et al. 2008). In general, IsoPs are considered as 'gold standard' biomarkers for oxidative stress and, in particular, lipid peroxidation (Comporti et al. 2008). IsoPs are mainly excreted in urine; several studies have shown that levels of IsoPs increase in certain disorders or experimental conditions such as Alzheimer's disease and chronic obstructive pulmonary disease (Helmersson et al. 2009; Praticò et al. 2002).

The COX pathway exists in two isoforms, COX-1 (encoded by a constitutively expressed gene) and COX-2 (encoded by an immediate early response gene), and responsible for the production of prostaglandins (PGs), prostacyclin and thromboxanes (TXs) (Chang et al. 2005). The cytochrome P450 epoxygenase pathway produces hydroxyeicosatetraenoic acid (HETEs) and epoxides (Harizi et al. 2008). There are several LOX enzymes (5-LOX, 12-LOX, 15-LOX) that convert AA into diverse hydroperoxyeicosatetraenoic acids (HPETEs) and HETEs (Harizi et al. 2008). Cells are highly selective as to the specific eicosanoids that they synthesise, however, the amounts generated are altered by the activation state and the physiological conditions of the specific tissues in which they reside (Dennis & Norris 2015).

1.4.1 LOX products

LOX are the main enzymes that generate lipid signalling molecules (Massey & Nicolaou 2013). The first step of leukotriene (LT) biosynthesis involves the conversion of AA into HETEs by LOX enzymes (Harizi & Gualde 2005). Activation of 5-LOX, in neutrophils, can subsequently lead to the conversion of 5-HETE into leukotriene LTA_4 which is a precursor of LTB_4, cysteinyl-LTs (CysLTs); including LTC_4, LTD_4 and LTE_4, and lipoxins (LXs) (Bäck 2007). The numbers denoted on the enzymes are derived by the number of the carbon on which the alcohol group is attached to the molecule; the products are named in the same way. 5-LOX, 12-LOX and 15-LOX convert AA into 5-HETEs, 12-HETEs and 15-HETEs, respectively (Figure 1.9a). Two separate classes of LTs are formed based on structural differences; firstly, cysLTs form one group based on the cysteine residue in the structure, secondly, LTB_4 contains two hydroxyl groups instead of amino acids (Brink 2007). 5-LOX is expressed by neutrophils and mast cells, 12-LOX is expressed in monocytes and platelets and 15-LOX is expressed by macrophages under certain circumstances and eosinophils (Clark et al. 2011; Alanne-Kinnunen et al. 2014).

Leukotrienes are potent mediators of inflammation and can initiate, amplify or dampen inflammatory responses (Bäck 2007). Generally, during inflammation eicosanoids will be present and the LOX products act as modifiers of vascular permeability (LTs), chemoattractants (LTB_4), and induce smooth muscle cell proliferation (CysLTs) (Parameswaran et al. 2002, Bäck 2007). Lipoxins (LXs) play an active role in promoting resolution of inflammation whereby they control entry of neutrophils to sites of inflammation (Schwab & Serhan 2006).

The biological effects of LTB_4 are primarily targeted towards inflammatory cells. LTB_4 is a potent chemotactic agent for neutrophils and macrophages. With neutrophils, LTB_4 also increases the production of toxic oxygen species and release of granule enzymes and up-regulates membrane adhesion molecule expression (T Lämmermann et al 2013).

LTB_4 stimulates action and cytokine release; on macrophages and lymphocytes, and is also found in inflammatory exudate and tissue (S Kaku et al 2001).

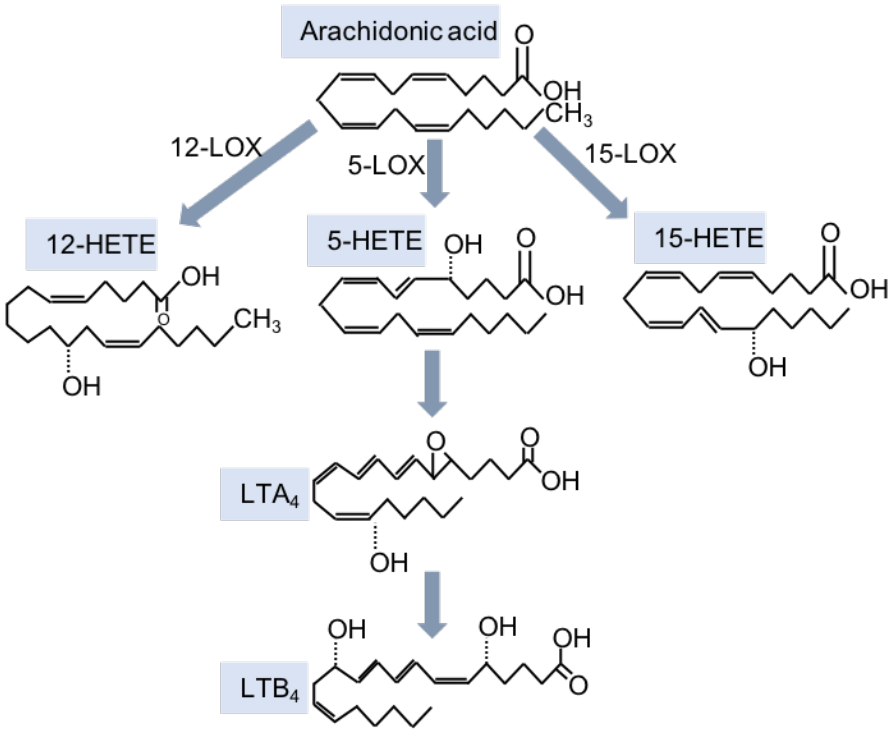


Figure 1.9a Selected eicosanoid structures in the lipoxygenase (LOX) pathway.

CysLTs are mediators associated with allergic inflammation; they are potent bronchoconstrictors, powerful inducers of vascular leakage and are important inflammatory mediators for asthma (Okunishi et al. 2004). Upon exposure to a specific allergen, LTC_4, LTD_4 and LTE_4 are released from the lung tissues of an asthmatic subject, which would suggest a pathophysiological role in immediate hypersensitivity reactions (Samuelsson et al. 1987). Specifically, LTC_4 and LTD_4 have been shown to be potent inducers of airway obstruction (Holroyde et al 1981). While LTC_4 and LTD_4 are very short lived in *vivo*, LTE_4 is stable and the only cysLT excreted in the urine (Drazen et al 1992).

1.4.2 COX products

PGs are a group of structurally related molecules that are generated by cells in response to stimuli, and regulate cellular growth, differentiation and homeostasis (Figure 1.9b) (Shibata et al. 2002). PGs are derived from fatty acids, namely AA, which is firstly converted into an unstable endoperoxide intermediate by cyclooxygenases and subsequently converted into one of several related products through the action of specific PG synthetases, these products include PGD_2, PGE_2, $PGF_{2\alpha}$, prostacyclin (PGIs) and thromboxane A_2 (Shibata et al. 2002; Dennis & Norris 2015). PGs are physiologically present in body fluids, however, arachidonate metabolism is highly increased under several pathological conditions, including hyperthermia, infection and inflammation (Shibata et al. 2002).

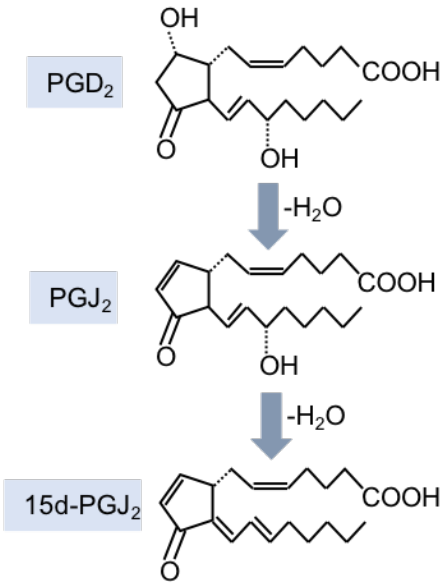


Figure 1.9b The PGD_2 metabolic pathway

1.4.3 Measuring eicosanoids

The quantification of eicosanoids must be carefully characterised, as they are chemically and biologically complex lipid mediators. There is a marked interest in the development of high-throughput analytical methods for targeted determination of eicosanoid panels for clinical use (Ferreiro-Vera et al. 2011; Dennis & Norris 2015). Quantitative analysis of eicosanoids in biological samples calls for detection techniques that should be highly accurate, sensitive and specific. There are several approaches that can be used to determine these mediators, including immunoassays (ELISA), high-performance liquid chromatography-ultraviolet detection (HPLC-UV) (trienes e.g. leukotrienes (235nm) and dienes e.g HETE (270nm) absorb at different UV maxima due to different chromophores), Gas chromatography-tandem mass spectrometry (GC-MS/MS) and HPLC-MS/MS. ELISA is a plating technique that passively binds antibodies. However, eicosanoids are lipids that are poorly immunogenic and so these ELISAs are not as sensitive or specific as ELISAs for proteins such as cytokines (Morgan et al. 2010). The use of these immunoassays also carries a degree of immunological cross-reactivity among commercially available eicosanoid antibodies (Ferreiro-Vera et al. 2011). To overcome such problems, techniques such as solid-liquid transfer to an organic solvent for tissue and liquid-liquid or solid phase extraction (SPE) for biofluids can be used to isolate eicosanoids from proteins. Additionally, a chromatographic step would be required for individual separation of eicosanoids to avoid immunological cross-reactivity (Ferreiro-Vera et al. 2011).

Lipidomics-based assays can be described as being ‘targeted’, where lipids to be monitored are known before the analysis. An example of a targeted assay is the multiple reaction monitoring (MRM) method described below. The shotgun approach is a lipidomics-based assay that is described as ‘untargeted’ and is a more exploratory, qualitative assessment of the lipids (Harkewicz & Dennis 2011). A mass spectral full scan will search new mass-to-charge ratio peaks, often used when hoping to detect novel lipid species.

HPLC with UV detection is mostly useful when dealing with a relatively large amount of eicosanoids that are present in a clean biological sample (containing conjugated doubles bonds, such as mono-, di-, or tri-hydroxy lipids from the LOX pathway) (Maskrey & O'Donnell 2008). Also, distinguishing isomers such as 5- and 15-HETE with UV is difficult. However, very specific separation can be achieved using HPLC to separate on the basis of retention time followed by MS analysis of the analyte, therefore, HPLC-MS/MS is a highly-sensitive technique (Maskrey & O'Donnell 2008). Coupling liquid chromatography to the mass spectrometer and employing MRM mode (see Figure 1.10 for detailed schematic of MRM), is highly selective and highly sensitive. If a specific molecule is eluting from the chromatographic column at any time during the analysis, its specified MRM pair can be detected and recorded (Harkewicz & Dennis 2011). The development of complex methods have shown that hundreds of lipid mediators, such as eicosanoids, can be monitored through their defined MRM pairs in a single analysis lasting only 20 min (Harkewicz & Dennis 2011). The ever-expanding field of lipidomics has provided fertile ground to discover and characterise novel lipid species (Clark et al. 2011), and mass spectrometry will continue to play a vital role in this area.

1.5 Cytokines

Cytokines are intracellular signalling proteins (defined as >0.5kDa and <30kDa) essential for the activation, differentiation and control of the immune system (Hiscott & Ware 2011; Anderstam et al. 2012). Cytokines, including interleukins, interferons, lymphokines, adipokines and chemokines, can work alone or in conjunction with each other, have multiple functions and act on several different cell types eliciting distinct developmental or functional effects (Hiscott & Ware 2011). Innate immune cells produce and release cytokines as part of a critical response to inflammation and infection in the body (Lacy & Stow 2011). The synthesis and release of cytokines is a carefully controlled and sequentially orchestrated process, which allows the immune system to function

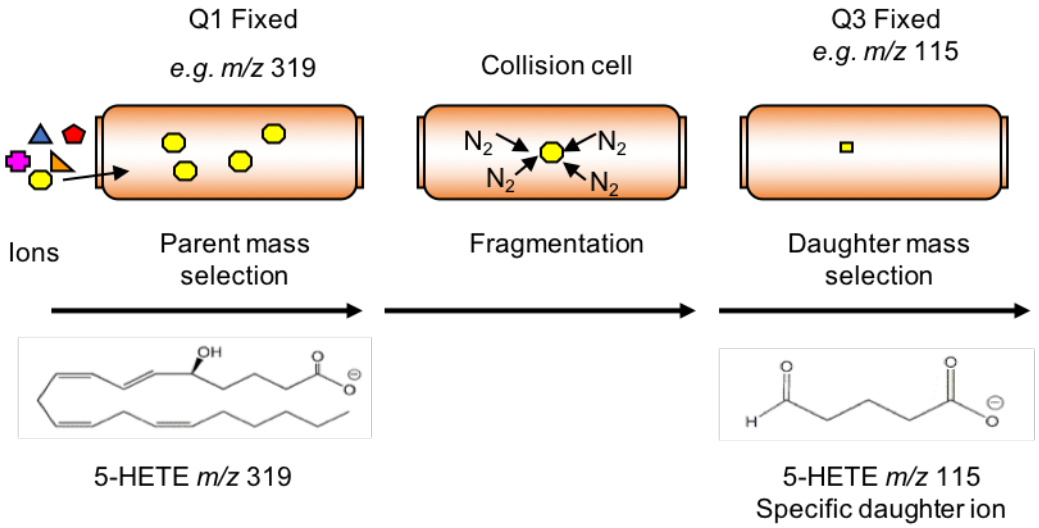


Figure 1.10 Mass spectrometry multiple reaction monitoring (MRM) scan. 5-HETE, which is an eicosanoid produced by neutrophils, will be used as an example. The parent ion of a specific mass is selected for detection, in this case 5-HETE parent ion has a mass to charge ratio of 319. This enters the collision cell where it is bombarded with nitrogen and fragments. It then moves onto Q3 where the specific daughter ion of a fixed mass, in this case *m/z* 115, is selected for detection.

appropriately (Lacy & Stow 2011). Thus, after innate immune cells have produced an initial cytokine cascade, mounting inflammatory or allergic responses, tight regulation later ensures the responses efficiently subside (Hu & Ivashkiv 2009). Proinflammatory cytokines also serve to generate adaptive immune responses by means of T lymphocyte recruitment and activation (Iwasaki & Medzhitov 2010).

Residing at the frontline of defence in immunity, cells of the innate immune system comprising white blood cells including neutrophils, monocytes, dendritic cells, eosinophils, and basophils along with tissue-resident mast cells and macrophages control opportunistic invasion from a wide range of viral, bacterial, fungal and parasitic pathogens (Iwasaki & Medzhitov 2010; Lacy & Stow 2011). This is coordinated somewhat by the plethora of cytokines and chemokines released by the innate immune cells, which include TNF, IFN-γ, TGF and interleukins such as IL-1β, IL-4, IL-6, IL-10, that subsequently communicate with other cells and thereby direct immune responses (Lacy & Stow 2011). Cytokines exert their effect through a matching cell-surface receptor, for example tumour necrosis factor (TNF) binding protein and IL-6 receptor (IL-6R), which when activated, initiate a cascade of immune-related events (Anderstam et al. 2012).

Complement- or immunoglobulin receptor-mediated signalling or pathogen-induced cellular receptors; including pattern recognition receptors such as TLRs, can directly evoke cytokine secretion (Hu & Ivashkiv 2009; Iwasaki & Medzhitov 2010). A highly potent trigger of cytokine release is the Gram-negative bacterial coat component LPS, which activates the cellular response through TLR4, and is the main factor for toxic shock syndrome and sepsis (Se et al. 2008; Lacy & Stow 2011).

1.5.1 Cytokine generation in neutrophils

Neutrophils produced a wide range of cytokines and chemokines, but despite being a first line defence in innate immunity, their precise intracellular locations are not known

and surprisingly little is known about the way in which their secretory organelles (azurophilic granules, specific granules, tertiary granules and secretary vesicles) contribute to cytokine trafficking and release (Borregaard et al. 2007; Lacy & Stow 2011). Upon immuno-histological examination of mature peripheral blood neutrophils it is suggested that TGF-α, TNF, IL-6, IL-12 and CXCL2/IL-8 are stored within peroxidase-negative organelles, which may be specific or tertiary granules or endosomal secretary vesicles (azurophilic granules are peroxidase-positive) (Calafat et al. 1997; Borregaard et al. 2007; Lacy & Stow 2011). Soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs), including vesicle-associated membrane proteins (VAMP)-2, VAMP-7, syntaxin-4, syntaxin-6 and synaptosome-associated protein (SNAP-23) are expressed in neutrophils and are involved in the regulation of trafficking and release of a number of these secretory organelles (Martín-Martín et al. 2000; Mollinedo et al. 2006; Logan et al. 2006).

1.5.2 Cytokine generation in macrophages

Macrophages, and dendritic cells (DC), do not possess the typical secretory granules seen in other innate immune cells such as neutrophils, eosinophils and natural killer cells (NK), therefore, constitutive exocytosis is the predominate mechanism for which cytokines are released from these cells (Figure 1.11) (Stow et al. 2009). TNF is a potent proinflammatory cytokine secreted by many innate immune cells, particularly activated macrophages, but also neutrophils, eosinophils, mast cells, DCs and NK cells, and is one of the most comprehensively studied pathways for cytokine release (Calafat et al. 1997; Shurety et al. 2000; Lacy & Stow 2011). There are varying pathways for exocytic release of TNF between these cell types, and in macrophages TNF is only released by constitutive

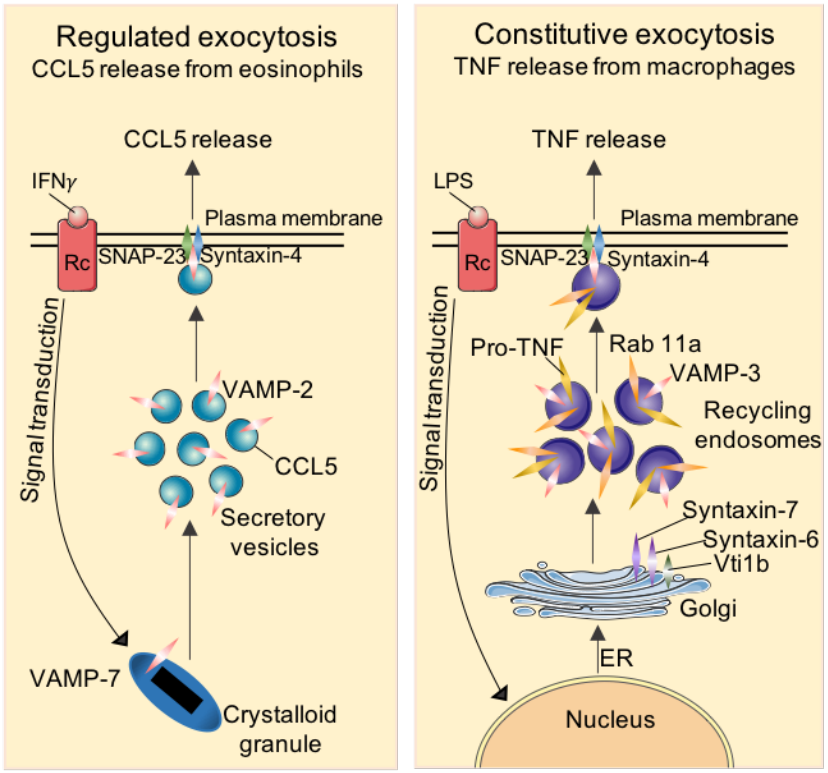


Figure 1.11 Regulated and constitutive exocytosis of cytokines. Representative pathways are shown for SNARE-mediated regulated (eosinophil, left) and constitutive (macrophage, right) release of CCL5/RANTES and TNF, respectively. Regulated exocytosis involves ligand-receptor signalling to secretory granules or vesicles, which contain preformed cytokines, that are mobilized to the cell surface for release. Constitutive exocytosis is instead dependent on receptor-mediated transcription events in the nucleus, leading to trafficking via golgi through recycling endosomes to the cell surface. Picture adapted from Lacy & Stow 2011, made using Servier Medical Art.

exocytosis after being synthesised in response to inflammatory stimuli (Shurety et al. 2000). Other pro- and anti-inflammatory cytokines produced and secreted by macrophages through constitutive exocytosis include IL-6, IL-10 and IL-12 (Shurety et al. 2000; Stow et al. 2009). The release of TNF-α from macrophages ensues within 30 minutes of pathogen recognition, following gene transcription and RNA translation, establishing this mediator as an early regulator of immune response (Schulte et al. 2013).

Cytokines that lack the N-terminal signal sequence required for endoplasmic reticulum (ER) entry, are synthesised in the cytoplasm before being released by the a cell in a non-classic secretory pathway (Nickel 2003). Examples of such cytokines include IL-1β, IL-15, IL-18 and macrophage inhibitory factor, where numerous potential yet diverse routes for these cytokines to cross the plasma membrane and exit the cell have been proposed (Figure 1.12) (Nickel 2003; Lacy & Stow 2011).

The potent proinflammatory cytokine IL-1β plays a crucial role in mediating inflammation and host responses to infection, in spite of this, its mechanism of release remains amongst the least understood (Eder 2009). The biologically inactive pro-interleukin-1β (pro-IL-1β) is the precursor to IL-1β and is synthesised directly in the cell cytoplasm to generate mature, biologically active IL-1β by caspase-1 before being released directly into the extracellular environment (Eder 2009; Lacy & Stow 2011). However, the point of exit for IL-1β, whether it be crossing the plasma membrane directly or by entering or associating with intracellular membranes remains controversial (Eder 2009; Lacy & Stow 2011).

Arrays of cytokines are released by cells of the immune system and currently play a pivotal role at the forefront of medicine, with a surge in popularity as targets for therapeutic antibodies and as causative factors in the pathogenesis of a large number of diseases associated with inflammation (Taylor & Feldmann 2009; Apostolaki et al. 2010).

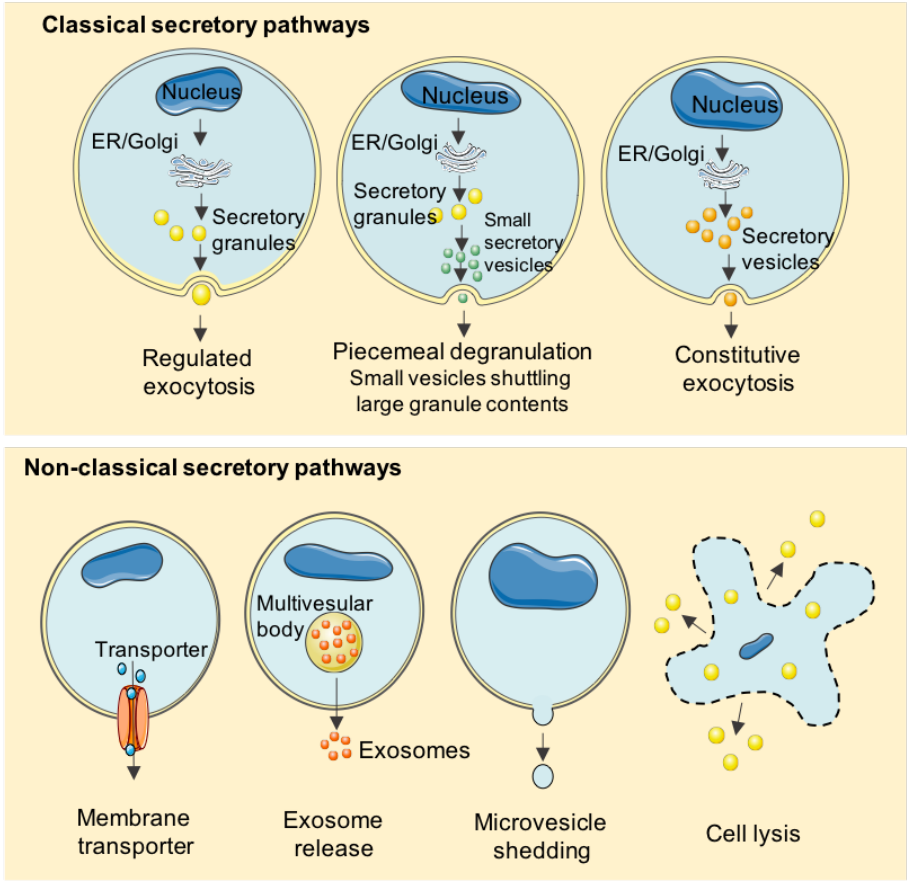


Figure 1.12 Differing secretory pathways for varying modes of release for cytokines. All cells have at least one variation on the classical secretory pathway depicted in the top panel. All of these pathways have multiple transport steps, each requiring sets of trafficking machinery molecules to execute carrier budding, movement and membrane fusion. The bottom panel shows a non-classical secretory pathway which involve movement of cytokines from their point of synthesis in the cytoplasm to the external milieu. Figure adapted from Lacy & Stow 2011; drawn using Servier Medical Art.

The ability to elucidate stimuli that are able to induce cytokine synthesis in our cells can bring us greater knowledge and understanding into the pathogenesis of diseases in which our innate immune cells represent the first cell type to encounter and interact with the etiologic agent; in turn helping to identify characteristic stimuli-specific responses of our cells (Tecchio et al. 2014).

The proinflammatory cytokines, tumour necrosis factor-α (TNF-α) and the interleukin-1 family (including IL-1α and IL-1β), are amongst the most well-studied cytokines in inflammation and infectious diseases such as sepsis and meningitis and have also been implicated in other non-infectious diseases including atherosclerosis, rheumatoid arthritis and Alzheimer's disease (Jawien 2008; Schulte et al. 2013). Both of these cytokines have been reported to have a link with sepsis in both humans and animal models. For example, administration of the bacterial endotoxin LPS showed increased levels of both proteins (Cannon et al. 1990; Schulte et al. 2013). The production of these proinflammatory cytokines by immune cells starts a continuum of cell stimulation, acting on target cells such as neutrophils and promoting macrophage differentiation and activation, ultimately prolonging their survival and enhancing their proinflammatory response during infection (Schulte et al. 2013).

1.5.3 Measuring cytokines

Detection of the spectrum of pro- and anti-inflammatory cytokines can be examined using several methods. One such method is using real-time quantitative polymerase chain reactions (Q-PCR). This involves the measurement of cytokine mRNA transcript abundance, detecting many different cytokines from a relatively small sample amount. One of the main disadvantages is that the presence of RNA does not always accurately reflect protein levels. For example, secretion of some cytokines (such as IL-4 and IL-10) is partially regulated at the translational level, where others are regulated post-translationally (such as IL-1 and IL-18) (Amsen et al. 2009). Another disadvantage of RNA-based detection of cytokines is that identification of cellular sources of the cytokines requires isolation of different cell types, which may be

difficult in some instances. Also, although PCR is sensitive, if there are only small numbers of cells present in tissue samples that generate the cytokines of interest, the limit of detection may not be reached (Amsen et al. 2009).

The presence of cytokines can also be determined by enzyme-linked immunosorbent assays (ELISAs). This method allows detection of secreted cytokines at the protein level. ELISAs are commonly chosen for their accuracy and sensitivity of detection. They possess the ability to specifically detect a protein within a protein network in comparison to high-performance liquid chromatography (HPLC) (Aly et al. 2011). High specificity is ensured for the detection of the target analyte as each assay is tested for cross-reactivity and interference with a panel of analyte-related molecules (R&D DuoSet ELISA development systems). A sandwich immunoassay is used to detect sample antigen between two layers of antibodies (capture and detection antibodies) and enables the quantification of cytokine secretion in cell supernatant. Sandwich ELISAs are preferred over competitive ELISAs as they result in less variable results. A plate reader is then used to measure the absorbance of the plate wells to determine the presence and quantity of antigen. A disadvantage to using ELISAs is that sufficient quantities of tissue fluids or supernatant are not always easily obtained. This coupled with cellular consumption of cytokines means that actual cytokine levels may be underestimated.

1.6 Biomarker isolation and identification

Biomarkers are characteristic biological molecules that provide independent diagnostic or prognostic value by reflecting normal or diseased states within the body (Stearns-Kurosawa et al. 2011). Such molecules can be detected and measured, for example, in blood, tissue or urine, and represent a marker of a biological process commonly used to diagnose disease and guide therapies (Stearns-Kurosawa et al. 2011).

The inflammatory response is complex, paving the way for the development of a biomarker approach towards accurate diagnosis for patients with bacterial infections. A key element in the survival rate of severe infections, such as meningitis

and sepsis, is early therapy. This being the case, a biomarker should reduce the time taken to confirm a diagnosis, distinguish between an infectious and non-infectious origin of inflammation, discriminate between viral and bacterial infections and evaluate a suitable control for the infection (Kobeissi & Zanotti-Cavazzoni 2010).

As mentioned above, antibiotic resistance is a major public healthcare challenge and strategies to promote a judicious use of antibiotics is urgently needed. The use of empiric broad-spectrum antibiotics is frequently initiated in critically ill patients presenting with systemic inflammation due to the time-consuming diagnostic challenges. Often a prolonged course of antibiotics is administered despite the absence of microbiological data supporting diagnosis of bacterial infection.

Several studies have evaluated various diagnostic biomarkers of systemic bacterial infection including ferritin, haptoglobin, interleukin 6, C-reactive protein (CRP) and procalcitonin (PCT) (Harbarth et al. 2001; Garnacho-Montero et al. 2014; Meynaar et al. 2011).

Currently there are diagnostic tests that can identify between bacterial and viral pathogens and therefore improve distinction between bacterial and viral infection. The most well studied biomarkers are PCT and CRP. These markers represent the most promising results to date as clinical markers for the diagnosis of bacterial infection (Han et al. 2015). However, their sensitivity and specificity are still questioned (Hausfater et al. 2002). PCT is normally secreted by the C cells of the thyroid in response to hyper-calcaemia, and is the pre-hormone of calcitonin (Simon et al. 2004). Under normal conditions, negligible serum PCT concentrations are detected (Whicher et al. 2001). It is believed that PCT is produced by the liver and peripheral blood mononuclear cells, and modulated by LPS and proinflammatory cytokines (Hausfater et al. 2002). Despite its lack of specificity, CRP level measurements are frequently used to differentiate diagnosis of bacterial infection (Hausfater et al. 2002). CRP is an acute-phase reactant that is synthesised by the liver, mainly in response to IL-6, which is produced not only during infection but also in many types of inflammation (Ridker 2003). It binds to polysaccharides in pathogens, activating the classic complement pathway (Simon et al. 2004). Overall, it has been

observed that PCT levels were more accurate markers for bacterial infection than were CRP levels, both when differentiating bacterial infections from non-infective causes of inflammation and when differentiating bacterial infections from viral infections (Simon et al. 2004).

While severe bacterial infections typically trigger the production of multiple mediators such as eicosanoids, cytokines and other inflammatory proteins, surprisingly, few studies have assessed the utility of a combination of biomarkers in the diagnosis of bacterial infection (Han et al. 2015). The use of multiple biomarkers will undoubtedly provide a more accurate strategy to diagnose bacterial infection. In addition, in the case of critically ill patients where timely administration of empiric broad-spectrum antibiotics significantly reduces mortality, the greatest clinical advantage of biomarker signatures may be to support clinical decisions to discontinue antibiotics when no bacterial infection is identified. There are initial efforts into potential biomarker algorithms to identify patients with suspected sepsis at very low likelihood of bacterial infection (Han et al. 2015). Studies aiming to identify potential markers of infection could contribute to the development of specific biomarker algorithms and further enhance and refined their accuracy.

Early identification of bacterial infection is still challenging for clinicians. The most precise way to diagnose bacterial infections is through blood cultures, which can take several days and are often inconclusive. The effort to determine if biomarkers can differentiate between bacterial pathogens would prove to be an invaluable practice. Using potential eicosanoid and cytokine readouts as biomarkers for bacterial infection could allow for faster administration of targeted antibiotics at the earliest opportunity, increasing positive long-term patient outcomes. Current practice uses broad-spectrum antibiotics to a presumed pathogen, which increases resistance and therefore reduces scope for treatment. The identification of profile markers explicitly for species-specific bacterial infections, that can be generated at the bedside as an on-the-spot system would be invaluable to assist in current medical practice.

1.7 Aims

This study aims to determine the nature of the relationship between bacterial species and eicosanoid and cytokine signalling in the early stages of infection with the host. It is hoped that further understanding of the eicosanoid and cytokine profiling during specific bacterial infections will identify biomarker fingerprints that could be used as early predictors of specific bacterial infections.

It is hypothesised that the profile of the eicosanoid and cytokine responses generated during bacterial infections varies depending on the nature of the bacterial pathogen.

The primary aims of the work presented in this thesis are:

1. To investigate the eicosanoid and cytokine response of neutrophils and macrophages when exposed to individual bacterial components.
2. To develop an infection model for live bacterial exposure to phagocytic cells.
3. To establish the relationship between bacterial strain and eicosanoid and cytokine signalling following infection, with a view to determining early profiles of infection.

Chapter 2

General Methods

2. General methods

The materials and methods defined in this section are non-specific and are relevant to the results presented in multiple chapters of this thesis. Detailed descriptions of the specific materials and methods for individual chapters will be given at the start of each chapter.

2.1 Chemicals

Unless stated otherwise, all chemicals used for the experiments described were purchased from Sigma-Aldrich Company Ltd (Poole, Dorset, UK), and all solvents used for the experiments described were purchased from Fisher Scientifics UK Ltd (Loughborough, Leicestershire, UK).

2.2 General buffers and solutions

All buffers were made from dry chemicals on the bench for the purpose of human neutrophil isolation and were filter sterilised by passing the buffer through a 0.2 micron syringe filter (Corning) in a tissue culture hood and were stored frozen at -20°C.

2.2.1 Preparation of phosphate buffered saline (PBS)

One Dulbecco's PBS (no calcium, no magnesium) tablet (Gibco) added to 100mL double-distilled H_2O, pH 7.4. *Note: one tablet dissolved in 200mL water yields 0.01M phosphate buffer, 0.0027M KCl and 0.137M NaCl*.

2.2.2 Preparation of citrate PBS

2.8% citrate (*w/v*); 2.8g sodium citrate tribasic in 100mL PBS, pH 7.3. 0.4% citrate (*w/v*); 0.4g sodium citrate tribasic in 100mL PBS, pH 7.3.

2.2.3 Preparation of hypotonic saline, 0.2%

0.2g NaCl in 100mL double-distilled H_2O.

2.2.4 Preparation of hypertonic saline, 1.6%

1.6g NaCl in 100 mL double-distilled H_2O.

2.2.5 Preparation of Krebs (Ca^{2+} free)

Hepes buffered Krebs [50mM HEPES, 100mM NaCl, 5mM KCl, 1mM NaH_2PO_4, 2mM glucose] was prepared and adjusted to pH 7.4 with NaOH. The buffer was filter sterilised and stored at -20°C.

2.3 Reagents

Eicosanoid analytes and deuterated internal standards used from Cayman Chemical (Michigan, USA) Human and Mouse TLR1-9 Agonist Kit, a set of known agonists for human and mouse TLR1 to TLR9, were purchased from InvivoGen.

Human TLR agonist kit (tlrl-kit1hw; InvivoGen, Toulouse, France). All descriptions are taken from the product information sheet for this product.

TLR1-9 Agonist Kit Content:

- Pam3CSK4 - TLR1/2 agonist

Pam3CSK4 is a synthetic tripalmitoylated lipopeptide that mimics the acylated amino terminus of bacterial lipoproteins. Pam3CysSerLys4 (Pam3CSK4) is a potent activator of the proinflammatory transcription factor NF-κB. Recognition of Pam3CSK4 is mediated by TLR2 which cooperates with TLR1 through their cytoplasmic domain to induce the signalling cascade leading to the activation of NF-κB.

- HKLM – Heat-*Killed Listeria monocytogenes* - TLR2 agonist

HKLM is a freeze-dried heat-killed preparation of *Listeria monocytogenes* (LM), a facultative intracellular Gram-positive bacterium. Infection with LM induces a strong nonspecific response characterized by the secretion of proinflammatory cytokines such as TNF-α. This response is mediated by TLR2. Stimulation with HKLM induces immediate activation of NF-kB and the production of proinflammatory cytokines.

- Poly (I:C) - TLR3 agonist

Poly(I:C) is a synthetic analogue of double-stranded RNA (dsRNA), a molecular pattern associated with viral infection.

- *Escherichia coli* K12 Lipopolysaccharide (LPS) - TLR4 agonist

Lipopolysaccharide (LPS), the major structural component of the outer wall of Gram-negative bacteria, is a potent activator of the immune system. LPS recognition is mediated by TLR4 which forms a complex with MD2 and CD14 leading the production of proinflammatory cytokines such as TNF-α through the MyD88 pathway. LPS signalling also involves a MyD88-independent cascade that mediates the expression of IFN-inducible genes via the adaptor TRIF.

- *Salmonella typhimurium* Flagellin - TLR5 agonist

Flagellin is the major component of the bacterial flagellar filament, which confers motility on a wide range of bacterial species. Flagellin is a potent stimulator of innate immune responses in a number of eukaryotic cells and organisms, including both mammals and plants. In mammals, flagellin is recognized by TLR5 and triggers defence responses both systemically and at epithelial surfaces. Flagellin induces the activation of NF-kB and the production of cytokines and nitric oxide depending on the nature of the TLR5 signalling complex.

- FSL-1 - TLR6/2 agonist

FSL-1 (Pam2CGDPKHPKSF) is a synthetic lipoprotein that represents the N-terminal part of the 44-kDa lipoprotein LP44 of *Mycoplasma salivarium*. The framework

structure of FSL-1 is the same as that of MALP-2, a *Mycoplasma fermentans* derived lipopeptide (LP), but they differ in the amino acid sequence and length of the peptide portion. FSL-1 is recognized by TLR2 and TLR6 inducing a MyD88-dependent signalling cascade that leads to the activation of NF-kB and the production of proinflammatory cytokines.

- Imiquimod - TLR7 agonist

Imiquimod (R837), an imidazoquinoline amine analogue to guanosine, is an immune response modifier with potent indirect antiviral activity. Imiquimod is an approved treatment for external genital warts caused by human papillomavirus infection.

- ssRNA40 - TLR8 agonist

ssRNA40 is a 20-mer phosphothioate protected single-stranded RNA oligonucleotide containing a GU-rich sequence15. ssRNA40 is complexed with the cationic lipid LyoVecTM (ratio 1:2), to protect it from degradation and facilitate its uptake, and lyophilized to generate ssRNA40/LyoVec. When complexed to cationic lipids, ssRNA can substitute for viral RNAs in inducing TNF-α and IFN-α production in peripheral blood mononuclear cells.

- ODN2006- TLR9 agonist

CpG ODNs are synthetic oligonucleotides containing unmethylated CpG dinucleotides in particular sequence contexts that induce strong immunostimulatory effects through the activation of TLR9. Two types of CpG ODNs have been described. Type A (or D) ODNs preferentially activate plasmacytoid dendritic cells (pDC) to produce IFN-α, whereas type B (or K) ODNs induce the proliferation of B cells and the secretion of IgM and IL-6.

Mouse TLR agonist kit (InvivoGen, tlrl-kit1mw). All descriptions are as above unless otherwise stated.

TLR1-9 Agonist Kit Content:

- Pam3CSK4 -TLR1/2 agonist
- HKLM – Heat-Killed *Listeria monocytogenes* - TLR2 agonist
- Poly (I:C) - TLR3 agonist
- *Escherichia coli K12* Lipopolysaccharide (LPS) - TLR4 agonist
- *Salmonella typhimurium* Flagellin - TLR5 agonist
- FSL-1 - TLR6/2 agonist
- Imiquimod - TLR7 agonist
- ODN1826 - TLR9 agonist

CpG dinucleotides in particular sequence contexts that induce strong immunostimulatory effects through the activation of TLR9. ODN1826 is the optimal motif to induce the proliferation of B cells.

2.4 Isolation of human neutrophils from peripheral blood

Whole blood was taken by venepuncture from the antecubital fossa, from healthy volunteers free from non-steroidal anti-inflammatory drugs for at least fourteen days. Clinical supplies (needles etc.) were obtained from clinical sterile stores (Cardiff School of Pharmacy and Pharmaceutical Sciences, SPPS). Ethical permission for all donations was obtained from the local research ethics committee (SPPS) (1314-19), and all human participants gave written informed consent. Human neutrophils were isolated using the methods described by Thomas and colleagues (Thomas et al. 2014). Briefly, blood was drawn through a 21-gauge butterfly needle directly into a syringe containing 2.8% citrate PBS and Hetasep (Stemcell Technologies) at a blood:citrate:Hetasep ratio of 10:2:2. The erythrocytes were allowed to sediment at room temperature for 35-50 minutes and the upper plasma layer removed and under laid with Lymphoprep (Stemcell Technologies) at a plasma Lymphoprep ratio of 2:1. This was centrifuged at 800 g for 20 minutes at 4°C (Heraeus, Megafuge-40R centrifuge). The monocytic cells at the interface were removed and discarded along with all the remaining plasma and Lymphoprep and the pelleted cells were recovered with 0.4% citrate PBS and transferred to a clean centrifuge tube. This was then centrifuged at 400g for 5 minutes at 4°C. The supernatant was discarded, and the red

blood cells were lysed by hypotonic shock. The cells were re-suspended in hypotonic saline solution (ice-cold 0.2% saline) before restoring osmolality after 35-50 seconds (by addition of an equal volume of ice cold 1.6% saline) after which the solution was centrifuged at 400 g for 5 minutes at 4°C. The hypotonic shock procedure was repeated until no further red colour was visible (typically 3 cycles). The final pellet was re-suspended in Krebs buffer and the neutrophils were diluted to a concentration of $1x10^7$ neutrophils per mL (the concentration was measured by trypan blue exclusion using a haemocytometer under an inverted microscope).

2.4.1 Neutrophil activation

Human neutrophils were isolated as per section 2.5.1 and used at 0.5ml of 0.5×10^6 cells/ml. The cells were incubated for 5 minutes at 37°C before the addition of 100 µl of activating agents' calcium ionophore A23187 (10µM final concentration) or fMLP (1µM final concentration) combined with 2.5 mM $CaCl_2$ and 1.25 mM $MgCl_2$ for between 2 minutes and 60 minutes. Activating agents were prepared as per Table 2.1.

Table 2.1 Activating agent preparations.

Agonist mix	**Krebs (µl)**	**Agonist (µl)**	**$MgCl_2$ (µl)**	**$CaCl_2$ (µl)**
$MgCl_2$ + $CaCl_2$ (untreated)	900	-	50	50
Calcium Ionophore A23187	875	25	50	50
fMLP	895	5	50	50

When neutrophils were exposed to TLR agonists, the cells were pre-incubated with agonists for 30 minutes at 37°C prior to the addition of 100 µl of the fMLP preparation for a further 2 minutes.

Following incubation, 500 µl samples for cytokine analysis were transferred into a fresh eppendorf tube and frozen at -80°C until required.

2.5Preparation of eicosanoid standard curve

Internal standard (IS) was prepared by diluting 12-HETE-$_{d8}$ stock to 1 ng/µl with methanol (MeOH). 20 pg/µl of the standard was then mixed with 980 µl of MeOH. The analyte mix was prepared as per Table 2.2 and the final volume was made up to 300 µl with MeOH.

Table 2.2 Internal standard and analyte dilutions.

	Stock	**Stock concentration**	**Add**
IS: 12-HETE-$_{d8}$	25µg in 250µl	100ng/µl	20µl of 100ng/µl to get 1ng/µl (in 980µl MeOH)
Analytes:			
5-HETE	100µg in 1ml	100ng/µl	6µl of 100ng/µl to get 2ng/µl final (in 300µl)
8-HETE	25µg in 250µl	100ng/µl	6µl of 100ng/µl to get 2ng/µl final (in 300µl)
11-HETE	25µg in 250µl	100ng/µl	6µl of 100ng/µl to get 2ng/µl final (in 300µl)
12-HETE	250µg in 2.5ml	100ng/µl	6µl of 100ng/µl to get 2ng/µl final (in 300µl)
15-HETE	25µg in 250µl	100ng/µl	6µl of 100ng/µl to get 2ng/µl final (in 300µl)
PGE_2	1mg/ml	1mg/ml	6µl of 100ng/µl to get 2ng/µl final (in 300µl)
PGD_2	1mg/ml	1mg/ml	6µl of 100ng/µl to get 2ng/µl final (in 300µl)
LTB_4	25µg in 250µl	100ng/µl	6µl of 100ng/µl to get 2ng/µl final (in 300µl)
LTE_4	25µg in 250µl	100ng/µl	6µl of 100ng/µl to get 2ng/µl final (in 300µl)
TxB2	1mg/ml		6µl of 100ng/µl to get 2ng/µl final (in 300µl)

To create the standard curve the analyte mix was diluted as per Table 2.3. 100 µl of the IS mix was added to 100 µl of each analyte dilution. 10 µl of each dilution was then injected onto the column to create the standard curve.

Table 2.3 Standard curve preparation.

#	**Dilution**	**Concentration**	**Final Concentration**	**Amount on column (final)**
1	Original analyte mix (6µl each with 240µl MeOH)	2ng/µl	1ng/µl	10ng
2	150µl of (1) with 150µl MeOH	1pg/µl	0.5pg/µl	5ng
3	60µl of (2) with 240µl MeOH	0.2ng/µl	0.1ng/µl	1ng
4	150µl of (3) with 150µl MeOH	0.1ng/µl	50pg/µl	500pg
5	60µl of (4) with 240µl MeOH	20pg/µl	10pg/µl	100pg
6	150µl of (5) with 150µl MeOH	10pg/µl	5pg/µl	50pg
7	60µl of (6) with 240µl MeOH	2pg/µl	1pg/µl	10pg
8	150µl of (7) with 150µl MeOH	0.5pg/µl	0.5pg/µl	5pg
9	60µl of (8) with 240µl MeOH	0.2pg/µl	0.1pg/µl	1pg

2.6Extraction of lipids with acidified hexane: isopropanol

Following treatment, lipids were extracted immediately from the supernatant using the extraction methods described by Thomas and colleagues (Thomas et al. 2014). For quantitation 5 µl of an internal standard was added to the cells. The internal standard is known amounts of compound added and detected in all samples, which allow the signal from the unknown analyte to be quantified when compared to the signal from the standard.

Lipids were extracted by adding a solvent mixture (1M acetic acid, propan-2-ol, hexane; 2:20:30; vol/vol/vol) at a ratio of 2.5 mL solvent mixture per millilitre of supernatant and vortexing for 1 minute. A further 2.5 mL of hexane was added followed by vortexing and centrifugation at 300g for 5 minutes at 4°C. Lipids were carefully recovered from the upper hexane layer and transferred into a clean glass tube. The lipids were then re-extracted from the lower layer by the addition of 2.5 mL of hexane, vortexing and centrifuging at 300 g for 5 minutes at 4°C. The upper hexane layer was recovered and combined with the first hexane layer. The samples were dried using nitrogen gas evaporation, dissolved in methanol (MeOH), and stored at -80°C before analysis by HPLC/MS/MS.

2.7Solid phase extraction

2.7.1 Sample preparation

The cells were analysed for intracellular eicosanoid generation. Cell samples underwent prior lipid extraction as described in section 2.6. The 100 µl sample (resuspended in MeOH) was added to 1700 µl of HPLC grade water and supplemented with 200 µl of MeOH to bring the total volume of MeOH to 15% by volume. 45 µl of acetic acid was then added to the sample mixture. Internal standard was added during the hexane-lipid extraction.

The media were analysed for extracellular eicosanoid release. After stimulation 1 mL of media was removed from the cell sample, 700 µl of HPLC grade water was added followed by 300 µl of MeOH to bring the total volume of MeOH to 15% by volume.

45 µl of acetic acid was then added to the sample mixture and 10 µl internal standard. Samples were centrifuged for 5 minutes at 3000 rpm to remove cellular debris, and then purified.

2.7.2 Extraction

Eicosanoids were extracted using Sep-Pak Vac 6cc (500mg) tC18 cartridges (Waters, UK; WAT036790) Columns were washed with 12 mL of MeOH and then 6 mL of HPLC grade water with 0.25% acetic acid. After applying the sample, the columns were washed with 10 mL of water followed by 6 mL of hexane. Columns were then allowed to dry for 20 minutes. Samples were eluted with 8 mL of methyl formate and then dried under vacuum at 30°C, before being dissolved in 100 µl of MeOH. Samples were stored at -80°C before analysis by HPLC/MS/MS.

2.8MultiQuant software

MultiQuant software was used for mass spectrometry integration and quantitation of detected analytes (Cardiff Lipidomic Group, School of Medicine).
User manual: https://sciex.com/documents/community/support/mq-glp-software-reference-guide.pdf.

2.9Cytokine concentration determination

2.9.1 Enzyme-linked immunosorbent assay

A DuoSet enzyme-linked immunosorbent assay (ELISA) development system (R&D Systems Europe, Ltd. Abingdon, UK) was used to measure the secretion of natural pro-inflammatory cytokines (IL-1β, TNFα, IL-6 and IL-8). The ELISA was performed as described by the manufacturers' guidelines. Typically, a 96-well plate (Greiner, Austria) was coated with 4 µg/mL of the capture antibody, sealed and incubated overnight at room temperature. The plate was aspirated/washed with wash buffer (PBST, 0.05% Tween®20 in PBS), inverted and blotted. Wells of the plate were then blocked with reagent diluent (RD, 1% BSA in 1xPBS), for a minimum of 1-hour at room

temperature. Aspiration of the plate was repeated before samples and standards were added. Samples were retrieved from -80°C storage and allowed to thaw on ice. Standards were tested using a seven-point standard curve, using 2-fold serial dilutions from the highest concentration provided (dilute using RD). A negative control (RD) was also included and the plate was incubated at room temperature for 2-hours. The plate was aspirated and blotted before adding 200 ng/mL of biotin labelled detection antibody in RD and subsequently incubated at room temperature for 2-hours. Aspiration of the plate was repeated, before adding enzyme-labelled streptavidin horseradish-peroxidase, diluted 1:40, to each well. The plate was incubated at room temperature, in the dark for 20 minutes, before the final aspiration. Enzyme-substrate solution (20 mg o-phenylenediamine dihydrochloride (OPD) and 20 µl 30% H_2O_2 in 0.1M citric acid and 0.2M phosphate) was added to each well and further incubated in the dark for 20-minutes at room temperature before the stop solution (2.5M H_2SO_4) was added. The optical density of each well was determined immediately, using a microwell reader set to 490 nm (Tecan, Infinite F50).

2.10 Statistical analysis

In Chapters 3 and 4 data were analysed using a one-way ANOVA and Chapters 5 and 6 data were analysed using a two-way ANOVA. When a significant main effect was reported a post-hoc Tukey analysis with Bonferroni corrections was used to detect individual group differences. Prior to carrying out the ANOVAs a test for normal distribution of data (Shapiro-Wilk; Tukey box-plots) were carried out. If data violated these tests the data were transformed by square root and transformed data were used for analysis. Where data remained not normally distributed following transformation or when data were unable to be transformed due to 0 values within the data set a non-parametric equivalent of the one-way ANOVA, Kruskal-Wallis test was used to determine main effects with Dunn's non-parametric comparison with Bonferroni corrections for post-hoc analysis to compare individual differences.

Chapter 3

Identification of 5-HETE and proinflammatory cytokines from isolated human neutrophils

3.1 Introduction

As previously discussed in Chapter 1, the ability for the host to recognise microbial pathogens is an essential element for the innate immune response. Cells of the innate immune system express various receptors both extracellularly and intracellularly, with the potential to directly recognise microbial or endogenous signals. Neutrophils express a large number of cell surface receptors as well as providing rapid deployment and effector functions of the innate immune response to infection. As part of their response they are able to generate immune-modulating signals, the composition of which is dependent on the nature of the pathogen. To do this the cell is equipped with a range of reporter systems, which recognise and respond to an extensive repertoire of pathogen-associated molecular patterns (PAMPs) (Prince et al. 2011). The system focussed upon in this body of work is comprised of toll-like receptors (TLRs), which were formerly discussed in Chapter 1.

Chapter 1 also discusses the necessity for rapid differentiation between bacterial aetiology of infection for a decision on empirical antibiotic treatment. It has previously been shown that primary human neutrophils possess bacterial toll-like receptors which generate proinflammatory cytokines, and have the ability to produce lipid mediators in response to bacterial components (Clark et al. 2011). For these reasons, primary human neutrophils provide a good model to investigate the relationship between lipid and cytokine generation under agonist-activated conditions, in addition to the fact that neutrophils are the predominate immune cell in human blood and are typically the first leukocyte recruited to the site of trauma or infection.

The focus of this research was to employ a targeted lipidomic approach to show that isolated human neutrophils generate 5-lipoxygenase (5-LOX) lipids, as discussed in Chapter 1, when activated with bacterial products. At the time, the most established technique in the Cardiff Lipidomic Group, School of Medicine, employed a Q-Trap mass spectrometer instrument (Applied Biosystems 4000) operating in multiple

reaction-monitoring (MRM) mode and is considered to be one of the most sensitive systems available. It is important to highlight that during the course of this PhD study and in keeping with the fast-moving developments within the lipidomics field, Chapters 5-6 show how technical and methodological aspects of the targeted lipidomics approach have been further enhanced. Nonetheless, techniques used in this chapter were optimised at the time of study.

The aim of this chapter was to determine whether different TLR agonists initiate distinct lipid and cytokine profiles, which could be used as a robust indicator of the early innate immune response to bacterial infection. An earlier study by Clark and colleagues found that human neutrophils, when exposed to various agonists including TLR binding agents, generated the eicosanoid 5-HETE (Clark et al. 2011). The results of this study indicated that the profile of 5-HETE eicosanoids varied in response to different classes of pathogens. Chapter 3 takes this approach a stage further by employing the whole array of bacterial TLR agonists to look at neutrophil responses and determine any variation between components.
A further aim is to characterise the profile of cytokines and eicosanoids produced by human neutrophils following exposure to bacterial TLR agonists with a view to determining which play a key role in early inflammatory signalling.

3.1.1 Specific aims

The specific aims of the work in this chapter are therefore:

1. To examine the effects of calcium ionophore A23187 (non-receptor agonist) and bacterial peptide N-formylmethionyl-leucyl-phenylalanin (fMLP) (receptor-based agonist) on isolated human neutrophils.
2. To establish mass spectrometry detection of neutrophil 5-HETE formation.
3. To determine the contribution of toll-like receptor agonist priming of isolated neutrophils on cytokine and eicosanoid signalling.

3.2 Specific material and methods

3.2.1 Isolation and activation of human neutrophils

Human neutrophils were isolated from a healthy donor, as previously described in Chapter 2 section 2.4, resuspended in a small volume of Ca^{2+}-free Krebs buffer, counted and kept on ice. Neutrophils ($1.5x10^5$ cells) were incubated for 5 minutes at 37°C before the addition of the activating agents calcium ionophore (A23187; 10 µM) or fMLP (1 µM) followed by 2.5 mM $CaCl_2$ and 1.25 mM $MgCl_2$ for between 2 minutes and 60 minutes.

When priming agents were used (Table 3.1) (InvivoGen, tlrl-kit1hw) neutrophils ($1.5x10^5$ cells) were pre-incubated for 30 minutes at 37°C in the presence of the TLR agonists, before the addition of the activating reagent fMLP (1 µM), 2.5 mM $CaCl_2$ and 1.25 mM $MgCl_2$ for a further 2 minutes. Following incubation, deuterated internal standard, $12HETED_8$ (5 ng/mL) (Cayman Chemicals, 334570), was added to all samples before subsequent lipid extraction, as described in Chapter 2, section 2.6. For cytokine analysis, isolated human neutrophils ($1.5x10^5$ cells) were primed separately with the six bacterial TLR agonist for 30 minutes at 37°C before being stimulated with fMLP (1 µM) for 4 hours (times points were indicated during optimisation). Samples were transferred into fresh Eppendorf tubes and frozen at -80°C until required.

Table 3.1 Toll-like receptor (TLR) agonist working concentrations used in isolated human neutrophil exposure.

Agonist	TLR Ligand	Working Concentration
N-palmitoyl-S-[2,3-bis(palmitoyloxy)-(2R,2S)-propyl]-Cys- [S]-Ser-[S]-Lys (4) trihydrochloride (Pam3CSK4)	1/2	100 ng/mL
heat-killed *Listeria monocytogenes* (HKLM)	2	10^8 HKLM/mL
E. coli K12 lipopolysaccharide (LPS)	4	1 µg/mL
Salmonella typhimurium (Flagellin)	5	1 µg/mL
S-(2,3- bispalmitoyloxypropyl)-Cys-Gly-Asp-Pro-Lys-His-Pro- Ser-Phe (FSL-1)	6/2	100 ng/mL
synthetic oligodeoxynucleotide 1826, 5'-TCCATGACGTTCCTGAC-GTT-3' (ODN 2006)	9	5 µM

3.2.2 Reverse-phase LC/MS/MS of lipids

The dried lipid extract was separated using liquid chromatography (LC)-MS/MS. Samples were separated on a C18 Spherisorb ODS2 HPLC Column (5 µm, 150 x 4.6 mm; Waters, Hertfordshire, UK), at a gradient of 50 to 90% mobile phase B over 15 minutes. Mobile phases A and B were made up of solvent mixtures water, acetonitrile, acetic acid at a ratio of 75:25:0.1; *v/v/v* and methanol, acetonitrile, acetic acid at a ratio of 60:40:0.1; *v/v/v*, respectively. Samples were injected with an auto-sampler and the sample chamber temperature was set at 4°C. The injection volume was 10µL with a flow rate of 1mL/minute. Electrospray mass spectra were obtained on a Q-Trap instrument (Applied Biosystems 4000 Q-Trap; Cardiff School of Medicine, Lipidomics Group) operating in the negative mode. Products were analysed in the multiple-reaction monitoring (MRM) mode transitions from the parent ion (*m/z* 319) to the daughter ion 115 (5-HETE $[M-H]^-$) every 0.60 seconds. The area under the curve for the parent ion to the 115 ion was integrated and normalised to the internal standard for 5-HETE. For quantification of the lipid, a standard curve was generated with purified 12-HETE. The limit of detection of the internal standard was determined to be 5 pg.

3.2.3 Enzyme-linked immunosorbent assay for cytokines

Double antibody sandwich enzyme-linked immunosorbent assays (ELISAs) were used to measure the protein concentration generated by isolated neutrophils. The analysis was performed using R&D systems Human DuoSet ELISA kits to quantify the concentration of four proinflammatory analytes; IL-1β (DY201), IL-6 (DY206), IL-8 (DY208) and TNF-α (DY210). The minimal detection concentrations (pg/mL) are as follows: IL-1β; 3.9, IL-6; 9.4, IL-8; 31.2, and TNF-α; 15.6. The ELISA kits were used with an ancillary reagent pack (R&D systems, DY008) to allow for uniform application of reagents, according to the manufacturer's instructions. The amount of cytokine was calculated using a standard curve generated from a known amount of the analyte provided with the kit.

3.2.4 Statistical analysis

Statistical analysis was carried out as described in Chapter 2, section 2.10.

Both the 5-HETE and cytokine responses to each TLR agonist came from the isolated neutrophils of one individual donor. Due to the large variation between donor responses, data were normalised where values were expressed as a mean percentage of the untreated (where the untreated value was 0 a value of 0.01 was given). Data were expressed on a log scale to enable identification of any patterns and allow comparison and statistical analysis between donors. Normalising data allows the comparison of values in a way that eliminates the effects of certain outside influences.

3.3 Results

3.3.1 Time course of 5-HETE expression

3.3.1.1 Responses of calcium ionophore A23187-activated neutrophils

Calcium ionophore A23187 (10 µM) was employed as a known chemical activation agent to confirm 5-HETE generation from human neutrophils isolated from fresh peripheral blood (Figure 3.1)(Clark et al. 2011). As can be seen from Figure 3.1A, precursor LC/MS/MS identified that exposure to calcium ionophore A23187 for 2 minutes stimulated the production of 5-HETE (~47 ng/10^6cells). The level of 5-HETE expression then reduced to 18 and 15 ng/10^6cells over the time course of 15 and 60 minutes, respectively.

3.3.1.2 Responses of fMLP activated neutrophils

After confirming 5-HETE generation with calcium ionophore A23187, the next consideration was to corroborate that 5-HETE could be generated and detected when neutrophils were stimulated with the well-characterised formylated bacterial

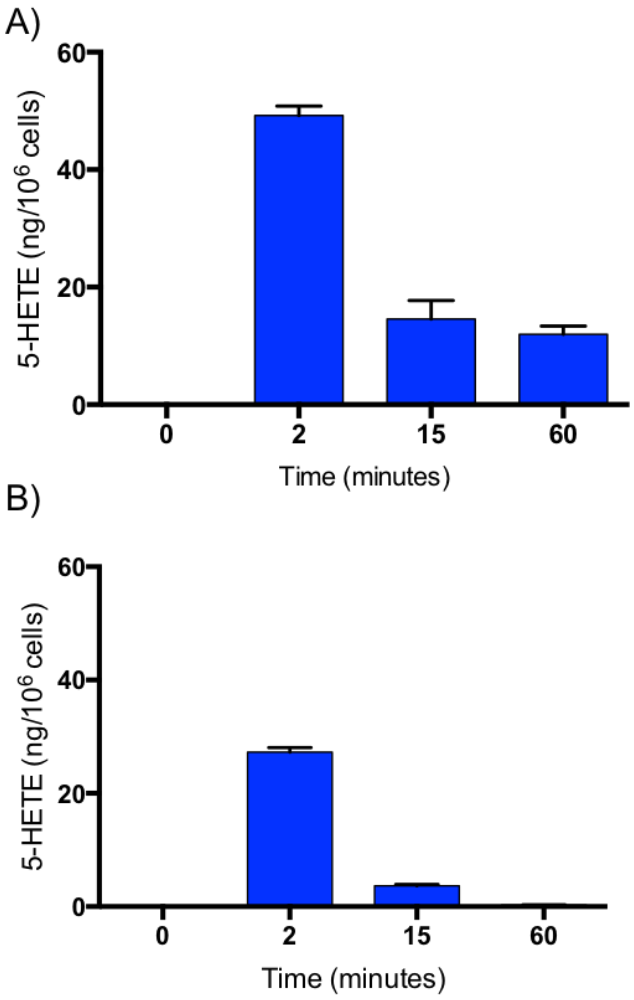


Figure 3.1 Generation of 5-HETE by isolated human neutrophils in response to A) calcium ionophore A23187 and B) fMLP activation. Neutrophils were activated for 0, 2, 15 and 60 minutes with **A)** calcium ionophore A23187 (10 μM) and **B)** fMLP (1 μM) before being incubated at 37°C and analysed using liquid chromatography/mass spectrometry tandem mass spectrometry (LC/MS/MS). *n* = 1, data presented from one donor carried out in triplicate. Data represented as mean ± S.E.M.

peptide, fMLP (1 μM) over time (Clark et al. 2011). fMLP activation of isolated neutrophils from the same donor for 2, 15 and 60 minutes also showed a similar response to calcium ionophore A23187, where the most substantial formation of 5-HETE was after 2 minutes of incubation generating ~ 28 ng/10^6cells, followed by a rapid decline to < 5 ng/10^6cells at 15 minutes and undetectable levels at 60 minutes (Figure 3.1B). The level of expression was also of a similar order of magnitude to that seen from ionophore-activated cells. These results are consistent with those previously published by Clark *et al*., 2011. For this reason, and because the focus was on bacterial generation of inflammatory mediators, in all subsequent experiments, bacterial peptide fMLP was used as the agonist for neutrophil activation at a time point of 2 minutes.

3.3.2 Neutrophil exposure to TLR bacterial agonists

3.3.2.1 Gram-positive TLR1/2 agonist Pam3CSK4

Normalised data for 5-HETE production showed that priming of human neutrophils with Gram-positive lipoprotein Pam3CSK4 resulted in no significant increase upon subsequent activation with fMLP compared to the fMLP control (P>0.05) (Figure 3.2A). Both fMLP treated neutrophils with and without Pam3CSK4 were significantly increased compared to untreated and Pam3CSK4 treated cells alone (P's < 0.001). Treatment of neutrophils with Pam3CSK4 in the absence of fMLP induced a small production of 5-HETE, 12-fold lower than levels seen when they were combined (P<0.001) (Figure 3.2A).

Normalised data indicate stimulation of human neutrophils with Pam3CSK4 alone resulted in the generation of TNF-α, IL-1β and IL-8. Although not significant (P>0.05), TNF-α and IL-8 showed an apparent 6 and 0.5-fold increase, respectively, when neutrophils were primed with Pam3CSK4 with the addition of fMLP, compared to the agonist alone (Figure 3.3Ai, Aiii). This additive effect was not seen for IL-1β (Figure 3.3Aii). fMLP-treated neutrophils showed no significant cytokine production. IL-6 was below the limit of detection.

Individual donor responses to the agonist are shown for information (Figure 3.2B-3.3Bi-iii).

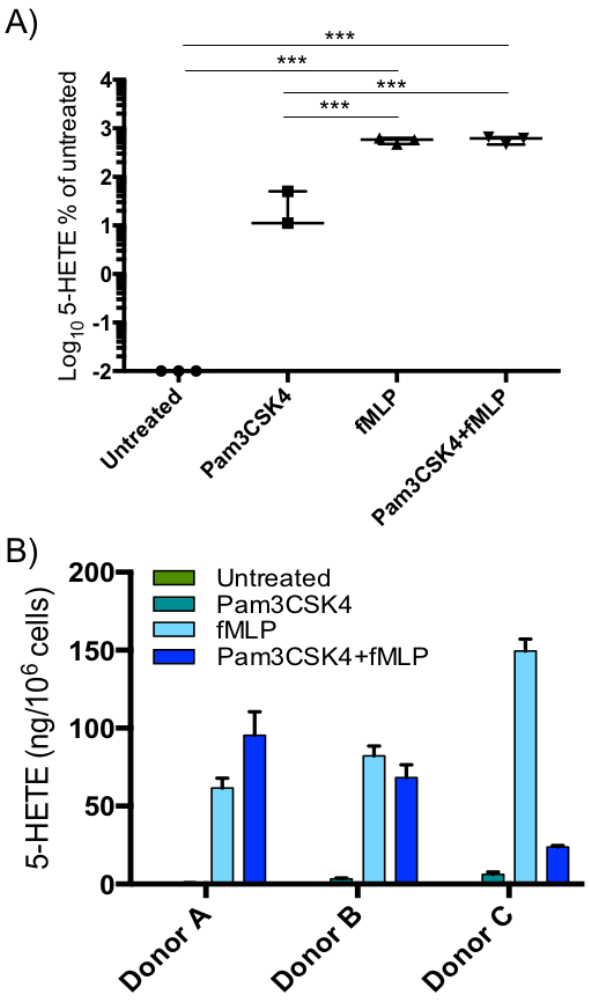


Figure 3.2 Generation of 5-HETE by Pam3CSK4 (TLR1/2) exposure to human neutrophils following fMLP activation. Isolated neutrophils were primed with Pam3CSK4 (100 ng/mL) for 30 minutes at 37°C before being stimulated with fMLP (1 μM, 2 minutes). Results show **A)** Normalised data expressed as a mean percentage of the untreated cells, where untreated is 0.01. Data are displayed on a logarithmic scale therefore, only values > 0 can be plotted. Bars represented as median with range. Data were analysed using a one-way ANOVA with a post hoc Tukey analysis. No significance = P > 0.05, *** = P < 0.001. *n* = 3. **B)** Data presented as mean individual donor responses. Pam3CSK4 priming of neutrophils with or without fMLP activation and also fMLP alone. Data represented as mean ± S.E.M.

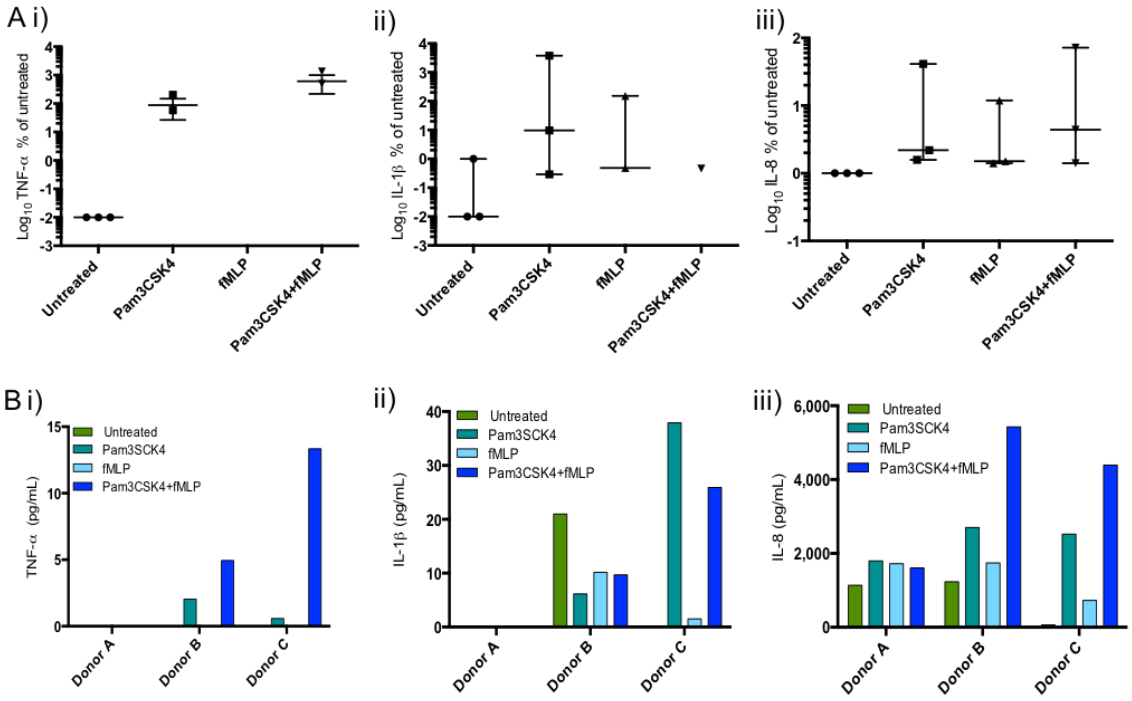


Figure 3.3 Generation of proinflammatory cytokines by Pam3CSK4 (TLR1/2) exposure to human neutrophils following fMLP activation. Isolated neutrophils were primed with Pam3CSK4 (100ng/mL) for 30 minutes at 37°C before being stimulated with fMLP (1μM, 4 hours). Results show **Ai-iii)** Normalized data for individual donor responses for the generation of TNF-α, IL-1β and IL-8 respectively, expressed as a mean percentage of the untreated. For normalised data untreated is 0.01 unless there is a real value. Data are displayed on a logarithmic scale therefore, only values > 0 can be plotted. Bars represented as median with range. **Ai)** Data were analysed using a one-way ANOVA with a post hoc Tukey analysis. $P > 0.05$. $n = 3$. **Aii-iii)** Data were analysed using a non-parametric Kruskal-Wallis test with a Dunn's comparison with Bonferroni corrections for post-hoc analysis. $P > 0.05$. $n = 3$. **Bi-iii)** Individual donor production of TNF-α, IL-1β and IL-8 respectively, when primed with Pam3CSK4 with or without fMLP activation and also fMLP alone.

3.3.2.2 Gram-positive TLR2 agonist heat-killed Listeria monocytogenes (HKLM)

Normalised data for 5-HETE production showed priming of human neutrophils with Gram-positive agonist HKLM had no significant increase when activated with fMLP compared to the fMLP control (P>0.05) (Figure 3.4A). HKLM-treated neutrophils showed minor production of 5-HETE, over 150-fold less than that seen when subsequently activated with fMLP (Figure 3.4A).

Normalised data showed that HKLM induced TNF-α and IL-8 generation from neutrophils, which, although not significant, had an apparent 1-fold increase when the agonist was combined with fMLP, for both cytokines (P>0.05) (Figure 3.5Ai, Aiii). HKLM stimulated generation of IL-1β, which was shown to decrease > 6-fold when treatment was combined with fMLP (Figure 3.5Aii). fMLP-treated neutrophils did not induce TNF-α or IL-1β production (Figure 3.5Ai-ii) but produced IL-8 at a 0.5-fold decrease compared to the agonist alone (P>0.05) (Figure 3.5Aiii). IL-6 was below the limit of detection.
Individual donor responses to the agonist are shown for information (Figure 3.4B-3.5Bi-iii).

3.3.2.3 Gram-negative TLR4 agonist lipopolysaccharide (LPS)

Normalised data for 5-HETE production showed that priming of human neutrophils with Gram-negative agonist LPS followed by activation with fMLP resulted in a non-significant, 0.25-fold increase compared to the fMLP control (P>0.05) (Figure 3.6A). LPS alone did not generate 5-HETE.
When data were normalised, neutrophils exposed to LPS showed TNF-α generation, which had an apparent 3-fold decrease with the addition of fMLP (Figure 3.7Ai). LPS alone also stimulated IL-1β generation; the agonist combined with fMLP and fMLP alone did not show any significant response (P>0.05) (Figure 3.7Aii). IL-8 was generated in response to LPS, fMLP and the combined treatment. There was no significant difference shown

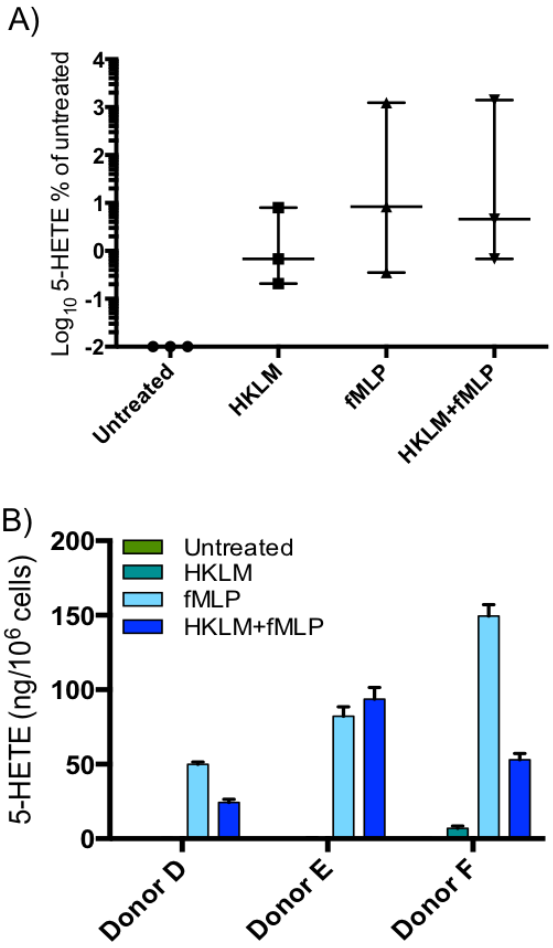


Figure 3.4 Generation of 5-HETE by HKLM (TLR2) exposure to human neutrophils following fMLP activation. Isolated neutrophils were primed with HKLM (10^8 cells/mL) for 30 minutes at 37°C before being stimulated with fMLP (1 μM, 2 minutes). Results show **A)** Normalised data expressed as a mean percentage of the untreated cells. Untreated = 0.01. Data displayed on a logarithmic scale therefore, only values > 0 can be plotted. Bars represented as median with range. Data were analysed using a one-way ANOVA with a post hoc Tukey analysis. $P > 0.05$. $n = 3$. **B)** Data presented as mean individual donor responses. HKLM priming of neutrophils with or without fMLP activation and also fMLP alone. Data represented as mean ± S.E.M.

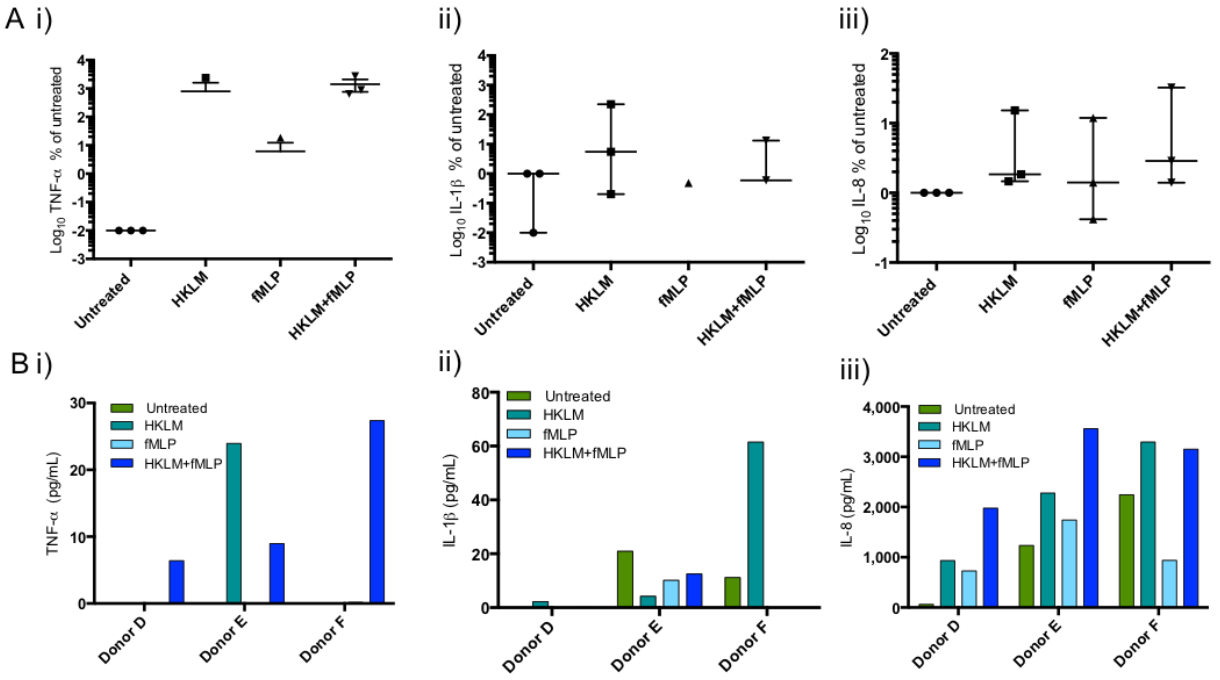


Figure 3.5 Generation of proinflammatory cytokines by HKLM (TLR2) exposure to human neutrophils following fMLP activation. Isolated neutrophils were primed with HKLM (10^8 cells/mL) for 30 minutes at 37°C before being stimulated with fMLP (1 μM, 4 hours). Results show **Ai-iii)** Normalized data for individual donor responses for the generation of TNF-α, IL-1β and IL-8 respectively, expressed as a mean percentage of the untreated. For normalised data untreated is 0.01 unless there is a real value. Data displayed on a logarithmic scale therefore, only values > 0 can be plotted. Bars represented as median with range. **Ai-ii)** Data were analysed using a non-parametric Kruskal-Wallis test with a Dunn's comparison with Bonferroni corrections for post-hoc analysis. $P > 0.05$. $n = 3$. **Aiii)** Data were analysed using a one-way ANOVA with a post hoc Tukey analysis. $P > 0.05$. $n = 3$. **Bi-iii) I**ndividual donor production of TNF-α, IL-1β and IL-8 respectively, when primed with HKLM with or without fMLP activation and also fMLP alone.

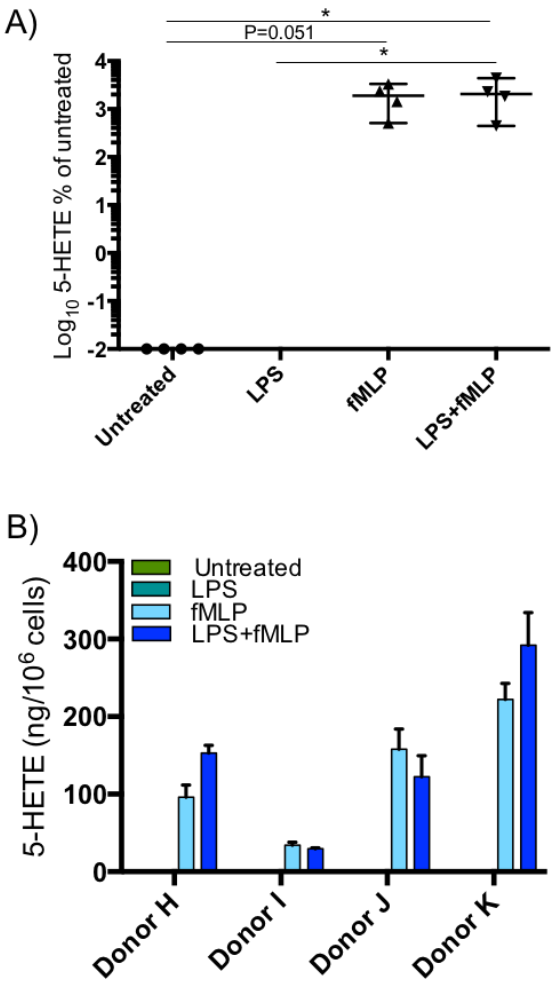


Figure 3.6 Generation of 5-HETE by LPS (TLR4) exposure to human neutrophils following fMLP activation. Isolated neutrophils were primed with LPS (1 µg/mL) for 30 minutes at 37°C before being stimulated with fMLP (1 µM, 2 minutes). Results show **A)** Normalised data expressed as a mean percentage of the untreated cells, where untreated is 0.01. Data displayed on a logarithmic scale therefore, only values > 0 can be plotted. Bars represented as median with range. Data were analysed using a one-way ANOVA with a post hoc Tukey analysis. No significance = P > 0.05, * = P < 0.05. *n* = 3. **B)** Data presented as mean individual donor responses. LPS priming of neutrophils with or without fMLP activation and also fMLP alone. Data represented as mean ± S.E.M.

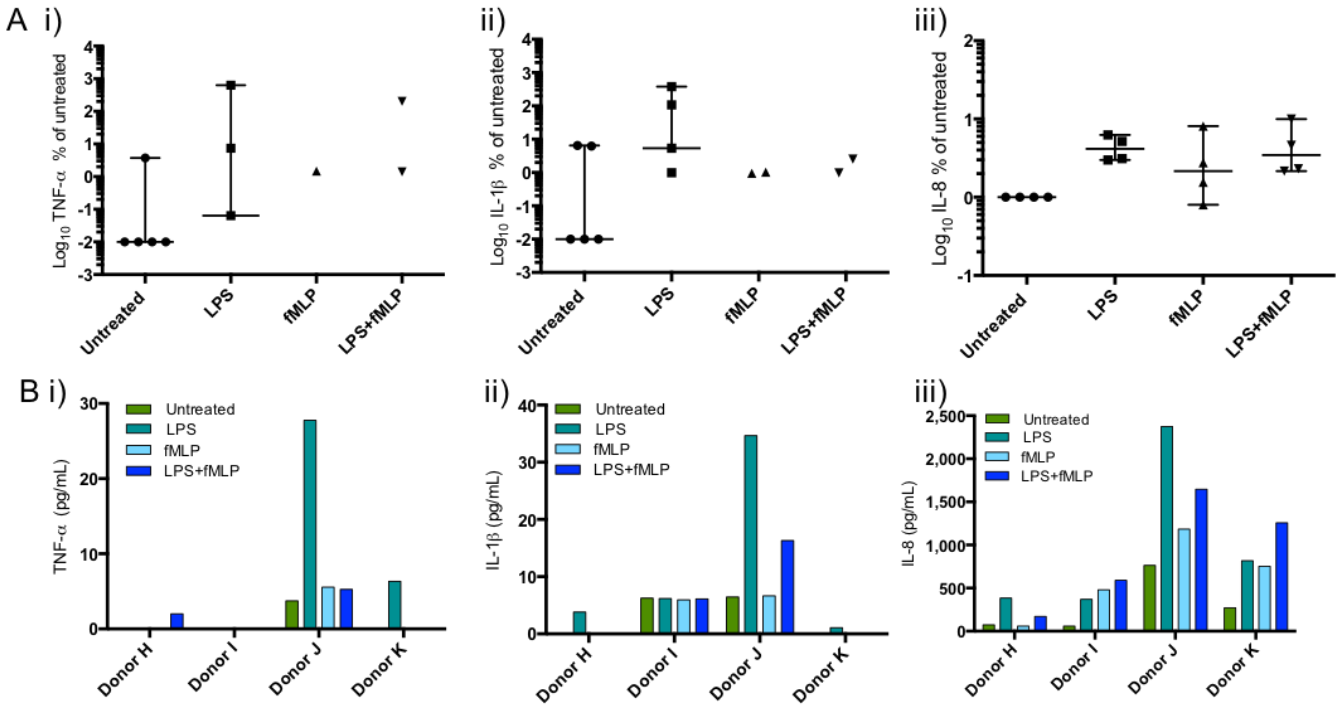


Figure 3.7 Generation of proinflammatory cytokines by LPS (TLR4) exposure to human neutrophils following fMLP activation. Isolated neutrophils were primed with LPS (1 μg/mL) for 30 minutes at 37°C before being stimulated with fMLP (1 μM, 4 hours). Results show **Ai-iii)** Normalized data for individual donor responses for the generation of TNF-α, IL-1β and IL-8 respectively, expressed as a mean percentage of the untreated. For normalised data untreated is 0.01 unless there is a real value. Data displayed on a logarithmic scale therefore, only values > 0 can be plotted. Bars represented as median with range. **Ai)** Data were analysed using a non-parametric Kruskal-Wallis test with a Dunn's comparison with Bonferroni corrections for post-hoc analysis. $P > 0.05$. $n = 3$. **Aii-iii)** Data were analysed using a one-way ANOVA with a post hoc Tukey analysis. $P > 0.05$. $n = 3$. **Bi-iii)** Individual donor production of TNF-α, IL-1β and IL-8 respectively, when primed with LPS with or without fMLP activation and also fMLP alone.

between any of these conditions for cytokine generation (P>0.05). IL-6 was below the limit of detection.
Individual donor responses to the agonist are shown for information (Figure 3.6B-3.7Bi-iii).

3.3.2.4 Gram-negative TLR5 agonist Flagellin

Although not significant, normalised data for 5-HETE production indicated that priming of neutrophils with *Salmonella typhimurium* flagellin followed by fMLP activation showed an inhibitory effect of ~ 0.3-fold, compared to fMLP alone (P>0.05) (Figure 3.8A). The agonist alone did not generate any significant 5-HETE compared to the untreated neutrophils (P>0.05). fMLP alone (P<0.01) and fMLP combined with flagellin (P<0.05) showed a significant increase in 5-HETE generation compared to untreated neutrophils and neutrophils exposed to flagellin alone.

For cytokines, normalised data showed that, flagellin induced TNF-α generation from neutrophils; levels remained the same with the addition of fMLP (Figure 3.9Ai). fMLP alone did not generate TNF-α. There was no significant difference shown between any of the conditions for cytokine generation of TNF-α (P>0.05). Flagellin stimulated the generation of IL-8, where levels showed a non-significant 0.8-fold apparent increase compared to fMLP alone and a significant 2-fold increase compared to flagellin combined with fMLP (Figure 3.9Aii). Neutrophils primed with flagellin before fMLP activation showed a significant increase in IL-8 generation compared to neutrophils alone (P<0.01). IL-1β and IL-6 were below the limit of detection.
Individual donor responses to the agonist are shown for information (Figure 3.8B-3.9Bi-iii).

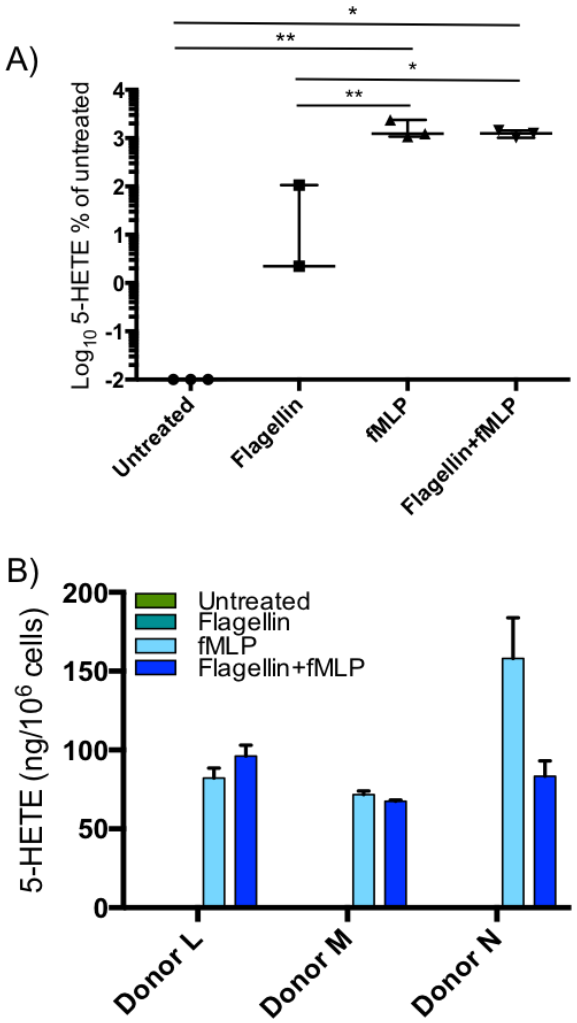


Figure 3.8 Generation of 5-HETE by flagellin (TLR5) exposure to human neutrophils following fMLP activation. Isolated neutrophils were primed with flagellin (1 µg/mL) for 30 minutes at 37°C before being stimulated with fMLP (1 µM, 2 minutes). Results show **A)** Normalised data expressed as a mean percentage of the untreated, where untreated is 0.01. Data displayed on a logarithmic scale therefore, only values > 0 can be plotted. Bars represented as median with range. Data were analysed using a one-way ANOVA with a post hoc Tukey analysis * = $P < 0.05$, ** = $P < 0.01$. *n* = 3. **B)** Data presented as mean individual donor responses. Flagellin priming of neutrophils with or without fMLP activation and also fMLP alone. Data represented as mean ± S.E.M.

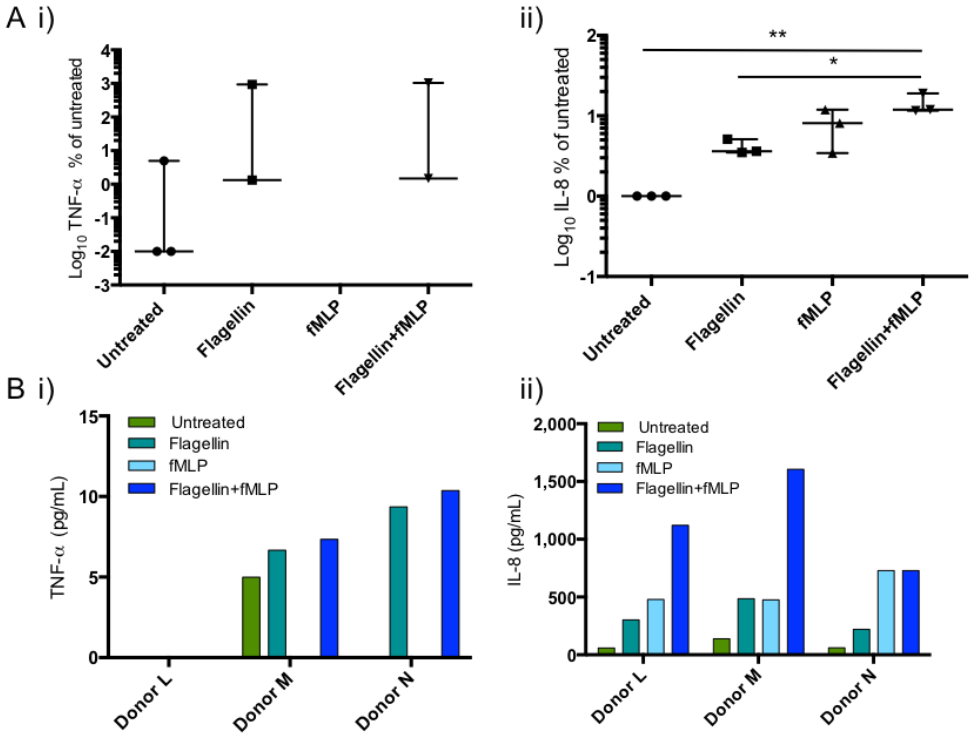


Figure 3.9 Generation of proinflammatory cytokines by flagellin (TLR5) exposure to human neutrophils following fMLP activation. Isolated neutrophils were primed with flagellin (1 µg/mL) for 30 minutes at 37°C before being stimulated with fMLP (1 µM, 4 hours). Results show **Ai-ii)** Normalized data for individual donor responses for the generation of TNF-α and IL-8 respectively, expressed as a mean percentage of the untreated. For normalised data untreated is 0.01 unless there is a real value. Data displayed on a logarithmic scale therefore, only values > 0 can be plotted. Bars represented as median with range. **Ai)** Data were analysed using a non-parametric Kruskal-Wallis test with a Dunn's comparison with Bonferroni corrections for post-hoc analysis. P > 0.05. *n* = 3. **Aii)** Data were analysed using a one-way ANOVA with a post hoc Tukey analysis. * < 0.05, ** < 0.01. *n* = 3. **Bi-ii) I**ndividual donor production of TNF-α and IL-8 respectively, when primed with flagellin with or without fMLP activation and also fMLP alone.

3.3.2.5 Gram-positive TLR6/2 agonist FSL

Normalised data for 5-HETE production showed that priming of human neutrophils with Gram-positive *Mycoplasma salivarium* lipoprotein FSL had no significant increase upon subsequent activation with fMLP compared to the fMLP control ($P>0.05$) (Figure 3.10A). Treatment of neutrophils with FSL alone induced a small production of 5-HETE, > 35-fold lower than levels seen when combined with fMLP (Figure 3.10A). Neutrophils exposed to fMLP alone and FSL combined with fMLP showed a significant increase in 5-HETE generation compared to untreated neutrophils and neutrophils exposed to the agonist alone ($P's<0.0001$).

When data were normalised, neutrophils exposed to FSL showed TNF-α generation, which had a 2-fold increase with the addition of fMLP (Figure 3.11Ai). fMLP alone did not produce TNF-α. FSL also induced IL-8, which showed a > 1-fold apparent increase when the agonist was combined with fMLP (Figure 3.11Aii). fMLP induced IL-8 generation. There was no significant difference shown between any of these conditions for cytokine generation ($P>0.05$). IL-1β and IL-6 were below the limit of detection.

Individual donor responses to the agonist are shown for information (Figure 3.10B-3.11Bi-iii).

3.3.2.6 Gram-negative and Gram-positive TLR9 agonist ODN

Although non-significant, normalised data for 5-HETE production showed that priming of human neutrophils with intracellular Gram-positive and -negative oligonucleotide ODN followed by fMLP activation showed an apparent inhibitory effect of ~ 0.3-fold, compared to fMLP alone (Figure 3.12A). The agonist combined with fMLP generated 5-HETE with an apparent ~ 17-fold increase compared to the agonist alone, this increase was not significant ($P>0.05$) (Figure 3.12A). The agonist combined with fMLP did show a significant increase ($P<0.05$) in 5-HETE generation when compared to the neutrophils alone. fMLP treated neutrophils generated 5-

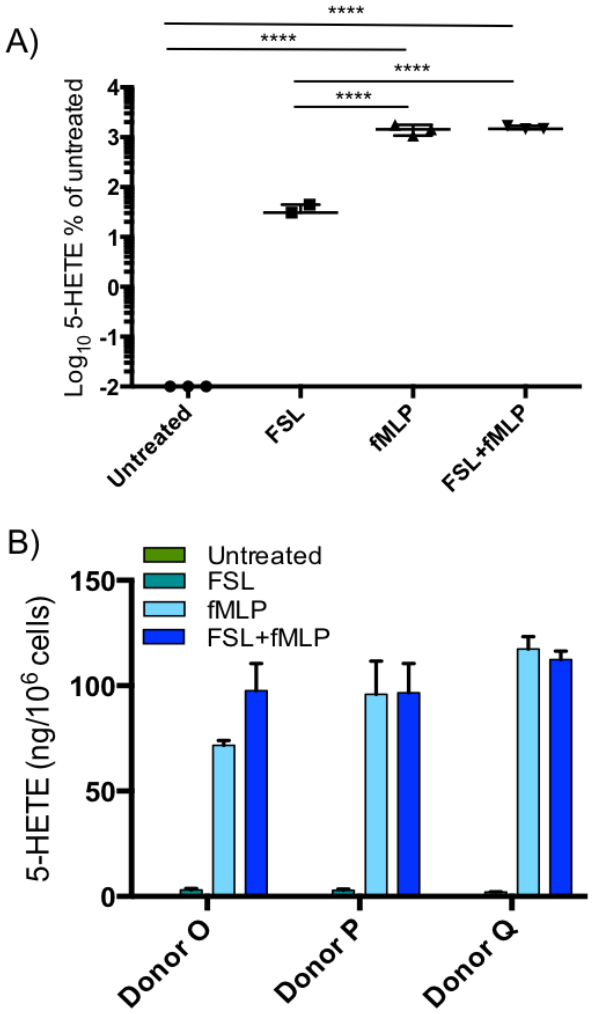


Figure 3.10 Generation of 5-HETE by FSL (TLR6/2) exposure to human neutrophils following fMLP activation. Isolated neutrophils were primed with FSL (100 ng/mL) for 30 minutes at 37°C before being stimulated with fMLP (1 μM, 2 minutes). Results show **A)** Normalised data expressed as a mean percentage of the untreated, where untreated is 0.01. Data displayed on a logarithmic scale therefore, only values > 0 can be plotted. Bars represented as median with range. Data were analysed using a one-way ANOVA with a post hoc Tukey analysis **** = $P < 0.0001$. $n = 3$. **B)** Data presented as mean individual donor responses. FSL priming of neutrophils with or without fMLP activation and also fMLP alone. Data represented as mean ± S.E.M.

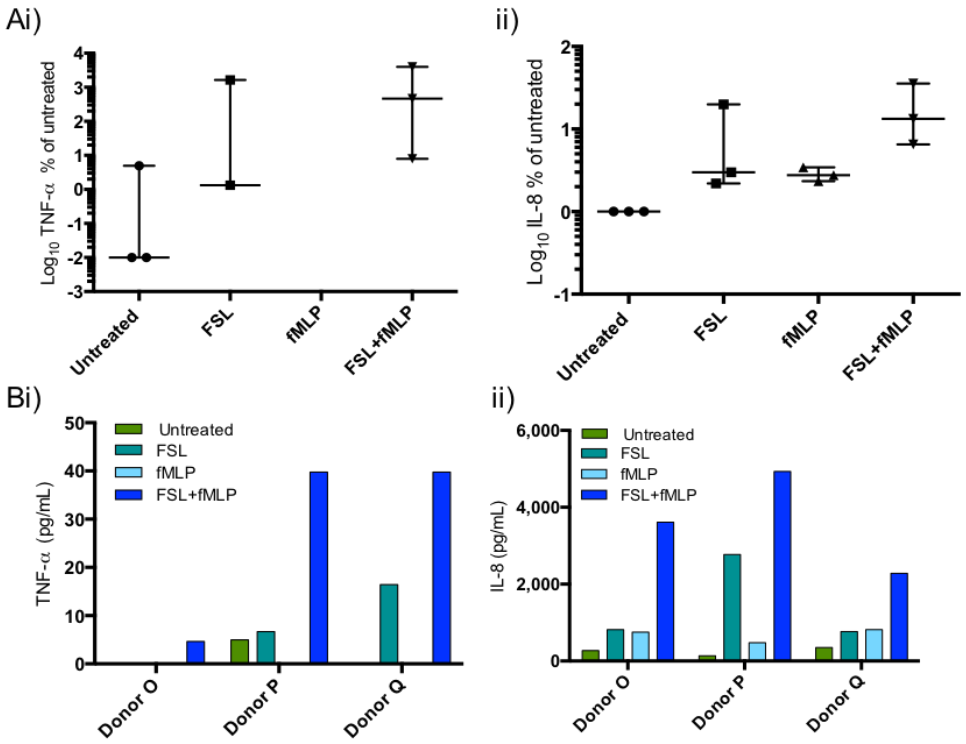


Figure 3.11 Generation of proinflammatory cytokines by FSL (TLR6/2) exposure to human neutrophils following fMLP activation. Isolated neutrophils were primed with FSL (100 ng/mL) for 30 minutes at 37°C before being stimulated with fMLP (1 μM, 4 hours). Results show **Ai-ii)** Normalized data for individual donor responses for the generation of TNF-α and IL-8 respectively, expressed as a mean percentage of the untreated. For normalised data untreated is 0.01 unless there is a real value. Data displayed on a logarithmic scale therefore, only values > 0 can be plotted. Bars represented as median with range. **Ai)** Data were analysed using a non-parametric Kruskal-Wallis test with a Dunn's comparison with Bonferroni corrections for post-hoc analysis. P > 0.05. *n* = 3. **Aii)** Data were analysed using a one-way ANOVA with a post hoc Tukey analysis. P > 0.05. *n* = 3. **Bi-ii)** Individual donor production of TNF-α and IL-8 respectively, when primed with FSL with or without fMLP activation and also fMLP alone.

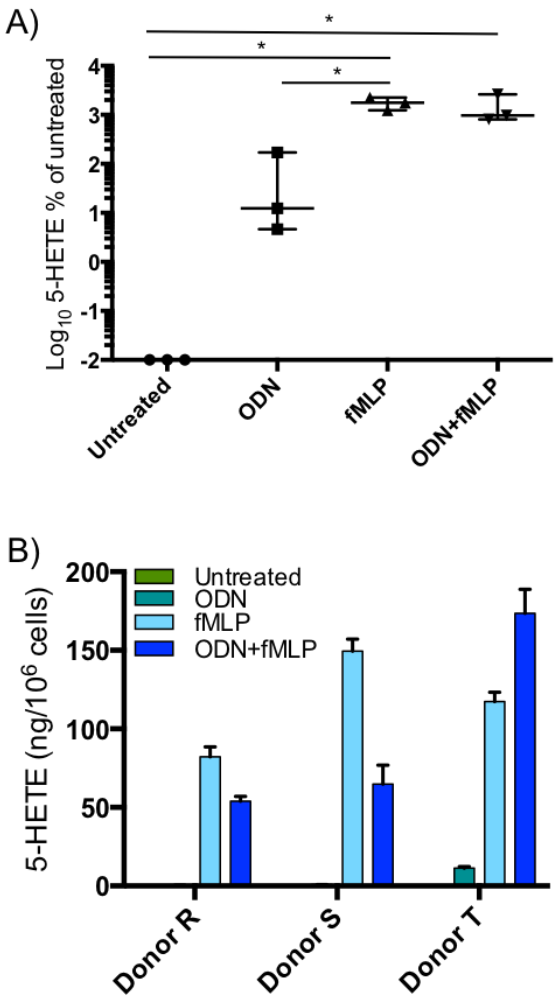


Figure 3.12 Generation of 5-HETE by ODN (TLR9) exposure to human neutrophils following fMLP activation. Isolated neutrophils were primed with ODN (5 μM) for 30 minutes at 37°C before being stimulated with fMLP (1 μM, 2 minutes). Results show **A)** normalised data expressed as a mean percentage of the untreated, where untreated is 0.01. Data displayed on a logarithmic scale therefore, only values > 0 can be plotted. Bars represented as median with range. Data were analysed using a one-way ANOVA with a post hoc Tukey analysis * = $P \leq 0.05$. *n* = 3. **B)** Data presented as mean individual donor responses. ODN priming of neutrophils with or without fMLP activation and also fMLP alone. Data represented as mean ± S.E.M.

HETE, which was significantly increased compared to the neutrophils alone and the neutrophils exposed to the agonist alone (P's<0.05).

Normalised data indicate, stimulation of human neutrophils with ODN alone generated of TNF-α, IL-8 and IL-6. Although not significant, TNF-α, IL-8 and IL-6 showed an apparent 1, 2.5 and 7-fold increase, respectively, when neutrophils were primed with ODN with the addition of fMLP, compared to the agonist alone (P>0.05) (Figure 3.13Ai-iii). fMLP-treated neutrophils showed no significant cytokine production (P>0.05). IL-1β was below the limit of detection.
Individual donor responses to the agonist are shown for information (Figure 3.12B-3.13Bi-iii).

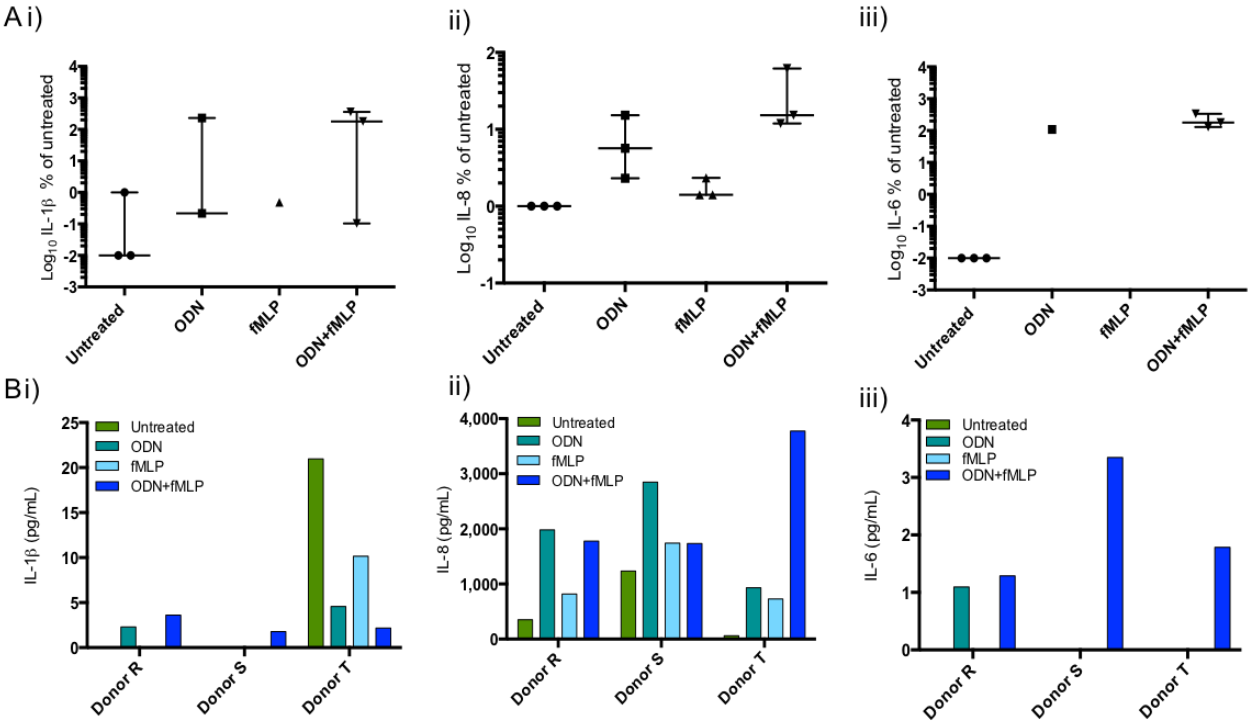


Figure 3.13 Generation of proinflammatory cytokines by ODN (TLR9) exposure to human neutrophils following fMLP activation. Isolated neutrophils were primed with ODN (5 μM) for 30 minutes at 37°C before being stimulated with fMLP (1 μM, 4 hours). Results show **Ai-iii)** Normalized data for individual donor responses for the generation of TNF-α, IL-8 and IL-6 respectively, expressed as a mean percentage of the untreated. For normalised data untreated is 0.01 unless there is a real value. Data displayed on a logarithmic scale therefore, only values > 0 can be plotted. Bars represented as median with range. **Ai&iii)** Data were analysed using a non-parametric Kruskal-Wallis test with a Dunn's comparison with Bonferroni corrections for post-hoc analysis. P > 0.05. *n* = 3. **Aii)** Data were analysed using a one-way ANOVA with a post hoc Tukey analysis. P > 0.05. *n* = 3. **Bi-iii)** Individual donor production of TNF-α, IL-8 and IL-6 respectively, when primed with ODN with or without fMLP activation and also fMLP alone.

3.4 Discussion

Work presented in this chapter describes the effect of individual bacterial TLR agonists on freshly isolated human neutrophils. The first series of experiments used a targeted lipidomics approach to compare the ability of calcium ionophore A23187 and fMLP to stimulate the production of the eicosanoid 5-HETE.

It has been shown previously, *in vitro*, that neutrophil 5-HETE is generated via the FPR1 receptor by the bacterial peptide fMLP (Clark et al. 2011). In the preliminary time course experiment the most substantial production of 5-HETE, by the isolated neutrophils, occurred following a 2 minute incubation in the presence of either the positive control, calcium ionophore, or fMLP. This rapid rate of formation could be suggestive of an autocrine mode of action, which is consistent with the observations seen by Clark *et al*., 2011. It cannot be determined from these data if the peak of formation is before 2 minutes, or whether levels are already declining by this point. Further experiments could investigate this by sampling at shorter time points. The level of 5-HETE expression reduced over the time course of 15 and 60 minutes, respectively, suggesting possible metabolism of 5-HETE over time.
For all subsequent experiments the bacterial peptide fMLP was used, to stimulate cells for 2 minutes, as it is able to activate all the classic functional activities of leukocytes, and acts independently to the TLR system (Pan et al. 2000).

3.4.1 Human neutrophil generation and detection of 5-HETE

Once the optimal time point of neutrophil activation was established, experiments then went on to analyse the generation of 5-HETE by isolated human neutrophils when primed with a comprehensive set of bacterial TLR agonists. TLR signalling is an important factor in host defence, playing a crucial role against a wide range of microorganisms (Bauer 2008).

Neutrophils have a wide array of effects in response to cell receptor TLR ligation, including cytokine production, priming, receptor expression and phagocytosis (Hayashi et al. 2003). The most studied TLRs are TLR2 and TLR4, mediating responses to Gram-positive and Gram-negative bacteria, respectively. TLR2 creates a heterodimer with TLR1 to detect triacylated peptides (Pam3CSK4) or TLR6 to detect diacylated peptides (FSL), whereas TLR4 recognises the lipid A component of lipopolysaccharide (LPS) (Prince et al. 2011).

TLR5 recognises bacterial flagellin while TLR9 which is an intracellular receptor response to bacterial DNA (ODN) which contains short sequences of non-methylated CpG dinucleotides that are at least 20-fold more common in bacterial than vertebrate DNA (Hayashi et al. 2001; Jozsef et al. 2006).

The most meaningful comparisons made within this body of work are between the neutrophils primed with the TLR agonist then activated with fMLP or exposed to fMLP alone. This is because it carries the most biological relevance, mimicking what would happen during infection as the host would be exposed to multiple pathogen derived signals. It was observed that there were no significant differences in 5-HETE production between these groups for any of the TLR agonists. This indicates that 5-HETE generation does not vary depending on the nature of the bacterial agonist, however, there may be several contributing factors.

Work here indicated that treatment of isolated neutrophils with *Escherichia coli* K12 LPS alone (TLR4 agonist), failed to stimulate the production of detectable 5-HETE. It may be the case that LPS alone does not prime human neutrophils (Hayashi et al. 2003; Kurt-jones et al. 2002). Commercially available LPS contains a significant amount of bacterial lipopeptide, a known TLR2 agonist and thus should be viewed as a dual TLR2 and TLR4 agonist (Hayashi et al. 2003). However, this is not the case for the LPS employed in this study as ultra-pure LPS was used.

It is known that treatment with the immune modulator granulocyte-macrophage colony-stimulating factor (GM-CSF) primes neutrophils to response to LPS (Kurt-Jones et al. 2002). Kurt-Jones *et al*., also described the synergistic effect of GM-CSF treatment prior to TLR2 stimulation, indicating that TLR2 expression was also

increased after GM-CSF treatment. This finding supports the fact that in this study neutrophils exposed to TLR1/2 agonist Pam2CSK4 showed little to no 5-HETE formation in response to the agonist alone. This lack of expression seen after treatment with the agonists alone was seen across all TLR agonists. This could indicate that cells may need pre-treatment with an immune modulator, such as GM-CSF, to increase receptor expression and induce an eicosanoid response to all bacterial agonists. It has been suggested by Hallett & Lloyds that priming is a laboratory artefact. However, they go on to discuss that in a physiological situation it is hard to envisage neutrophils being directly exposed to an activating stimulus at a high concentration without first being exposed to a lower concentration (Hallett & Lloyds 1995). Therefore, transition from resting to activated may possibly proceed *via* an intermediate primed state.

In vitro, priming with either Gram-negative LPS or Gram-positive Pam3CSK4, HKLM and FSL before subsequent fMLP activation showed a slight additive effect in terms of 5-HETE production when data were normalised, although not significant. This suggests that additional bacterial factors could enhance 5-HETE generation, in response to these TLRs. Clark *et al*., showed that levels of 5-LOX lipids generated in vitro and in vivo were remarkably similar, suggesting that 5-LOX-derived lipids will occur in response to Gram-positive and -negative organisms, stimulated by bacterial factors and thus is probably a common response to acute infection.

In contrast to the other agonists, when data were normalised, both the fixed concentrations of TLR5 flagellin and TLR9 ODN showed a non-significant decrease in fMLP-dependent formation of 5-HETE. This suggests that the concentration of the agonists employed may need to be increased to look at the dose response of these cells when exposed to the agonists, or that the exposure time for these specific agonists may need to be extended to see a greater response. It is also possible that the neutrophil cell type requires a co-stimulation for activation of the cell response, or synergy between TLRs for activation to be increased (Kurt-jones et al. 2002; Clark et al. 2011).

3.4.2 Human neutrophil generation of proinflammatory cytokines

In conjunction with 5-HETE formation, I also examined the ability of isolated human neutrophils to produce the following proinflammatory cytokines, TNF-α, IL-1β, IL-6 and IL-8, when exposed to the bacterial TLR agonists. In contrast to 5-HETE, it was seen that cytokine generation was not fMLP-dependent. Cytokine production showed a more consistent response between donors compared to lipid generation. It is unknown whether this response would mirror physiology as to date there are no published studies comparing cytokine and lipid profiles generated from human donors. However, the responses suggest that there may be potential to use both mediators as a double marker of infection, using cytokine expression as a well-characterised initial readout followed by the possibility to confirm microbial species with HETE formation.

The proinflammatory cytokines TNF-α and IL-1β are two of the most potent cytokines implicated in infectious and non-infectious diseases (Schulte et al. 2013). They both amplify the inflammatory cascade, in a autocrine and paracrine manner, by activating macrophages to secrete other inflammatory cytokines (IL-6 and IL-8), lipid mediators and reactive oxygen species (Schulte et al. 2013). The minimal levels of TNF-α and IL-1β seen with Pam3CSK4 generation may imply that agonist concentrations need to be increased to look at a dose response of the cells to agonist exposure. However, HKLM was used at its maximal working concentration (InvivoGen, tlrl-kit1hw) and is unlikely to induce a greater TNF-α response. As previously suggested it is also possible that the neutrophil cell type requires a co-stimulation for activation of the cell response (Clark et al. 2011), or that the number of neutrophils employed in our studies need to be increased. Neutrophils primed with Pam3CSK4 before being stimulated with fMLP showed an increased production of TNF-α but this was not shown to be significant. fMLP alone was not able to stimulate TNF-α formation which is consistent with findings that showed a relatively high concentration of fMLP (1 μM), matching that employed in this study, suppresses the production of TNF-α (Divisio 1998). Although it has been reported that saturation of responsiveness of the FPR1 fMLP receptor occurs at low concentrations of fMLP (Zigmond et al. 1982), it

has been shown that the regulation of many significant biological functions of neutrophils requires high concentrations of the formyl peptide (Elbim et al. 1993; Tomhave et al. 1994), thus justifying the dose employed.

The TLR agonists LPS (TLR4), flagellin (TLR5) and FSL (TLR6/2) also showed low induction levels of TNF-α. Flagellin and FSL had no detectable generation of IL-1β. It has been shown that in some cases various TLRs work synergistically to produce a response, this could be the case, for example, with Gram-negative LPS and flagellin (Mizel et al. 2003). As previously mentioned, it may also be plausible that working concentrations of the agonists need to be increased to enable us to see a greater response. Even though formylated peptides are well known proinflammatory molecules, primed neutrophils with LPS showed little to no formation of TNF-α when exposed to fMLP. It must also be taken into account that activated neutrophils also secrete many direct inhibitors, for example, the release of superoxide anions and/or lysosomal proteases that could affect the biological activity of TNF-α (Divisio 1998).

The intracellular TLR9 agonist ODN failed to produce detectable levels of TNF-α. This could be explained by evidence that has shown TLRs to interact in synergy or in a sequential fashion to amplify an innate immune response (Bhan et al. 2007). Although minimal, ODN was the only TLR agonist seen to generate the proinflammatory cytokine IL-6. This cytokine has a variety of biological functions, the main one being to mediate an acute phase response to inflammatory stimuli characterised by an induced fever, leucocytosis and release of hepatic acute phase proteins such as C-reactive protein (Schulte et al. 2013).

It has been speculated that low level of expression could be due to the purity of the isolated neutrophils, where high purity neutrophils (>99%) release detectable, but considerably less, IL-6 than standard neutrophil preparations (~5% contaminating cells) (Jozsef et al. 2006). However, the method of isolation used in this study provides the standard neutrophil preparation.

It may also be the case that the number of neutrophils used for TLR exposure needs to be increased to be able to detect higher levels, however, this would be difficult as individual donors produced varying amounts of neutrophils. Another possibility is

that the time point of formation differs between cytokines, for example one study showed that flagellin (TLR5) induced IL-6 production where levels peaked at 2-hours exposure and had declined by 4 hours (Hayashi et al. 2001).

The proinflammatory cytokine IL-8 was found to be secreted in abundant quantities by isolated human neutrophils stimulated with all bacterial TLR agonists. In all cases the agonists alone were able to generate the production of IL-8. This was also the case when neutrophils were exposed to fMLP without the TLR agonist, which corroborates finding that indicate fMLP is a potent inducer of IL-8 release in human neutrophils (Hidalgo et al. 2015). Four of the 6 bacterial agonists, Pam3CSK4, HKLM, Flagellin and FSL, showed an increased generation of IL-8 when neutrophils were primed prior to treatment with fMLP. This could indicate a possible synergy between TLR priming and fMLP activation, as it has been reported that the addition of fMLP increases NADPH oxidase activity and reactive oxygen species production, which favour the activation of the NF-kB transduction pathway involved in IL-8 release by neutrophils (Hidalgo et al. 2015). Normalised data showed that the Gram-positive ligands, Pam3CSK4, HKLM and FSL showed similar responses in terms of stimulating IL-8 generation compared to the other TLRs, which supports other findings that there is no difference between TLR ligands inducing release of IL-8 (Hilda & Das 2016).

3.4.3 Variability of neutrophil responses from different donors

This body of work proposes many factors that should be taken into consideration when interpreting the data. These include the diverse range of responses seen from individual donors following exposure of their neutrophils to TLR agonists. A retrospective power calculation was carried out to show how many donors would be needed in order to obtain significant results (TLR + fMLP Vs. fMLP). Based on the data collected in this chapter, and presuming donors are representative of the population, the study would require a sample size of 724 donors to achieve a power of 80% to declare that the conditions have significantly different means (i.e. a two sided p-value of less than 0.05) (Dhand & Khatkar 2014). With such a limited group of donors it is

also not possible to speculate whether any outliers within the donor responses (e.g. as seen with pam3CSK4) are in fact extreme outliers or are a genuine member of the population. This indicates that, as the donor cohort was extremely small, a much larger data set would need to be carried out to allow for the diverse variation between donors in a population and enable more significant comparisons between responses. This is an important point as the human race is outbred and thus observing a wide range of responses would be expected. The challenge in terms of predicting infection is determining which response is outside the 'normal' range.

Due to the ethics of the study presented in this chapter no information about the donors, such as gender and age, can be disclosed. Donors within this study were considered to be 'healthy donors'. This meant that they had not consumed alcohol within 12 hours of their morning blood draw and not taken non-steroidal anti-inflammatory drugs as they are known to inhibit the arachidonic acid cascade (Meirer et al. 2014). Donors were also required to have no known health conditions or diseases. For example, an individual with unstable asthma or someone who had suffered a recent asthmatic attack may present with elements of an infection, meaning that an inflammatory immune response would already be triggered (Lin et al. 2014). However, any possible underlying unknown conditions could have had an impact on their immune response.

Another factor to be considered is the varied ethnicities presented within an undefined donor population. This has been seen to demonstrate different characteristics, for example, people of African descent have been observed to have a reduced neutrophil count compared to individuals of European ancestry (Reich et al. 2009). Other influences to consider include age, gender, diet and lifestyle, which are all know to have an effect on the immune system function (Nakanishi et al. 2004; Oertelt-Prigione 2012). These factors further indicate the need for a larger donor pool within a defined population and could all be reasons for the diverse variation seen within donor responses in this body of work.

3.5 Conclusion

Work in this chapter observed that primary neutrophil exposure to bacterial TLR agonists stimulated the generation of the eicosanoid 5-HETE and proinflammatory cytokines. The TLR agonist results indicate that components derived from different bacteria had varying effects on the neutrophil 5-LOX pathway. This supports the idea that it may be possible to use LOX (or other eicosanoids) as an indicator of the presence of bacteria during an acute inflammatory response. This work also observes that all the TLR agonists explored in this study were able to induce neutrophils to secrete cytokines, and supports the concept that neutrophils form a link between innate and adaptive immune systems as cytokines are mediators between various immune cells during an immune response.

The response of primary human neutrophils was examined as they play a central role in the human immune response to infection. Although primary neutrophils have a limited lifespan, they do offer a number of advantages over a cell line, specifically, that they are more cost-effective compared to using a cell line; they carry a lower risk of gene mutations; and are isolated directly from the tissue and therefore resemble primary tissue characteristic and the capture the genetic diversity of individual donors thus increasing their biological relevance (Welser-Alves 2015).

Indeed, it is this diversity and the wide range of “normal” responses generated by the human race that represents the principal challenge when seeking to identify a specific response indicative of bacterial infection.

To be able to get a real understanding of the biological readouts seen in this chapter a much larger cohort of donors would need to be analysed to allow for the variability seen within a population. A further issue which arose during this chapter of study was the unpredictable quantity of neutrophils isolated from the peripheral blood of individual donors, which in some cases was too low to support planned experiments.

These issues coupled with the need to markedly increase the donor pool to account for the natural variation within the population lead us to investigate the feasibility of employing a human immortalised cell line THP-1 to characterise the responses to individual TLR agonists (Chapter 4).

Chapter 4

Detecting the production of 15-HETE and proinflammatory cytokines by human THP-1 monocyte derived macrophages

4.1 Introduction

There are numerous methods used to study macrophages *in vitro*, with the most common being either the use of primary peripheral blood mononuclear cells (PBMCs) or monocyte cell lines with standardised degrees of differentiation. As demonstrated in Chapter 3 primary cells are highly variable, which is why monocyte cell line populations are frequently used as models. As previously mentioned, when isolated from human whole blood donations, cells are limited in numbers. This, coupled with the fact that primary tissue macrophages cannot be readily expanded *ex vivo*, limits use of these primary cells when needed in protocols requiring large numbers of cells (Daigneault et al. 2010). It is also important to produce a standardised population of cells due to the wide variation in responses seen in primary cells, as demonstrated in Chapter 3. The use of cell lines are advantageous as they present a homogeneous genetic background which reduces the variability in cell phenotype. However, it is important to note differences in their differentiated state can lead to variations in cell responses and therefore experiments may not always accurately predict the behaviour of differentiated tissue macrophages (Daigneault et al. 2010; Lund et al. 2016). Cell lines are also very sensitive to good culture conditions, and in some cases can undergo differentiation as a result of inconsistencies within their culture conditions, which can subsequently affect the outcome of the study (Aldo et al. 2012).

A possible model system for neutrophils is the human leukaemia cell line HL-60, which can be stimulated with agents such as dimethyl sulfoxide (DMSO) to differentiate into neutrophil-like cells (Hauert et al. 2002). However, conflicting data suggest that these differentiated cells are limited in their capacity to respond to stimuli such as formylated peptides, which also activate neutrophils, as seen in Chapter 3 (Mangelsdorf et al. 1984). There are several examples of monocytic cell lines, which include human U937, HL-60 or THP-1 (Aldo et al. 2012) and mouse RAW 264.7 cells (Schrimpe & Wright 2009). Human THP-1 cells are the most widely employed to investigate the function and expression of primary human macrophages during disease (Lund et al. 2016). THP-1 cells are a human monocytic leukaemia-

derived cell line, acquired from the peripheral blood of a one-year-old male with acute monocytic leukaemia (ECACC 2016). These cells are differentiated into monocyte-derived macrophages using phorbol 12-myristate 13-acetate (PMA), and are accepted as a model to study immune response because of the similarities in their responses, which closely resemble those of primary human macrophages (Chanput et al. 2010; Lund et al. 2016).

It has been reported that expression of 15-lipoxygenase (15-LOX) and proinflammatory markers have been used to look at correlations during ischemic heart disease and atherosclerosis; cells employed included isolated human peripheral blood monocytes and subsequent cell culture into macrophages, isolated human carotid plaque macrophages and differentiated THP-1 cells (Magnusson et al. 2012; Wuest et al. 2012; Hultén et al. 2010; Danielsson et al. 2008). Literature has also shown that overexpression of 15-LOX in THP-1 monocyte-derived macrophages generates 15-HETE when infected with recombinant adenoviral vectors (Danielsson et al. 2008). These studies show that macrophages have been seen to generate markers of non-infectious and infectious disease. The major advantages of using THP-1 cells over primary cells is their homogeneous genetic background which abolishes donor variability, and they are easily accessible and obtained without contamination with other blood components (Schildberger et al. 2013). Therefore, this readily available cell line will be employed in this body of work, with the appreciation that extrapolation of the results for *in vivo* settings will be with care.

This chapter proposed to establish a growing culture of human THP-1 monocyte cells, which could be differentiated into monocyte-derived macrophages, and used as a standardised population of cells to produce readouts in response to bacterial treatment. Initial experiments aimed to use a lipidomic approach, described in Chapter 3, to show that differentiated human THP-1 cells generate 15-lipoxygenase (15-LOX) derived lipids, when activated with the stimuli calcium ionophore A23187 and fMLP. As detailed in Chapter 1, calcium ionophore A23187 promotes calcium mobilisation and subsequent translocation of the enzyme, 15-LOX, to the inner membrane surface, and is a commonly use stimulus for the generation of these lipids

in human and animal macrophages (Hammond & O'Donnell 2012). The potent formylated chemotactic peptide, fMLP, is similar in structure to oligopeptide products of bacterial metabolism (Holian & Daniele 1981). Both neutrophils and macrophages are known to display the specific formyl peptide receptor for fMLP, allowing cells to respond to infection/inflammation (Shrivastava 2007).

As discussed in Chapter 1, innate immune cells produce and release an array of cytokines in response to inflammation and infection in the body, and modulation of this inflammatory signalling during systemic infection has been considered a possible means to improve survival (Osuchowski et al. 2006; Lacy & Stow 2011). Activated monocytes, macrophages and neutrophils generate cytokines, chemokines, cell surface receptor/adhesion proteins and other molecules as part of an immune response essential to host defence during bacterial infection (Z. K. Pan et al. 2000). As previously discussed, proinflammatory cytokines, including interleukin-1beta (IL-1β), interleukin-8, interleukin-6 and tumour necrosis factor (TNF), are some of the most well studied proteins that regulate inflammation (Anderstam et al. 2012). Techniques will be employed to confirm cytokine generation alongside eicosanoids.

4.1.1 Aims of this chapter

The first aim of the work reported in this chapter was to use a lipidomic approach to determine whether the human monocytic THP-1 cell line generated 15-lipoxygenase (15-LOX) lipids, when differentiated into THP-1 monocyte-derived macrophages and thus allow me to use these cells as an *in vitro* model for macrophage bacterial response. The second aim was to confirm the production of selected proinflammatory cytokines from differentiated THP-1 cells, in response to LPS stimulation.

The objectives were:

1. To establish a growing culture of human THP-1 monocyte cells and successfully differentiate them into THP-1 monocyte-derived macrophages.

2. To examine the effects of calcium ionophore A23187, using mass spectrometry detection of THP-1 macrophage 15-HETE formation.
3. To use ELISAs to analyse the cell supernatant after stimulation to detect proinflammatory cytokine generation.

4.2 Specific materials and methods

4.2.1 Human monocyte THP-1 cell culture

The cell line was purchased from Public Health England's European Collection of Authenticated Cell Cultures (ECACC 88081201). Sigma-Aldrich Company Ltd (Gillingham, Dorset, UK) supplied all cell culture reagents unless otherwise stated.

THP-1 cells were maintained in RPMI-1640 supplemented with heat-inactivated foetal calf serum (FCS; 10% *v/v*) and 2mM L-glutamine (ThermoFisher Scientifics, Gibco 25030-081) (referred to hereafter as THP-1 media). The cells were grown in a humidified incubator at 37°C in the presence of 5% CO_2.

4.2.1.1 Revival of stock THP-1 cells from liquid nitrogen storage

Cells were removed from liquid nitrogen storage and placed on ice to allow gentle thawing. Routine culture media (RMPI-1640 with 10% heat-treated FCS and 2mM L-glutamine) was added to the cells and centrifuged at 300g for 5 minutes. The supernatant was removed, and the cells were re-suspended in fresh culture media before being transferred to a T-25 cell culture flask (Sigma-Aldrich). The flask was stood upright and incubated at 37°C in the presence of 5% CO_2.

4.2.2 Human monocyte THP-1 cell differentiation

To optimise differentiation, cells were exposed to varying concentrations of phorbol 12-myristate 13-acetate (PMA) ranging from 0.5-100 μmol/L over a time course of

24-72 hours (stock PMA was dissolved in dimethyl sulfoxide (DMSO)). Optimisation showed that exposing the cells to 0.5 µmol/L PMA for both 24 and 48 hours had the best rate of differentiation. Differentiation was determined by adherence. For all subsequent experiments THP-1 cells were differentiated in accordance with these findings.

For differentiation into a macrophage phenotype, THP-1 cells (used at 5 x 10^5 cells/0.5mL) were incubated for 24 and 48 hours with PMA (0.5 µmol/L) in a 24 well tissue culture plate (Corning). After 24-hours non-adherent cells were removed by gently washing the cells in fresh THP-1 media when looking for cytokine secretion or RPMI-1640 media alone when looking for eicosanoid generation. Adherent cells were incubated and allowed to recover in the appropriate fresh THP-1 media or RPMI-1640 for a further 30 minutes prior to use.

4.2.3 THP-1 cell fluorescent staining and microscopy

THP-1 cells ($2.5x10^5$) were grown in glass bottom dishes (MatTek, ThermoFisher Scientifics), either untreated or PMA-treated (0.5 µmol/L) (as above) for both 24 and 48-hours. Cells were washed and re-suspended in phenol red-free RPMI 1640, 10% FCS-HI, 1% L-glutamine. Hoechst 33342 (ThermoFisher Scientifics) nucleic acid stain is a semi-permeant counter stain that emits blue fluorescence when bound to dsDNA. Hoechst was prepared by diluting the stock solution 1:10,000 in media. Sufficient staining solution was added to cover the cells (~ 1mL) and incubated (37°C, 5% CO_2) for 5-10 minutes, protected from light. The Hoechst was subsequently removed from the cells by washing them in fresh media. Non-differentiated THP-1 cells were spun at 150g for 3 minutes as they are non-adherent. The cells were then counter stained with CellMask™ Orange (Life Technologies, C10045), which is a plasma membrane stain that allows a fast and uniform labelling of the plasma membrane for standard fluorescence microscopy. Stock was diluted 1:10,000 in media and 1 mL was added to the cells before being incubated for a further 3

minutes. Cell were then washed again with fresh media and replaced with approximately 1 mL media and incubated.

Fluorescent images were acquired using a Leica DMIRB inverted microscope equipped with a 40x oil objective (Cardiff School of Pharmacy). Ultraviolet (UV) light was used to activate the Hoechst (excitation wavelength 360 nm), which emits blue fluorescence between a wavelength of 460 to 490 nm. Green light was used to activate the CellMask™ Orange (excitation wavelength 554 nm), which emits orange fluorescence 567 nm. Fluorescent images were detected using Alexa 350 spectrum to identify the nucleus and Cy5-666 spectrum to identify the plasma membrane. Differential interference contrast (DIC) microscopy was performed using a Leica DMIRB inverted microscope equipped with 10x objective and using the brightfield mode with a "Ph1" setting on the phase selector ring.

4.2.4 Generation and identification of 15-HETE

To generate 15-HETE from these cells, subsequent to differentiation, THP-1 monocyte-derived macrophages were stimulated with either calcium ionophore (A23187; 10 µM) or fMLP (1 µM) and 2.5mM $CaCl_2$ and 1.25mM $MgCl_2$ for 2, 15 or 60 minutes. Samples for 15-HETE analysis were then extracted using lipid hexane extraction described in Chapter 2, section 2.6. Reverse-phase LC/MS/MS of lipids was then carried out to identify generation of 15-HETE. The specific method used is the same as that described in Chapter 3 section 3.1.3, however, to quantify the detections of 15-HETE the MRM transition used was 319-219.

4.2.5 Generation and identification of proinflammatory cytokines

Enzyme-linked immunosorbent assays (ELISAs) were used to determine any cytokine expression from differentiated THP-1 monocyte-derived macrophages in response to different stimuli. Differentiated cells were incubated with fMLP (1 µM) and calcium ionophore A23187 (10 µM) over a 60 minutes time course to confirm the production

of proinflammatory cytokines. Following incubation, cell-free supernatants were transferred into fresh Eppendorf tubes for subsequent cytokine analysis. Cytokine analysis was carried out using the R&D Systems Human DuoSet ELISAs described in Chapter 3, section 3.1.4.

Cytokine secretion was also identified following *E. coli* K12 lipopolysaccharide (LPS) activation. THP-1 cells were differentiated for 24 hours with PMA (0.5 µmol/L), cells were washed in RPMI-1640 before being exposed to varying concentrations of LPS (1, 10, 100 ng/mL) for 0, 1, 4 and 24 hours. Samples were analysed for cytokine production using ELISAs as previously described.

To determine cytokine secretion under basal conditions, THP-1 cells were differentiated with PMA (0.5 µmol/L) for 24 hours. Cells were subsequently washed in fresh media and the supernatant was collected over a time course of 4 hours. Samples were analysed as above.

4.2.6 Statistical analysis

Statistical analysis was carried out as described in Chapter 2, section 2.10.

4.3 Results

4.3.1 Optimisation of THP-1 cell differentiation

A standardised human monocyte cell line (THP-1) was employed to characterise the eicosanoid and cytokine response of different bacterial agonists. Human THP-1 monocyte cells were cultured as previously described. Originally, cells were differentiated using methods provided in Connon *et al*. 2003. In this protocol, cells were incubated (37°C) for 3 hours in the presence of PMA (0.5 µmol/L) to allow for

cell differentiation. However, it was found that after this period of time most cells had not adhered to the plate therefore indicating a lack of cell differentiation.

Optimisation indicated that THP-1 cells plated in 24-well plates at 0.5 x 10^6 cells/well with 0.5 µmol/L PMA and incubated for 24 and 48 hours were the optimum conditions for cell differentiation. Cell adherence was indicative of differentiation. Following incubation, cell supernatant containing PMA was removed and cells were washed in RPMI-1640 media alone and allowed to recover for 30 minutes at 37°C. When analysing the generation of eicosanoids, cells were washed in RPMI-1640 media alone, as culture media with foetal calf serum contains high levels of all hydroxyicosatetraenoic acids (HETE), which were not seen in RMPI-1640 alone (Figure 4.1). Therefore, for all subsequent experiments activation of the cells was done in RPMI-1640 alone.

4.3.2 PMA-Induced differentiation of THP-1 monocyte into macrophages

THP-1 cells were differentiated into macrophages by the treatment with 0.5 µmol/L PMA for 24 hours. The treatment induced the typical features of macrophages, represented by cell adhesion, spread morphology, increased granularity and irregular nucleus shape, as detected by brightfield and fluorescent microscopy (Figure 4.2). Differentiation of monocytes into macrophages show an association with a reduction on the nucleocytoplasmic ratio due to an increase in cytoplasmic volume

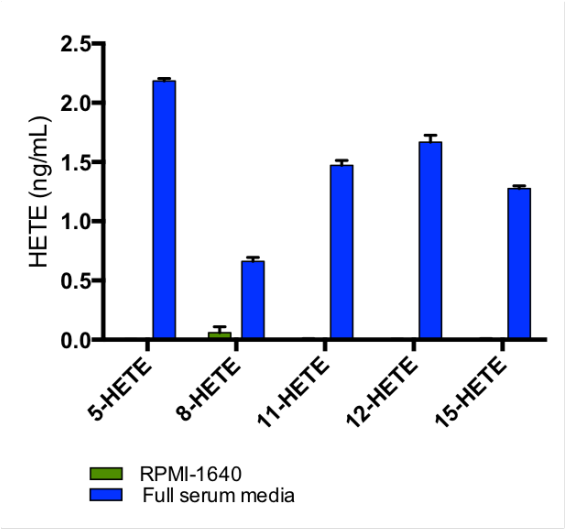


Figure 4.1 Analysis of hydroxyicosatetraenoic acids (HETEs) in RPMI-1640 media alone and full serum culture media (RPMI-1640, 10% heat-inactivated foetal calf serum (FCS), 2mM L-glutamine). Lipids were extracted from RPMI-1640 and full serum media by hexane lipid extraction (Chapter 2, section 2.6) and analysed using HPLC-MS/MS. N= 2, data represented as mean ± S.E.M.

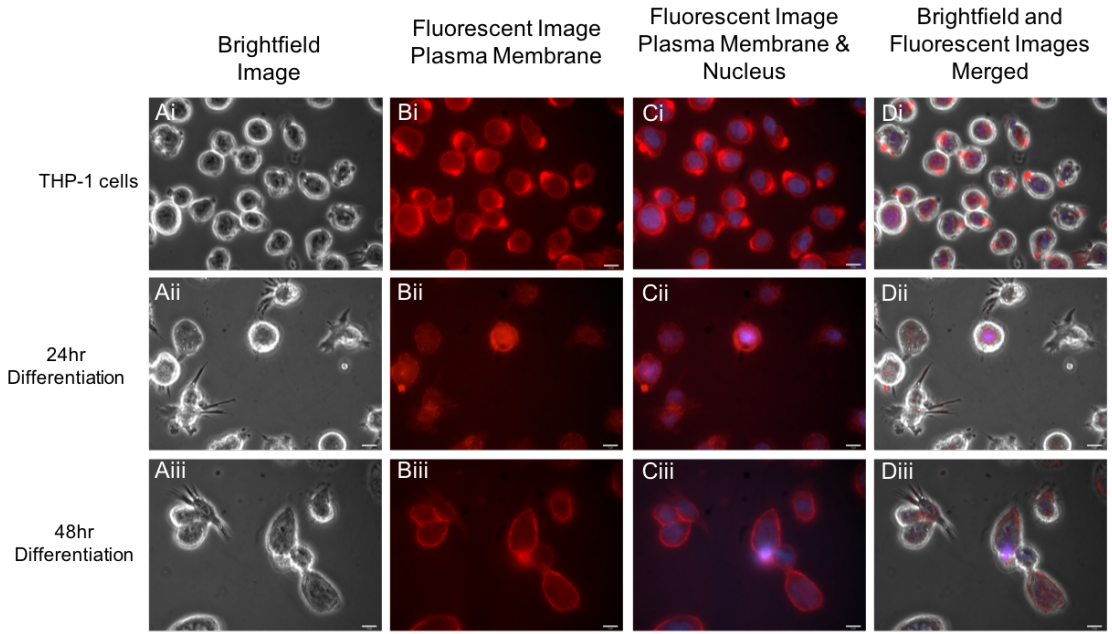


Figure 4.2 Brightfield and fluorescent microscopy images of undifferentiated THP-1 monocyte cells and 24 and 48 hour differentiated THP-1 monocyte-derived macrophages. Images indicate cell morphological changes induced by PMA (0.5 μmol/L) treatment. **Ai-iii)** Differential interference contrast (DIC) microscopy was performed using a Leica DMIRB inverted microscope equipped with 10x objective and using the brightfield mode with a "Ph1" setting on the phase selector ring. Brightfield microscopy relies on the differences in absorption of light due to differences in densities between various parts of the cell. **B-Ci-iii)** A Leica DMIRB inverted microscope was used with a 40x oil objective. Fluorescent microscopy was used to increase resolution. Fluorophores were used to fluorescently label the **Bi-iii)** plasma membrane (orange) and **Ci-iii)** nucleus (blue) of the cells, creating a more visible contrast and making it possible to see a much more detailed cell structure. The approximate fluorescence excitation/emission maxima are 554/567 nm and 360/460-490 nm for the plasma membrane and nucleus respectively. **Di-iii)** A merged overlay of both bright field and fluorescent images. Scale bar = 15μm.

(Daigneault et al. 2010). Undifferentiated cells are spherical in shape (Figure 4.2 Ai) with an average diameter of 16 µm (Chitra et al. 2014). As expected, differentiated THP-1 monocyte-derived macrophages increased their cytoplasmic volume to an average of diameter of 24 µm (Figure 4.2 A-Bii-iii) compared to THP-1 monocytes (Figure 4.2 A-Bi). PMA treatment also enhances the adherence and granularity of the THP-1 cells with monocyte-derived macrophages displaying an amoeboid and granular morphology (Figure 4.2 Aii-iii) relative to untreated cells (Figure 4.2 Ai). Through observation there appears to be increased granularity, which results from an increase in certain membrane bound organelles (Daigneault et al. 2010). Macrophage differentiation also induces an irregular nucleus shape (Figure 4.2 Cii-iii) compared to monocyte cells (Figure 4.2 Ci).

4.3.3 Basal levels of cytokine expression in differentiated THP-1 cells

The basal line status of the culture, prior to any stimulation, is a critical factor to establish for the differentiated cell response. THP-1 cell cytokine secretion after differentiation with PMA (0.5 μ mol/L) over a time course of 4 hours demonstrated basal levels of each proinflammatory cytokine TNF-α, IL-1β, IL-8 and IL-6 (Figure 4.3). A similar pattern of increased secretion over time was shown across all proteins, between 0 and 4 hours. Cytokine secretion typically increased over time, with the highest seen at 4 hours. This increase was significantly different for TNF-α, IL-1β and IL-8 at 2 and 4 hours compared to time 0, ($P<0.05$, $P<0.05$ and $P<0.0001$, respectively). Concentrations of IL-8 were considerably enhanced, reaching ~2500 pg/mL at 4 hours, which indicates a 7 and 24-fold increase compared to TNF-α and IL-1β, respectively. IL-6 displayed the lowest levels of generation peaking at ~15 pg/mL. IL-6 had no significant increased at any point compared to time 0, ($P>0.05$).

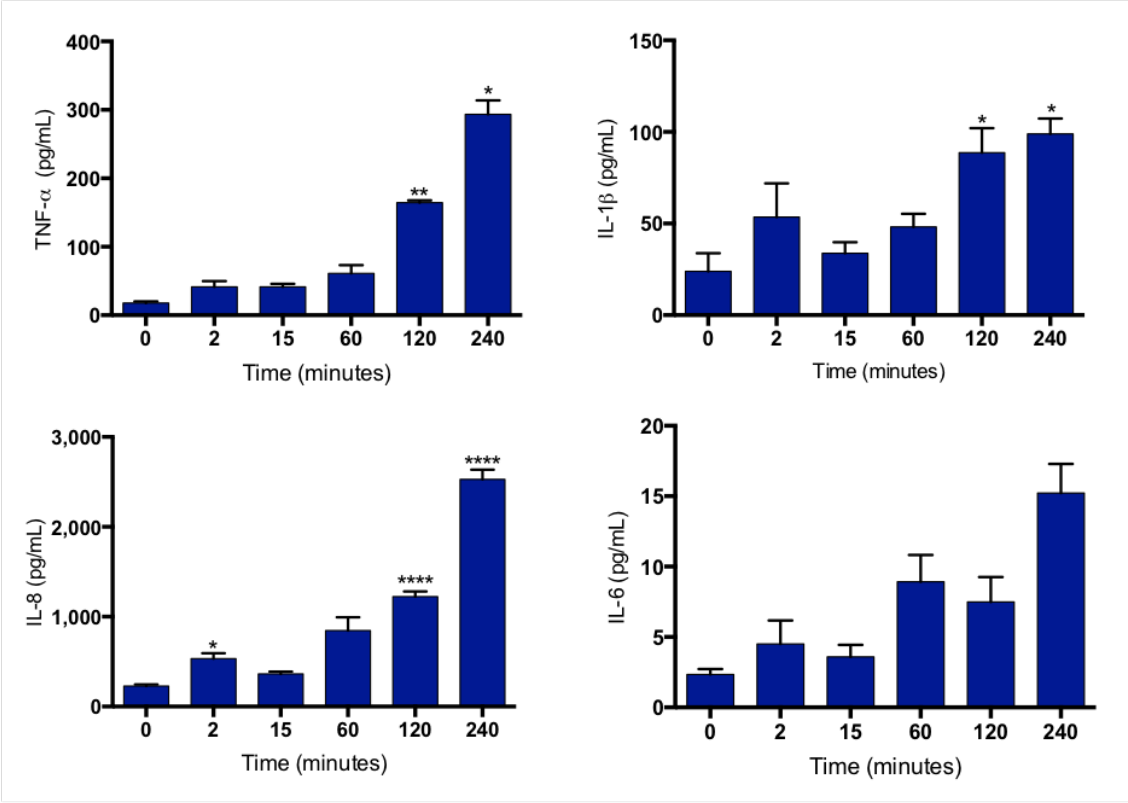


Figure 4.3 THP-1 monocyte-derived macrophage generation of proinflammatory cytokines TNF-α, IL-1β, IL-8 and IL-6 under basal conditions. THP-1 cells were differentiated with PMA (0.5 μmol/L) for 24 hours at 37°C. Cells were washed and media was replaced with fresh media. Supernatant was removed over a 4 hour time course to look for cytokine expression. Data were analysed using a one-way ANOVA followed by a Tukey's multiple comparison test for each time point. * Significantly different from time 0. * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$, N=3, data represented as mean ± S.E.M.

4.3.4 Human THP-1 cell generation and identification of 15-HETE eicosanoid

The 24 and 48 hour differentiated cells were stimulated with the positive control calcium ionophore A23187 (10 µM) agonist, which is routinely used to induce expression in macrophages (Wilson et al. 1993), at 37°C for 2, 15 and 60 minutes. This narrow time course was used as an initial look at eicosanoid formation as previous data, in Chapter 3, showed rapid eicosanoid generation from human neutrophils at 2 minutes. Deuterated internal standard 12HETEd_8 (5ng/mL) was added to all samples followed by lipid extraction as described in Chapter 2, section 2.6.

In time course studies of activated THP-1 monocyte-derived macrophages; 24 and 48 hour cell differentiation predominantly showed minimal (~0.01 ng/10^6cells) 15-HETE formation when cells were incubated with calcium ionophore A23187 for 2, 15 and 60 minutes (Figure 4.4). Cells differentiated for 24 hours showed a >100-fold increase in 15-HETE generation (~1.7 ng/10^6cells) after 15 minute treatment with calcium ionophore A23187 compared to the other times points. Cells differentiated for 48 hours showed an 8-fold increase (~0.09 ng/10^6cells) at 2 minutes. However, no significant generation of 15-HETE was seen with the positive control calcium ionophore A23187 ($P > 0.05$).

To confirm cell activation, 24 hour differentiated THP-1 cells were exposed to agonists previously seen in studies to generate cytokines, calcium ionophore A23187 (10 µM) and fMLP (1 µM) (Wilson et al. 1993; Shrivastava 2007), in the presence of Ca^{2+} (2.5 mM) and Mg^{2+} (1.25 mM) for 2, 15 and 60 minutes. Aliquots of supernatant were assessed for cytokine generation (Figure 4.5).

Differentiated control THP-1 cells were shown to generate all four proinflammatory cytokines; TNF-α, IL-1β, IL-8 and IL-6 (Figure 4.5). THP-1 macrophages exposed to each agonist showed no increase in generation of TNF-α at 2 or 15 minutes compared

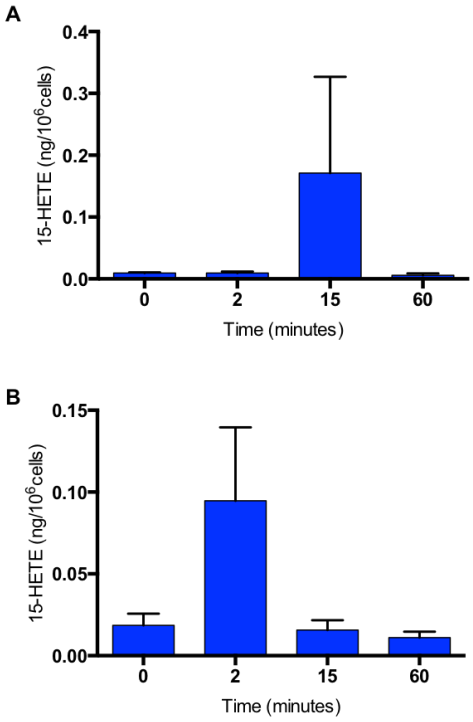


Figure 4.4 Generation of 15-HETE by differentiated human THP-1 monocyte cells in response to calcium ionophore A23187 activation. Time course activation of human THP-1 cells by calcium ionophore A23187 (10μM) for 2, 15 and 60 minutes following differentiation with PMA (0.5 μmol/L) for **A)** 24 hours and **B)** 48 hours. Cells were incubated at 37°C and analysed using LC-MS/MS. Data were analysed using a one-way ANOVA followed by a Tukey's multiple comparison test for each HETE. N=3, data represented as mean ± S.E.M. No significant increase was seen in 15-HETE generation when stimulated with the positive control, calcium ionophore A23187, $P > 0.05$.

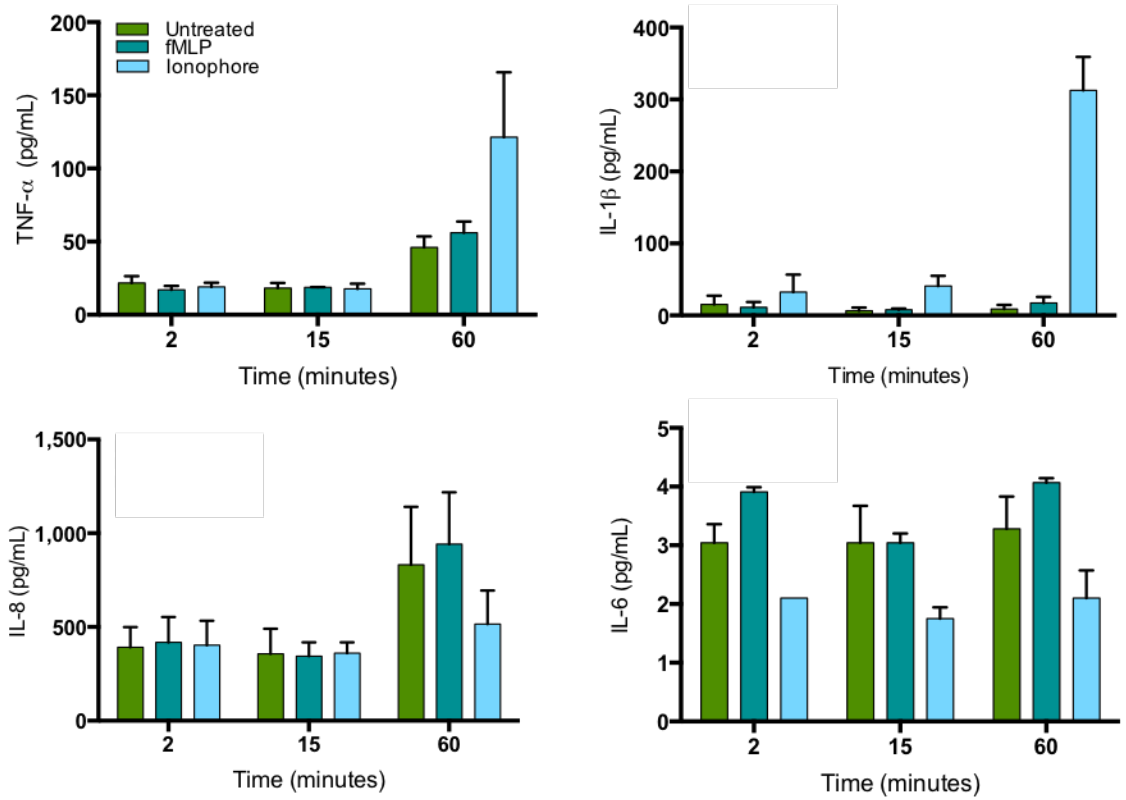


Figure 4.5 Generation of proinflammatory cytokines TNF-α, IL-1β, IL-8 and IL-6 by monocyte-derived macrophages in response to bacterial agonists fMLP and calcium ionophore A23187. THP-1 cells were differentiated with PMA (0.5 μmol/L) for 24-hours at 37°C before being further incubated with fMLP (1μM) or calcium ionophore (10μM) for 2, 15 and 60 minutes. N=2, data represented as mean ± S.E.M.

to the untreated cells, at ~20 pg/mL. The fMLP treated cells and untreated THP-1 cells showed a proportionate increased to ~50 pg/mL in TNF-α at 60 minutes. Although not significant, calcium ionophore A23187 induced increased expression of both TNF-α (~125 pg/mL) and IL-1β (~320 pg/mL) at 60 minutes compared to both untreated and fMLP treated cells ($P>0.05$). Low levels of IL-1β expression were apparent at 2 and 15 minutes with all treatments, with levels between 20-30 pg/mL. THP-1 macrophages showed the highest generation of IL-8 compared to all other cytokines. IL-8 generation was comparable for all treatments over time at 2 and 15 minutes (~400 pg/mL). However, IL-8 was seen to increase 2-fold for untreated and fMLP treated cells at 60 minutes but remained unaltered in the presence of calcium ionophore A23187. Minimal basal levels of IL-6 were seen in untreated cells over time (~3 pg/mL). fMLP and calcium ionophore A23187 had no major effect on IL-6 production.

4.3.5 THP-1 cell expression after LPS treatment

As cytokine expression is usually seen under longer exposure times than those previously described in section 4.3.4 (Daigneault et al. 2010), a longer time course was carried out over 24 hours. Here, I wanted to confirm cytokine expression under a longer time course of exposure and determine whether varying concentrations of LPS (1, 10 and 100 ng/mL) had an effect on the generation of cytokines in differentiated THP-1 monocyte-derived macrophages.

Treatment with 100 ng/mL LPS, at 24 hours, showed the biggest generation of cytokines (Figure 4.6). Cells treated with 1ng/mL LPS showed the same cytokine response as the untreated cells. Cell exposure to 10 and 100 ng/mL LPS showed increased TNF-α and IL-1β generation compared to the untreated cells at 4 and 24 hours. TNF-α increased more than 2-fold from ~1300 pg/mL at 4 hours to 24 hours, when cells were exposed to 100 ng/mL LPS. IL-1β generation showed a similar pattern of generation to TNF-α, with levels peaking at ~1700 pg/mL when cells were

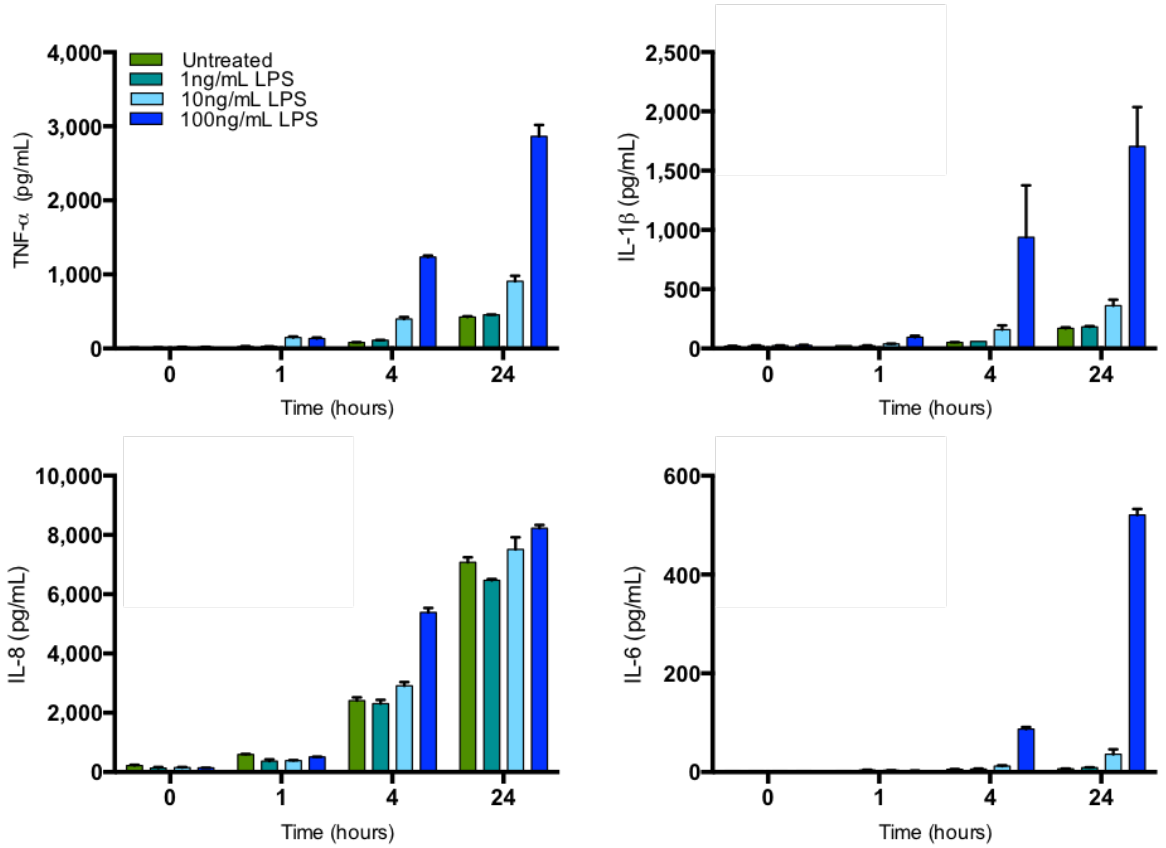


Figure 4.6 THP-1 monocyte-derived macrophage challenged with varying concentrations of *E. coli* K12 lipopolysaccharide (LPS) over time to observe the generation of proinflammatory cytokines TNF-α, IL-1β, IL-8 and IL-6. THP-1 cells were differentiated with PMA (0.5 µmol/L) for 24 hours at 37°C. Cells were washed and media was replaced with fresh media before the addition of LPS (1, 10 and 100 ng/mL) over a time course of 24-hours. N=2, data represented as mean ± S.E.M.

exposed to 100 ng/mL LPS for 24 hours. THP-1 cells showed moderate production of IL-6 with the most substantial increase being 500 pg/mL seen at 24-hours with 100 ng/mL LPS, which was 4-fold bigger than that seen at 4 hours. IL-8 had the biggest generation amongst the selected cytokines with levels reaching ~8000 pg/mL at 24 hours. At 4 hours exposure, untreated and 1 and 10 ng/mL LPS treated cells all showed about the same generation of IL-8 at ~2900 pg/mL. However, at 24 hours IL-8 expression reached its highest levels of ~8000 pg/mL but was consistent across all treatments.

4.4 Discussion

The first aim of this chapter was to use a lipidomic approach to determine whether the human monocytic THP-1 cell line generated 15-LOX lipids, when differentiated into THP-1 monocyte-derived macrophages and thus allow me to use these cells as an *in vitro* model for macrophage bacterial response. The second aim was to confirm the production of selected proinflammatory cytokines from differentiated THP-1 cells, in response to LPS stimulation.

4.4.1 The effect of culture conditions on THP-1 cells

Initial experiments aimed to establish a growing culture of human THP-1 monocyte cells, which could be differentiated into monocyte-derived macrophages, and used as a standardised population of cells to produce readouts of lipid and cytokines in response to bacterial treatment.

4.4.1.1 Identification of the presence of lipids in culture medium

Foetal calf serum (FCS) is the sterile serum obtained from the clotted blood of a bovine foetus. It contains numerous factors that are needed for the survival and proliferation of mammalian cells in culture. Therefore, it was important to establish

the composition of the media used to culture the cells before looking for lipid generation as they may already be present. It was identified that full serum media contained increased levels of HETEs compared to RPMI-1640 alone. This is because coagulated blood contains oxidised lipids. When clotting occurs activated platelets and leukocytes rapidly produce oxidised lipids through the enzymatic action of lipoxygenase (Sarah N. Lauder et al. 2017). Data confirmed that THP-1 media containing FCS showed an array of oxidised lipids compared to RPMI-1640 alone, subsequent agonist exposure was carried out using RPMI-1640 alone.

4.4.1.2 Cell differentiation with phorbol myristate acetate

PMA is a phorbol ester, a class of tumour promoting compounds that can influence cell maturation and promote cell surface expression of numerous antigens characteristic of human mononuclear phagocytes (Nolfo & Rankin 1990). PMA is one of the most common reagents used to induce THP-1 monocyte differentiation into macrophage-like cells. To confirm that PMA induced differentiation, microscopy images were taken of THP-1 monocytes and 24 and 48 hour PMA-differentiated cells. As expected, untreated THP-1 cells were non-adherent, spherical cells. Treatment with PMA showed the typical hallmarks of macrophages, including cell adherence, which were consistent with that seen by Gatto *et al*., 2017. The phenotype of cells has been seen to vary between studies under PMA stimulations, but this can be attributed to concentration of PMA used or time of incubations (Aldo et al. 2012). Therefore, to minimise this, for all subsequent data cell differentiation was kept consistent at 24 hour PMA treatment.

THP-1 cells were differentiated in the presence of 0.5 µmol/L PMA. This concentration of PMA was previously seen to differentiate THP-1 cells by Connon *et al*., 2003, although a lower concentration than that reported by others (Danielsson et al. 2008; Daigneault et al. 2010; Gatto et al. 2017). As previously mentioned, microscopy images at 24 and 48 hours showed the expected attributes of a macrophage-like cell. Using a lower concentration of PMA was more appropriate, as

it has been shown that the use of increased concentrations of PMA (16-540 nM) for THP-1 cell differentiation can be too high, leading to unusually expressed genes during the differentiation process and highly upregulated genes appear to overwhelm the effect of stimuli on the THP-1 macrophages (Park et al. 2007). Even though the THP-1 cell line is a widely accepted model for observing macrophage functions and responses to external stimulation *in vitro*, there is no current standardised protocol for the differentiation of THP-1 monocytes to macrophages using PMA (Lund et al. 2016). Here, PMA-induced differentiation of THP-1 monocyte cells into a macrophage phenotype was represented by cell adhesion and also confirmed through brightfield and fluorescent microscopy, which showed a characteristic spread in morphology and irregular nucleus shapes (Gatto et al. 2017).

4.4.1.3 Establishing the baseline status of the THP-1 culture

Next, I analysed the supernatant of PMA-treated THP-1 cells and determined the cytokine profile. Cytokine production may be induced through treatment with PMA, therefore it was important to establish a baseline level of expression over time of the PMA-differentiated cells, prior to any stimulation. This study found that PMA treated THP-1 cells did generate cytokines. It is not clear if THP-1 monocytes elicit this effect as they were not tested here, but published data suggests that treatment with PMA enhances the THP-1 cell cytokine profile compared to those without PMA treatment (Aldo et al. 2012). Data indicated a similar pattern of cytokine secretion that typically increased over time, with the highest levels seen at 4-hours. TNF-α, IL-1β and IL-8 all showed a significant increase in generation at 2 and 4-hours compared to time 0. Concentrations of IL-8 were considerably enhanced compared to the other responses and IL-6 displayed the lowest levels of generation. These levels were consistent with those found by Aldo *et al*. 2012. The increase in baseline expression levels are comparable between samples as cell culture conditions were kept consistent throughout.

4.4.2 THP-1 monocyte-derived macrophage 15-HETE generation

The initial time course looked at the optimal time point of cell differentiation for subsequent 15-HETE generation. Primary experiments indicated that 24 and 48 hours displayed the best time point for THP-1 cell differentiation in the presence of 0.5 µmol/L PMA.

Once optimum differentiation was established cells were treated to confirm induction of 15-HETE formation. Data indicated that THP-1 monocyte-derived macrophages generated low levels (<0.2 ng/10^6cells) of 15-HETE, when challenged with the positive control calcium ionophore A23187 and analysed using sensitive LC/MS/MS over a time course of 60 minutes. Calcium ionophore A23187 is a carboxylic acid antibiotic, which bypasses receptor-ligand interactions and selectively increases the permeability of cell membranes to calcium, in turn activating cells for the release of numerous mediators, including lipids (Nolfo & Rankin 1990). The time course of 60 minutes was used as previous data in chapter 3, along with that shown by Morgan *et al.,* 2009 and Clark *et al*., 2011, indicated rapid production of lipids upon cell stimulation. The most substantial amount of 15-HETE was formed after 24-hour differentiation with PMA, at 15 minutes exposure to calcium ionophore A23187.

It has previously been shown that PMA-treated THP-1 cells did not release leukotrienes, which are downstream products of 15-HETE metabolism, in response to 5 µM calcium ionophore A23187 for 30 minutes (Nolfo & Rankin 1990). This could suggest a possible dose response to the stimuli, as results in this chapter of work indicate that increasing the concertation of calcium ionophore A23187 to 10 µM did stimulate cells to produce eicosanoids, although in much smaller quantities than what would be expected for a positive control. The anticipated quantity of lipid generated by calcium ionophore A23187 would be similar to that shown by Morgan *et al.,* 2009, who demonstrated the formation of 12-HETE by murine macrophages in response to 10 µM A23187 to reach 12 ng/10^6 cells after 15 minutes stimulation. This value indicates a 59-fold increase compared to the THP-1 lipid generation, in

response to calcium ionophore A23187, seen in this chapter. Calcium ionophore A23187 stimulus is routinely used in experimental conditions as a positive control to assess the maximal quantities of eicosanoids released from white blood cells, which is why it was selected to establish the overall capacity of THP-1 macrophages to release 15-HETE. It could be a possibility that 15-LOX expression may be enhanced upon interleukin-4 (IL-4) stimulation of the cells before agonist treatment (Wuest et al. 2012; Lauder et al. 2017). However, it might be the case that larger quantities of 15-LOX derived lipids can only be seen from THP-1 cells engineered to over express 15-LOX (Danielsson et al. 2008).

To confirm that the lack of 15-HETE generation, in the cells and supernatant together, was real and not due to a lack of activation, the next step was to assess the cell supernatant, under the same conditions, for proinflammatory cytokine generation.

4.4.2.1 THP-1 cell confirmation of activation

The human THP-1 monocyte cell line has been widely used to study the immune response of monocytes and monocyte-derived macrophages due to the similarity in responses when compared to peripheral blood mononuclear cells (Seshadri et al. 2007; Chanput et al. 2010; Wang et al. 2012). To confirm THP-1 cell activation by calcium ionophore A23187 and receptor mediated fMLP, supernatants were also analysed for cytokine generation. Exposing 24 hour differentiated THP-1 monocyte-derived macrophages to the non-physiological calcium ionophore A23178 and the bacterial peptide fMLP for 2, 15 and 60 minutes showed that there was an increase in TNF-α and IL-1β following treatment with calcium ionophore A23187 in comparison to untreated and fMLP-treated cells at 60 minutes. This finding suggests that an increase in intracellular calcium is one mechanism by which the TNF-α and IL-1β genes can be activated.

The lack of cytokine generation seen by fMLP could be because cytokines are produced over a longer time of exposure to treatment, as indicated by Pan *et al*. who showed that fMLP resulted in a time-dependent production of IL-1β with a notable

increase in secretion detected after 60 minutes stimulation. Although not significant, IL-1β secretion by macrophages in this study showed the most substantial increase. IL-1β is one of the main pathological mediators of inflammatory disease, and during microbial infection, blood monocytes and tissue macrophages serves as major producers of IL-1β (Z. K. Pan et al. 2000). IL-8 was the most generated cytokine and also indicated, at 60 minutes, the pattern seen in IL-6. These findings were also shown by Wilson *et al*. who indicated that calcium ionophore A23187 induced IL-8 gene expression and protein secretion in THP-1 cells.

The time course of activation showed clear generation of all cytokines in response to both untreated and treated cells. These data confirm that 24 hour differentiated THP-1 cells do release detectable levels of the proinflammatory cytokine when stimulated with calcium ionophore A23187, as expected, and therefore had been activated when looking for 15-HETE generation, thus proving that the lack of 15-HETE is a real result.

However, as 60 minutes was the longest time point in this experiment, the effect of calcium ionophore A23187 and fMLP on cytokine generation after this time is unknown. This time course was used as an initial study to confirm cell activation during lipid analysis, and a previous study had shown detectable cytokine levels between 0 and 60 minutes (Z. K. Pan et al. 2000). The time course of exposure for the THP-1 cells could be increased to give an additional indication of the pattern of release over 24 hours. Although not the aim here, increasing the time point of incubation with stimuli will correlate with the literature, which has shown human monocytes and monocyte-derived macrophages as well as THP-1 cells being exposed to various bacterial components for up to 30-hours (Seshadri et al. 2007; Chanput et al. 2010; Daigneault et al. 2010). Therefore, it was thought appropriate to examine the cytokine generation of the THP-1 macrophage-like cells over a longer time course, in response to a well-known bacterial agonist *E. coli* K12 lipopolysaccharide (LPS).

4.4.3 Assessment of the proinflammatory response of differentiated THP-1 cells following LPS stimulation

To confirm activation of alternative pathways, cells were exposed to varying concentrations of the toll-like receptor (TLR) agonist LPS over a 24 hour time course. Concentrations of LPS used were taken from those described in other studies (Park et al. 2007; Lund et al. 2016), making data comparable and allowing further confirmation that the cells were behaving as expected in terms of proinflammatory response, therefore indicating the lack of 15-HETE generation to be true.

The binding of the TLR4 ligand LPS to macrophages activates a plethora of signalling cascades. As would be expected the biggest increase was seen across all cytokines when cells were challenged with 100 ng/mL LPS for 24 hours. These data are consistent with findings demonstrating THP-1 macrophages respond to varying concentrations of proinflammatory stimulation (Park et al. 2007). Interestingly, TNF-α showed the biggest increase after 24 hours treatment with 100 ng/mL LPS compared to 4 hours. This was not consistent with Lund *et al*. who stated that TNF secretion levels were maximal at 4 hours post stimulation with LPS. However, findings in this chapter were consistent with those of Park *et al*. who showed increased protein concentrations, notably TNF, after 24 hour LPS treatment. This work confirmed that THP-1 cells were behaving as expected when differentiated into macrophage-like cells, and further indicates the lack of 15-HETE to be a real result.
Due to time pressures, it was not possible to carry out a lipid extraction and LC/MS/MS analysis of LPS stimulated cells to check for any 15-HETE generation. However, published data has seen that LPS stimulates cyclooxygenase (COX) induction but not LOX (Pfau et al. 2000).

4.5 Conclusions

There are numerous advantages to be seen from employing a cell line, such as THP-1, compared to using primary cells, namely having a homogeneous genetic

background minimises the degree of variability that can be seen in the cell phenotype.

This chapter of results indicate that PMA differentiated THP-1 monocyte-derived macrophages do not generate sufficient levels ($\geq$12 ng/10^6 cells, Morgan et al. 2009) of the eicosanoid 15-HETE through the 15-lipoxygenase-enzyme pathway when exposed to the positive control calcium ionophore A23187. Though, they can be induced to generate the proinflammatory cytokines TNF-α, IL-1β, IL-8 and IL-6, in response to calcium ionophore A23187 and LPS.

The absence of sufficient 15-HETE generation by THP-1 monocyte-derived macrophages supports the idea that this standardised cell line cannot be used as a suitable human macrophage cell model to look at human cell eicosanoid responses to bacterial infection. Also, despite the wide acceptance of this cell line as a suitable model for looking at macrophage function and responses, as previously mentioned, the lack of a standard protocol for cell differentiation, would make it difficult to compare results between studies (Lund et al. 2016). However, the use of a cell line does reduce variability and enables a larger quantity of cells to be obtained and used over a shorter time frame.

For these reasons, an alternative murine cell line was employed based on RAW 264.7 macrophages which have been genetically engineered with the 12/15-LOX pathways. This cell line, which was developed by Professor Valerie O'Donnell's Lipidomics Group at Cardiff University, has been extensively characterised and is known to generate eicosanoids in response to stimuli, therefore representing a suitable model for future studies as described in the next chapter.

Chapter 5

Detecting eicosanoids and proinflammatory cytokines generated by 12/15-LOX transfected RAW 264.7 murine macrophages

5.1 Introduction

Chapter 1 describes how macrophages contribute to innate immunity through methods which include phagocytosis, secretion of inflammatory mediators, antigen processing and presentation to cells of the adaptive immune system (Sigola et al. 2016). Macrophages are a major source of lipid and protein mediators, and respond to a variety of stimuli by generating various eicosanoids and cytokines (Norris & Dennis 2014; Berghaus et al. 2010).

As described in Chapter 4, multiple monocyte-lineage cell lines are available, including the murine macrophage-like RAW 264.7 cell line. Notably, these cell lines display fundamental differences compared to primary cells, due to their continuous growth in culture resulting in permanent alterations in their genes. These alterations may have an effect on the signalling cascades activated by microbial ligands (Hartley et al. 2008). A comparison of murine models and humans also presents additional obvious differences in comparative disease pathology and healing, together with physiology and anatomy (e.g., bone, skin and blood), which must be taken into account (Chamberlain et al. 2009). However, factors including cost effectiveness and abundant gene variants mean that murine models are often employed in survey and pre-clinical studies as a first step in the transition between *in vitro* models and pre-clinical trials (Chamberlain et al. 2009). The increased prevalence of the murine model in pre-clinical *in vivo* studies has led to the widespread use of murine cells as *in vitro* models (Nieves & Moreno 2006; Vigo et al. 2005). This is significant as the use of the same species for both *in vivo* and *in vitro* work allows for the use of identical reagents and permits comparisons of the data.

The RAW 264.7 murine macrophage cell line, the most commonly used mouse macrophage cell line in medical research (Hartley et al. 2008), was established from a tumour induced by Abelson murine leukaemia virus and has been used in numerous studies as a model of primary macrophages (Buczynski et al. 2007; Lee et al. 2016; Berghaus et al. 2010). RAW 264.7 macrophages have been shown to express bacterial TLR1, TLR2, TLR4, TLR5 and TLR6 on their surface, in addition to the intracellular

expression of bacterial TLR9 (Applequist et al. 2002). This cell line would have been ideal for my studies; however, native RAW 264.7 macrophages do not possess the relevant enzymes to make LOX-derived lipids.

The work reported in this chapter was carried out in collaboration with Professor Valerie O'Donnell's Lipidomics Group, Cardiff School of Medicine. RAW 264.7 murine macrophages had been transfected with a vector containing the 12/15-LOX gene. This overexpression allows the cells to generate 12/15-LOX lipids, which could potentially be enhanced upon stimulation and thus mimicking macrophages *in vivo*, making the cells an excellent model for the proposed study (paper in preparation). These cells also carry the ampicillin resistant gene as well as a puromycin resistant gene to make them a stable cell line. The cells carrying this 12/15-LOX vector will be referred to as RAW 264.7 ALOX macrophages. The RAW 264.7 Control macrophages will refer to cells that have also been transfected and are ampicillin and puromycin resistant, but the vector does not have the 12/15-LOX gene.

The Lipidomics Group have also developed a robust liquid chromatography/tandem mass spectrometry method that can identify and quantitate 110 different eicosanoids and fatty acids in a single analysis (paper in preparation). This method was applied to the TLR agonist-stimulated and non-stimulated transfected RAW 264.7 macrophages. It was proposed that if an initial profile could be established in response to the TLR agonists, these cells would then be employed to determine profiles in response to whole, live bacteria as they would activate multiple TLRs at once.

5.1.1 Aims and objectives

The first aim of this chapter was to employ a targeted lipidomic approach to profile the basal eicosanoid content of the transfected RAW 264.7 ALOX macrophages compared to the RAW 264.7 Control macrophages. Previous work carried out by Buczynski *et al.,* 2007 and Lee *et al*., 2016 described how liquid-chromatography-

MS/MS profiles of eicosanoids produced by native RAW 264.7 macrophages varied when treated with various agonists, including TLRs. Therefore, work presented in this chapter exposes RAW 264.7 Control and ALOX macrophages to the whole array of individual bacterial TLR agonists. This was to confirm that the transfected ALOX macrophages generated the lipids of interest.

The second aim of this chapter was to demonstrate whether individual bacterial TLR agonists induced varying responses, in terms of their eicosanoid and proinflammatory cytokines generation, in RAW 264.7 ALOX macrophages with a view to determining which TLR agonists play a key role in early inflammatory signalling.

The specific objectives of the work in this chapter are therefore:

1. Confirm the distinct eicosanoid profile generated by RAW 264.7 Control and ALOX macrophages.
2. Establish mass spectrometry detection of RAW 264.7 ALOX macrophage eicosanoid formation.
3. Determine the response to TLR agonist treatment.
4. Compare the cytokine and eicosanoid signalling when exposed to different TLR agonists.

5.2 Specific materials and methods

The work presented in this chapter was carried out in Professor Valerie O'Donnell's laboratory, Cardiff Lipidomics Group, Division of Infection and Immunity, School of Medicine by myself.

5.2.1 Construction of recombinant ALOX plasmid and optimisation of transgene expression

Murine RAW 264.7 macrophages were transfected with pMXs-IRES-Puro retroviral plasmid, with (RAW 264.7 ALOX macrophages) and without (RAW 264.7 Control macrophages) the 12/15-LOX gene, using standard cloning techniques (Sambrook et al. 1989) by Dr Victoria Tyrell, Cardiff Lipidomics Group. The plasmid contains both the ampicillin resistance gene (Amp^r) and puromycin resistance gene ($Puro^r$). Transfection of the RAW 264.7 macrophages was carried out prior to the start of my project and were fully characterised by Professor Valerie O'Donnell's Lipidomics Group.

5.2.2 Transfected murine RAW 264.7 macrophage culture

Transfected macrophages were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with-heat inactivated foetal calf serum (FCS; 10% *v/v*), penicillin-streptomycin-glutamine (1% *v/v*) and puromycin dihydrochloride (150 µl/ 500 mL DMEM) (referred to hereafter as RAW media). The cells were grown in a humidified incubator at 37°C in the presence of 5% CO_2.

5.2.2.1 Revival of stock macrophages from liquid nitrogen storage

Cells were removed from liquid nitrogen storage and placed on ice to allow gentle thawing. Routine RAW media was added to the cells and centrifuged at 300g for 5

minutes. The supernatant was removed, and the cells were re-suspended in fresh RAW media before being transferred to a T-25 cell culture flask (Sigma-Aldrich). The cells were incubated overnight at 37°C in the presence of 5% CO_2.

5.2.3 Comparison of basal eicosanoid expression in RAW 264.7 Control and ALOX macrophages

To establish any basal levels of eicosanoids generated by both the RAW 264.7 Control and ALOX macrophages, cells were seeded in 6-well plates and left untreated at 37°C for 1 hour ($5x10^6$ cells/well) and 24 hours ($4x10^6$ cell/well) in RPMI-1640 alone. RAW media was not used as it contained FCS, which contains oxidised lipids as seen in Chapter 4. Cells and supernatant were collected separately. Following incubation, both cell and supernatant samples had deuterated internal standard mix added (5 µl of 1 ng/µl stock), prepared as detailed in Appendix I. The cell samples went through lipid extraction (described in Chapter 2, section 2.6). Subsequently, all samples (cells and supernatant) underwent solid phase extraction (SPE) as described in Chapter 2 section 2.7.

5.2.4 RAW 264.7 ALOX macrophage exposure to bacterial agonists

The RAW 264.7 ALOX murine macrophages were plated in 6-well culture plates with 4 mL of RAW media (5×10^6 cells/well for 30 minute exposure, 4×10^6 cells/well for 24 hour exposure), and allowed to adhere overnight. Once adhered, the RAW media was replaced with 1 or 4 mL RPMI-1640 alone, for the 30 minute and 24 hour time points respectively, and stimulated with the bacterial toll-like receptor (TLR) agonists (InvivoGen, tlrl-kit1mw). The bacterial TLR agonists used were TLR1/2 Pam3SCK4, TLR2 heat-killed *Listeria monocytogenes* (HKLM), TLR4 *E. coli* K12 lipopolysaccharide (LPS), TLR5 *Salmonella typhimurium* flagellin, TLR6/2 FSL, and TLR9 synthetic ODN 1826. Agonists were added at the doses shown in Table 5.1. Cells were incubated at 37°C in a humidified 5% CO_2 atmosphere for either 30 minutes or 24 hours (these time points were optimised previously by characterisation of the cells in Professor

Valerie O'Donnell's Lipidomics Group). Cells and supernatant underwent subsequent extraction as described in section 5.2.3.

Table 5.1 Toll-like receptor (TLR) agonist working concentrations used in RAW 264.7 ALOX macrophage exposure.

Agonist	TLR Ligand	Working Concentration
Pam3CSK4	1/2	300 ng/mL
HKLM	2	10^8 HKLM/mL
LPS	4	100 ng/mL
Flagellin	5	1 μg/mL
FSL-1	6/2	100 ng/mL
ODN 1826	9	5 μM

5.2.5 Lipidomic profiling of fatty acids and eicosanoids

The lipid extract was separated using liquid chromatography (LC)-MS/MS. Samples were separated on a ZORBAX RRHD Eclipse Plus C18 column (1.8 µm, 150 x 2.1 mm; Agilent, UK), at a solvent gradient shown in Table 5.2 over 22 minutes. Solvent A was made up of 5% solvent B, water, glacial acetic acid at a ratio of 5:95:0.1; *v/v/v*. Solvent B was made up of the solvent mixture acetonitrile, methanol, acetic acid at a ratio of 80:15:0.1; *v/v/v*. Samples were injected with an auto-sampler and the sample chamber temperature was set at 4°C. The injection volume was 5 µl with a flow rate of 1 mL/minute. Electrospray mass spectra were obtained on a Q-Trap instrument (Applied Biosystems 6500 Q-Trap; Cardiff School of Medicine, Lipidomics Group) operating in the negative mode. Products were analysed using targeted multiple-reaction monitoring (MRM) mode transitions from the parent ions to the daughter ions every 0.60 seconds, scanning for 110 eicosanoids and fatty acids. The area under the curve for the parent ion to the daughter ion was integrated and normalised to the corresponding deuterated internal standard for that analyte. For quantification of the lipid, a standard curve was generated with purified lipids as described in Appendix ll. The limit of detection of the internal standard was determined to be 5 pg.

Table 5.2 Solvent gradient used for Liquid chromatography MS/MS method.

Solvent (%)		Time (minutes)
A	B	
70	30	0.01
70	30	1.00
65	35	4.00
32.5	67.5	12.5
0	100	17.5
0	100	21.00
70	30	21.01
70	30	22.50

5.2.5.1 Heatmap correlation

Heatmaps were generated using the pheatmap package in R Studio using hierarchical clustering to group similar lipids. Data were first normalised to the mean of the unstimulated control values for each lipid. Lipids were colour-coded according to their lipid groups.

5.2.6 Proinflammatory cytokine analysis

Double antibody sandwich enzyme-linked immunosorbent assay (ELISA) was used to measure cytokine concentrations. The analysis was performed using R&D systems Mouse DuoSet ELISA kits to quantify the concentration of three proinflammatory analytes; IL-1β (DY401), IL-6 (DY406) and TNF-α (DY410). The minimal detection concentrations were as follows: IL-1β; 15.6 pg/mL, IL-6; 15.6 pg/mL and TNF-α; 31.2 pg/mL. The ELISA kits were used with an ancillary reagent pack (R&D systems, DY008) according to the manufacturer's instructions. The amount of cytokine was calculated using a standard curve generated from a known amount of the analyte provided with the kit.

5.3 Results

5.3.1 Baseline profiling in RAW 264.7 Control and ALOX macrophages

5.3.1.1 Basal eicosanoid expression in RAW 264.7 ALOX macrophages

Out of the 110 analyte transitions, 30 were detected, using this method, across the untreated samples and are displayed in the heatmap (Figure 5.1). All subsequent analysis will only discuss lipids that showed the greatest difference between untreated and activated cells, out of the 30 that were detected (Table 5.3). In the heatmaps, an increase in abundance, compared to other lipids detected, is denoted in red, whereas a decrease in abundance is denoted in blue. Deeper tones indicate greater differences, with comparisons for individual lipids made across samples. Where the enzymes responsible for synthesising the lipid were known, lipids were labelled as being derived from their specific pathways.

5.3.1.1.1 Basal generation of eicosanoids in RAW 264.7 Control and ALOX macrophages

The highest abundance of eicosanoids were generated intracellularly at 1 hour for both RAW 264.7 Control and ALOX macrophages (Figure 5.1). RAW 264.7 Control macrophages generated mainly COX-derived lipids. Whereas, the RAW 264.7 ALOX macrophages generated 12/15-LOX-derived lipids, which are not detected in Control macrophages.

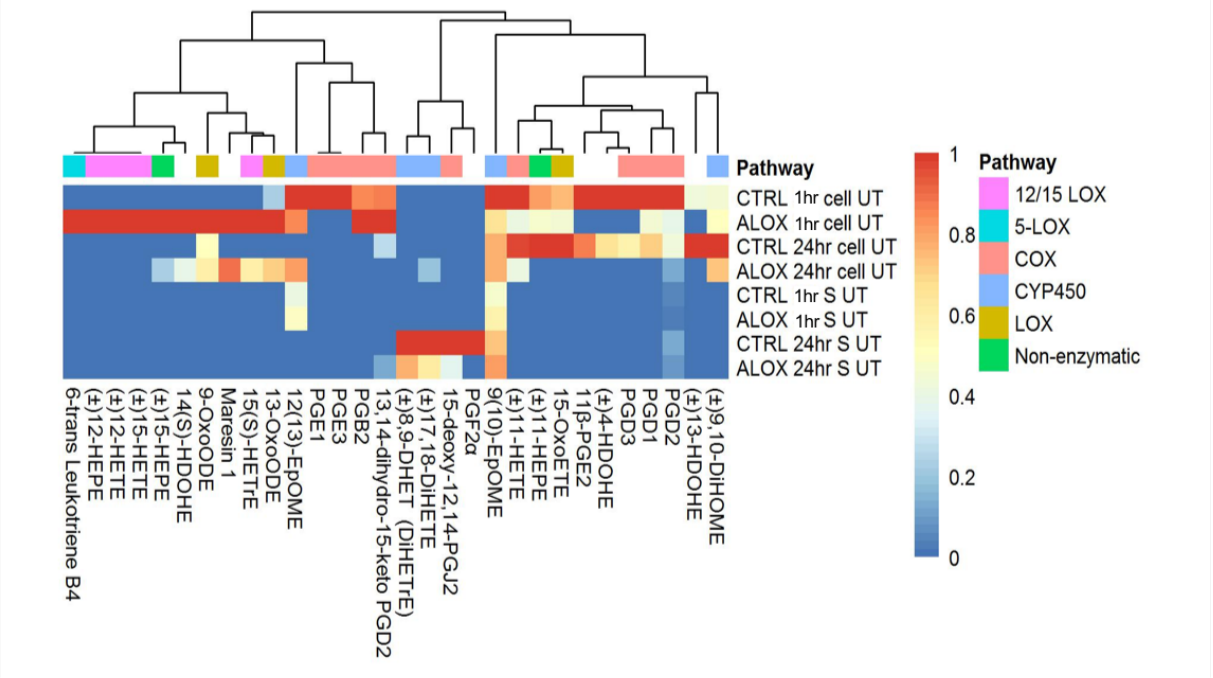


Figure 5.1 Lipidomic profiling of eicosanoids defines their enzymatic origin and regulatory networks. Data indicate targeted lipidomic analysis of RAW control (CTRL) and RAW ALOX (ALOX) macrophages that were seeded and left untreated for 1 hour ($5x10^6$ cells/well) and 24 hours ($4x10^6$ cells/well) before lipids were extracted and analysed by liquid chromatography-MS/MS scanning for 110 different lipids, as described in the specific methods of this chapter. The cells (cell) and supernatant (S) were extracted and analysed separately. The heatmap was generated using the pheatmap package in R Studio and lipids colour-coded according to lipid groups. Levels of treatment response are normalised to the highest value per lipid in the group and colour-coded to represent increasing amount of lipid (from blue (no or low amount of lipid) to red (increased amount of lipid)). Deeper tones indicate greater differences. Scale shows change relative to the most abundant lipid within that group. Lipids are colour-coded by their pathway and clustered by similarity in overall responses to the treatment. *n* = 9 with 2 replicates. UT=untreated.

Table 5.3. The 8 most abundant eicosanoids detected under basal conditions in RAW 264.7 ALOX cells. Multiple reaction monitoring (MRM), deionising potential (DP), collision energy (CE), retention time (RT), internal standard (IS).

No.	Compound Name	MRM Transitions		DP	CE	RT (min)	IS
		Q1	Q3				
1	(±)12-HETE	319.2	167.202	-60	-19	13.91	12(S)-HETE-d8
2	9(10)-EpOME	295.3	171.1	-80	-21	14.86	11(12)-EET (EpETrE) -d11
3	12(13)-EpOME	295.3	195.2	-80	-19	14.74	11(12)-EET (EpETrE) -d11
4	6-trans Leukotriene B4	335.2	195.101	-65	-23	9.89	Leukotriene B4-d4
5	PGD1	353.3	317.202	-55	-16	6.65	Prostaglandin D2-d4
6	PGD2	351.2	271.302	-50	-22	6.61	Prostaglandin D2-d4
7	PGD3	349.3	269.201	-50	-17	5.26	Prostaglandin D2-d4
8	PGE3	349.3	269.2	-60	-17	4.86	Prostaglandin E2-d4

At 1 hour untreated Control macrophages displayed higher levels of lipids generated by the COX pathway, compared to ALOX macrophages (Figure 5.1). These compounds include COX-derived prostaglandin D_1 (PGD_1) (69 vs 30 pg/10^6cells), prostaglandin D_2 (PGD_2) (955 vs 662 pg/10^6cells), and prostaglandin E_3 (PGE_3) (5 vs 0 pg/10^6cells).

Lipids generated were mainly seen intracellularly and not extracellularly (Figure 5.1). The only lipids seen at 1 hour in the supernatant, for both cell types, were 9(10)-EpOME (between 51-63 pg/10^6cells) and 12(13)-EpOME (between 161-193 pg/10^6cells), generated from the CYP450 pathway.

Quantification of intracellular 12-HETE and PGD_2 eicosanoids generated by RAW 264.7 Control and ALOX macrophages at 1 hour is shown in Figure 5.2. ALOX macrophages showed generation of 12/15-LOX-derived lipids compared to Control macrophages (Figure 5.1). Detection of 12-HETE generated by RAW 264.7 Control and ALOX macrophages at 1 and 24 hours is shown in Figure 5.3A. ALOX macrophages showed a significant increase ($P<0.001$) in intracellular 12/15-LOX-derived 12-HETE (800 pg/10^6cells) compared to Control macrophages. 12-HETE was not detected in Control macrophages. Extracellular 12-HETE was not detected in the supernatant from both Control and ALOX macrophages.

Detection of PGD_2 generated by RAW 264.7 Control and ALOX macrophages at 1 and 24 hours is shown in Figure 5.3B. Both Control and ALOX macrophages generated COX-derived PGD_2. Higher levels of PGD_2 were detected intracellularly for both Control (938 pg/10^6cells) and ALOX (537 pg/10^6cells) macrophages, compared to extracellular detection which showed 17 and 125 pg/10^6cells, respectively. There were no statistically significance observations between any of the groups in this data set ($P>0.05$).

Additionally, the oxidation metabolite $PGF_{2\alpha}$ was only identified in Control cell supernatant at 24 hours (86 pg/10^6cells). Untreated macrophages showed a lower

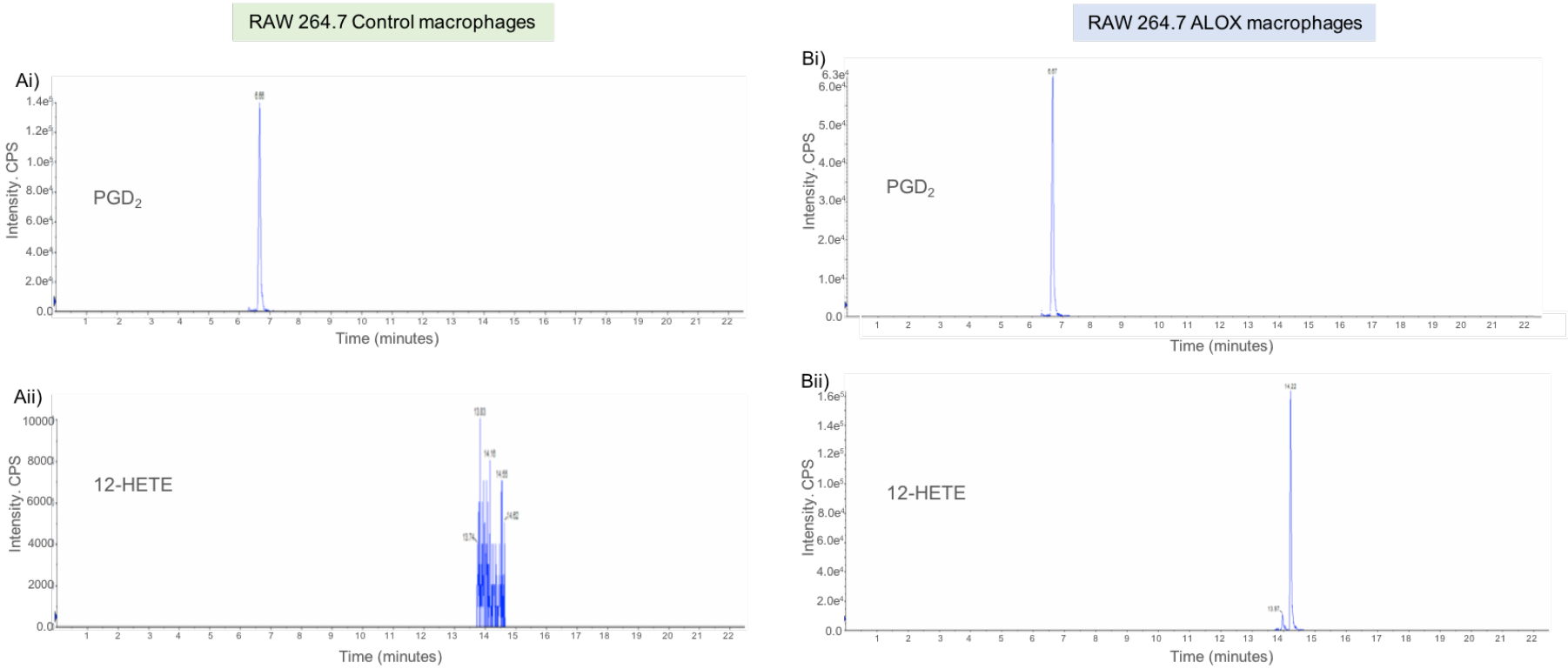


Figure 5.2 Quantification of intracellular PGD_2 and 12-HETE eicosanoids generated by RAW 264.7 Control and ALOX macrophages at 1 hour. Chromatographs showing **Ai)** basal PGD_2 detection from RAW 264.7 Control cells. **Aii)** no basal 12-HETE detected from RAW 264.7 Control cells. **Bi)** basal PGD_2 detection from RAW 264.7 ALOX cells. **Bii)** basal 12-HETE detection from RAW 264.7 ALOX cells. PGD_2 retention time (RT) = 6.66 minutes. 12-HETE RT = 14.22 minutes. To confirm reliability of lipid peak, chromatographic peaks must be at least 3 times the intensity of the background noise, displaying at least 6 detection points during peak elution.

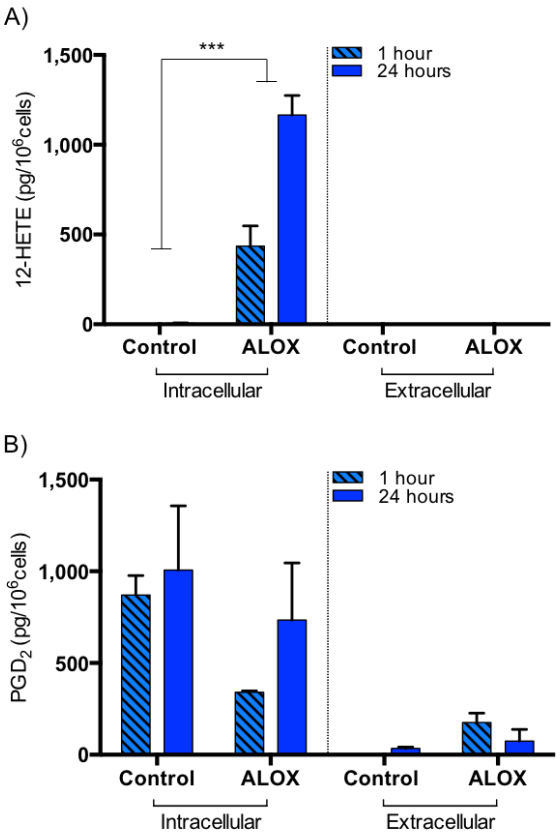


Figure 5.3 Comparison of the baseline generation of eicosanoids from RAW 264.7 Control and ALOX macrophages. A) 12/15-ALOX derived-lipid only shown to be present in RAW 264.7 ALOX macrophages. Data were analysed using a two-way ANOVA, Control vs ALOX, $P < 0.001$. **B)** Generation of the COX-derived lipid PGD_2. Data were analysed using a two-way ANOVA, Control vs ALOX, $P>0.05$. No significant interactions were observed. $n = 9$ with 2 replicates. Data represented as mean ± S.E.M.

abundance of eicosanoids were generated at 24 hours (Figure 5.1). COX and CYP450-derived lipids including 15-deoxy-^{12,14}-PGJ_2 (20-57 pg/10^6cells), 17,18-DiHETE (2-15 pg/10^6cells) and 8,9-DHET (5-11 pg/10^6cells) were the only metabolites identified in the supernatant of both cell types after 24 hours; basal levels were higher in the Control macrophages compared to the ALOX macrophages (Figure 5.1).

All subsequent data presented in this chapter represents an *n* of 1, therefore, no statistical analysis could be carried out to indicate any significant differences between the TLR agonists.

5.3.1.2 Cytokine generation from RAW 264.7 Control and ALOX macrophages

Untreated RAW 264.7 Control and ALOX macrophages had levels of TNF-α of 84 and 129 pg/10^6cells, respectively (Figure 5.4A). Levels of IL-6 were below the limit of detection in untreated RAW 264.7 Control and ALOX macrophages (Figure 5.4B). Unexpectedly, upon stimulation, the cytokine generation between the two cell types was not similar (Figure 5.4). Treatment with each agonist lead to higher cytokine generation in ALOX macrophages over the Control macrophages. IL-1β was below the limit of detection.

All subsequent experiments were carried out using RAW 264.7 ALOX macrophages as they are a suitable model for looking at both eicosanoids and cytokines. Even though differences in cytokine levels were observed between the two cell lines it has previously been reported that native RAW 264.7 macrophages, stimulated with LPS and Pam3CSK4 gave similar levels of TNF-α to RAW 264.7 ALOX macrophages, suggesting that the transfection with the 12/15-LOX gene did not alter the cytokine generation from the RAW 264.7 ALOX cells (Berghaus et al., 2010). Therefore, the RAW 264.7 macrophages were considered an appropriate model as they are a closer representation of *in vivo* macrophages.

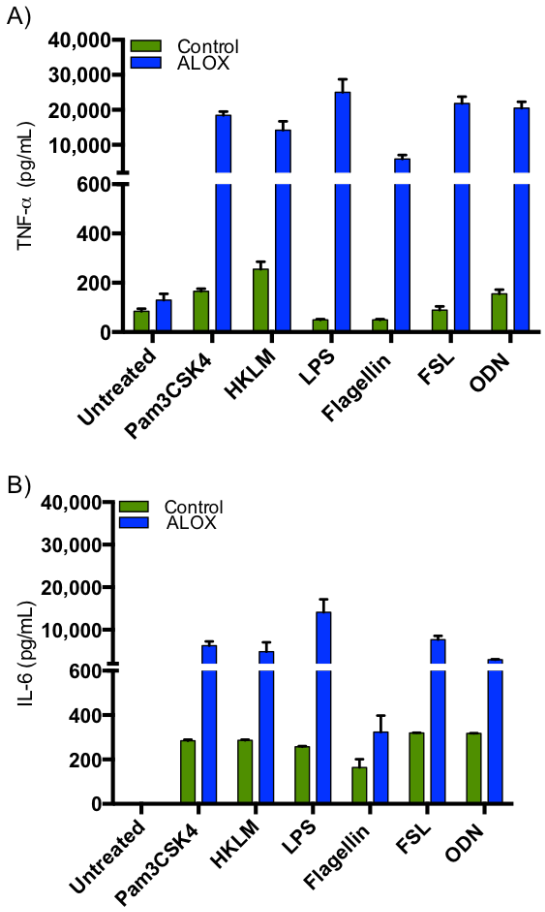


Figure 5.4 Comparison of proinflammatory cytokines TNF-α and IL-6 produced by RAW 264.7 Control and ALOX macrophages in response to bacterial toll-like receptor (TLR) agonists. RAW Control and ALOX macrophages were seeded at $4x10^6$ cells/well and either left untreated or were stimulated with bacterial TLR agonists for 24 hours. Supernatant was analysed for cytokine generation using enzyme-linked immunosorbent assays, as described in the specific methods of this chapter. IL-1β was below the limit of detection. *n* = 1 with 3 replicates. Data represented as mean ± S.E.M.

5.3.2 Eicosanoid generation from RAW 264.7 ALOX macrophages during bacterial toll-like receptor activation

Out of the 110 eicosanoid and fatty acid transitions, (as described in Appendix lll) 39 were within detectable limits of the mass spectrometer across the samples, when exposed to TLR agonists (Figure 5.5). Untreated cells provided a baseline of lipid mediators generated by RAW 264.7 ALOX macrophages (Figure 5.5A). All subsequent analysis will only comment on lipids that showed the highest abundance out of the 39 that were detected (Table 5.4).

5.3.2.1 Gram-positive TLR1/2 agonist Pam3CSK4

Treatment with the TLR1/2 agonist, Pam3CSK4, showed a higher abundance of lipids intracellularly at 24 hours compared to 30 minutes (Figure 5.5B). 12/15-LOX derived lipids, including 12-HETE, 15-HETE and 12-HEPE, were only detected intracellularly at 369, 76 and 62 pg/10^6cells, respectively. Negligible amounts of lipid were expressed extracellularly by the cells at 30 minutes. Only COX- and CYP450-derived lipids, such as thromboxane B2, 8-iso PGE_2, PGD_2, 8,9, DHET and 17,18-DiHETE were seen extracellularly at 24 hours, showing levels of 83, 349, 27,172, 39 and 104 pg/10^6cells, respectively.

5.3.2.2 Gram-positive TLR2 agonist heat-killed Listeria monocytogenes (HKLM)

Treatment with the TLR2 agonist, HKLM, showed the highest abundance of lipids both intracellularly and extracellularly at 24 hours (Figure 5.5C) compared to other TLR responses. Most 12/15-LOX derived lipids were detected intracellularly, including 12-HETE, 15-HETE and 15-HETrE showing levels of 396, 89 and 33 pg/10^6cells, respectively. Negligible amounts of lipid were expressed extracellularly by the cells at 30 minutes. Lipids detected extracellularly at 24 hours included

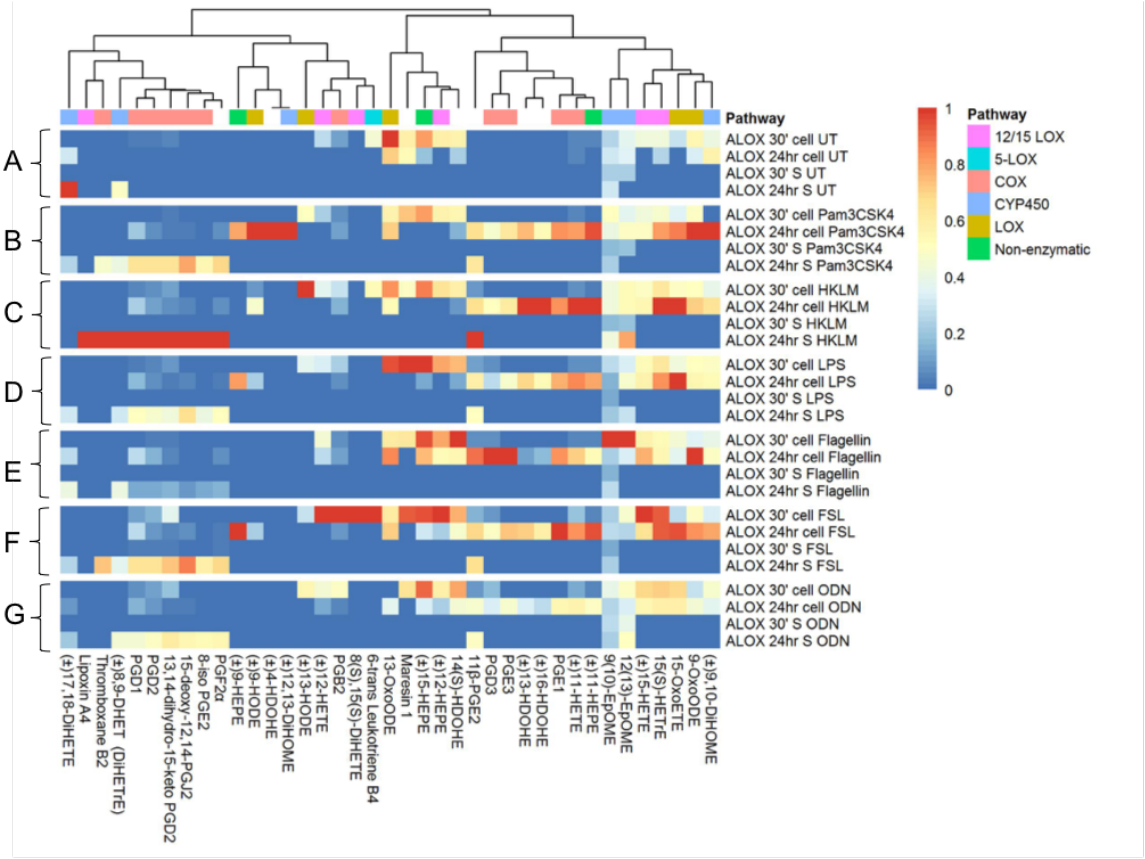


Figure 5.5 Lipidomic profiling of eicosanoids generated by RAW 264.7 ALOX macrophages exposed to bacterial stimulation. Data indicate targeted lipidomic analysis of ALOX macrophages that were seeded and either left **A)** untreated or were **B-G**) stimulated with bacterial toll-like receptor (TLR) agonists for 30 minutes ($5x10^6$ cells/well) and 24 hours ($4x10^6$ cells/well) before lipids were extracted and analysed by liquid chromatography-MS/MS scanning for 110 different lipids, as described in the specific methods of this chapter. The cells (cell) and supernatant (S) were extracted and analysed separately. The heatmap was generated using the pheatmap package in R Studio and lipids colour-coded according to lipid groups. Levels of treatment response are normalised to the highest value per lipid in the group and colour-coded to represent increasing amount of lipid (from blue (no or low amount of lipid) to red (increased amount of lipid)). Deeper tones indicate greater differences. Scale shows change relative to the most abundant lipid within that group. Lipids are colour-coded by their pathway and clustered by similarity in overall responses to the treatment. *n* = 1 with 3 replicates. UT=untreated.

Table 5.4. The 14 most significant eicosanoids detected in RAW 264.7 ALOX cells exposed to TLR agonists. Multiple reaction monitoring (MRM), deionising potential (DP), collision energy (CE), retention time (RT), internal standard (IS).

No.	Compound Name	MRM Transitions		DP	CE	RT (min)	IS
		Q1	Q3				
1	(±)12-HETE	319.2	167.202	-60	-19	13.91	12(S)-HETE-d8
2	(±)15-HETE	319.2	179.2	-65	-18	14.11	15(S)-HETE-d8
3	(±)12-HEPE	317.2	167.201	-50	-20	12.69	12(S)-HETE-d8
4	15(S)-HETrE	321.2	221.2	-70	-21	14.29	13(S)-HODE-d4
5	(±)8,9-DHET (DiHETrE)	337.2	127.1	-70	-25	12.14	15(S)-HETE-d8
6	(±)17,18-DiHETE	335.3	247.2	-65	-24	9.97	Leukotriene B4-d4
7	9(10)-EpOME	295.3	171.1	-80	-21	14.86	11(12)-EET (EpETrE) -d11
8	12(13)-EpOME	295.3	195.2	-80	-19	14.74	11(12)-EET (EpETrE) -d11
9	PGD1	353.3	317.202	-55	-16	6.65	Prostaglandin D2-d4
10	PGD2	351.2	271.302	-50	-22	6.61	Prostaglandin D2-d4
11	PGB2	333.3	175.1	-60	-24	8.82	Prostaglandin D2-d4
12	8-iso PGE2	351.201	271.001	-55	-21	5.94	Prostaglandin E2-d4
13	15-deoxy-12,14-PGJ2	315.2	271.2	-65	-18	12.44	20-HETE-d6
14	PGF2α	353.2	309.2	-85	-24	5.89	Prostaglandin F2α-d4

CYP450-derived 9(10)-EpOME (49 pg/10^6cells) and 12(13)-EpOME (236 pg/10^6cells) and COX-derived PGD_2 (41,627 pg/10^6cell).

5.3.2.3 Gram-negative TLR4 agonist lipopolysaccharide (LPS)

Cell exposure to the TLR4 agonist, LPS, showed the majority of lipids were generated intracellularly (Figure 5.5D). 12/15-LOX derived lipids were only detected intracellularly, including 12-HETE (354 pg/10^6cells) at 30 minutes. Negligible amounts of lipid were expressed extracellularly by the cells at 30 minutes. Only COX- and CYP450-derived lipids, including PGD_2, 8-iso PGE_2, 9(10)-EpOME and 12(13)-EpOME, were seen extracellularly at 24 hours showing levels of 19,728, 257, 17 and 77 pg/10^6cells, respectively.

5.3.2.4 Gram-negative TLR5 agonist Flagellin

The TLR5 agonist, flagellin, showed that the majority of lipids were generated intracellularly (Figure 5.5E). 12/15-LOX-derived lipids, including 12-HETE and 15-HETE were only detected intracellularly at levels of 494 and 103 pg/10^6cells, respectively. Only COX- and CYP450-derived lipids were seen extracellularly at 24 hours, these included PGD_1 (165 pg/10^6cells), PGD_2 (5,746 pg/10^6cells), 17,18-DiHETE (169 pg/10^6cells) and 9(10)-EpOME (15 pg/10^6cells). Treatment with flagellin also showed the lowest abundance of lipids extracellularly compared to other TLR responses.

5.3.2.5 Gram-positive TLR6/2 agonist FSL

The heatmap shows that treatment with FSL (TLR6/2 agonist) had the highest abundance of lipids intracellularly at 30 minutes (Figure 5.5F), compared to other lipid responses. 12/15-LOX-derived lipids, including 12-HETE, 15-HETE and 15-HETrE were only detected intracellularly at levels of 1081, 179, 65 pg/10^6cells. Negligible

amounts of lipid were expressed extracellularly by the cells at 30 minutes. Only COX- and CYP450-derived lipids were seen extracellularly at 24 hours, these included PGD_1 (878 pg/10^6cells), PGD_2 (26,953 pg/10^6cells) and 15-deoxy-12,14 PGJ_2 (10,477 pg/10^6cells), and 9(10)-EpOME (12 pg/10^6cells).

5.3.2.6 Gram- positive and -negative TLR9 agonist ODN

Treatment with TLR9, ODN, show the lowest abundance of lipids extracellularly compared to other TLR responses (Figure 5.5G). 12/15-LOX-derived lipids, including 12-HETE, 15-HETE and 12-HEPE, were only detected intracellularly at levels of 480, 125 and 63 pg/10^6cells, respectively. Negligible amounts of lipid were expressed extracellularly by the cells at 30 minutes. Only COX- and CYP450-derived lipids were seen extracellularly at 24 hours, these included PGD_2 (20,687 pg/10^6cells), 8,9-DHET (88 pg/10^6cells), 8-iso PGE_2 (334 pg/10^6cells) and $PGF_2\alpha$ (4,132 pg/10^6cells).

5.3.2.7 Comparisons between responses to TLR agonists for selected lipids

The majority of the lipids generated were detected intracellularly. This was the case for 15-HETE, 12-HETE and its downstream metabolite 6-trans LTB_4 generation. All agonists did not generate higher amounts of 12/15-LOX-derived 12-HETE production over untreated levels (296 pg/10^6cells) at 30 minutes cell analysis, except FSL TLR6/2 agonist (~1100 pg/10^6cells) (Figure 5.6A).

15-HETE showed the same pattern of response at 30 minutes in the cells but with an ~3-fold decrease in generation compared to 12-HETE (Figure 5.6B). Intracellular 15-HETE production remained constant at 24 hours.

The potential side product of the COX oxidation of arachidonic acid, 11-HETE, was detected intracellularly at 24 hours (Figure 5.6C). All TLR agonist treatments showed

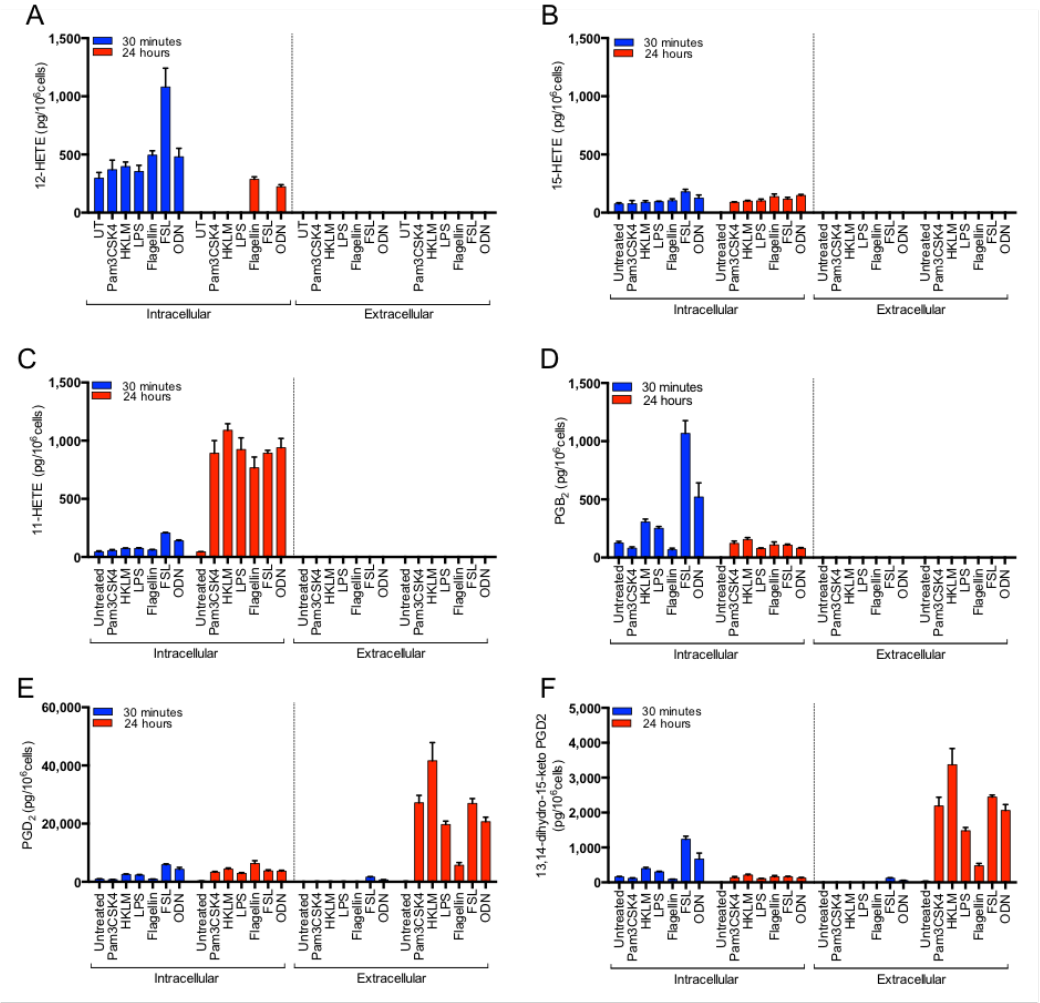


Figure 5.6 Generation of eicosanoids in response to TLR agonists. RAW 264.7 ALOX macrophages were exposed to TLR agonists for 30 minutes and 24 hours. The cells (intracellular) and supernatant (extracellular) were collected and analysed separately. **A-B)** Generation of 12/15-LOX lipids was confirmed by 12-HETE and 15-HETE production. **C-F)** representative eicosanoids generated by the COX pathway. **A-D)** Eicosanoid generation is displayed on the same Y-axis scale, whereas **E** and **F** are shown on differing scales. *n* = 1 with 3 replicates. Data represented as mean ± S.E.M.

the same levels of 11-HETE generation as seen with 15-HETE generation at 30 minutes, but levels were approximately 20-fold higher compared to the control levels of 11-HETE (~44 pg/10^6cells) at 24 hours.

Another COX derived lipid, PGB_2, was also detected intracellularly and indicated an apparent increase in generation in response to TLR6/2 FSL (1066 pg/10^6cells) at 30 minutes, compared to all other TLR agonist treatments, which generated levels less than half of this value (Figure 5.6D).

There were higher levels of COX-derived lipids in the supernatant after 24 hours exposure to the TLR agonists, these included PGD_1, PGD_2, $PGF_{2\alpha}$, 13, 14-dihydro-15-keto PGD_2 and 15-deoxy-12, 14-PGJ_2 (Figure 5.5B-G). The most abundant eicosanoid generated was PGD_2. Gram-positive HKLM showed the most intense abundance of the COX-derived eicosanoids, namely PGD_2, which was expressed at 41,627 pg/10^6cells (Figure 5.5C, 5.6E). Gram-positive Pam3CSK4 and FSL showed levels at 27,172 and 26,953 pg/10^6cells respectively, whereas Gram-negative LPS and Flagellin generated lower amounts at 19,577 and 5,746 pg/10^6cells, respectively. Intracellular Gram-negative TLR9, ODN, showed levels similar to LPS. The downstream metabolite of PGD_2, 13, 14-dihydro-15-keto PGD_2, showed the same pattern of expression for each TLR agonist but with over 3-fold lower levels generated compared to PGD_2 (Figure 5.6F).

Looking at the heatmap (Figure 5.5) there are consistent patterns of lipid expression between all TLR agonists. Interestingly, 30 minutes showed the highest abundance of 12/15-LOX-derived lipids inside the cells and levels were not detectable at 24 hours in the supernatant. Whilst, at 24 hours, mainly COX-derived lipids were the highest generated lipids in the extracellular supernatant.

5.3.3 Bacterial toll-like receptor induction of proinflammatory cytokines from RAW 264.7 ALOX macrophage

RAW 264.7 ALOX macrophage generated the proinflammatory cytokines TNF-α and IL-6 (Figure 5.7). IL-6 was seen to be TLR agonist-dependant as negligible amounts were detected in untreated cells. TNF-α was generated in higher amounts compared to IL-6, however, the pattern of expression was similar in response to each TLR agonist. IL-1β was below the limit of detection (data not shown).

5.3.3.1 TNF-α generation

Gram-negative TLR4 agonist LPS induced the highest level of TNF-α (25,032 pg/mL), whereas, Gram-negative TLR5 agonist Flagellin showed the lowest level of TNF-α (5,935 pg/mL) (Figure 5.7A). Gram-positive TLR6/2 agonist FSL and Gram-negative TLR9 agonist ODN generated similar levels of TNF-α (21,834 and 20,469 pg/mL, respectively) (Figure 5.7A). The Gram-positive TLR1/2 agonist Pam3CSK4 and TLR2 agonist HKLM showed slightly lower levels at 18,425 and 14,187 pg/mL, respectively (Figure 5.7A).

5.3.3.2 IL-6 generation

Gram-negative TLR4 agonist LPS again induced the highest level of IL-6 (14,099 pg/mL), whereas, Gram-negative TLR5 agonist Flagellin showed the lowest level of IL-6 (324 pg/mL) (Figure 5.7B). Gram-positive agonists TLR1/2 PAM3CSK4 and TLR6/2 agonist FSL and generated similar levels of IL-6 (6,287 and 7678 pg/mL, respectively) (Figure 5.7B). The Gram-positive TLR2 agonist HKLM and Gram-negative TLR9 agonist ODN showed slightly lower levels at 4841 and 2,983 pg/mL, respectively (Figure 5.7B).

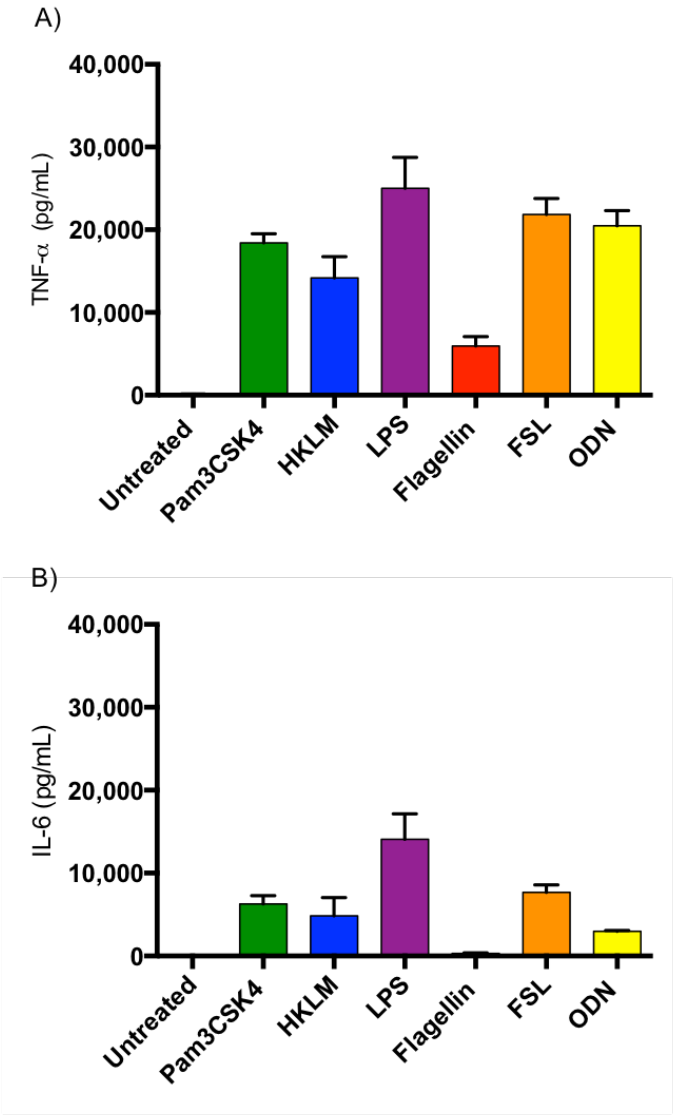


Figure 5.7 Comparison of responses generated by RAW 264.7 ALOX macrophages when exposed to bacterial toll-like receptor (TLR) agonists. RAW ALOX macrophages were seeded at $4x10^6$ cells/well and either left untreated or were stimulated with bacterial TLR agonists for 24 hours. Supernatant was analysed for cytokine generation using enzyme-linked immunosorbent assays, as described in the specific methods of this chapter. **A)** Generation of TNF-α by RAW 264.7 ALOX macrophages exposed to bacterial TLR agonists. **B)** Generation of IL-6 by RAW 264.7 ALOX macrophages exposed to bacterial TLR agonists. IL-1beta was below the limit of detection. *n* = 1 with 3 replicates. Data represented as mean ± S.E.M.

5.4 Discussion

The work in this chapter represents the first ever measure of lipids and cytokines generated from the 12/15-LOX transfected RAW 264.7 murine macrophages exposed to an array of bacterial TLR agonists. The initial aim in this body of work was to employ a targeted lipidomic approach to profile the basal eicosanoid content of the 12/15-LOX transfected RAW 264.7 ALOX macrophages compared to the RAW 264.7 Control macrophages. The next aim of this chapter was to characterise the generation of lipids and cytokines by RAW 264.7 ALOX macrophages when exposed to individual TLR agonists, to determine any varying responses between agonists.

5.4.1 Comparison of baseline expression between RAW 264.7 Control and ALOX macrophages

Work carried out by Buczynski et al., 2007, described how RAW 264.7 murine macrophages were stimulated with 16 different agonists of TLRs, G protein-coupled receptors and purinergic receptors. This study showed that 15 different eicosanoids were prodcued through the COX and 5-LOX pathway and were detected either intracellularly or in the media following stimulation with the agonist. No significant differences were seen in the COX metabolite profiles of RAW 264.7 macrophages exposed to the agonists and only agonists capable of creating a sustained Ca^{2+} influx were capable of activating the 5-LOX pathway in these cells; this didn't include TLR agonists for the lipids looked at in this paper (Buczynski et al. 2007).

The first part of the work carried out in this chapter evaluated the basal eicosanoid profile in RAW 264.7 Control and ALOX macrophages. 12-HETE and 15-HETE are synthesised by 12-LOX and 15-LOX, respectively, from arachidonic acid (AA). The enzyme 12/15-LOX generates different isoforms of lipids depending on the species. In man, 12/15-LOX will generate 15-HETE whereas in mice it generates 12-HETE. Therefore, in work carried out with the RAW 264.7 ALOX murine macrophages 12/15-

LOX activity was measured as formation of 12-HETE. Several prostaglandins (PGs), such as PGD_2, PGE_2 and $PGF_{2\alpha}$, are synthesised by COX.

Results here showed the RAW 264.7 Control macrophages, containing the empty vector, had basal levels of COX-derived lipids without the presence of any 12/15-LOX products. These findings were also demonstrated by Buczynski *et al*. 2007, who showed unstimulated RAW 264.7 cells did not generate 15-HETE but did produced COX-derived PGs. Buczynski *et al*. showed PGD_2 to have the highest abundance compared to other lipids, which was also the case for the RAW 264.7 Control macrophages here.

Comparing the findings from Buczynski et al. 2007 to the work carried out in this chapter confirms that the RAW 264.7 cells carrying the empty vector remain unaltered in terms of their lipid generation as they behave the same as the standard RAW 264.7 cell line. This is important as it demonstrates that insertion of the empty vector itself does not interfere with the lipid generation of the cells when compared to the original RAW 264.7 cells.

Analysis of the lipids detected from the RAW 264.7 ALOX macrophages confirmed that these cells significantly upregulated the generation of 12/15-LOX-derived lipids compared to the RAW 264.7 Control macrophages, where no 12/15-LOX-derived lipids were detected. These findings are important as they confirm that the transfected RAW 264.7 Control macrophages with the empty vector and the RAW 264.7 ALOX macrophages carrying the 12/15-LOX gene remain unaltered in terms of their natural lipid generation, as they behave the same as the standard RAW 264.7 cell line.

5.4.2 Comparison of proinflammatory cytokine generation between RAW 264.7 Control and ALOX macrophages

An initial comparison of the cytokine profile of both the RAW 264.7 Control and ALOX macrophages was established. It was predicted that these cells would generate the

same cytokine profile as each other because the transfection shouldn't alter the cytokine production. Data indicated that the RAW 264.7 Control macrophages generated cytokine levels that were between 50-100-fold less than those seen by the RAW 264.7 ALOX macrophages, when exposed to the TLR agonists. However, one study showed that treatment of native RAW 264.7 cells with the TLR1/2 agonist Pam3CSK4 and the TLR4 agonist LPS generated ~6000 pg/mL and ~14,000 pg/mL TNF-α, respectively (Berghaus et al. 2010). These levels are almost 100-fold greater than those seen from the RAW 264.7 Control cells. A possible explanation for the difference seen between the RAW 264.7 Control and ALOX cells may be that the LOX products generated by the RAW 264.7 ALOX macrophages could upregulate cytokine production compared to the RAW 264.7 Control cells (Professor Valerie O'Donnell, personal communication). As this data set is a *n* of 1, with 3 replicates it would need to be repeated to make further conclusions.

Both cell types generated TNF-α and IL-6. The proinflammatory cytokine IL-1β was below the limit of detection, this finding matched that seen by Merly & Smith, 2005, who showed that upon stimulation RAW 264.7 macrophages did not generate levels of IL-1β detectable by ELISA. IL-8 was not tested for in this data set as mice do not carry the IL-8 gene (Asfaha et al. 2013).

Once it was confirmed that the RAW 264.7 ALOX macrophages generated 12/15-LOX derived-lipids, the next step was to expose these cells to bacterial TLR agonists and establish any patterns in their responses.

5.4.3 RAW 264.7 ALOX macrophage lipid profiling

As previously described in Chapter 1, macrophages express a large number of distinct TLRs, which when stimulated activate cytosolic phospholipase A_2 ($cPLA_2$) to liberate AA from membrane phospholipids and generate an eicosanoid response by distinctly different pathways (Buczynski et al. 2007). By employing an array of bacterial stimuli, it was anticipated that the macrophages would generate a number of different

eicosanoid profiles that could indicate the presence of agonist-specific responses. This hypothesis was tested by stimulating the RAW 264.7 ALOX macrophages with 6 different bacterial agonists targeting TLRs.

Of the 110 eicosanoid and fatty acid transitions, 39 were detectable from the RAW 264.7 ALOX cells. It was found that, for most of the lipids produced, the same eicosanoid profile was generated for the 6 bacterial agonists tested. This profile was dominated by COX products, with PGD_2 produced in greatest amount and its dehydration products 15d PGJ_2 and 15d PGD_2 also seen. These results seen in the RAW 264.7 ALOX cells are consistent with those demonstrated by Buczynski *et al*. 2007, who found that 14 of the 16 agonists used, which included TLRs, to stimulate RAW 264.7 cells generated the same eicosanoid profile. Buczynski *et al*. 2007, also confirmed that out of the 60 lipids they scanned for, the COX-derived PGD_2 and its metabolites generated the highest levels.
For long term stimulation (24 hours) of RAW 264.7 ALOX cells with the TLR4 agonist LPS, the observation of increased COX products is also consistent with finding by Lee *et al*. 2016, who showed that levels of COX-dervied lipids dramatically increase over this time period, whereas 5-LOX activity was impaired by subsequent nitric oxide production.

When comparing the cell responses to the TLRs on the heatmap it could be observed that TLR2 HKLM and TLR6/2 FSL displayed a more intense abundance of lipids compared to the other TLRs, however, when looking at the actual amounts generated these data didn't appear to be suggestive of any substantial differences.
The uniformity of the eicosanoid profile in response to these TLR agonists was particularly striking as it has been shown that macrophages obtained from *in vivo* sources yield different and unexpected eicosanoid profiles, which was demonstarted by studies that modulate diet (Trebino et al. 2005; Wang et al. 2006). Furthermore, another study (Ragolia et al. 2005), which compared human atherosclerotic plaques, identified that macrophages in unstable plaques have significantly higher levels of PGE_2 and PGD_2 synthase than stable plaque macrophages. The notable difference between the *in vivo* macrophages and the RAW 264.7 cells is that the differentiation

process occurs in different enviroments. Also, the eicosanoid profile generated between human and mouse models are not directly comparable.

As described in Chapter 1, eicosanoids are produced rapidly after the initiation of inflammation. They are subsequently released by the cell to act in an autocrine or paracrine manor. During these studies, eicosanoid levels both inside and outside the cell were examined. One finding that was apparent was the elevated intracellular eicosanoid levels compared to the extracellular environment. For example, 12-HETE and 15-HETE were only detected intracellularly and surprisingly not detected extracellularly. It is possible that the RAW 264.7 ALOX macrophages retain these lipids inside the cell as they are not part of their normal processing and therefore lack the dedicated export pathways.

Increased intracellular levels of eicosanoids were also confirmed by the observations of Balsinde and colleagues (Balsinde et al. 1995) who saw increases in intracellular AA-derived lipids following [^{3}H]AA labelling of phospholipids and stimulation of P388D$_1$ murine macrophages. This increase was also seen by Buczynski et al. 2007 who reported the AA inside RAW 264.7 cells was ~4 times greater than the amount detected outside. This could be due to immortalised cells being different to primary macrophages as they undergo manipulation altering original characteristics and functions so that they proliferate indefinitely.

Interestingly, results in this chapter show that increased levels of COX-derived prostaglandins and their metabolites were mainly detected extracellularly after 24 hour agonist exposure to the RAW 264.7 ALOX cells. As previously mentioned, this could be due to the increased COX expression over time when exposed to stimuli (Lee et al. 2016). It may also be the case that as these cells characteristically have the COX enzyme, they also possess the correct machinery to facilitate transport out of the cell.

5.4.4 Cytokine generation following toll-like receptor stimulation of RAW 264.7 ALOX macrophage

TLR activation initiates multiple changes in the cell, which include the induction of gene expression, changes in protein levels and changes in protein phosphorylation levels (Buczynski et al. 2007). As well as looking at the changes in eicosanoid release when the RAW 264.7 ALOX macrophages were activated by six bacterial TLR agonists, I also examined the induction of proinflammatory cytokines after 24 hour treatment.

The RAW 264.7 ALOX macrophages generated similar levels of TNF-α to those seen by Berghaus *et al*. 2010, who exposed RAW 264.7 cells to the TLR4 agonist LPS and the TLR1/2 agonist Pam3CSK4.

Flagellin is the natural ligand of TLR5 and resulted in the lowest generation of both cytokines. It has been seen that treatment of RAW 264.7 cells with flagellin does not change the protein levels of the receptor (Palazzo et al. 2008) and therefore ceases to promote the expression of the specific receptor, dampening the response compared to the other TLRs. It has also been shown that the RAW 264.7 macrophage cell line expresses similar TLR transcripts with the exception of TLR5, which was down regulated in comparison (Applequist et al. 2002). It could also be possible that TLRs work in synergy to generate a response, as it has been shown that RAW 264.7 cells present higher levels of the TLR4 receptor when treated with the TLR9 ligand ODN (Palazzo et al. 2008). Therefore, there are a number of reasons why flagellin induced the lowest abundance of cytokines.

No major difference was seen in the cytokine generation when the RAW 264.7 ALOX macrophages were exposed to individual TLR agonists. However, experiments would need to be repeated in order to draw any further conclusions.

5.5 Conclusion

Using this novel RAW 264.7 transfected cell line enabled me to generate an initial profile of mediator expression when cells were exposed to different bacterial TLR

agonists. No major differences were seen in the lipid and cytokine generation within this data set when the RAW 264.7 ALOX cells were exposed to individual TLR agonists. Therefore, it was not possible to identify profiles for individual TLR agonists.

Individual TLR agonists were used to look for initial differences in lipid and cytokine profiles. However, during an infection multiple TLR agonists would be present. This further supports the idea of using whole bacteria, which contain multiple TLR agonists, and looking at the responses generated by the RAW 264.7 ALOX macrophages.

This body of work confirmed that the RAW 264.7 ALOX macrophages were a functional cell line able to generate 12/15-LOX-derived lipids. It may be the case that carrying out further experiments using different combinations of TLR agonists would demonstrate any synergistic effects between agonists, which may give rise to distinct patterns within the lipid and cytokine profiles. However, the number of possible combinations which could be investigated was very large and might still not show differences. Therefore, I decided to expose these cells to whole, live bacteria. This would enable me to see whether the profile of lipids and eicosanoids varied in response to whole bacteria as opposed to individual components, as would be the case during bacterial infection thus creating more pathophysiologically relevant conditions.

Chapter 6

Detecting eicosanoids and proinflammatory cytokines generated by RAW 264.7 ALOX macrophages upon stimulation with whole bacteria

6.1 Introduction

Chapter 5 confirmed the ability of RAW 264.7 ALOX macrophages to produce detectable levels of eicosanoids and cytokines following exposure to individual bacterial TLR agonists. These cells were employed in this chapter to characterise the innate immune response to different classes of bacterial pathogen with a view to identifying specific responses that could potentially be used as diagnostic fingerprints.

As described in Chapter 1, the host defence against invading pathogens is mediated by the acute inflammatory response (Russell & Schwarze 2013). This response depends upon the innate immune recognition of unique molecules emanating from the bacteria (Gioannini & Weiss 2007). Chapter 1 explains how the inflammatory response couples the detection of these specific molecules to physiological alterations that characterise acute inflammation (Beutler et al. 2006). It is important that the inflammatory response includes fast recognition and mounts a response shortly after invasion of small amounts of bacteria, due to the potentially rapid extracellular proliferation of certain invading organisms (Gioannini & Weiss 2007). This action should materialise well before antigen-specific immune recognition of the invading pathogen has developed.

Chapter 1 also explains how macrophages play an important role in controlling bacterial infection since they are one of the earliest components of the host defence systems to be activated (Daigneault et al. 2010). The outcome of the interaction between bacteria and macrophages (i.e., survive or destroyed) contributes to determining whether the infected host will eventually develop the disease associated with the bacteria, along with other host factors, microbial characteristics and timeliness of administration of appropriate antimicrobial therapy (Jin et al. 2010; Simor et al. 2016).

There is a significant variety of bacteria causing infections, including opportunistic and hospital-acquired microorganisms. Chapter 1 details the difference between

these Gram-positive and Gram-negative bacteria. Some of the most frequently isolated bacteria that commonly cause infection include Gram-positive *Staphylococcus aureus* (*S. aureus*) and Gram-negative *Escherichia coli* (*E. coli*) and *Klebsiella pneumoniae* (Klebsiella) (Ramachandran 2014).

Gram-positive *S. aureus*, in particular methicillin-resistant *S. aureus* (MRSA), remains one of the most prevalent bacterial pathogens worldwide (Bal et al. 2017). The past decade has seen a decrease in health-care-associated MRSA infections worldwide, but despite this, MRSA bloodstream infections continue to be associated with substantial morbidity and mortality, prolonged hospital stays and excess healthcare costs (Köck et al. 2010; Gastmeier et al. 2012; Simor et al. 2016). It was also initially thought that Gram-negative organisms were the major cause of bacterial sepsis, however, it has been shown that it is Gram-positive bacteria, such as *S. aureus*, that are the most common cause (Martin et al. 2003). The significance and pathogenic nature of Gram-positive MRSA make this resistant bacterium an excellent choice to employ when establishing an *in vitro* model of bacterial infection.

The most common Gram-negative infectious bacteria in patients of all ages is *E. coli* (Uzodi et al. 2017). *E. coli* is the leading cause of urinary tract infections (UTI) in children and bacteremia and meningitis in infants (Mackenzie 2014; Edlin et al. 2013; Greenhow et al. 2014). A major concern is the emergence of antimicrobial resistance in *E. coli*. Globally, there is an increased incidence of antimicrobial-resistant *E. coli* infections, which contribute to poor patient outcomes, and increased healthcare costs (Swami et al. 2012; Uzodi et al. 2017). These multi-drug resistant strains are a significant health threat as they have proliferated faster than the development of new antibiotics, ultimately leading to a lack of therapeutic agents to treat these highly resistant strains (Swami et al. 2012; Uzodi et al. 2017).

As well as *E. coli*, the Gram-negative bacteria *Klebsiella pneumoniae* (Klebsiella) is also a leading cause of serious infection in neonates, neutropenic cancer patients and other patients with underlying diseases (Kim et al. 2002). Klebsiella is an acute cause of infections in multiorgan systems, and is the third most commonly isolated

bacterium from the blood of sepsis patients (Huang et al. 2013). Incidence of hypervirulent Klebsiella has risen worldwide over the past few decades and it has been deemed the next superbug (Shon et al. 2013; Zhang et al. 2016). These strains have the capacity to cause severe and metastatic infections in young and healthy individuals, such as pyogenic liver abscesses and endophthalmitis and are even more antibiotic-resistant than *E. coli* (Kim et al. 2002; Zhang et al. 2016).

The bacteria employed in this chapter of work were Gram-positive MRSA, Gram-negative *E. coli* and Gram-negative Klebsiella. The strains of bacteria were carefully chosen as they are highly pathogenic, in order to set up the worst-case scenario of infection to look for initial marker profiles, and as previously mentioned are some of the most common causes of bacterial infections. Their ability to develop multi-drug resistant strains deems them 'superbugs' (Huang et al. 2013), and it is clear that there is a direct need for faster detection of these bacteria as a lack of early appropriate treatment is associated with high mortality and further increases the risk of resistance (Simor et al. 2016).

Inappropriate treatment of patients with antibiotics when they are not infected with a bacterium has been shown to have an adverse effect on the patient and can lead to the emergence of antibiotic-resistant bacteria (Cassell & Mekalanos 2007). Failure to treat an individual with a bacterial infection also has dire consequences (Gauer 2013). The types of antibiotics used to treat bacterial infections differ depending on their type i.e. Gram-positive versus Gram-negative and their antibiotic sensitivity. Normally, in cases such as sepsis, patients will be given type-specific, broad-spectrum treatment, thus the need to know what type of bacteria is responsible for the infection. In most hospitals in the UK clinicians will have information about the antibiotic susceptibility of the different classes of bacterial pathogens, therefore, if they know the nature of the infecting organism they can make an informed decision as to which antibiotics to use. Hence the desire to rapidly determine the nature of the infecting bacterium.

Establishing profiles of inflammatory markers, such as eicosanoids and cytokines, specific to individual bacteria causing an infection could lead to faster treatment, increased patient survival rates and lower risk of bacteria becoming increasingly drug-resistant.

To characterise the interaction between the different bacteria and macrophages, this chapter focuses on detecting distinct profiles of eicosanoids and cytokines generated by RAW 264.7 ALOX macrophages in response to the highly antibiotic-resistant bacterial species, MRSA, *E. coli* and Klebsiella.

6.1.1 Aims of this chapter

The first aim of this chapter was to analyse the cytokine generation between RAW 264.7 Control and ALOX macrophages to determine if exposure to whole bacteria altered the cytokine generation. As the cells have been engineered to generate the 12/15-LOX-derived lipids there should be no effect on the cytokine generation and thus be the same between the two cell types.

The second aim of this chapter exposed the RAW 264.7 ALOX macrophages to live Gram-positive and Gram-negative bacteria in order to identify if varying eicosanoid and cytokine responses are induced with a view to identifying pathogen specific signatures.

The specific objectives of the work in this chapter are therefore:

1. To establish the cytokine profiles generated by the RAW 264.7 ALOX macrophages are not altered by the presence of the 12/15-LOX gene.
2. To employ mass spectrometry to determine the eicosanoid profiles generated by RAW 264.7 Control and ALOX macrophages following exposure to bacteria.
3. To employ an ELISA-based method to determine the cytokine profiles generated by RAW 264.7 Control and ALOX macrophages following exposure to bacteria.

6.2 Specific materials and methods

The transfected murine RAW 264.7 macrophage cell lines used in this chapter of work are the same as those described in Chapter 5, section 5.2.1. Culture conditions and media (which will be referred to hereafter as RAW media) are also consistent with those described in Chapter 5, section 5.2.2.

6.2.1 Bacteria strains and growth conditions

The bacteria selected for this study were the Gram-positive methicillin-resistant *Staphylococcus aureus* (MRSA) National Collection of Type Cultures (NCTC) strain reference 12497, Gram-negative *Escherichia coli* (*E. coli*) extensively resistant NCTC strain reference 8003 and a multi-drug resistant Gram-negative *Klebsiella pneumoniae* (Klebsiella) 20 clinical isolate from Pakistan known to cause serious invasive disease. Bacteria were checked for purity using the streak plate method and grown on Luria-Berani (LB) agar (Fisher Scientifics, BP1425-500) at 37°C for 18 hours.

6.2.2 Maintenance of stocks and cultures

Bacteria were recovered from ceramic bead stocks (Fisher Scientifics, 12817755), which had been stored at -80°C since preparation from stocks received from NCTC or clinical isolate from Pakistan. After growth in LB broth for 18±2 hours at 37°C and 120 rpm (shaking incubator; IKA®KS3000-i-control), purity was assessed by Gram staining and strain identity of colonies from LB agar sub-cultured and confirmed by matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF MS) (Public Heath Wales Microbiology Division), as standard protocol for Cardiff University stocks.

6.2.2.1 Preparation of initial glycerol stocks of bacteria

Glycerol stocks of verified strains were prepared by streaking the bacteria onto LB agar plates and incubated at 37°C for 18-24 hours. Plates were checked visually for uncontaminated growth. Three to five morphologically identical colonies were suspended in 20 mL LB broth and incubated for 18±2 hours at 37°C and 120 rpm. The bacterial culture was mixed with an equal volume of sterile 20% *v*/*v* glycerol and stored in 1 mL aliquots at -80°C.

Stock maintenance was confirmed after three days by inoculating LB broth with three random samples of each organism, before being checked for purity by sub-culture onto LB agar for 18±2 hours at 37°C.

For use in this work, fresh working cultures of each organism were produced once monthly by reviving one rapidly thawed glycerol stock in LB broth incubated at 37°C and 120 rpm for 18±2 hours (shaking incubator; IKA®KS3000-i-control). A 10 µl loopful of bacterial culture were streaked onto LB agar plates, incubated at 37°C for 18-24 hours and stored agar side up at 4°C for a maximum of 6 weeks.

6.2.3 Experimental growth conditions

Bacteria were picked from single colonies and grown in LB broth (Sigma Aldrich, L3022) at 37°C, 120 rpm (shaking incubator; IKA®KS3000-i-control) to exponential growth phase. They were then washed in sterile phosphate-buffered saline (PBS) twice before being resuspended in RPMI-1640. Suspensions were adjusted to optical density (OD) 0.329, 0.198 and 0.199 for MRSA, *E. coli* and Klebsiella, respectively at wavelength 625 nm, which gave approximately 10^1 colony forming units per millilitre (cfu/mL) (JENWAY 6305 Spectrophotometer). The adjusted bacteria suspension was then added to the cells in volumes shown in Table 5.1 to obtain a 10:1 bacterial:cell ratio.

Table 5.1 Volumes for bacterial exposure model

Bacteria	OD for 10:1 bug cell ratio +/- 0.01 OD	Volume of RPMI (µl)		Volume of bacterial suspension (µl)	
		1 hour	24 hours	1 hour	24 hours
MRSA	0.329	900	3920	100	80
E. coli	0.198	950	3960	50	40
Klebsiella	0.199	950	3960	50	40

Once the OD was reached, drop counts were used to determine the number of bacteria in each suspension. Drops of 10 µl were spotted onto LB agar to determine cfu/mL, according to the Miles and Misra method (Miles & Misra 1938). Bacteria were stored at 4°C prior to exposure to macrophages.

6.2.4 RAW 264.7 ALOX macrophage infection model

The RAW 264.7 ALOX murine macrophages were plated in 6-well culture plates with 4 mL of RAW media (5 x 10^6 cells/well for 1 hour exposure, 4 x 10^6 cells/well for 24 hour exposure), and allowed to adhere overnight (time points were determined during infection model optimisation). Once adhered the RAW media was replaced with 1 or 4 mL RPMI-1640 alone, for the 1 hour and 24 hour time points respectively, and stimulated with the bacteria (MRSA, E. coli or Klebsiella). Bacteria were prepared as described in section 6.2.3 to be added to the cells at a 10:1 bug/cell ratio. Cells were incubated at 37°C in a humidified 5% CO_2 atmosphere for either 1 hour or 24 hours. (I had previously optimised these timepoints during characterisation of the cells). Cells and supernatant were collected separately. Supernatants were filtered using a 0.2-micron filter to remove bacteria. Following incubation, both cell and supernatant samples had deuterated internal standard mix added (75 nM), preparation described in Appendix l. The cell samples went through a hexane lipid extraction (described in Chapter 2, section 2.6). Subsequently, all samples (cells and supernatant) underwent SPE, as detailed in Chapter 2, section 2.7.

6.2.5 Lipidomic profiling of fatty acids and eicosanoids

Samples were analysed using the targeted lipidomic method described in Chapter 5, section 5.2.5.

6.2.5.1 Heatmap correlation

The heatmap was generated as described in Chapter 5, section 5.2.5.1.

6.2.6 Proinflammatory cytokine analysis

Protein concentration was measured using the double antibody sandwich enzyme-linked immunosorbent assay (ELISA), as described in Chapter 5, section 5.2.6.

6.2.7 Statistical analysis

Statistical analysis was carried out as described in Chapter 2, section 2.10.

6.3 Results

6.3.1 Comparison of cytokine generation in RAW 264.7 Control and ALOX macrophages

Chapter 5, section 5.3.1, defined the basal eicosanoid expression of RAW 264.7 Control and ALOX macrophages. Initial experiments in this chapter aimed to show that insertion of the 12/15-LOX gene in the ALOX macrophages did not alter the cytokine expression compared to the Control macrophages.

6.3.1.1 Gram-positive bacteria MRSA

Untreated RAW 264.7 Control and ALOX macrophages showed basal levels of TNF-α at 1021 and 1130 pg/mL, respectively (Figure 6.1Ai). Generation of IL-1β and IL-6 for untreated RAW 264.7 Control and ALOX macrophages was below the limit of detection (Figure 6.1Aii-iii).

Upon stimulation, there was a significant increase ($P<0.05$) in TNF-α generation in ALOX macrophages compared to the Control macrophages, where values showed 79,095 and 50,479 pg/mL, respectively (Figure 6.1Ai). There were no significant differences ($P>0.05$) seen between the RAW 264.7 Control and ALOX macrophage generation of IL-1β (207 and 181 pg/mL, respectively) and IL-6 (178 and 228 pg/mL, respectively) when cells were exposed to MRSA (Figure 6.1Aii-iii). MRSA alone showed TNF-α, IL-1β and IL-6 to be below the limit of detection.

6.3.1.2 Gram-negative bacteria E. coli

Untreated RAW 264.7 Control and ALOX macrophages showed basal levels of TNF-α at 321 and 140 pg/mL, respectively (Figure 6.1Bi). Untreated RAW 264.7 Control and

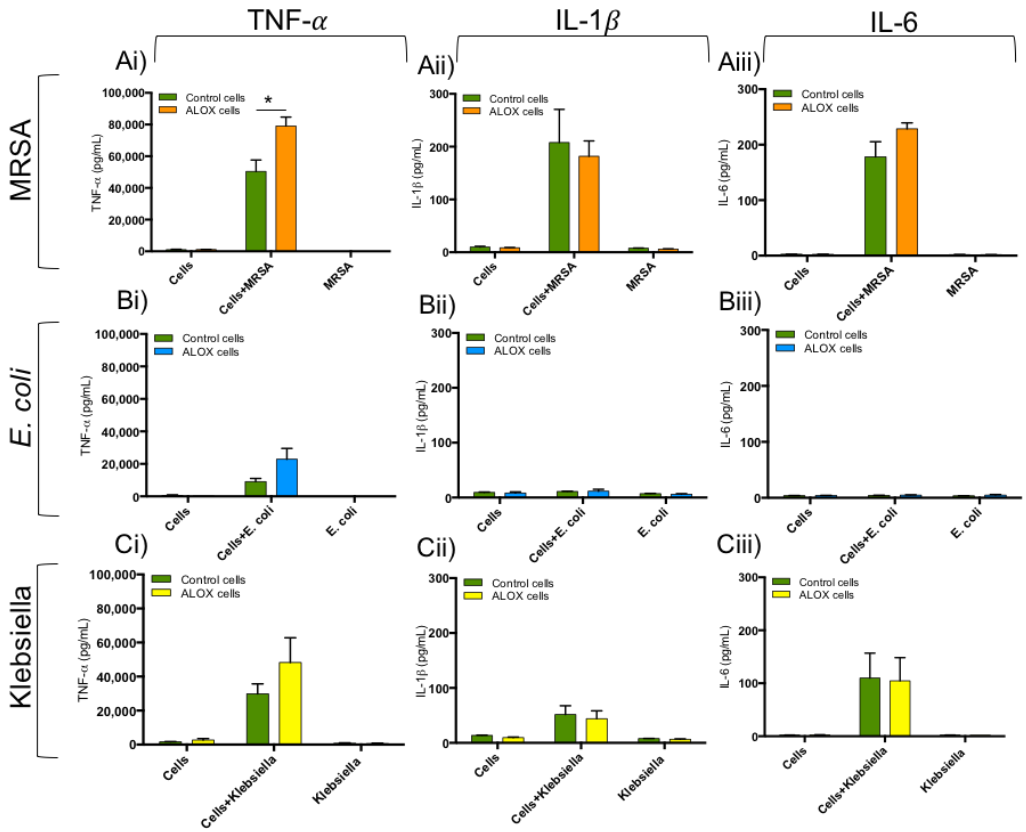


Figure 6.1 Comparison of proinflammatory cytokines TNF-α, IL-1β and IL-6 produced by RAW 264.7 Control and ALOX macrophages in response to whole live bacteria. RAW 264.7 Control and ALOX macrophages were seeded at $4x10^6$ cells/well and either left untreated (Cells) or were stimulated with bacteria (Cells+Bacteria) for 24 hours. RPMI media was inoculated with the bacteria alone (MRSA, *E. coli* or Klebsiella) as a control. Supernatants were analysed for cytokine generation using enzyme-linked immunosorbent assays, as described in the specific methods of this chapter. Data were analysed using an independent samples t test. * = $P<0.05$. *n* = 3 with 2 replicates. Data represented as mean ± S.E.M. No significant difference = $P>0.05$.

ALOX macrophages showed basal levels of cytokine generation for IL-1β and IL-6 to be below the limit of detection (Figure 6.1Bii-iii).

When cells were exposed to *E. coli*, there was no significant difference (P>0.05) in TNF-α generation between the RAW 264.7 Control and ALOX macrophage (Figure 6.1Bi). Even though the average value for TNF-α detection from ALOX macrophages (22,856 pg/mL) was higher upon stimulation than the Control macrophages (9,118 pg/mL), it was not significantly different due to the variation between the replicates (P>0.05). The RAW 264.7 Control and ALOX macrophages exhibited no detectable generation of IL-1β and IL-6 upon stimulation (Figure 6.1Bii-iii). *E. coli* alone showed TNF-α, IL-1β and IL-6 to be below the limit of detection.

6.3.1.3 Gram-negative bacteria Klebsiella

Untreated RAW 264.7 Control and ALOX macrophages showed basal levels of TNF-α at 1628 and 2675 pg/mL, respectively (Figure 6.1Bi). Generation of IL-1β and IL-6 for untreated RAW 264.7 Control and ALOX macrophages was negligible (Figure 6.1Cii-iii).

Upon stimulation, there was no significant increase (P>0.05) in TNF-α, IL-1β or IL-6 generation in ALOX macrophages (48,305, 43, 104 pg/mL, respectively) compared to the Control macrophages (29,881, 51, 109 pg/mL, respectively). Klebsiella alone showed TNF-α, IL-1β and IL-6 to be below the limit of detection.

Lipid analysis of basal eicosanoid levels in Chapter 5, section 5.3.1, determined that RAW 264.7 ALOX macrophages, containing the 12/15-LOX gene, generated 12/15-LOX-derived lipids compared to the RAW 264.7 Control macrophages.
In this chapter of work, both cell types gave similar responses in terms of the production of cytokines upon bacterial stimulation. The only significant difference between the two cell types was seen in TNF-α generation, upon treatment with MRSA (section 1.3.1.1). Given that these were such high levels compared to all other

cytokine generation, and that both cell types gave similar levels of response for all of the other test conditions, the RAW 264.7 ALOX macrophages were used for all subsequent experiments to provide a suitable model similar to *in vivo* macrophages.

6.3.2 Eicosanoid generation from RAW 264.7 ALOX macrophages upon stimulation with live bacteria

Out of the 110 eicosanoid and fatty acid transitions (Appendix lll) 41 showed detectable levels of expression when exposed to whole bacteria (Figure 6.2). All subsequent analysis will only discuss lipids that showed the greatest difference between the untreated and activated cells out of the 41 that were detected (Table 6.2). The greatest difference was defined as lipids that showed a significant difference or change in abundance between groups. Untreated cells provided a baseline of basal lipid mediators generated by RAW 264.7 ALOX macrophages (Figure 6.2A).

6.3.2.1 Gram-positive bacteria MRSA

The highest levels of 12/15-LOX-derived lipids, such as 12-HETE, 12-HEPE and 8,15-DiHETE were detected intracellularly at 1 hour showing values of 4993, 917 and 394 pg/10^6cells, respectively (Figure 6.2B). Stimulation with MRSA resulted in increased detection of intracellular lipids derived from the LOX pathways at 24 hours, these included LOX-derived lipids 13-HODE (6,872 pg/10^6cells), 13-OxoODE (1,847 pg/10^6cells) and 9-HODE (533 pg/10^6cells). Although an apparent increase was seen in 13-HODE detection from 1 hour to 24 hours, it was not found to be statistically significant ($P>0.05$) (Figure 6.3). At 24 hours, the level of expression was higher than that seen for the Gram-negative bacteria, but this difference was not statistically significant ($P>0.05$) (Figure 6.3). Negligible amounts of lipid were detected extracellularly from the cells

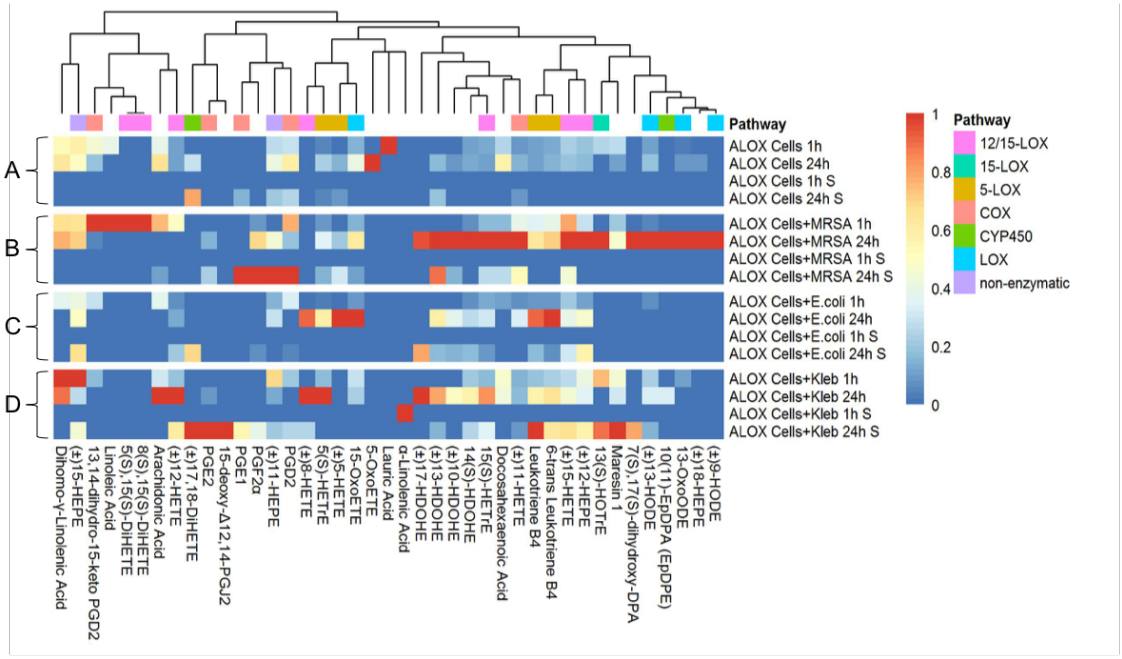
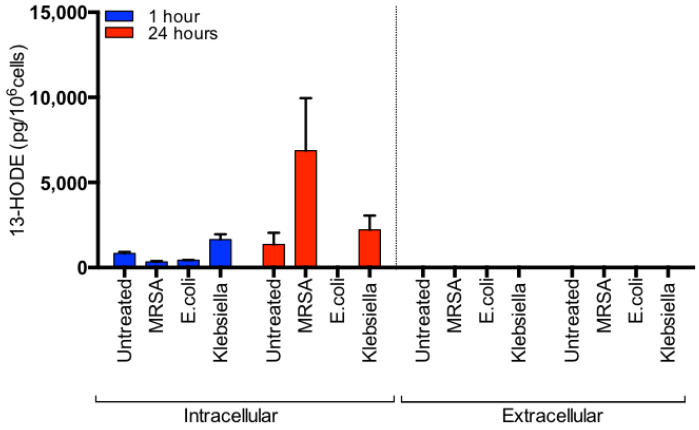


Figure 6.2 Lipidomic profiling of eicosanoids generated by RAW ALOX (ALOX) macrophages exposed to whole live bacterial stimulation. Data indicate targeted lipidomic analysis of ALOX macrophages that were seeded and either left **A)** untreated or were individually stimulated with bacteria **B)** Methicillin-resistant *Staphylococcus aureus* (MRSA), **C)** *Escherichia coli* (*E. coli*) or **D)** *Klebsiella pneumoniae* (Kleb) for 1 hour ($5x10^6$ cells/well) and 24 hours ($4x10^6$ cells/well) before lipids were extracted and analysed by liquid chromatography-MS/MS scanning for 110 different lipids, as described in the specific methods of this chapter. 41 out of 110 lipids were identified. The cells (cells) and supernatant (S) were extracted and analysed separately. The heatmap was generated using the pheatmap package in R Studio and lipids colour-coded according to lipid groups. Levels of treatment response are normalised to the highest value per lipid in the group and colour-coded to represent increasing amount of lipid (from blue (no or low amount of lipid) to red (increased amount of lipid)). Deeper tones indicate greater differences. Scale shows change relative to the most abundant lipid within that group. Lipids are colour-coded by their pathway and clustered by similarity in overall responses to the treatment. ALOX cells alone *n* = 9 with 2 replicates. ALOX cells exposed to bacteria *n* = 3 with 2 replicates. UT=untreated.

Table 6.2. The 10 most abundant eicosanoids detected in RAW 264.7 ALOX cells exposed to whole bacteria. Multiple reaction monitoring (MRM), deionising potential (DP), collision energy (CE), retention time (RT), internal standard (IS).

No.	Compound Name	MRM Transitions		DP	CE	RT (min)	IS
		Q1	Q3				
1	(±)12-HETE	319.2	167.202	-60	-19	13.91	12(S)-HETE-d8
2	(±)12-HEPE	317.2	167.201	-50	-20	12.69	12(S)-HETE-d8
3	15(S)-HETrE	321.2	221.2	-70	-21	14.29	13(S)-HODE-d4
4	(±)9-HODE	295.2	171.1	-85	-23	13.34	13(S)-HODE-d4
5	(±)13-HODE	295.2	195.2	-85	-23	13.28	13(S)-HODE-d4
6	13-OxoODE	293.2	195.1	-85	-25	13.72	13(S)-HODE-d4
7	8(S),15(S)-DiHETE	335.2	235.2	-65	-22	9.63	Leukotriene B4-d4
8	Leukotriene B4	335.2	195.1	-70	-23	10.22	Leukotriene B4-d4
9	PGD2	351.2	271.302	-50	-22	6.61	Prostaglandin D2-d4
10	PGE2	351.2	271.3	-60	-19	6.2	Prostaglandin E2-d4



Statistical comparisons	Intracellular		Extracellular	
	1 hour	24 hours	1 hour	24 hours
UT vs. MRSA	NS	NS	NS	NS
UT vs. E. coli	NS	NS	NS	NS
UT vs. Klebsiella	NS	NS	NS	NS
MRSA vs. E. coli	NS	NS	NS	NS
MRSA vs. Klebsiella	NS	NS	NS	NS
E. coli vs. Klebsiella	NS	NS	NS	NS
	Intracellular		Extracellular	
UT vs. UT	NS		NS	
MRSA vs. MRSA	NS		NS	
E. coli vs. E. coli	NS		NS	
Klebsiella vs. Klebsiella	NS		NS	

Figure 6.3 Generation of LOX-derived lipid 13-HODE in response to whole live bacteria. RAW 264.7 ALOX macrophages were exposed to TLR agonists for 1 hour and 24 hours. The cells (intracellular) and supernatants (extracellular) were collected and analysed separately. Data were analysed using a mixed measures ANOVA. P>0.05. *n* = 3 with 2 replicates. Data represented as mean ± S.E.M. UT = untreated, NS = not significant (P>0.05).

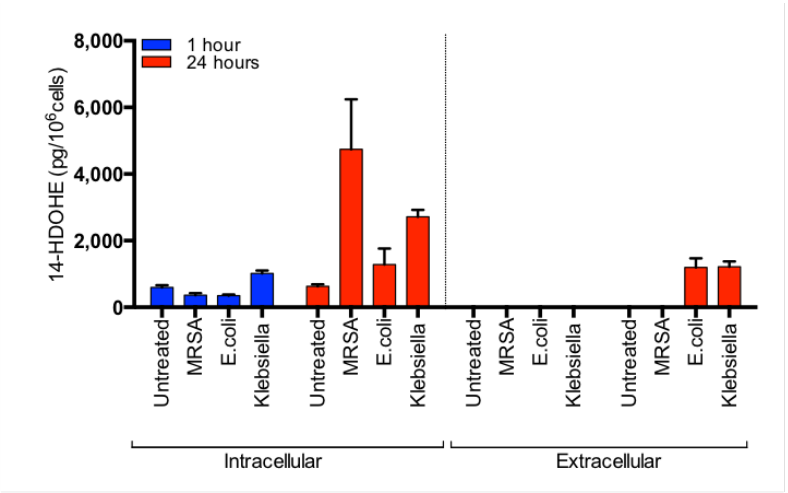
at 1 hour. Mainly COX-derived-lipids such as PGE_2 and PGD_2 were seen extracellularly at 24 hours, showing values of 193 and 1,190 pg/10^6cells, respectively (Figure 6.2B).

6.3.2.2 Gram-negative bacteria E. coli

A lower level of lipids were generated intracellularly at 1 hour, compared to 24 hours (Figure 6.2C). The majority of lipids detected were generated intracellularly at 24 hours including 12/15-LOX-derived lipids 12-HETE (1,212 pg/10^6cells) and LTB_4 (1,021 pg/10^6cells) (Figure 6.2C). Negligible amounts of lipid were expressed extracellularly by the cells at 1 hour. 12/15-LOX-derived lipids such as 12-HETE, LTB_4 and 15-HETrE were also detected extracellularly at 24 hours, showing levels of 2,649, 231 and 177 pg/10^6cells (Figure 6.2C). Extracellular detection of 14-HDOHE, following exposure to *E. coli*, significantly increased over time ($P<0.05$) (Figure 6.4).

6.3.2.3 Gram-negative bacteria Klebsiella

The heatmap indicates that treatment with Klebsiella showed increased lipid generation at 24 hours, both intracellularly and extracellularly, compared to 1 hour (Figure 6.2D). Intracellular and extracellular 12/15-LOX-derived 12-HETE significantly increased ($P<0.001$) at 24 hours (10,078 and 6,224 pg/10^6cells, respectively) compared to 1 hour (1,951 pg/10^6cells and below the limit of detection, respectively) (Figure 6.2D). Negligible amounts of eicosanoids were expressed extracellularly by the cells at 1 hour. Abundance of 14-HDOHE significantly increased extracellularly over time ($P<0.01$) (Figure 6.4).



Statistical comparisons	Intracellular		Extracellular	
	1 hour	24 hours	1 hour	24 hours
UT vs. MRSA	NS	NS	NS	NS
UT vs. E. coli	NS	NS	NS	NS
UT vs. Klebsiella	NS	NS	NS	NS
MRSA vs. E. coli	NS	NS	NS	NS
MRSA vs. Klebsiella	NS	NS	NS	NS
E. coli vs. Klebsiella	NS	NS	NS	NS
	Intracellular		Extracellular	
UT vs. UT	NS		NS	
MRSA vs. MRSA	NS		NS	
E. coli vs. E. coli	NS		*	
Klebsiella vs. Klebsiella	NS		**	

Figure 6.4 Generation of 14-HDOHE in response to whole live bacteria. RAW 264.7 ALOX macrophages were exposed to TLR agonists for 1 hour and 24 hours. The cells (intracellular) and supernatants (extracellular) were collected and analysed separately. Data were analysed using a mixed measures ANOVA. * = P<0.05, ** = P<0.01. *n* = 3 with 2 replicates. Data represented as mean ± S.E.M. UT = untreated, NS = not significant (P>0.05).

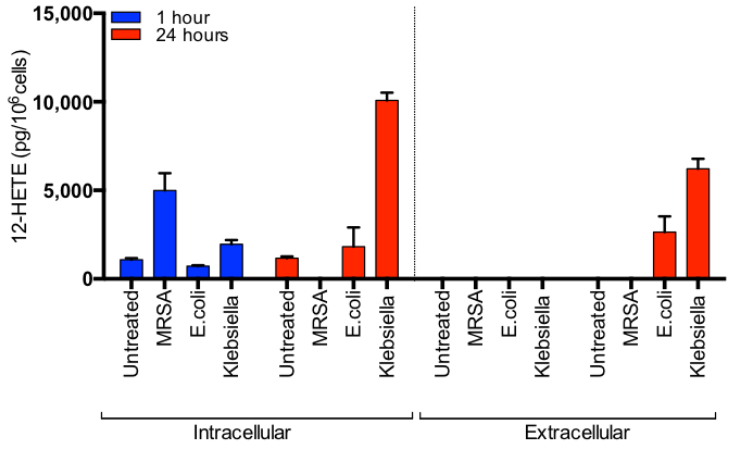
6.3.3 Comparison between the RAW 264.7 ALOX macrophage responses to individual bacteria

The highest abundance of the lipids generated were seen intracellularly, at 24 hours cell exposure to the bacteria (Figure 6.2). Overall Gram-positive MRSA generated the highest abundance of lipids intracellularly compared to Gram-negative *E. coli* and Klebsiella at 24 hours (Figure 6.2B). RAW 264.7 ALOX macrophages stimulated with Klebsiella showed the highest overall abundance of extracellular lipid generation at 24 hours, compared to MRSA and *E. coli* (Figure 6.2D). Cell exposure to *E. coli* showed the lowest overall generation of lipids compared to MRSA and Klebsiella (Figure 6.2C). Some 12/15-LOX derived lipids were also seen extracellularly at 24 hours; both *E. coli* and Klebsiella showed that this was the case for 15-HETE (328 and 611 pg/10^6cells, respectively), 12-HETE (2649 and 6224 pg/10^6cells receptively) and its downstream metabolite LTB_4 (231 and 1124 pg/10^6cells, respectively) generation.

6.3.3.1 12/15-LOX-derived lipid 12-HETE

12-HETE was generated by all bacteria intracellularly at 1 hour, showing levels of 4,993, 717 and 1,951 pg/10^6cells for MRSA, *E. coli* and Klebsiella, respectively (Figure 6.5, Table 6.3A). MRSA treatment showed a significant increase ($P<0.05$) compared to the untreated cells (1,081 pg/10^6cells). There was no significant difference ($P>0.05$) seen with either *E. coli* or Klebsiella intracellularly at 1 hour compared to the untreated cells. MRSA showed a significant increase ($P<0.05$) in 12-HETE compared to E. coli intracellularly at 1 hour. No other significant differences were seen within this group. There were no statistically significance observations seen extracellularly at 1 hour ($P>0.05$).

Intracellularly at 24 hours, Klebsiella showed a significant increase ($P<0.001$) in 12-HETE (10,178 pg/10^6cells) compared to the untreated (1,193 pg/106cells), MRSA- (not detected) and *E. coli*- (1,818 pg/10^6cells) treated cells (Figure 6.5, Table 6.3A).



Statistical comparisons	Intracellular		Extracellular	
	1 hour	24 hours	1 hour	24 hours
UT vs. MRSA	*	NS	NS	NS
UT vs. E. coli	NS	NS	NS	NS
UT vs. Klebsiella	NS	***	NS	**
MRSA vs. E. coli	*	NS	NS	NS
MRSA vs. Klebsiella	NS	***	NS	**
E. coli vs. Klebsiella	NS	***	NS	*
	Intracellular		Extracellular	
UT vs. UT	NS		NS	
MRSA vs. MRSA	***		NS	
E. coli vs. E. coli	NS		*	
Klebsiella vs. Klebsiella	***		***	

Figure 6.5 Generation of 12-HETE in response to whole live bacteria. RAW 264.7 ALOX macrophages were exposed to TLR agonists for 1 hour and 24 hours. The cells (intracellular) and supernatants (extracellular) were collected and analysed separately. Generation of 12/15-LOX lipids was confirmed by 12-HETE. Data were analysed using a mixed measures ANOVA. * = P<0.05, ** = P<0.01, *** = P<0.001. *n* = 3 with 2 replicates. Data represented as mean ± S.E.M. UT = untreated, NS = not significant (P>0.05).

A)

Intracellular						
Eicosanoid (pg/10^6cells)	MRSA (Gram-positive)		*E. coli* (Gram-negative)		Klebsiella (Gram-negative)	
	1 hour	24 hours	1 hour	24 hours	1 hour	24 hours
12-HETE	4993	ND	717	1818	1951	10,078
11-HETE	1435	3684	264	1247	565	654
13-HODE	337	6872	426	ND	1642	2218
14-HDOHE	360	4745	349	1284	1015	2719
17-HDOHE	ND	1159	ND	ND	ND	1223
PGD2	917	229	394	ND	256	ND

B)

Extracellular						
Eicosanoid (pg/10^6cells)	MRSA (Gram-positive)		*E. coli* (Gram-negative)		Klebsiella (Gram-negative)	
	1 hour	24 hours	1 hour	24 hours	1 hour	24 hours
12-HETE	ND	ND	97	1021	ND	2649
11-HETE	ND	ND	ND	ND	ND	305
13-HODE	ND	ND	ND	ND	ND	ND
14-HDOHE	ND	ND	ND	795	ND	1215
17-HDOHE	ND	ND	ND	976	ND	ND
PGD2	ND	1190	ND	54	ND	305

C)

Cytokine (pg/mL)	MRSA	*E. coli*	Klebsiella
TNF-α	79,095	22,856	48,305
IL-1β	181	ND	43
IL-6	228	ND	104

Highest level of lipid at 1 hour
Highest level of lipid or cytokine at 24 hours

Table 6.3 Summary profiles highlighting potential distinct markers of bacterial infection. A) intracellular and **B)** extracellular detection of eicosanoids exposed to Gram-positive and –negative bacteria at 1 and 24 hours. **C)** overall cytokine responses. Highlighted cells show lipids and cytokines that had significantly increased or were in greatest abundance compared to cell exposure with the other bacteria. ND = not detected.

Klebsiella also showed a significant increase in 12-HETE (6,224 pg/10^6cells) extracellularly at 24 hours compared to untreated (P<0.01), MRSA- (P<0.01) and *E. coli*- (P<0.05) treated cells; 12-HETE was not detected in untreated and MRSA-treated samples and showed a value of 2,649 pg/10^6cells in E. coli treated cells.

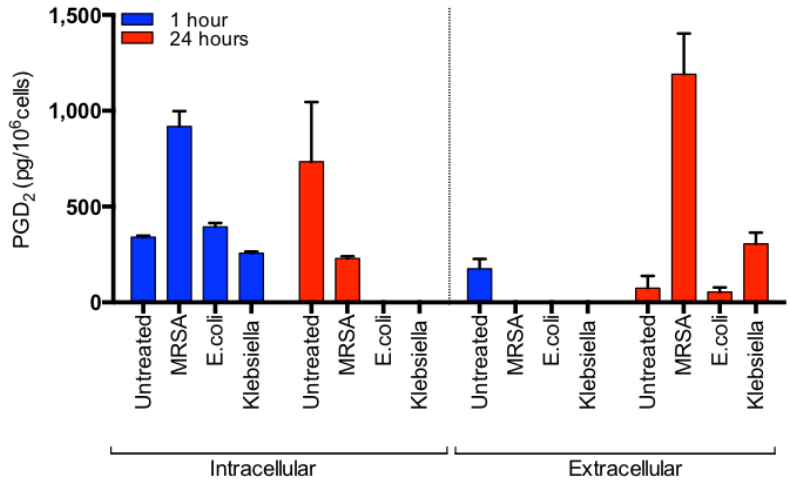
Generation of intracellular 12-HETE by MRSA showed a significant decrease over time (P<0.001), however, Klebsiella indicated a significant increase over time, from 1 hour to 24 hours (P<0.001) (Figure 6.5).
Generation of extracellular 12-HETE by *E. coli* and Klebsiella showed a significant increase over time, from 1 hour to 24 hours (P<0.05, P<0.001, respectively) (Figure 6.5).

6.3.3.2 COX-derived lipid PGD_2

There were increased levels of COX-derived lipids in the supernatant at 24 hours exposure to the bacteria, these included PGD_2, $PGF_{2\alpha}$, PGE_1 and 13, 14-dihydro-15-keto PGD_2 (Figure 6.2).

PGD_2 was generated by all bacteria intracellularly at 1 hour (Figure 6.6). Only MRSA treatment (917 pg/10^6cells) showed a significant increase (P<0.001) in PGD_2 compared to the untreated cells (340 pg/10^6cells) intracellularly at 1 hour. MRSA showed a significant increase (P<0.001) in PGD_2 compared to E. coli and Klebsiella intracellularly at 1 hour, which showed values of 394 and 256 pg/10^6cells, respectively (Table 6.3A). There were no statistically significance observations seen extracellularly at 1 hour (P>0.05).

Intracellularly at 24 hours, both Gram-negative bacteria, E. coli and Klebsiella showed a significant decrease (P<0.05) in PGD_2 (not detected) compared to the untreated cells (694 pg/10^6cells) (Figure 6.6). MRSA showed a significant increase (1,190



Statistical comparisons	Intracellular		Extracellular	
	1 hour	24 hours	1 hour	24 hours
UT vs. MRSA	***	NS	NS	*
UT vs. E. coli	NS	*	NS	NS
UT vs. Klebsiella	NS	*	NS	NS
MRSA vs. E. coli	***	NS	NS	*
MRSA vs. Klebsiella	***	NS	NS	*
E. coli vs. Klebsiella	NS	NS	NS	NS
	Intracellular		Extracellular	
UT vs. UT	*		NS	
MRSA vs. MRSA	***		***	
E. coli vs. E. coli	*		NS	
Klebsiella vs. Klebsiella	NS		NS	

Figure 6.6 Generation of COX-derived lipid PGD_2 in response to whole live bacteria. RAW 264.7 ALOX macrophages were exposed to TLR agonists for 1 hour and 24 hours. The cells (intracellular) and supernatants (extracellular) were collected and analysed separately. Data were analysed using a mixed measures ANOVA. * = P<0.05, ** = P<0.01, *** = P<0.001. *n* = 3 with 2 replicates. Data represented as mean ± S.E.M. UT = untreated, NS = not significant (P>0.05).

pg/10^6cells) in PGD_2 extracellularly at 24 hours compared to untreated (292 pg/10^6cells), E. coli- (54 pg/10^6cells) and Klebsiella- (305 pg/10^6cells) treated cells (P's<0.05) (Table 6.3B).

There was a significant increase (P<0.05) in intracellular PGD_2 seen in untreated cells over time. Generation of intracellular PGD_2 by MRSA and E. coli showed a significant decrease over time (P<0.001, P<0.05, respectively) (Figure 6.6).
Generation of extracellular PGD_2 by MRSA showed a significant increase over time, from 1 hour to 24 hours (P<0.001) (Figure 6.6).

The differences seen intracellularly at 24 hours between the lipid responses upon cell exposure to the different bacteria are summarised in Figure 6.7 (Table 6.3A). For MRSA and Klebsiella, data indicate that the overall pattern of response (excluding 12-HETE) is similar, however, levels are approximately 3-fold higher in MRSA compared to Klebsiella stimulated cells. 12-HETE is shown to be significantly increased (P<0.01; P<0.05) (Figure 6.5) in response to Klebsiella compared to MRSA and *E. coli,* respectively. Gram-negative *E. coli* did not show a similar pattern of response to the MRSA or the other Gram-negative bacteria Klebsiella (Figure 6.7).

6.3.4 Generation of proinflammatory cytokines by RAW 264.7 ALOX macrophages exposed to whole bacteria

RAW 264.7 ALOX macrophage generated the proinflammatory cytokines TNF-α, IL-1β and IL-6 (Figure 6.8). Earlier cytokine analysis (section 6.3.1) compared generation between RAW 264.7 Control and ALOX macrophages to confirm that the 12/15-LOX gene insertion had no effect on cytokine responses. This section will now focus on bacteria specific cytokine responses by RAW 264.7 ALOX macrophages.

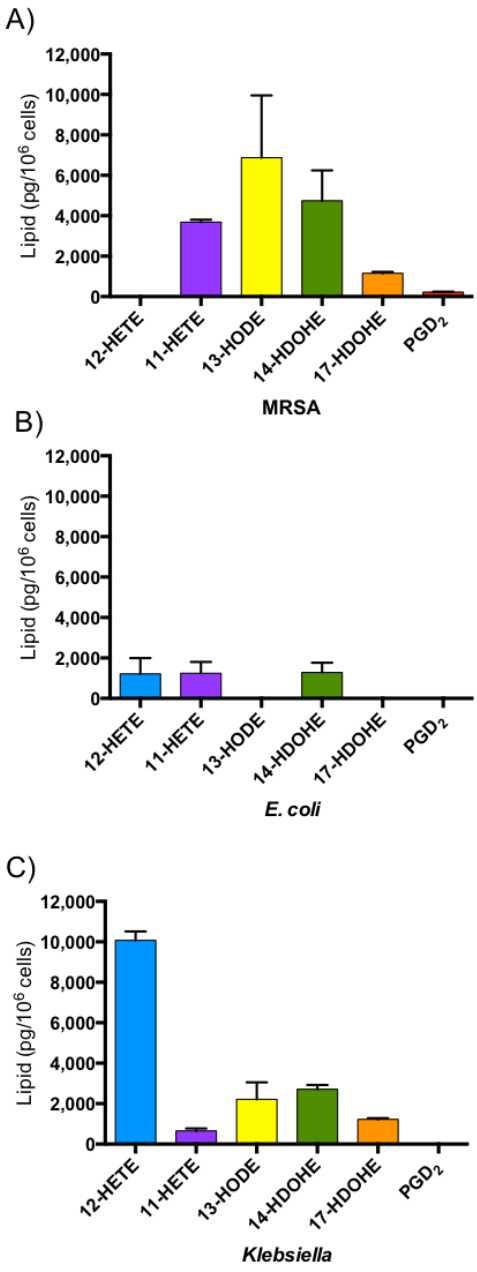
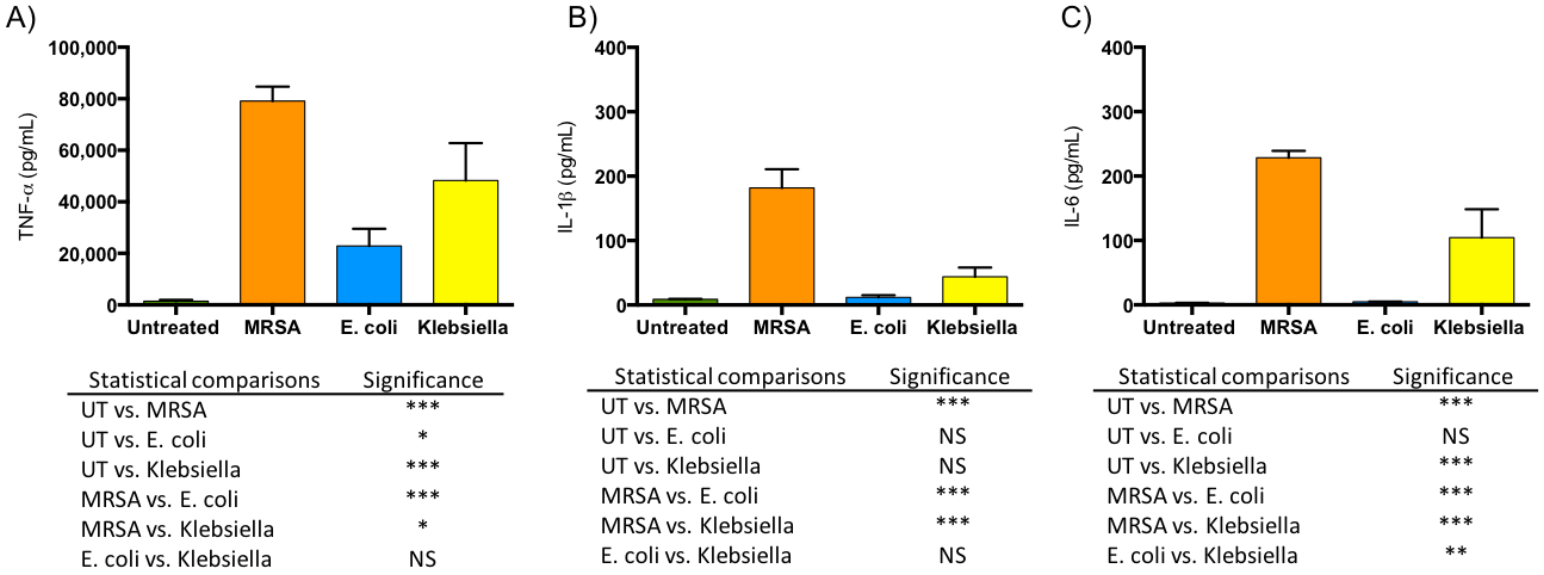


Figure 6.7 Summary profile of the top 6 most detected lipids. Data illustrate the profiles of the 6 most abundant lipids detected from RAW 264.7 ALOX macrophages intracellularly at 24 hours bacterial exposure to **A)** MRSA **B)** *E. coli* **C)** Klebsiella. Data represented as mean ± S.E.M. N=3. Statistical analysis is shown in Figures 6.3-6.6.



Statistical comparisons	Significance
UT vs. MRSA	***
UT vs. E. coli	*
UT vs. Klebsiella	***
MRSA vs. E. coli	***
MRSA vs. Klebsiella	*
E. coli vs. Klebsiella	NS

Statistical comparisons	Significance
UT vs. MRSA	***
UT vs. E. coli	NS
UT vs. Klebsiella	NS
MRSA vs. E. coli	***
MRSA vs. Klebsiella	***
E. coli vs. Klebsiella	NS

Statistical comparisons	Significance
UT vs. MRSA	***
UT vs. E. coli	NS
UT vs. Klebsiella	***
MRSA vs. E. coli	***
MRSA vs. Klebsiella	***
E. coli vs. Klebsiella	**

Figure 6.8 Comparison of cytokine responses generated by RAW 264.7 ALOX macrophages when exposed to whole live bacteria. RAW 264.7 ALOX macrophages were seeded at $4x10^6$ cells/well and either left untreated or were stimulated with bacterial for 24 hours. Supernatants were analysed for cytokine generation using enzyme-linked immunosorbent assays to look for the proinflammatory cytokines. **A)** TNF-α **B)** IL-1β **C)** IL-6. Data were analysed using a one-way ANOVA. An overall group difference was reported for each cytokine tested P's<0.001. Post-hoc Tukey's analysis with Bonferroni corrections was used for individual group comparisons. * = P<0.05, ** = P<0.01, *** = P<0.001. *n* = 3 with 2 replicates. Data represented as mean ± S.E.M. UT = untreated, NS = not significant (P>0.05).

6.3.4.1 Production of TNF-α

There was a significant increase in TNF-α production when cells were exposed to MRSA (P<0.001; 79,096 pg/mL), E. coli (P<0.05; 22,856 pg/mL) and Klebsiella (P<0.001; 48,305 pg/mL) compared to the untreated cells (1,315 pg/mL) for 24 hours (Figure 6.8A). Gram-positive MRSA was shown to significantly increase TNF-α generation compared to the Gram-negative bacteria E. coli and Klebsiella (P<0.001, P<0.05, respectively) (Figure 6.8A, Table 6.3C).

6.3.4.2 Production of IL-1β

Treatment of RAW 264.7 ALOX macrophage with Gram-positive bacteria MRSA (181 pg/mL) showed a significant increase in IL-1β generation compared to untreated, *E. coli*- and Klebsiella-treated cells (P's<0.001) at 24 hours; untreated and *E. coli*-treated cells showed levels below the limit of detection and Klebsiella was 43 pg/mL (Figure 6.8B, Table 6.3C). There were no other statistically significant observations between groups (P>0.05).

6.3.4.3 Production of IL-6

The RAW 264.7 ALOX macrophage generated a significantly increased amount of IL-6 when treated with MRSA (228 pg/mL) compared to untreated, *E. coli*- and Klebsiella-treated cells (P's<0.001); untreated and *E. coli*-treated cells showed levels below the limit of detection and Klebsiella was 104 pg/mL (Figure 6.8C, Table 6.3C). Upon stimulation with Klebsiella, the RAW 264.7 ALOX macrophage showed a significant increase in IL-6 compared to untreated and *E. coli*-treated cells (P<0.001, P<0.01, respectively) (Figure 6.8C). Treatment of cells with *E. coli* generated negligible levels of IL-6, below the limit of detection.

6.4 Discussion

This chapter of work represents the first ever measure of a lipid and cytokine biomarker series studied in an infection model of macrophages. The initial aim in this chapter was to analyse the cytokine generation between RAW 264.7 Control and ALOX macrophages in order to determine if the cell transfection altered the cytokine generation. The next aim was to expose the RAW 264.7 ALOX macrophages to live Gram-positive and Gram-negative bacteria to identify if pathogen specific eicosanoid and cytokine responses were induced.

6.4.1 Comparison of basal expression between RAW 264.7 Control and ALOX macrophages

As previously demonstrated in Chapter 5, section 5.3.1.1.1, initial experiments were carried out to establish basal levels of eicosanoid and cytokine expression by the RAW 264.7 Control and ALOX macrophages. As mentioned, the activity of 12/15-LOX in the RAW 264.7 ALOX cells was measured by the formation of 12-HETE, which was not seen in the RAW 264.7 Control macrophages and therefore confirms the presence of the gene in the RAW 264.7 ALOX cells.

6.4.1.1 Proinflammatory cytokine generation

An initial comparison of the cytokine profile of both the RAW 264.7 Control and ALOX macrophages was established. As previously mentioned, it was expected that these cells would generate the same cytokine profile as each other because the introduction of the ALOX gene was not expected to effect cytokine production.
Interestingly, this wasn't the case in Chapter 5 (Figure 5.4), where data showed up to a 100-fold increase in TNF-α cytokine generation from TLR-stimulated RAW 264.7 ALOX macrophages compared to the Control cells. However, in this body of work further investigation showed that there was no significant difference in the cytokine generation between the RAW 264.7 Control and ALOX macrophages when exposed

to E. coli and Klebsiella, both of which are Gram-negative bacteria. Treatment of cells with MRSA showed a significant increase in TNF-α generation in the RAW 264.7 ALOX macrophages compared to the Control. A potential explanation for this is that the LOX products generated by the MRSA-stimulated RAW 264.7 ALOX macrophages upregulated cytokine production compared to the MRSA-stimulated RAW 264.7 Control macrophages (Professor Valerie O'Donnell, personal communication).

The average basal levels of TNF-α generated by both the RAW 264.7 Control and ALOX macrophages (990 and 1,315 pg/mL, respectively) were shown to be similar to basal levels in native RAW 264.7 macrophages (~1,500 pg/mL) in work carried out by Berghaus et al. 2010. This implies that the transfection has not changed the cytokine generation of these cells.

Both cell types generated the proinflammatory cytokines TNF-α, IL-1β and IL-6. Unlike TNF-α, basal levels of IL-1β and IL-6 produced by RAW 264.7 Control and ALOX cells were below the limits detectable by ELISA. This finding also matches that seen by Chamberlain et al. 2009, who showed only TNF-α expression at the mRNA level in untreated RAW 264.7 macrophages but not IL-1β and IL-6.

The next step was to expose these cells to whole, live bacteria and establish any patterns in their responses with a view to identifying pathogen specific signatures.

6.4.2 Lipid profiling of RAW 264.7 ALOX macrophage exposure to live bacteria

The use of various eicosanoid species as biomarkers for disease and pathophysiological conditions has previously been reported (Higashi et al. 2010; Drozdovszky et al. 2014). For example, Mal et al., (2011) showed that endogenous levels of arachidonic acid, PGE_2 and 12-HETE were significantly altered in cancerous mucosae; indicating inflammation is correlated with tumorigenesis. It was also reported by Rago & Fu, (2013) that it was possible to distinguish between three groups of patients (healthy individuals, hypertensive patients and severe

atherosclerotic patients) by quantities of several eicosanoids in human plasma. The results showed that lower levels of 8-HETE, LTB_4, 9-HODE and 13-HODE are potential biomarkers of severe heart disease. The metabolism of eicosanoids may differ depending on biological samples and pathophysiological events (Lee et al. 2016).

This chapter of work shows, with the use of a targeted lipidomic approach, that murine RAW 264.7 ALOX macrophages generate differing lipid profiles when co-cultured with Gram-positive and Gram-negative bacteria. Of the 110 eicosanoids and fatty acids transitions analysed, 41 were detectable from the RAW 264.7 ALOX cells. Analysis of data in the heatmap showed that Gram-positive MRSA had increased levels of lipids compared to the Gram-negative bacteria *E. coli* and Klebsiella. This is interesting as the Gram-positive TLR2 agonist HKLM and TLR6/2 agonist FSL used in Chapter 5, which are ligands present on MRSA, also displayed a more intense abundance of lipids compared to TLRs associated with Gram-negative bacteria. These findings contradict data that suggest Gram-negative infections induce a greater magnitude of inflammatory response than Gram-positive infections (Abe et al. 2010). The difference seen between the bacterial responses may be due to the varying degree of pathogenicity between the Gram-positive and -negative bacteria (Hoerr et al. 2012). It may also be the case that the Gram-negative bacteria have developed ways to evade earlier detection by the host, for example, by modifying the structure of the endotoxin lipid A, unique to Gram-negative bacteria, leading to greater resistance to host antimicrobial peptides and altering TLR4 recognition (Ramachandran 2014). This means that initially the Gram-negative bacteria may have not been detected by the RAW 264.7 ALOX macrophages, creating a delayed or dampened host response compared to the Gram-positive bacteria.

Another possible reason for the increased response to the Gram-positive compared to Gram-negative bacteria, may be due to the differences in their cell wall structure. This is because Gram-positive bacteria are bound by a single cell membrane containing peptidoglycan layers, which are approximately 50 times thicker than those of Gram-negative bacteria (Tietze et al. 2006). Peptidoglycans and lipoteichoic acid are two of the major components in Gram-positive bacteria, such as MRSA, and have been shown to stimulate inflammatory responses in a number of *in vivo* and *in*

vitro experimental models (Mattsson et al. 1993; Lawrence & Nauciel 1998) and therefore may be a reason why the increased cell response to MRSA is seen. In contrast to this, Gram-negative bacteria, including *E. coli* and Klebsiella, contain a thin peptidoglycan layer (i.e. cell wall) lying between two different cell membranes in addition to LPS, where the composition of LPS can vary depending on the bacterial origin (Tietze et al. 2006).

Chapter 5 describes the uniformity between the lipid profiles generated in response to individual TLR agonists, however, the intensities between responses differed. In this chapter of work, the overall lipid profiles generated by the RAW 264.7 ALOX macrophages was more distinct depending on the bacteria, suggesting it is possible to determine signatures of lipids in response to individual bacteria. This finding is supported by work carried out by Hoerr *et al*., 2012, who described how there are increased metabolic changes seen in the serum of mice infected with the Gram-postive bacteria *S. aureus* compared to the Gram-negative bacteria *E. coli*.

At 1 hour, generation of 12/15-LOX-derived 12-HETE was seen to significantly increase in reponse to MRSA stimulation compared to untreated and *E. coli*-treated cells. However, at 24 hours Klebsiella significantly increased 12-HETE compared to untreated, MRSA- and *E. coli*-treated cells. The significant differences seen in the generation of 12-HETE suggest that this lipid is formed in disease, where there may be a time-dependent factor for increases in production upon specific bacterial stimulation. These results represent a potential marker of infection that can not only distingustish between Gram-positive and -negative bacteria but also species within these classes.

The data also showed that generation of 12/15-LOX-derived 12-HETE was seen to increase >4- and 40-fold upon stimulation with MRSA and Klebsiella, respectively, compared to the Gram-positive and -negative TLR agonist treatments shown in Chapter 5. This suggests a possible synergistic effect induced by several pathogenic elements in the presence of whole bacteria (Tan et al. 2014), and emphasises the importance of looking at whole bacterial responses compared to individual

components. When relating whole bacterial responses to TLR agonists it is important to note that, whole bacteria is likely to have considerably higher levels of individual TLR agonists compared to the specific known amounts being delivered to the cells, shown in Chapter 5. It is also the case that multiple TLR agonists will be delivered with whole bacteria, which may interact with eachother in positive and negative way and thus the overall result is the sum of multiple stimulatory pathways.

In general, persistent bacterial infections trigger and sustain locally long-lasting inflammatory responses able to mediate dramatic changes in tissue remodelling (Leone et al. 2016). This sustained response was not seen for 12-HETE in response to MRSA, which was significantly increased at 1 hour and subsequently not detected at 24 hours. This may be due to metabolism of 12-HETE over time, as there was detection of its downstream metabolite LTB_4 at 24 hours. It may also be the case that administration of MRSA significantly increased levels of 12-HETE for longer than 1 hour before returning to baseline levels at 24 hours. To further explore this, a profile of product formation could be carried out over time for detected lipids, as shown by Clark et al. 2011, who examined *in vivo* generation of 5-HETE in a murine model of bacterial peritonitis induced by Gram-positive *Staphylococcus epidermidis* (*S. epidermidis*). It was found that administration of live *S. epidermidis* significantly increased levels of 5-HETE by 3 hours, peaking at 6 hours before returning to baseline at 18 hours. These results show that it could be possible that levels of 12-HETE may have further increased between 1 and 24 hours; additional time course investigations would need to be carried out in order to examine this theory.

When RAW 264.7 ALOX macrophages were exposed to live bacteria, the profiles were seen to be dominated by lipids derived from the LOX pathways. However, in most cases, such as 13-HODE, when looking at the actual amounts generated these data did not show significant differences. The increased products seen with the LOX-pathway do not correlate with the results of Buczynski *et al*. 2007, and those seen in Chapter 5, where it was shown that upon stimulation with TLR agonists the native RAW 264.7 macrophage and the RAW 264.7 ALOX macrophage profiles were

dominated by COX products, namely PGD_2. This again speaks to the importance of using whole bacteria to look at responses rather than individual components.

Upon stimulation with MRSA, PGD_2 was seen to be significantly increased compared to *E. coli* and Klebsiella treatments. Interestingly, levels of PGD_2 detected during exposure to live bacteria were up to 60-fold lower than those seen upon stimulation with individual TLR agonists. This may suggest that synergy between receptors activated in the presence of whole, live bacteria decreases PGD_2 generation. It may also be possible that the potent proinflammatory chemoattractant, PGD_2, has a significantly faster rate of metabolism when initiating an immune response during exposure to live bacteria (Murata & Maehara 2016). This may be because after an initial proinflammatory response is mounted, PGD_2 then undergoes rapid dehydration into PGs such as 15-deoxy-^{12,14}-PGJ_2, which have been suggested to exert anti-inflammatory effects (Shibata et al. 2002; Norris et al. 2011). This hypothesis is only true for Klebsiella-treated cells as they were the only group to show an increase in 15-deoxy-^{12,14}-PGJ_2 at 24 hours compared to PGD_2. However, the quantities of 15-deoxy-^{12,14}-PGJ_2 were very small by comparison with conventional PGs (Bell-Parikh et al. 2003) and therefore may need to be detected using an alternative lipidomic method.

Between the two Gram-negative bacteria, Klebsiella generated increased lipid detection compared to *E. coli*. This is interesting as the Klebsiella strain produces a capsule which one might expect to dampen innate responses due to inhibition of phagocytosis (Tomás et al. 2015). A possible explanation for this may be due to the heterogeneous lipopolysaccharide structures, which possess differences between and within bacterial species, in the length and position of the acyl chains in the lipid A portion of the lipopolysaccharide, the length and polarity of the polysaccharide tail, and the formation of supramolecular structures, (Lüderitz et al. 1984; Brandenburg 1993). The differences detected between the responses of the Gram-negative bacteria suggest that the effect of interaction between the pathogen and the immune cells is not limited to just differences between Gram-positive and -negative bacteria but also incorporates differences between species within that group. These

results are interesting as they are the first steps in determining potential lipid signatures for specific bacterial infections.

6.4.3 Proinflammatory cytokine generation following bacterial stimulation of RAW 264.7 ALOX macrophages

Bacterial stimulation of the cell initiates multiple changes, including gene expression and changes in protein levels (Leone et al. 2016). Cytokine expression is recognised as one of the early events following infection by a variety of pathogens (Kim et al. 2007). It has been reported that cytokines, such as TNF-α, IL-1β and IL-6 are proinflammatory *in vitro* and *in vivo* (Feldmann et al. 1991). As well as looking for changes in eicosanoid generation, the induction of proinflammatory cytokines after a 24 hour treatment with live bacteria was also examined.

The RAW 264.7 ALOX macrophages exposed to Gram-positive MRSA generated significantly increased expression of TNF-α, IL-1β and IL-6 compared to the Gram-negative *E. coli* and Klebsiella treatments. Klebsiella also generated significantly increased levels of IL-6 compared to *E. coli*. These findings suggest that there are differences in host responses to pathogenic microorganisms and virulence mechanisms should be considered in the treatment of patients with bacterial infection (Abe et al. 2010). Overall cell stimulation with *E. coli* resulted in the lowest cytokine generation compared to the other bacteria, which may indicate that *E. coli* has a cytokine inhibitory response.

Levels of TNF-α were shown to be increased compared to TLR agonist exposure in Chapter 5. Upon cell exposure to Gram-positive MRSA, levels of TNF-α detected were ~4-fold more than those generated by the Gram-positive TLR agonists. Gram-negative *E. coli* and Klebsiella generated levels that were similar or $\geq$2-fold greater, respectively, than those induced by the Gram-negative TLR agonists. This finding again suggest it is possible to establish a distinct pattern of response to different species of bacteria. It also further drives the idea of receptor synergy in the presence

of whole, live bacterial, emphasising the importance of using whole bacteria to generate a response (Tan et al. 2014). TNF-α is known to increase during inflammation and infection (Lv et al. 2014). Given that TNF-α generation was much higher than the other proinflammatory cytokines, with a significant difference between the Gram-positive and -negative bacteria, these results could indicate it is a possible marker to differentiate between bacterial infections.

Interestingly, IL-6 generation showed levels of up to a 40-fold decrease when cells were stimulated with live bacteria compared to individual TLR agonists. IL-6 is induced by TNF-α, and appears in the circulation soon after the initial TNF-α response (Alexandraki & Palacio 2010). Therefore, it may be the case that increasing the time of exposure to greater than 24 hours would see an increase in IL-6 levels detected. This is possible as IL-6 has been seen to have a longer half-life than TNF-α and remains elevated in the presence of various diseases (Porter 1997; Alexandraki & Palacio 2010).

IL-1β and TNF-α are two cytokines primarily generated by activated macrophages in response to infection and injury (Dama et al. 1992). In this study, IL-1β was the lowest detected cytokine. Chapter 5 showed that IL-1β was below the limit of detection for the ELISA when cells were exposed to TLR agonists. These finding correlate with those seen by Dama *et al*., 1992, who looked at the cytokine levels of forty critically ill patients with documented infections and found that IL-1β was not detected in any patient serum samples.

6.5 Conclusion

MRSA, *E. coli* and Klebsiella are all important pathogens that can cause potentially lethal systemic infections of mammalian hosts. An important approach to dissect aspects of the infection process has been to characterise the interaction between these bacteria and cultured cells. This study investigated the consequences of co-culture of RAW 264.7 ALOX macrophages with Gram-positive or -negative bacteria in

order to analyse the effects of eicosanoid and cytokines signalling molecules in response to this interaction.

The results here suggest that there are distinctions not only between Gram-positive and -negative bacterial responses but also between species. Further investigation into these initial eicosanoids and cytokines profiles are required to improve and increase our understanding. This will require greater focus on factors such as specific pathogen assault and severity of trauma.

Application of refined methodologies in lipidomics and other similar approaches are crucial to advance the understanding of the role of eicosanoids in health and disease (Dennis & Norris 2015). The immune response is a complex network. A key element in the survival rate in severe cases such as sepsis or bacterial meningitis is early therapy. Work here has initiated an investigation into the cytokine and eicosanoid storm that accompanies classic inflammation to provide a new insight into novel strategies for early detection of bacterial infection and inflammation. The next stage of this work would be to obtain patient samples with known infections to see if there is any correlation between the responses of infected patients and healthy individuals. The ability to now analyse over a hundred eicosanoids and fatty acids, alongside a handful of well-characterised cytokine responses, provides a wealth of possibilities for understanding the immune response to bacterial infections. This could prove to become an extremely valuable clinical tool to distinguish between bacterial infections to better inform physicians and aid with faster appropriate treatment ultimately leading to better clinical outcomes.

Chapter 7

General Discussion

7 General discussion

The work presented in this thesis has aimed to identify pathogen-specific signatures of eicosanoids and cytokines that may represent useful prognostic indicators of bacterial infection. This was done with a view to identifying bacteria-specific bio-signatures which could be used to underpin the development of rapid diagnostics which would allow clinicians to administer the correct antibiotics for patients with bacterial infections at the earliest opportunity, therefore increasing positive patient outcomes and reducing antibiotic resistance.

In order to understand the complex networks of eicosanoid metabolism and signalling at the physiological level, the examination of individual cell contributions is required, which can then be applied to whole tissues. Advanced lipidomics strategies can be used to exploit the cell-specific signalling of eicosanoid pathway enzymes in neutrophils, macrophages and other immune cells. The presence of specific leukocytes is commonly used as an indicator of disease and its severity. Determining lipidomic profiles can potentially be used in addition to cell-specific protein markers such as cytokines to create a more specific read-out of disease. The complexity of eicosanoid biosynthesis is characteristic of the dynamic expression and intracellular compartmentalisation of biosynthetic machinery, which is dependent on time, conditions and cell type (Dennis & Norris 2015). Creating predictive models of eicosanoid and cytokine signalling to bacterial infection can provide faster point of care treatment increasing the chances of positive therapeutic outcomes.

The focus of this research was to employ a targeted lipidomic approach to investigate the profile of 5-LOX lipids from isolated human neutrophils when activated with each individual bacterial TLR agonist. The initial responses examined were those of primary human neutrophils, as they play a central role in the human immune response to infection. Earlier work carried out by Clark *et al*. 2011, identified that human neutrophils generated 5-HETE when exposed to various agonists including TLR1/2 and TLR4. Chapter 3 took this study a stage further by employing the whole array of bacterial TLR agonists to look at the neutrophil responses and establish

whether eicosanoid and cytokine responses varied between TLR components, with a view to determining which play a key role in early inflammatory signalling.

Initial lipidomic methods used in Chapter 3 only looked for the 5-HETE transition, subsequent developments in methods during the course of the project meant that in later chapters (5 and 6) 110 transitions were observed. It was seen that the eicosanoid 5-HETE was generated upon stimulation of primary neutrophils with bacterial TLR agonists. The results indicated that components derived from different bacteria had varying effects on the neutrophil 5-LOX pathway, however, these were not statistically significant. This work also showed that all the TLR agonists induced neutrophils to secrete proinflammatory cytokines, supporting the concept that neutrophils form a link between the innate and adaptive immune systems as cytokines are mediators between various immune cells during an immune response.

This body of work identified many factors that should be taken into consideration when interpreting data generated from isolated cells in an outbred human population. Namely, the diverse variability within the range of responses seen from individual donors following exposure of their neutrophils to TLR agonists. There are multiple factors that may have contributed to these innate immune differences seen across the host responses during treatment, including age, gender, diet, exercise, smoking status, any underlying comorbidities, circadian rhythm, stress and ethnicity. For example, in humans, one of the systems that responds to challenging circumstances is the immune system and several facets have been empirically associated with stress (Morey et al. 2016). During times of acute and chronic stress increases in blood levels of proinflammatory cytokines have been detected (Steptoe et al. 2007; Gouin et al. 2012). To try and overcome this, early morning blood draws were taken from donors as this was thought to be the least stressful time of day. However, this does not account for any underlying stresses they may be facing. Another study exploring the interaction of the host and pathogen factors on the innate immune response determined that both race/ethnicity and pathogen factors influenced the innate immune response (Nahid et al. 2018). This fuels the need to have access to donor characteristics, which may help explain the varied responses by

categorising the population used. Data presented in this thesis were obtained using a donor group that consisted of 20 volunteers. The retrospective power calculation for this study suggested that 724 donors would be needed to achieve a power of 80% to declare that the conditions have significantly different means. With such a limited group of donors used in this study, it is also not possible to speculate whether any outliers within the donor responses are in fact extreme outliers or are a genuine member of the population. This suggests that, as the donor cohort was extremely small, a much larger data set would need to be carried out to allow for the diverse variation between donors in a population and enable more significant comparisons between responses.

Indeed, it is this diversity and the wide range of "normal" responses generated by the human race that represents the principal challenge when seeking to identify a specific response indicative of bacterial infection. Therefore, a limitation of this study was the small, undefined donor population cohort and a much larger pool of donors would need to be analysed to allow for the variability seen within a population. It is recognised that amendments to the ethics of the study would be beneficial to allow for collection of factors such as ethnicity, age and gender from donors and permission for these to be disclosed to enable them to be related to the responses seen. This is an important point as the human race is outbred and thus it would be expected to observe a wide range of responses. The challenge in terms of predicting infection is determining a 'normal' range.

Primary scientific research with neutrophils presents a number of challenges due to their short half-life, tendency to become activated, and also their predisposition to spontaneously apoptose. This is coupled with donor variability in the number of neutrophils collected during a single blood draw; sometimes leading to inadequate amounts and therefore terminating subsequent experiments. All of these factors make conducting experiments to investigate the innate immune cell responses to infection challenging. As previously mentioned, however, their involvement in early inflammation and infection does make any advance in the understanding of their function very valuable, regardless of the magnitude.

In light of the issues surrounding the use of primary neutrophils, coupled with the need to markedly increase the donor cohort to account for the natural variation within the population, initial experiments lead to the employment of an immortalised cell line to characterise the responses to individual TLR agonists. Whilst going from primary human cells to an immortalised cell line is counterintuitive, it provided a more robust, reproducible model to look at the initial responses generated as they present with a homogenous genetic background, therefore eliminating this confounding factor seen using primary cells to gain an initial readout of responses to infection. It must be noted that cell lines may display fundamental differences compared to primary cells. This is due to their continuous growth in culture resulting in permanent alterations in their genes, which may have an effect on the signalling cascade (Hartley et al. 2008). However, the positives in this case outweigh the negatives, not only providing a better model, but also allowing faster establishment and optimisation for protocols that require large numbers of cells.

As mentioned in Chapter 4, a possible model system for neutrophils is the human leukaemia cell line HL-60, which can be differentiated into neutrophil cell lines. However, conflicting data suggest that these differentiated cells are limited in their capacity to respond to stimuli such as formylated peptides, which also activate neutrophils, as seen in Chapter 3. For example, one study showed that fMLP has no effect on differentiated HL-60 cells in chemokinesis assays (Meyer & Howard 1987). Whilst another study found that fMLP induced chemotaxis in differentiated HL-60 cells, but in much smaller numbers compared to neutrophils (Fontana et al. 1980). Therefore, the initial cell line used was the human THP-1 monocyte cell line. This myeloid cell line is the most widely employed to investigate the function and expression of primary human macrophages during disease (Lund et al. 2016). These cells were chosen as they could be differentiated into monocyte-derived macrophages, which work alongside neutrophils as first responders to infection, and are accepted as a model to study immune response because they closely resemble those of primary human macrophages. Alongside this, these cells were also reported to produce inflammatory markers in response to infectious and non-infectious disease.

As outlined in Chapter 1, macrophages express a large number of distinct TLRs, which when stimulated activates an eicosanoid response by distinctly different pathways. By employing an array of bacterial stimuli, it was anticipated that the macrophages would generate a number of different eicosanoid profiles in response to the TLR agonists with a view that they could indicate the presence of agonist-specific responses. Unlike using primary cells, seen in Chapter 3, this model eliminated the variability in donor responses due to the genetic background of the host and also enabled the use of a larger quantity of cells.

Despite THP-1 cells being a widely accepted model for observing macrophage functions and responses to external stimulation *in vitro*, the first hurdle faced here was that there was no current standardised protocol for the differentiation of THP-1 monocytes to macrophages using PMA. Through optimisation, cell differentiation was established and characterised through cell adhesion, however, the lack of a standardised protocol for differentiation would make it difficult to compare results between studies.

The results in this chapter of work (Chapter 4) went on to show that PMA differentiated THP-1 monocyte-derived macrophages did not generate biologically relevant amounts of 15-HETE through the 15-LOX-enzyme pathway when exposed to the positive control calcium ionophore A23187. The anticipated quantity of lipid generated by calcium ionophore A23187 would be similar to that shown by Morgan *et al.,* 2009, who demonstrated the formation of 12-HETE by murine macrophages in response to 10 µM A23187 to reach 12 ng/10^6 cells after 15 minutes stimulation. Calcium ionophore A23187 stimulus is routinely used in experimental conditions as a positive control to assess the maximal quantities of eicosanoids released from white blood cells, which is why it was selected to establish the overall capacity of THP-1 macrophages to release 15-HETE. However, the data did show calcium ionophore A23187 and LPS induced generation of proinflammatory cytokine TNF-α, IL-1β, IL-8 and IL-6. This confirmed that the cells had been activated, although the absence of 15-HETE generation by these cells supported the idea they were not a suitable macrophage model to carry forward and further explore eicosanoid responses to bacterial infection.

The use of a cell line was still considered advantageous over primary cells predominantly because they had a homogenous genetic background which minimises the degree of variability that can be seen in the cell phenotype, and they could also be generated in large quantities over a shorter period of time. For these reasons, an alternative macrophage cell line derived from mice was employed, RAW 264.7. These cells represent an immortalized phenotype derived from peritoneal tumours induced in BALB/c male mice by the Abelson murine leukaemia virus (Norris & Dennis 2014). As mentioned in Chapter 5, the specific cells used had been developed by Professor Valerie O'Donnell's Lipidomics Group at Cardiff University and were genetically engineered with the 12/15-LOX enzyme as native RAW 264.7 macrophages do not possess the relevant enzymes to make these 12/15-LOX lipids. This cell line, known as RAW 264.7 ALOX macrophages, had been extensively characterised and were known to generate eicosanoids in response to stimuli and therefore represented a suitable model capable of generating 12/15-LOX-derived lipids to establish initial responses to TLR agonists and subsequently determine the profiles in response to whole, live bacteria.

Work in this thesis showed the first ever measure of both lipids and cytokines generated from the same 12/15-LOX transfected RAW 264.7 murine macrophages exposed to an array of bacterial TLR agonists and live, pathogenic bacteria.

Initially as a control cell type, RAW 264.7 Control macrophages were employed alongside the ALOX macrophages. This cell line had been engineered with an empty vector to determine if the vector alone played a part in the responses of these cells. Studies carried out by Buczynski *et al*., showed that when native RAW 264.7 macrophages were stimulated with 16 different agonists, including TLR agonists, no significant differences were seen in the COX metabolite profiles. It was established that the RAW 264.7 Control cells behaved in the same way that Buczynski and colleagues demonstrated with the original RAW 264.7 cells when comparing their responses to TLR agonist exposure. Therefore, the RAW 264.7 Control cells are the same as the native RAW 264.7 cells.

As described in Chapter 5, the first part of the work carried out assessed the basal eicosanoid profile in RAW 264.7 Control and ALOX macrophages. As this is a murine cell line, 12-HETE was the lipid isoform generated by the 12/15-LOX enzyme and was used to measure its activity. Results showed that the RAW 264.7 Control macrophages generated basal levels of COX-derived lipids without the presence of any 12/15-LOX products. The profile of basal lipids detected in the RAW 264.7 Control macrophages was also demonstrated in the native RAW cells by Buczynski *et al*. 2007. This confirmed that the insertion of the empty vector into the cells did not alter the lipid signalling and therefore the 12/15-LOX-derived lipids could be attributed to the expression of the 12/15-LOX enzyme.

Subsequent analysis of basal lipid detection from RAW 264.7 ALOX macrophages showed that these cells significantly upregulated the generation of 12/15-LOX-derived lipids compared to the RAW 264.7 Control macrophages, where no 12/15-LOX-derived lipids were detected. There was also no significant difference seen in the detection of COX-derived lipid PGD_2. These results were important as they confirmed that the transfected RAW 264.7 Control macrophages with the empty vector and the RAW 264.7 ALOX macrophages carrying the 12/15-LOX gene remain unaltered in terms of their innate lipid generation, as they behave the same as the standard RAW 264.7 cell line.

Another control measure for the transfected cells was to compare the generation of proinflammatory cytokines, in response to TLR agonists and to whole bacteria, between the RAW 264.7 Control and ALOX macrophages. It was predicted that the profiles generated by the two cell lines would be comparable as the transfection shouldn't alter the cytokine production. Interestingly, data presented in Chapter 5 showed that upon TLR agonist exposure the RAW 264.7 Control macrophages generated cytokine levels that were between 50-100-fold less than those seen by the RAW 264.7 ALOX macrophages. It was speculated that a possible reason for this difference between the two cell lines may be that the LOX products generated by the RAW 264.7 ALOX cells could upregulate cytokine production compared to the RAW 264.7 Control cells. However, a study by Berghaus et al. 2010, showed that original RAW 264.7 macrophages treated with TLR1/2 and TLR4 agonists generated cytokine

levels closer to values seen by the RAW 264.7 ALOX cells when treated with the same agonists. As the RAW 264.7 ALOX macrophages were closer in respones to the native RAW 264.7 cells and they had been seen to generate 12/15-LOX-derived lipids, it was justified that the RAW 264.7 ALOX macrophages could be used for all subsequent experiments to provide a suitable model similar to *in vivo* macrophages. Nevertheless, it is still noted that this set of data is an *n* of 1 and would need to be repeated in order to make further conclusions.

Chapter 6 subsequently provided evidence that the cytokines generated by the RAW 264.7 Control and ALOX cell types in response to whole bacteria did show a similar response to each other. The only significant difference between the two cell types was seen in TNF-α generation, upon treatment with MRSA. However, as these were such high levels compared to all other cytokine generation, and that both cell types gave similar levels of response for all of the other test conditions, it was again justified to employ the RAW 264.7 ALOX macrophages to determine cell responses to whole bacteria.

Advanced methodologies developed within Professor Valerie O'Donnell's Lipidomics Group enabled the application of a specifically designed method using a targeted lipidomics approach to analyse 110 eicosanoid and fatty acid transitions. Sample analysis saw 39 detectable analytes from the RAW 264.7 ALOX macrophages, and for the most part, the same eicosanoid profile was generated for the 6 bacterial agonists tested. The profiles seen were dominated by COX products, with PGD_2 seen in the greatest amount, which was consistent with that demonstrated by Buczynski *et al*. 2007. In the work presented in Chapter 6, the concentrations of TLR agonists used were based on the amounts seen to be used throughout other studies that were still within the optimal range indicated on the InvivoGen TLR agonist kit used (Buczynski et al. 2007; Clark et al. 2011; InvivoGen, Toulouse, France). It may be the case that carrying out a dose response for each TLR agonist, using a variety of concentrations within the appointed range to specifically identify the optimum concentration, e.g. the EC50, could prove to generate more comparative responses between cells.

Using this novel RAW 264.7 transfected cell line enabled me to generate an initial profile of mediators expressed when cells were exposed to different bacterial TLR agonists. However, no major differences were seen in the lipid and cytokine generation within these data sets when the RAW 264.7 ALOX cells were exposed to individual TLR agonists. Looking at the overall cell responses generated in response to the TLR agonists on the heatmap, it could be observed that the Gram-positive agonists TLR2 HKLM and TLR6/2 FSL displayed a higher abundance (change in the heatmap colour of intensity) of lipids compared to the other TLR agonists. However, when looking at the actual amounts generated these data didn't appear to be suggestive of any substantial differences between the agonists.

Other *in vivo* studies have demonstrated that macrophages yield different eicosanoid profiles during disease (Trebino et al. 2005; Wang et al. 2006; Ragolia et al. 2005). However, many of these profiles are determined looking at non-infectious diseases, therefore, it could be plausible that there aren't distinct patterns generated during specific infectious insult. Notably, there are differences between the cells used in these studies such as the enviroment in which the *in vivo* macrophages differentiate is different to that of the RAW 264.7 cells, also the eicosanoid profile generated between human and mouse models are not directly comparable.

It is also important to remember that TLR agonists represent only one bacterial component and in reality multiple factors will interlink in the presence of whole bacteria. This work showed that cell reponses to TLR agonists were alike and therefore it was not possible to distinguish between responses in this way.

Alongside looking at eicosanoid release upon TLR agonist stimulation of RAW 264.7 ALOX macrohages, the induction of proinflammatory cytokines was also examined. As cytokines have been extensively studied and characterised it was decided to select three of the most dominate cytokines during inflammation to look at in parallel to the eicosnaoind profile after 24 hour TLR agonist treatment of RAW 264.7 ALOX macrophages. Findings here showed that again there were no major differences seen in cytokine generation when the RAW 264.7 ALOX macrophages were exposed to individual TLR agonists. It may be possible that a longer time course of exposure is needed in order to see differences in cytokine generation between agonists or

combination treatment of several agonists at once is needed. However, experiments would need to be repeated in order to draw any further conclusions.

Using the novel RAW 264.7 transfected cell line enabled the generation of an initial profile of mediator expression when cells were exposed to individual bacterial TLR components. Data indicated that it was not possible to identify profiles for individual TLR agonists. Another step to continue this work and further characterise the TLR agonists responses would be to expose the cells to combinations of the agonists, as during an infection multiple TLR agonists would be present at once. In fact, the presence of the whole bacteria during an infection would trigger a plethora of receptor-mediated responses in immune cells, as detailed in Chapter 1. To carry this investigation forward, it was decided that the natural next step was to expose the RAW 264.7 ALOX macrophages to whole, live bacteria, inevitably creating more pathophysiologically relevant conditions.

Chapter 6 employed three highly pathogenic, clinically relevant strains of bacteria to determine if there are differences seen in the eicosanoid and cytokine signalling between species during infection. For a first look at these signalling profiles, it was important to pick multi-drug resistant and extremely virulent species in order to induce an immune response during a worst-case clinical scenario. The bacteria used were Gram-positive bacteria MRSA and Gram-negative bacteria *E. coli* and Klebsiella. Chapter 1 explains the differences within the cell wall that distinguish between the Gram-positive and -negative bacteria and how some of the unique components within the cell wall act as PAMPs that are recognised by receptors and activate the immune cells.

Results showed that RAW 264.7 ALOX macrophages generated significantly different lipid and cytokine profiles when co-cultured with Gram-positive and Gram-negative bacteria. Overall, heatmap analysis showed Gram-positive MRSA had increased intracellular levels of lipids at 24 hour exposure compared to the Gram-negative bacteria *E. coli* and Klebsiella. These finding are interesting as they challenge data that suggest Gram-negative infections induce a greater inflammatory response

compared to Gram-positive infections (Abe et al. 2010). However, they do correlate with Chapter 5, which showed the Gram-positive TLR2 and TLR6/2 agonists, which are ligands present on MRSA, also had an increased level of lipids compared to the other TLRs. The possible reasons for the differences seen between the responses to the bacteria are discussed in more detail in Chapter 6.

Chapter 5 also saw the lipid profiles generated upon cell treatment with the TLR agonists to be uniform in their pattern of response, differing only in their intensities. However, the profiles generated in response to cell stimulation with whole bacteria showed the profiles to be more distinct depending on the species used, indicating that the presence of whole bacteria may reveal defined lipid signatures. Again, this reinforces the point that a bacteria is the sum of its TLR agonists and other immune-stimulatory factors.

Data showed that MRSA had higher levels of lipids, some of which were significantly increased, after 1 hour exposure to the cells compared to the Gram-negative bacteria. For example, the 12/15-LOX-derived lipid, 12-HETE, was significantly increased with MRSA after one hour compared to *E. coli*-treated cells. However, Klebsiella-treated cells showed 12-HETE to be significantly increased at 24 hours bacterial exposure to the cells compared to MRSA and *E. coli*. The differences seen here represent a potential marker of infection that can not only distinguish between Gram-positive and -negative bacteria but also species within these classes. They also indicate that there may be a time-dependant factor for increases in production upon specific bacterial stimulation. Therefore, further studies could benefit from a longer time course of activation with several timepoint intervals in between. This would allow for a greater understanding into the generation and metabolism of the lipids during an infectious insult. It is also important to note that during an immune response cells are called to the sight of infection and generate signalling molecules. During this time cells are constantly replenished, sustaining the response, which is not the case in an *in vitro* model. Another point to note is that taking measurements at the earlier time points, such as 1 hour, are not feasible in a diseased state setting as the likelihood of recognising an infection at this time is unrealistic. It is however,

important to use such time points to gain an initial understanding of early signalling and how it changes over time.

The importance of using whole bacteria was highlighted when comparing their generation of lipids and cytokines to that produced by TLR agonist stimulation. Data showed that 12-HETE, for example, was seen to increase >4- and 40-fold upon treatment with MRSA and Klebsiella, respectively, compared to TLR agonist exposure. Data also showed that TNF-α levels increased between 2-4-fold upon bacterial stimulation compared to the Gram-positive and -negative TLR agonists treatments. This is suggestive of a possible synergistic effect induced by several pathogenic elements in the presence of whole bacteria.

Equally, the use of whole bacteria showed that upon stimulation with MRSA the COX-derived lipid PGD_2 was seen to be significantly increased compared to exposure to *E. coli* and Klebsiella treatments. Interestingly, levels of PGD_2 detected during exposure to live bacteria were up to 60-fold lower than those seen upon stimulation with individual TLR agonists. This was also the case for the cytokine IL-6 which showed levels to be ~100-fold lower in bacteria-treated cells compared to the TLR agonists. These findings suggest that interactions between receptors activated in the presence of whole, live bacteria can lead to faster metabolism of mediators during an infectious insult. For examples, as suggested in Chapter 6, it may also be possible that the potent proinflammatory chemoattractant, PGD_2, has a significantly faster rate of metabolism when initiating an immune response during exposure to live bacteria (Murata & Maehara 2016). This could be because PGD_2 undergoes rapid dehydration into other PGs after an initial proinflammatory response is mounted. For IL-6, it may be that the time of exposure would need to be greater than 24 hours in order to see increased detection of IL-6 levels. This is because IL-6 is induced by TNF, and appears in circulation after the initial TNF response (Alexandraki & Palacio 2010). These data emphasise the significance of bacterial co-culture work compared to individual component analysis.

Chapter 1 describes how eicosanoids are produced rapidly after the initiation of inflammation. They are not typically stored within cells but rather synthesised as

required and released by the cell by simple diffusion, driven by pH and the membrane potential to act in an autocrine or paracrine manor (Schuster 2002). Although considerable experimental effort has been expanded towards understanding mechanisms of eicosanoid synthesis and signalling, little attention has been paid to the mechanisms by which they traverse biological membranes (Schuster 2002). The data presented in this thesis show that upon examination, eicosanoid levels inside and outside the cell varied. Data showed elevated intracellular eicosanoid levels compared to the extracellular environment. For example, 12-HETE was only detected intracellularly at 1 hour and, surprisingly, not detected extracellularly in response to TLR agonist stimulation. It was also seen that 12-HETE was significantly increased intracellularly at 1 hour for MRSA, and 24 hours for *E. coli* and Klebsiella, compared to extracellular levels. It may be the case that the engineered RAW 264.7 ALOX macrophages retain these lipids inside the cell as they are not part of their normal processing and therefore lack the dedicated export pathways. This must be considered when assessing the data using this model as this would have an impact on the method used to assess inflammatory mediator profiles, be it with isolation of cells or more realistically in a patient setting from whole blood or plasma. Therefore, now that the infection model is established, and it is apparent that there are differences in profiles generated, the next step would be to employ primary human macrophages which should be able to release lipids and analyse their responses to whole bacteria.

Work presented in Chapter 6 used refined methodologies to investigate the effect of co-culture of RAW 264.7 ALOX macrophages with highly pathogenic Gram-positive or -negative bacteria in order to analyse the effects of eicosanoid and cytokine signalling molecules in response to this interaction. Work here has taken the first step into investigating the eicosanoid and cytokine surge that accompanies classic inflammation to provide a new insight into novel strategies for early detection of bacterial infection and inflammation. It was apparent that the application of whole bacteria give differing mediator profiles not only between Gram-positive and -negative bacteria but also between species within these classes and this warrants further studies.

7.1 Future work

There are several lines of future research which could follow from the findings reported in this thesis.

The buffy coat is the fraction of an anticoagulated blood sample that contains most of the white blood cells and platelets following density gradient centrifugation. Using buffy coat is a means of obtaining human donors which can be used to isolate and culture primary human macrophages. Now that an established model of infection has been designed and implemented it can be applied to human macrophages to analyse the eicosanoid and cytokine profiles. The advantages of using buffy coats is that a large number of cells can be attained rapidly for use without the difficulty of recruiting individual donors. Using human cells also has the advantage of the data being more relatable to the human system and mechanisms of disease. Notably, the cells would have not been from a defined population and would not be genetically identical. Also, donor information would not be available when employing buffy coats from a bank and therefore may elude to similar issues raised when using primary human neutrophils in Chapter 3.

The RAW 264.7 ALOX macrophages are an established cell line now confirmed to generate distinct profiles of signalling molecules under induced infection. Now that cell-specific signalling has been determined, a novel approach could be to use a mouse model to analyse the profiles generated in a whole system. This would involve using a wild type mouse, which inherently has the 12/15-LOX enzyme, and inducing bacterial infection. A 12/15-LOX knock-out mouse could be generated as a control. Employing a mouse model could shed light on the cell interactions of signalling during infection. Having the interaction of a whole system may cause the macrophages to release the lipids they generate into the extracellular environment as opposed to being kept within the cells, as seen with the RAW 264.7 ALOX cells. An advantage of using a mouse model where the whole system is involved will mean that responses can be sustained as cells would be continuously generating mediators in response to infection. This would allow for assessment of profiles over longer periods of time,

which is important as infectious agents may be present in the body for several days before symptoms of infection become apparent. Therefore, findings could ultimately allow for early detection and treatment of bacterial infections.

Finally, the data in this thesis have provided evidence to show that profiles of eicosanoids and cytokines vary in response to infection. An additional step that could be looked at is using whole blood samples taken from patients with confirmed infection. Here, patient samples would be drawn, and cells could be isolated and/or whole blood used to analyse the signalling during a known infection. However, initial experiments would have to be run to make sure it is possible to isolate and analyse the required products using whole blood. With the appropriate ethics it may also be possible to gain access to patient records and correlate information (such as age, gender, comorbidities, ethnicity) to profiles seen. A disadvantage to using patient samples is that they may present with multiple comorbidities or infections at once. This would make it difficult to distinguish between individual profiles. In which case, the use of buffy coats would be more appealing as a first step and could be used as a model to look at infection with multiple bacteria at once. The use of patient samples also means that a healthy population of donors would need to be established. As previously mentioned it is the diversity and the broad range of 'normal' responses produced by the outbred human race that represents the fundamental challenge when looking to identify a specific response indicative of infectious disease, as demonstrated by the aforementioned power calculation in Chapter 3.

7.2 Conclusion

The overall effects of eicosanoid and cytokine storms on host responses and survival are likely to be context-dependent, and further improvements in our understanding will require greater focus on factors such as age, diet, genetic variation and comorbidities, in addition to the specific pathogenic assault and severity of trauma. The refined application of lipidomics methodologies and similar approaches will be crucial to advance our understanding of the role of eicosanoids in health and disease. Work in this thesis has identified potential markers of bacterial infection, such as 12-HETE, 14-HDOHE and TNF-α, which, along with future advances, could be used to develop novel strategies for clinicians, nurses and primary care staff to analyse patients suspected of bacterial infection at the bedside. Work here provides an insight of how the eicosanoid and cytokine storms are generated alongside each other to accompany classic inflammation during specific bacterial infection. The ability to distinguish between species of bacteria causing infection could prove invaluable, reducing the time taken to establish the cause of infection, ultimately leading to better patient outcomes.

8 References

Abe, R. et al., 2010. Gram-negative bacteremia induces greater magnitude of inflammatory response than Gram-positive bacteremia. *Critical Care*, 14(2).

Ahmad-Nejad, P. et al., 2002. Bacterial CpG-DNA and lipopolysaccharides activate toll-like receptors at distinct cellular compartments. *European Journal of Immunology*, 32(7), pp.1958–1968.

Akira, S., Uematsu, S. & Takeuchi, O., 2006. Pathogen recognition and innate immunity. *Cell*, 124(4), pp.783–801.

Alanne-Kinnunen, M. et al., 2014. Activated Human Mast Cells Induce LOX-1-Specific Scavenger Receptor Expression in Human Monocyte-Derived Macrophages. *PLoS ONE*, 9(9), p.e108352.

Aldo, P.B. et al., 2012. Effect of culture conditions on the phenotype of THP-1. *Am J Reprod Immunol*, 29(6), pp.997–1003.

Alexandraki, I. & Palacio, C., 2010. Gram-negative versus Gram-positive bacteremia: What is more alarmin(g)? *Critical Care*, 14(3).

Aly, S. et al., 2011. Nisin quantification by ELISA allows the modeling of its apparent diffusion coefficient in model cheeses. *Journal of Agricultural and Food Chemistry*, 59(17), pp.9484–9490.

Amsen, D., Visser, K.E. De & Town, T., 2009. Approaches to Determine Expression of Inflammatory Cytokines. *Methods of molecular biology*, 512, pp.1–27.

Anderson, K. V., Jürgens, G. & Nüsslein-Volhard, C., 1985. Establishment of dorsal-ventral polarity in the Drosophila embryo: Genetic studies on the role of the Toll gene product. *Cell*, 42(3), pp.779–789.

Anderstam, Bj., Lindholm, B. & Stenvinkel, P., 2012. CYTOKINES. In *Uremic Toxins*. pp. 259–277.

Apostolaki, M. et al., 2010. Cellular mechanisms of TNF function in models of inflammation and autoimmunity. *Curr Dir Autoimmun*, 11, pp.1–26.

Applequist, S.E., Wallin, R.P. a & Ljunggren, H.-G., 2002. Variable expression of Toll-like receptor in murine innate and adaptive immune cell lines. *International immunology*, 14(9), pp.1065–1074.

Asfaha, S. et al., 2013. Mice that express human interleukin-8 have increased mobilization of immature myeloid cells, which exacerbates inflammation and accelerates colon carcinogenesis. *Gastroenterology*, 144(1), pp.155–166.

Bäck, M., 2007. Leukotriene receptors: crucial components in vascular inflammation. *TheScientificWorldJournal*, 7, pp.1422–39.

Bal, A.M. et al., 2017. Future trends in the treatment of methicillin-resistant Staphylococcus aureus (MRSA) infection: An in-depth review of newer antibiotics active against an enduring pathogen. *Journal of Global Antimicrobial Resistance*, 10, pp.295–303.

Balsinde, J. et al., 1995. Inhibition of calcium-independent phospholipase A2 prevents arachidonic acid incorporation and phospholipid remodeling in P388D1 macrophages. *Proceedings of the National Academy of Sciences of the United States of America*, 92(18), pp.8527–31.

Bauer, S., 2008. Toll-Like Receptors (TLRs) and Innate Immunity. *SABiociences*, 183, pp.2–5.

Beceiro, A., Tomás, M. & Bou, G., 2013. Antimicrobial resistance and virulence: A successful or deleterious association in the bacterial world? *Clinical Microbiology Reviews*, 26(2), pp.185–230.

Bell-Parikh, L.C. et al., 2003. Biosynthesis of 15-deoxy-12,14- PGJ2 and the ligation of PPARγ. *Journal of Clinical Investigation*, 112(6), pp.945–955.

Berghaus, L.J. et al., 2010. Innate immune responses of primary murine macrophage-lineage cells and RAW 264.7 cells to ligands of Toll-like receptors 2, 3, and 4. *Comparative Immunology, Microbiology and Infectious Diseases*, 33(5), pp.443–454.

Beutler, B. et al., 2006. GENETIC ANALYSIS OF HOST RESISTANCE: Toll-Like Receptor Signaling and Immunity at Large. *Annual Review of Immunology*, 24(1), pp.353–389.

Bhan, U. et al., 2007. TLR9 Is Required for Protective Innate Immunity in Gram-Negative Bacterial Pneumonia: Role of Dendritic Cells. *The Journal of Immunology*, 179(6), pp.3937–3946.

Bone, R. et al., 1992a. ACCP/SCCM consensus conference Definitions for Sepsis and Organ Failure and Guidlines for the use of Innovative Therapies in Sepsis. *Chest*,

101(6), pp.1644–1655.

Bone, R. et al., 1992b. accplsccm consensus conference for Sepsis and Organ Failure and. *Chest*, 101, pp.1644–1655.

Borregaard, N., Sørensen, O.E. & Theilgaard-Mönch, K., 2007. Neutrophil granules: a library of innate immunity proteins. *Trends in Immunology*, 28(8), pp.340–345.

Brancato, S.K. & Albina, J.E., 2011. Wound macrophages as key regulators of repair: Origin, phenotype, and function. *American Journal of Pathology*, 178(1), pp.19–25.

Brandenburg, K., 1993. Fourier transform infrared spectroscopy characterization of the lamellar and nonlamellar structures of free lipid A and Re lipopolysaccharides from Salmonella minnesota and Escherichia coil. *Biophys. J.*, 64, pp.1215–1231.

Brink, C., 2007. Structural manipulation of eicosanoid receptors and cellular signalling. J. The scientific world. , 7, pp.1285–1306.

Brouwer, M.C., Tunkel, A.R. & Van De Beek, D., 2010. Epidemiology, diagnosis, and antimicrobial treatment of acute bacterial meningitis. *Clinical Microbiology Reviews*, 23(3), pp.467–492.

Buczynski, M.W. et al., 2007. TLR-4 and sustained calcium agonists synergistically produce eicosanoids independent of protein synthesis in RAW264.7 cells. *Journal of Biological Chemistry*, 282(31), pp.22834–22847.

Calafat, J. et al., 1997. Human Monocytes and Neutrophils Store Transforming Growth Factor-a in a Subpopulation of Cytoplasmic Granules. *Blood*, 90(3), pp.1255–1266.

Calfee, C.S. & Matthay, M.A., 2010. Culprits with evolutionary ties. , 464(March).

Cannon, J.G. et al., 1990. Circulating Interleukin-l and Tumor Necrosis Factor in Septic Shock and Experimental Endotoxin Fever. *The Journal of infectious diseases*, 161, pp.79–84.

Cantwell, R. et al., 2011. Saving Mothers' Lives: Reviewing maternal deaths to make motherhood safer: 2006-2008. The Eighth Report of the Confidential Enquiries into Maternal Deaths in the United Kingdom. *Bjog*, 118(1), pp.1–203.

Cassell, G.H. & Mekalanos, J., 2007. Development of Antimicrobial Agents in the Era of New and Reemerging Infectious Diseases and Increasing Antibiotic Resistance

Magnitude of the Problem. *RESEARCH OPPORTUNITIES FOR SPECIFIC DISEASES AND DISORDERS*, 285(5), pp.1–17.

Cavenagh, J., 2007. White blood cells. *Surgery (Oxford)*, 25(2), pp.61–64.

Chamberlain, L.M. et al., 2009. Phenotypic non-equivalence of murine (monocyte-) macrophage cells in biomaterial and inflammatory models. *Journal of Biomedical Materials Research - Part A*, 88(4), pp.858–871.

Chang, Y.W. et al., 2005. Differential regulation of cyclooxygenase 2 expression by small GTPases Ras, Rac1, and RhoA. *J Cell Biochem*, 96(2), pp.314–329.

Chanput, W. et al., 2010. Transcription profiles of LPS-stimulated THP-1 monocytes and macrophages : a tool to study inflammation modulating effects of food-derived compounds. *Food function, The royal society of chemistry*, 1, pp.254–261.

Chitra S, N, G. & T.S, L., 2014. Comparison of Differentiation To Macrophages in Isolated Monocytes From Human Peripheral Blood and Thp1 Cells. *Sri Ramachandra Journal of Medicine*, 7(1), pp.4–9.

Cho, I. & Blaser, M.J., 2012. The human microbiome: At the interface of health and disease. *Nature Reviews Genetics*, 13(4), pp.260–270.

Choi, Y. et al., 2013. PCR-reverse blot hybridization assay for screening and identification of pathogens in sepsis. *Journal of clinical microbiology*, 51(5), pp.1451–7.

Clark, S.R. et al., 2011. Esterified eicosanoids are acutely generated by 5-lipoxygenase in primary human neutrophils and in human and murine infection. *Blood*, 117(6), pp.2033–43.

Clark, S.R. et al., 2007. Platelet TLR4 activates neutrophil extracellular traps to ensnare bacteria in septic blood. *Nature medicine*, 13(4), pp.463–9.

Claus, D., 1992. A standardized Gram staining procedure. *World Journal of Microbiology & Biotechnology*, 8(4), pp.451–452.

Cogen, A.L., Nizet, V. & Gallo, R.L., 2008. Skin microbiota: A source of disease or defence? *British Journal of Dermatology*, 158(3), pp.442–455.

Cohen, J., 2002. The immunopathogenesis of sepsis. *Nature*, 420(6917), pp.885–891.

Comporti, M. et al., 2008. Isoprostanes and hepatic fibrosis. *Molecular Aspects of Medicine*, 29(1–2), pp.43–49.

Connon, C.J., Young, R.D. & Kidd, E.J., 2003. P2X7 receptors are redistributed on human monocytes after pore formation in response to prolonged agonist exposure. *Pharmacology*, 67(3), pp.163–168.

Cook, J.A., 2005. Eicosanoids. *Critical Care Medicine*, 33(Suppl), pp.S488–S491.

Croxen, M.A. & Finlay, B.B., 2010. Molecular mechanisms of Escherichia coli pathogenicity. *Nature Reviews Microbiology*, 8(1), pp.26–38.

Daigneault, M. et al., 2010. The identification of markers of macrophage differentiation in PMA-stimulated THP-1 cells and monocyte-derived macrophages. *PLoS ONE*, 5(1).

Dama, P. et al., 1992. Cytokine Serum Level During Severe Sepsis in Human IL-6 as a Marker of Severity. *Annals of Surgery*, 215(4), pp.356–362.

Daniels, R., 2011. Surviving the first hours in sepsis: getting the basics right (an intensivist's perspective). *The Journal of antimicrobial chemotherapy*, 66 Suppl 2, pp.ii11-23.

Daniels, R., 2009. The incidence, mortality and eiconomic burden of sepsis. In: NHS Evidence emergency and urgent care. *NHS Library, Central alerting system*.

Danielsson, K.N. et al., 2008. 15-Lipoxygenase-2 expression in human macrophages induces chemokine secretion and T cell migration. *Atherosclerosis*, 199(1), pp.34–40.

Das, U.N., 2011. Infection, inflammation, and polyunsaturated fatty acids. *Nutrition*, 27(10), pp.1080–1084.

Dellinger, R.P. et al., 2013. Surviving Sepsis Campaign: International Guidlines for Management of Severe Sepsis and Septic Shock: 2012. *Critical Care Medicine*, 41(2), pp.580–637.

Delves, P.J. & Roitt, I.M., 2000. First of Two Parts THREE LEVELS OF DEFENSE. *Advances in immunology*, 343(1), pp.37–50.

Dennis, E.A. & Norris, P.C., 2015. Eicosanoid storm in infection and inflammation. *Nature Reviews Immunology*, 15(8), pp.511–523.

Dhand, N.. & Khatkar, M.., 2014. Statulator: An online statistical calulator. Sample Size Calculator for Comparing Two Independent Means. Available at: http://statulator.com/SampleSize/ss2M.html.

Divisio, M.A.I., 1998. N-formyl-methionyl-leucyl-phenylalanine (fMLP) inhibits

tumour necrosis factor- alpha (TNF-a) production on lipopolysaccharide (LPS) -stimulated human neutrophils. *Methods*, pp.39–47.

Doorduijn, D.J. et al., 2016. Complement resistance mechanisms of Klebsiella pneumoniae. *Immunobiology*, 221(10), pp.1102–1109.

Dorward, D.A. et al., 2015. The Role of Formylated Peptides and Formyl Peptide Receptor 1 in Governing Neutrophil Function during Acute In fl ammation. *The American Journal of Pathology*, 185(5), pp.1172–1184.

Drifte, G. et al., 2013. Innate immune functions of immature neutrophils in patients with sepsis and severe systemic inflammatory response syndrome. *Critical care medicine*, 41(3), pp.820–32.

Drozdovszky, O., Barta, I. & Antus, B., 2014. Sputum eicosanoid profiling in exacerbations of chronic obstructive pulmonary disease. *Respiration*, 87(5), pp.408–415.

Dunkelberger, J.R. & Song, W.-C., 2009. Complement and its role in innate and adaptive immune responses. *Cell Research*, 20, p.34.

ECACC, 2016. ECACC General Cell Culture Collection: THP-1. *European Collection of Authenticated Cell Culture (ECACC)*, p.THP-1 ECACC 88081201. Available at: http://www.phe-culturecollections.org.uk/products/celllines/generalcell/detail.jsp?refId=88081201&collection=ecacc_gc.

Eder, C., 2009. Mechanisms of interleukin-1beta release. *Immunobiology*, 214(7), pp.543–553.

Edlin, R.S. et al., 2013. Antibiotic resistance patterns of outpatient pediatric urinary tract infections. *Journal of Urology*, 190(1), pp.222–227.

Edwards, R. & Harding, K.G., 2004. Bacteria and wound healing. *Curr Opinion in Infectious Diseases*, 17(February), pp.91–96.

Ehrlich, P.L.., 1898. *Die Anaemie*, Wein. Kessinger Publishing Compay.

Elbim, B.C. et al., 1993. Priming of Polymorphonuclear Neutrophils by Tumor Necrosis Factor. , pp.0–7.

Feldmann, M. et al., 1991. Cytokine Assays: Role in Evaluation of the Pathogenesis of Autoimmunity. *Immunological Reviews*, 119(1), pp.105–123.

Ferreiro-Vera, C. et al., 2011. Automated targeting analysis of eicosanoid

inflammation biomarkers in human serum and in the exometabolome of stem cells by SPE-LC-MS/MS. *Analytical and Bioanalytical Chemistry*, 399(3), pp.1093–1103.

Ferrer, R.M.D.P. et al., 2014. Empiric Antibiotic Treatment Reduces Mortality in Severe Sepsis and Septic Shock From the First Hour: Results From a Guideline-Based Performance Improvement Program*. *Critical care medicine*, 42(8), pp.1749–1755.

Fontana, J.A. et al., 1980. Development of chemotactic responsiveness in myeloid precursor cells: studies with human leukemia cell line. *Proc. Natl. Acad. Sci. USA*, 77, pp.3664–3668.

Foster, T.J., 2004. The Staphylococcus aureus 'superbug.' *Journal of Clinical Investigation*, 114, pp.1693–1696.

Fullerton, J.N., O'Brien, A.J. & Gilroy, D.W., 2014. Lipid mediators in immune dysfunction after severe inflammation. *Trends in immunology*, 35(1), pp.12–21.

Garnacho-Montero, J. et al., 2003. Impact of adequate empirical antibiotic therapy on the outcome of patients admitted to the intensive care unit with sepsis. *Critical care medicine*, 31(12), pp.2742–2751.

Garnacho-Montero, J. et al., 2014. Prognostic and diagnostic value of eosinopenia, C-reactive protein, procalcitonin, and circulating cell-free DNA in critically ill patients admitted with suspicion of sepsis. *Critical Care*, 18(3), pp.1–9.

Garnacho-Montero, J. et al., 2006. Timing of adequate antibiotic therapy is a greater determinant of outcome than are TNF and IL-10 polymorphisms in patients with sepsis. *Critical care (London, England)*, 10(4), p.R111.

Gastmeier, P. et al., 2012. Decreasing healthcare-associated infections (HAI) is an efficient method to decrease healthcare-associated Methicillin-resistant S.aureus (MRSA) infections Antimicrobial resistance data from the German national nosocomial surveillance system KISS. *Antimicrobial Resistance and Infection Control*, 1(1), p.3.

Gatto, F. et al., 2017. PMA-Induced THP-1 Macrophage Differentiation is Not Impaired by Citrate-Coated Platinum Nanoparticles. *Nanomaterials*, 7(10), p.332.

Gauer, R.L., 2013. Early recognition and management of sepsis in adults: The first six

hours. *American Family Physician*, 88(1), pp.44–53.

Geissmann, F. et al., 2010. Development of monocytes, macrophages and dendritic cells. *Science*, 327(5966), pp.656–661.

Genin, M. et al., 2015. M1 and M2 macrophages derived from THP-1 cells differentially modulate the response of cancer cells to etoposide. *BMC cancer*, 15, p.577.

Gioannini, T.L. & Weiss, J.P., 2007. Regulation of interactions of Gram-negative bacterial endotoxins with mammalian cells. *Immunologic Research*, 39(1–3), pp.249–260.

Gotts, J.E. & Matthay, M.A., 2016. Sepsis: pathophysiology and clinical management. *Bmj*, 353(i1585), pp.1–20.

Gouin, J.P. et al., 2012. Chronic stress, daily stressors, and circulating inflammatory markers. *Health Psychology*, 31(2), pp.264–268.

Greenhow, T.L. et al., 2014. The Changing Epidemiology of Serious Bacterial Infections in Young Infants. *The Pediatric Infectious Disease Journal*, 33(6), pp.595–599.

de Haas, C.J.C. et al., 2004. Chemotaxis Inhibitory Protein of *Staphylococcus aureus* , a Bacterial Antiinflammatory Agent. *The Journal of Experimental Medicine*, 199(5), pp.687–695.

Hallett, M.B. & Lloyds, D., 1995. Neutrophil priming: the cellular signals that say “amber” but not “green.” *Immunology Today*, 16(6), pp.264–268.

Hammond, V.J. & O’Donnell, V.B., 2012. Esterified eicosanoids: Generation, characterization and function. *Biochimica et Biophysica Acta - Biomembranes*, 1818(10), pp.2403–2412.

Han, J.H. et al., 2015. Use of a combination biomarker algorithm to identify medical intensive care unit patients with suspected sepsis at very low likelihood of bacterial infection. *Antimicrobial Agents and Chemotherapy*, 59(10), pp.6494–6500.

Harbarth, S. et al., 2001. and Interleukin-8 in Critically Ill Patients Admitted. *American Journal of Respiratory and Critical Care Medicine*, 164, pp.396–402.

Harbarth, S. et al., 2003. Inappropriate initial antimicrobial therapy and its effect on survival in a clinical trial of immunomodulating therapy for severe sepsis.

American Journal of Medicine, 115(7), pp.529–535.

Harizi, H., Corcuff, J.-B. & Gualde, N., 2008. Arachidonic-acid-derived eicosanoids: roles in biology and immunopathology. *Trends in molecular medicine*, 14(10), pp.461–9.

Harizi, H. & Gualde, N., 2005. The impact of eicosanoids on the crosstalk between innate and adaptive immunity: The key roles of dendritic cells. *Tissue Antigens*, 65(6), pp.507–514.

Harkewicz, R. & Dennis, E.A., 2011. Applications of Mass Spectrometry to Lipids and Membranes. *Annual Review of Biochemistry*, 80(1), pp.301–325.

Hartley, J.W. et al., 2008. Expression of infectious murine leukemia viruses by RAW264.7 cells, a potential complication for studies with a widely used mouse macrophage cell line. *Retrovirology*, 5, pp.6–11.

Hauert, A.B. et al., 2002. Differentiated HL-60 cells are a valid model system for the analysis of human neutrophil migration and chemotaxis. *The International Journal of Biochemistry & Cell Biology*, 34(7), pp.838–854.

Hauser, C.J. et al., 2000. Major Trauma Enhances Store-Operated Calcium Influx in. , 48(4).

Hausfater, P. et al., 2002. Usefulness of Procalcitonin as a Marker of Systemic Infection in Emergency Department Patients: A Prospective Study. *Clinical Infectious Diseases*, 34(7), pp.895–901.

Hawkey, P.M. & Jones, A.M., 2009. The changing epidemiology of resistance. *Journal of Antimicrobial Chemotherapy*, 64(SUPPL.1).

Hayashi, F. et al., 2001. The innate immune response to bacterial flagellin is mediated by Toll- like receptor 5. *Nature*, 410(6832), p.1099–103.

Hayashi, F., Means, T.K. & Luster, A.D., 2003. Toll-like receptors stimulate human neutrophil function. , 102(7), pp.2660–2669.

Helmersson, J. et al., 2009. Low dietary intake of beta-carotene, alpha-tocopherol and ascorbic acid is associated with increased inflammatory and oxidative stress status in a Swedish cohort. *The British journal of nutrition*, 101(12), pp.1775–1782.

Hidalgo, M.A. et al., 2015. FMLP-Induced IL-8 release is dependent on NADPH oxidase in human neutrophils. *Journal of Immunology Research*, 2015.

Higashi, N. et al., 2010. Profile of eicosanoid generation in aspirin-intolerant asthma and anaphylaxis assessed by new biomarkers. *Journal of Allergy and Clinical Immunology*, 125(5), p.1084–1091.e6.

Hilda, J.N. & Das, S.D., 2016. TLR stimulation of human neutrophils lead to increased release of MCP-1, MIP-1α, IL-1β, IL-8 and TNF during tuberculosis. *Human Immunology*, 77(1), pp.63–67.

Hiscott, J. & Ware, C., 2011. Cytokines. *Current Opinion in Immunology*, 23(5), pp.561–563.

Hoerr, V. et al., 2012. Gram-negative and gram-positive bacterial infections give rise to a different metabolic response in a mouse model. *Journal of Proteome Research*, 11(6), pp.3231–3245.

Holian, A. & Daniele, R.P., 1981. Release of oxygen products from lung macrophages by N-formyl peptides. *Journal of Applied Physiology: Respiration, Environmental and Exercise Physiology*, 50, pp.736–740.

Holroyde M C, Altounyan, R.E.C. & Elliott, E. V, 1981. BRONCHOCONSTRICTION PRODUCED IN MAN BY LEUKOTRIENES C AND D. *The Lancet*, 318, pp.17–18.

Hu, X. & Ivashkiv, L.B., 2009. Cross-regulation of Signaling Pathways by Interferon-gamma: Implications for Immune Responses and Autoimmune Diseases. *Immunity*, 31(4), pp.539–550.

Huang, H. et al., 2013. Lipid-based signaling modulates dna repair response and survival against Klebsiella pneumoniae infection in host cells and in mice. *American Journal of Respiratory Cell and Molecular Biology*, 49(5), pp.798–807.

Hultén, L.M. et al., 2010. 15-Lipoxygenase-2 is expressed in macrophages in human carotid plaques and regulated by hypoxia-inducible factor-1α. *European Journal of Clinical Investigation*, 40(1), pp.11–17.

Huo, Y. et al., 2017. HHS Public Access. , 197(5), pp.1–13.

Hutchins, N. a. et al., 2014. The new normal: Immunomodulatory agents against sepsis immune suppression. *Trends in Molecular Medicine*, 20(4), pp.224–233.

Indesmith, L.I.S. a L. et al., 2003. Human susceptibility and resistance to Norwalk virus infection. *Nat Med*, 9(5), pp.548–553.

Iwasaki, A. & Medzhitov, R., 2010. Regulation of adaptive immunity by the innate immune system. *Science (New York, N.Y.)*, 327(5963), pp.291–295.

J M Drazen et al, 1992. Recovery of leukotriene E4 from the urine of patients with urine obstruction. *American Journal of Respiratory and Critical Care Medicine*, 146, pp.104–108.

Jawien, J., 2008. New insights into immunological aspects of atherosclerosis. *Polskie Archiwum Medycyny Wewnetrznej*, 118(3), pp.127–131.

Jin, Y. et al., 2010. Tracking bacterial infection of macrophages using a novel red-emission pH sensor. *Analytical and Bioanalytical Chemistry*, 398(3), pp.1375–1384.

Jozsef, L. et al., 2006. Activation of TLR-9 Induces IL-8 Secretion through Peroxynitrite Signaling in Human Neutrophils. *The Journal of Immunology*, 176(2), pp.1195–1202.

Kendall, A.C. & Nicolaou, A., 2013. Bioactive lipid mediators in skin inflammation and immunity. *Progress in Lipid Research*, 52(1), pp.141–164.

Kim, J.-B. et al., 2007. Inhibition of LPS-induced iNOS, COX-2 and cytokines expression by poncirin through the NF-kappaB inactivation in RAW 264.7 macrophage cells. *Biological & pharmaceutical bulletin*, 30(12), pp.2345–2351.

Kim, M. et al., 2011. Neutrophil survival and c-kit+ -progenitor proliferation in Staphylococcus aureus – infected skin wounds promote resolution. *Blood*, 117(12), pp.3343–3352.

Kim, Y. et al., 2002. Bloodstream Infections by Extended-Spectrum ᴺL -Lactamase-Producing Escherichia coli and Klebsiella pneumoniae in Children : Epidemiology and Clinical Outcome. *Society*, 46(5), pp.1481–1491.

Kobeissi, Z. a. & Zanotti-Cavazzoni, S.L., 2010. Biomarkers of sepsis. *Yearbook of Critical Care Medicine*, 2010(7), pp.227–228.

Köck, R. et al., 2010. Methicillin-resistant Staphylococcus aureus (MRSA): burden of disease and control challenges in Europe. *Euro surveillance : bulletin européen sur les maladies transmissibles = European communicable disease bulletin*, 15(41), p.19688.

Kolaczkowska, E. & Kubes, P., 2013. Neutrophil recruitment and function in health and inflammation. *Nature Reviews Immunology*, 13(3), pp.159–175.

Krieg, A.M., 2002. CPG MOTIFS IN BACTERIAL DNA AND THEIR IMMUNE EFFECTS. *Annual Review of Immunology*, 20(1), pp.709–760.

Kumar, A. et al., 2006. Duration of hypotension before initiation of effective antimicrobial therapy is the critical determinant of survival in human septic shock. *Critical care medicine*, 34(6), pp.1589–1596.

Kumar, H., Kawai, T. & Akira, S., 2009. Toll-like receptors and innate immunity. *Biochemical and Biophysical Research Communications*, 388(4), pp.621–625.

Kurt-jones, E.A. et al., 2002. Role of Toll-like receptor 2 (TLR2) in neutrophil activation : GM-CSF enhances TLR2 expression and TLR2-mediated interleukin 8 responses in neutrophils. *Blood*, 100(5), pp.1860–1868.

Lacy, P. & Stow, J.L., 2011. Cytokine release from innate immune cells : association with diverse membrane trafficking pathways. *Blood*, 118(1), pp.9–19.

Latz, E. et al., 2004. TLR9 signals after translocating from the ER to CpG DNA in the lysosome. *Nature Immunology*, 5, p.190.

Lauder, S.N. et al., 2017. Myeloid 12 / 15-LOX regulates B cell numbers and innate immune antibody levels in vivo. , (0), pp.1–16.

Lauder, S.N. et al., 2017. Networks of enzymatically oxidized membrane lipids support calcium-dependent coagulation factor binding to maintain hemostasis. *Science Signaling*, 10(507).

Lawrence, C. & Nauciel, C., 1998. Production of interleukin-12 by murine macrophages in response to bacterial peptidoglycan. *Infection and Immunity*, 66(10), pp.4947–4949.

Leavy, O., 2011. Immunotherapy: Stopping monocytes in their tracks. *Nature reviews immunology*, 11(715).

Lee, J.W. et al., 2016. UPLC-MS/MS-based profiling of eicosanoids in RAW264.7 cells treated with lipopolysaccharide. *International Journal of Molecular Sciences*, 17(4).

Lemaitre, B. et al., 1996. The dorsoventral regulatory gene cassette spatzle/Toll/Cactus controls the potent antifungal response in Drosophila adults. *Cell*, 86(6), pp.973–983.

Lemaitre, B., Reichhart, J.-M. & Hoffmann, J.A., 1997. Drosophila host defense: Differential induction of antimicrobial peptide genes after infection by various classes of microorganisms. *Proceedings of the National Academy of Sciences*, 94(26), pp.14614–14619.

Leone, L. et al., 2016. Klebsiella pneumoniae is able to trigger Epithelial-Mesenchymal Transition process in cultured airway epithelial cells. *PLoS ONE*, 11(1), pp.1–18.

Lerman, Y. V. et al., 2014. Sepsis lethality via exacerbated tissue infiltration and TLR-induced cytokine production by neutrophils is integrin ??3??1-dependent. *Blood*, 124(24), pp.3515–3523.

Levy, M.M. et al., 2003. 2001 SCCM/ESICM/ACCP/ATS/SIS International Sepsis Definitions Conference. *Crit Care Med.*, 31(0090–3493 (Print)), pp.1250–1256.

Limongi, D., D'Agostini, C. & Ciotti, M., 2016. New sepsis biomarkers. *Asian Pacific Journal of Tropical Biomedicine*, 6(6), pp.516–519.

Lin, C.K. et al., 2014. Current issues in donor health and safety. *ISBT Science Series*, 9, pp.222–227.

Logan, M.R. et al., 2006. A critical role for vesicle-associated membrane protein-7 in exocytosis from human eosinophils and neutrophils. *Allergy: European Journal of Allergy and Clinical Immunology*, 61(6), pp.777–784.

Lüderitz, O. et al., 1984. Lipopolysaccharides: Structural Principles and Biologic Activities. *Reviews of Infectious Diseases*, 6(4), p.428–431,.

Lund, M.E. et al., 2016. The choice of phorbol 12-myristate 13-acetate differentiation protocol influences the response of THP-1 macrophages to a pro-inflammatory stimulus. *Journal of Immunological Methods*, 430, pp.64–70.

Lv, B. et al., 2014. Tumor necrosis factor-α as a diagnostic marker for neonatal sepsis: A meta-analysis. *The Scientific World Journal*, 2014.

Ma, X.X. et al., 2002. Novel Type of Staphylococcal Cassette Chromosome mec Identified in Staphylococcus aureus Strains Novel Type of Staphylococcal Cassette Chromosome mec Identified in Community-Acquired Methicillin-Resistant Staphylococcus aureus Strains. *Antimicrobial agents and chemotherapy*, 46(4), pp.1147–1152.

Mackenzie, A., 2014. Epidemiology of bacteremia in febrile infants in the united states: Biondi E, Evans R, Mischler M, et al. Pediatrics 2013;132;990-6. *Journal of Emergency Medicine*, 46(3), p.446.

Madigan, M. et al., 2009. *Brock Biology of Microorganisms* 12th ed., Pearson Education Inc., Upper Saddle River, NJ.

Magnusson, L.U. et al., 2012. High expression of arachidonate 15-lipoxygenase and proinflammatory markers in human ischemic heart tissue. *Biochemical and Biophysical Research Communications*, 424(2), pp.327–330.

Mal, M. et al., 2011. Ultra-pressure liquid chromatography/tandem mass spectrometry targeted profiling of arachidonic acid and eicosanoids in human colorectal cancer. *Rapid Communications in Mass Spectrometry*, 25(6), pp.755–764.

Mangelsdorf, D.J. et al., 1984. 1 , 25-Dihydroxyvitamin D 3 -induced Differentiation in a Human Promyelocytic Leukemia Cell Line (HL-60) : Receptor-mediated Maturation to Macrophage-like Cells. *The journal of cell biology*, 98, pp.391–98.

Martin, G.S. et al., 2003. The epidemiology of sepsis in the United States from 1979 through 2000. *The New England journal of medicine*, 348(16), pp.1546–1554.

Martinez, F.O. & Gordon, S., 2014. The M1 and M2 paradigm of macrophage activation: time for reassessment. *F1000Prime Reports*, 6(March), pp.1–13.

Maskrey, B.H. & O'Donnell, V.B., 2008. Analysis of eicosanoids and related lipid mediators using mass spectrometry. *Biochemical Society transactions*, 36(Pt 5), pp.1055–1059.

Massey, K.A. & Nicolaou, A., 2013. Lipidomics of oxidized polyunsaturated fatty acids. *Free Radical Biology and Medicine*, 59, pp.45–55.

Mattsson, E. et al., 1993. Peptidoglycan and teichoic acid from Staphylococcus epidermidis stimulate human monocytes to release tumour necrosis factor-alpha, interleukin-1 beta and interleukin-6. *FEMS Immunology medical microbiology*, 7(3), pp.281–7.

Mazel, D. & Davies, J., 2016. Antibiotic resistance in. *Cellular and molecular life sciences*, 56, pp.742–754.

Meirer, K., Steinhilber, D. & Proschak, E., 2014. Inhibitors of the arachidonic acid cascade: Interfering with multiple pathways. *Basic and Clinical Pharmacology and Toxicology*, 114(1), pp.83–91.

Merly, L. & Smith, S.L., 2005. Pathogen recognition with Toll-like receptors. *Current Opinion in Immunology*, 17(4 SPEC. ISS.), pp.338–344.

Metchnikov, ilya, 1905. *Immunity in infectious diseases*, Cambridge University, London.

Metchnikov, E., 1939. *THE COMPARATIVE PATHOLOGY OF INFLAMMATION*,

Meyer, H. & Howard, T.H., 1987. Actin Polymerization and Its Relationship Response in Maturing Human By William H. Meyer and to Locomotion and Chemokinetic Promyelocytic Leukemia. *Blood*, 70(2), pp.363–367.

Meynaar, I.A. et al., 2011. In Critically Ill Patients, Serum Procalcitonin Is More Useful in Differentiating between Sepsis and SIRS than CRP, Il-6, or LBP. *Critical Care Research and Practice*, 2011, pp.1–6.

Miles, B.Y.A.A. & Misra, S.S., 1938. THE ESTIMATION OF THE BACTERICIDAL POWER OF THE BLOOD From the Department of Pathology , British Postgraduate Medical School. *J. Hyg*, 38, pp.732–749.

Mizel, S.B. et al., 2003. Induction of Macrophage Nitric Oxide Production by Gram-Negative Flagellin Involves Signaling Via Heteromeric Toll-Like Receptor 5/Toll-Like Receptor 4 Complexes. *The Journal of Immunology*, 170(12), pp.6217–6223.

Mollinedo, F. et al., 2006. Combinatorial SNARE Complexes Modulate the Secretion of Cytoplasmic Granules in Human Neutrophils. *The Journal of Immunology*, 177(5), pp.2831–2841.

Morey, J.N. et al., 2016. Current Directions in Stress and Human Immune Function. *Curr Opin Psychol*, 5, pp.13–17.

Morgan, A.H. et al., 2009. Phosphatidylethanolamine-esterified eicosanoids in the mouse. Tissue localization and inflammation-dependent formation in Th-2 disease. *Journal of Biological Chemistry*, 284(32), pp.21185–21191.

Morgan, A.H. et al., 2010. Quantitative assays for esterified oxylipins generated by immune cells. *Nature protocols*, 5(12), pp.1919–1931.

Moya, B. et al., 2008. Benefit of having multiple ampD genes for acquiring β-lactam resistance without losing fitness and virulence in Pseudomonas aeruginosa. *Antimicrobial Agents and Chemotherapy*, 52(10), pp.3694–3700.

Murata, T. & Maehara, T., 2016. Discovery of anti-inflammatory role of prostaglandin D2. *Journal of Veterinary Medical Science*, 78(11), pp.1643–1647.

Nahid, P. et al., 2018. Interplay of strain and race / ethnicity in the innate immune response to M . tuberculosis. *PLoS ONE*, 13(5), pp.1–16.

Nakanishi, N., Suzuki, K. & Tatara, K., 2004. Age-related Change in Relationship between White Blood Cell Count and Some Features of the Metabolic

Syndrome. *Industrial Health*, 42(3), pp.359–368.

Neu, H.C., 1992. The crisis in Antibiotic resistance BT - Science. , 257(AUGUST 1992), pp.1064–1073.

Nicholson, J.K. et al., 2012. Host-Gut Microbiota Metabolic Interactions. *Science*, 336(6086), pp.1262–67.

Nickel, W., 2003. The mystery of nonclassical protein secretion: A current view on cargo proteins and potential export routes. *European Journal of Biochemistry*, 270(10), pp.2109–2119.

Nicolaou, A., 2013. Eicosanoids in skin inflammation. *Prostaglandins Leukotrienes and Essential Fatty Acids*, 88(1), pp.131–138.

Nicolle, L.E., 2014. Catheter associated urinary tract infections. *Antimicrobial Resistance and Infection Control*, 3(1), pp.1–8.

Nieves, D. & Moreno, J.J., 2006. Role of 5-lipoxygenase pathway in the regulation of RAW 264.7 macrophage proliferation. *Biochemical Pharmacology*, 72(8), pp.1022–1030.

Nolfo, R. & Rankin, J. a, 1990. U937 and THP-1 cells do not release LTB4, LTC4, or LTD4 in response to A23187. *Prostaglandins*, 39(2), pp.157–165.

Norris, P.C. et al., 2011. Specificity of eicosanoid production depends on the TLR-4-stimulated macrophage phenotype. *Journal of Leukocyte Biology*, 90(3), pp.563–574.

Norris, P.C. & Dennis, E.A., 2014. A lipidomic perspective on inflammatory macrophage eicosanoid signaling. *Advances in Biological Regulation*, 54(1), pp.99–110.

Oertelt-Prigione, S., 2012. The influence of sex and gender on the immune response. *Autoimmunity Reviews*, 11(6–7).

Okunishi, K. et al., 2004. A Novel Role of Cysteinyl Leukotrienes to Promote Dendritic Cell Activation in the Antigen-Induced Immune Responses in the Lung. *The Journal of Immunology*, 173(10), pp.6393–6402.

Osuchowski, M.F. et al., 2007. Chronic sepsis mortality characterized by an individualized inflammatory response. *Journal of immunology (Baltimore, Md. : 1950)*, 179(1), pp.623–630.

Osuchowski, M.F. et al., 2006. Circulating cytokine/inhibitor profiles reshape the

understanding of the SIRS/CARS continuum in sepsis and predict mortality. *Journal of immunology (Baltimore, Md. : 1950)*, 177(3), pp.1967–1974.

Palazzo, M. et al., 2008. Cross-talk among Toll-like receptors and their ligands. *International Immunology*, 20(5), pp.709–718.

Pan, Z.K. et al., 2000. fMet-Leu-Phe stimulates proinflammatory cytokine gene expression in human peripheral blood monocytes: the role of phosphatidylinositol 3-kinase. *Journal of immunology (Baltimore, Md. : 1950)*, 164(1), pp.404–411.

Pan, Z.K. et al., 2000. fMet-Leu-Phe Stimulates Proinflammatory Cytokine Gene Expression in Human Peripheral Blood Monocytes: The Role of Phosphatidylinositol 3-Kinase. *The Journal of Immunology*, 164(1), pp.404–411.

Parameswaran, K. et al., 2002. Cysteinyl leukotrienes promote human airway smooth muscle migration. *American Journal of Respiratory and Critical Care Medicine*, 166(5), pp.738–742.

Parham, P., 2015. The Immune System. *Immunology*, 313(16), p.Chapter 14.

Parihar, A., Eubank, T. & Doseff, A., 2010. Monocytes and macrophages regulate immunity through dynamic networks of survival and cell death. *journal of innate immunology*, 2, pp.204–215.

Park, E.K. et al., 2007. Optimized THP-1 differentiation is required for the detection of responses to weak stimuli. *Inflammation Research*, 56(1), pp.45–50.

Patel, S., 2007. The Impact of MRSA on Wound Healing. *Wound Essentials*, 2, pp.144–148.

Pfau, J.C., Walker, E. & Card, G.L., 2000. Monoclonal antibodies to CD45 modify LPS-induced arachidonic acid metabolism in macrophages. *Biochimica et Biophysica Acta - Molecular Cell Research*, 1495(3), pp.212–222.

Philpott, D.J. & Girardin, S.E., 2004. The role of Toll-like receptors and Nod proteins in bacterial infection. *Molecular Immunology*, 41(11 SPEC. ISS.), pp.1099–1108.

Poltorak, A. et al., 1998. Defective LPS Signaling in C3H / HeJ and C57BL / 10ScCr Mice : Mutations in Tlr4 Gene. *Science*, 282, pp.2085–2089.

Porter, S.B., 1997. Current status of clinical trials with anti-TNF. *Chest*, 112(6 Supplement), p.339S.

Praticò, D. et al., 2002. Increase of Brain Oxidative Stress in Mild Cognitive

Impairment. *Archives of Neurology*, 59(6), p.972.

Prince, L.R. et al., 2011. The role of TLRs in neutrophil activation. *Current Opinion in Pharmacology*, 11(4), pp.397–403.

Pugin, J., 2012. How tissue injury alarms the immune system and causes a systemic inflammatory response syndrome. *Annals of Intensive Care*, 2(1), p.27.

Rabiet, M. et al., 2005. Leukocyte signaling Human mitochondria-derived N -formylated peptides are novel agonists equally active on FPR and FPRL1 , while Listeria monocytogenes -derived peptides preferentially activate FPR. , pp.2486–2495.

Rago, B. & Fu, C., 2013. Development of a high-throughput ultra performance liquid chromatography-mass spectrometry assay to profile 18 eicosanoids as exploratory biomarkers for atherosclerotic diseases. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, 936, pp.25–32.

Ragolia, L. et al., 2005. Accelerated glucose intolerance, nephropathy, and atherosclerosis in prostaglandin D2 synthase knock-out mice. *Journal of Biological Chemistry*, 280(33), pp.29946–29955.

Ramachandran, G., 2014. Gram-positive and gram-negative bacterial toxins in sepsis: a brief review. *Virulence*, 5(1), pp.213–8.

Reich, D. et al., 2009. Reduced neutrophil count in people of African descent is due to a regulatory variant in the Duffy antigen receptor for chemokines gene. *PLoS Genetics*, 5(1).

Ridker, P.M., 2003. Clinical application of C-reactive protein for cardiovascular disease detection and prevention. *Circulation*, 107(3), pp.363–369.

Rittirsch, D., Flierl, M.A. & Ward, P.A., 2008. Harmful molecular mechanisms in sepsis. *Nature reviews. Immunology*, 8(10), pp.776–87.

Robson, W.P. & Daniels, R., 2008. The Sepsis Six: helping patients to survive sepsis. , 17(2007), pp.2002–2004.

Rock, F.L. et al., 1998. A family of human receptors structurally related to Drosophila Toll. *Proceedings of the National Academy of Sciences*, 95(2), pp.588–593.

Russell, C.D. & Schwarze, J., 2013. The role of pro-resolution lipid mediators in infectious disease. *Immunology*, 141(2), pp.166–73.

S Kaku, S Yunioki, K Ohkura, M Sugano, M Nonaka, H.T. and K.Y., 2001. Interactions

of dietary fats and proteins on fatty acid composition of immune cells anf LTB4 production by peritoneal exudate cells of rats. , 65(2), pp.315–321.

Salomao, R. et al., 2008. Tlr Signaling Pathway in Patients With Sepsis. *SHOCK*, 30(13), pp.73–76.

Sambrook, J., Fritsch, E.F. & Maniatis, T., 1989. *Molecular Cloning: A Laboratory Manual, 2nd edition*, Cold Sring Harbor Laboratory, Cold Spring Harbor, NY.

Samuelsson, B. et al., 1987. Structures , Biosynthesis , and Biological Effects.

Schildberger, A. et al., 2013. Monocytes, Peripheral Blood Mononuclear Cells, and THP-1 Cells exhibit different cytokine expression patterns following stimulation with lipopolysaccharide. *Mediator of Inflammation*, 2013(697972), pp.1–10.

Schrimpe, A.C. & Wright, D.W., 2009. Differential gene expression mediated by 15-hydroxyeicosatetraenoic acid in LPS-stimulated RAW 264.7 cells. *Malaria journal*, 8, p.195.

Schulte, W., Bernhagen, J. & Bucala, R., 2013. Cytokines in sepsis: Potent immunoregulators and potential therapeutic targets - An updated view. *Mediators of Inflammation*, 2013.

Schuster, V.L., 2002. Prostaglandin transport. *Prostaglandins and Other Lipid Mediators*, 68–69, pp.633–647.

Schwab, J.M. & Serhan, C.N., 2006. Lipoxins and new lipid mediators in the resolution of inflammation. *Current Opinion in Pharmacology*, 6(4), pp.414–420.

Selvaraj, S.K. & Prasadarao, N. V., 2005. *Escherichia coli* K1 inhibits proinflammatory cytokine induction in monocytes by preventing NF-κB activation. *Journal of Leukocyte Biology*, 78(2), pp.544–554.

Seshadri, S. et al., 2007. Pyrin Levels in Human Monocytes and Monocyte-Derived Macrophages Regulate IL-1 Processing and Release. *The Journal of Immunology*, 179(2), pp.1274–1281.

Shibata, T. et al., 2002. 15-Deoxy-$\Delta^{12,14}$-prostaglandin J_2. *Journal of Biological Chemistry*, 277(12), pp.10459–10466.

Shimazu, R. et al., 1999. MD-2, a Molecule that Confers Lipopolysaccharide Responsiveness on Toll-like Receptor 4. *The Journal of Experimental Medicine*, 189(11), pp.1777–1782.

Shon, A.S., Bajwa, R.P.S. & Russo, T.A., 2013. Hypervirulent (hypermucoviscous)

Klebsiella Pneumoniae: A new and dangerous breed. *Virulence*, 4(2), pp.107–118.

Shrivastava, A., 2007. Activation of macrophages with N-formyl-methionyl-leucyl-phenylalanine: Involvement of protein kinase C and tyrosine kinase. *Indian Journal of Experimental Biology*, 45(9), pp.755–763.

Shurety, W. et al., 2000. Localization and post-golgi trafficking of tumor necrosis factor-alpha in macrophages. *J Interferon Cytokine Res*, 20(4), pp.427–438.

Sigola, L.B. et al., 2016. Effects of Toll-like receptor ligands on RAW 264.7 macrophage morphology and zymosan phagocytosis. *Tissue and Cell*, 48(4), pp.389–396.

Silva, M.T. & Correia-Neves, M., 2012. Neutrophils and macrophages: The main partners of phagocyte cell systems. *Frontiers in Immunology*, 3(JUL), pp.2008–2013.

Simon, L. et al., 2004. Serum procalcitonin and C-reactive protein levels as markers of bacterial infection: a systematic review and meta-analysis. *Clin Infect Dis*, 39(2), pp.206–17.

Simor, A.E. et al., 2016. Determinants of outcome in hospitalized patients with methicillin-resistant staphylococcus aureus bloodstream infection: Results From National Surveillance in Canada, 2008-2012. *Infection Control and Hospital Epidemiology*, 37(4), pp.390–397.

Singer, M. et al., 2016. The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3). *Jama*, 315(8), pp.801–10.

St-Onge, M. et al., 2007. Characterization of prostaglandin E2 generation through the cyclooxygenase (COX)-2 pathway in human neutrophils. *Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids*, 1771(9), pp.1235–1245.

Stafford, J.B. & Marnett, L.J., 2008. Prostaglandin E2 inhibits tumor necrosis factor-alpha RNA through PKA type I. *Biochemical and Biophysical Research Communications*, 366(1), pp.104–109.

Stearns-Kurosawa, Marcin F. Osuchowski, C.V. & Shinichiro Kurosawa, D.G.R. and D.J., 2011. The pathogenesis of sepsis. *Annu Rev. Pathol. Mech. Dis*, 16, pp.19–48.

Steinke, J.W. & Borish, L., 2006. Cytokines and chemokines. *Journal of Allergy and*

Clinical Immunology, 117(2 SUPPL. 2), pp.441–445.

Steptoe, A., Hamer, M. & Chida, Y., 2007. The effects of acute psychological stress on circulating inflammatory factors in humans: A review and meta-analysis. *Brain, Behavior, and Immunity*, 21(7), pp.901–912.

Stoppelkamp, S. et al., 2015. Identification of predictive early biomarkers for sterile-sirs after cardiovascular surgery. *PLoS ONE*, 10(8), pp.1–20.

Stow, J.L. et al., 2009. Cytokine secretion in macrophages and other cells: Pathways and mediators. *Immunobiology*, 214(7), pp.601–612.

Strassheim, D., Park, J.S. & Abraham, E., 2002. Sepsis: Current concepts in intracellular signaling. *International Journal of Biochemistry and Cell Biology*, 34(12), pp.1527–1533.

Struve, C. & Krogfelt, K.A., 2003. Role of capsule in *Klebsiella pneumoniae* virulence: lack of correlation between in vitro and in vivo studies. *FEMS Microbiology Letters*, 218(1), pp.149–154.

Sukumaran, S.K., Selvaraj, S.K. & Prasadarao, N. V, 2004. Inhibition of Apoptosis by Escherichia coli K1 Is Accompanied by Increased Expression of Bcl XL and Blockade of Mitochondrial Cytochrome c Release in Macrophages. *Society*, 72(10), pp.6012–6022.

Summers, C. et al., 2010. Neutrophil kinetics in health and disease. *Trends in Immunology*, 31(8), pp.318–324.

Swami, S.K. et al., 2012. Incidence of antibiotic-resistant Escherichia coli bacteriuria according to age and location of onset: A population-based study from Olmsted County, Minnesota. *Mayo Clinic Proceedings*, 87(8), pp.753–759.

T Lämmermann et al, 2013. Neutrophil swarms require LTB4 and inter grins at sites of cell death in vivo. *Nature medicine*, 498, pp.371–375.

Taanman, J., Street, R.H. & Nw, L., 1999. The mitochondrial genome : structure , transcription , translation and replication. , 1410.

Tan, R.S.T. et al., 2014. TLR cross-talk confers specificity to innate immunity. *International Reviews of Immunology*, 33(6), pp.443–453.

Tarlowe, M.H. et al., 2003. Inflammatory Chemoreceptor Cross-Talk Suppresses Leukotriene B 4 Receptor 1-Mediated Neutrophil Calcium Mobilization and Chemotaxis After Trauma. , 171, pp.2066–2073.

Tecchio, C., Micheletti, A. & Cassatella, M.A., 2014. Neutrophil-derived cytokines: Facts beyond expression. *Frontiers in Immunology*, 5(OCT), pp.1–7.

Thomas, C.P. et al., 2014. Identification and quantification of aminophospholipid molecular species on the surface of apoptotic and activated cells. *Nature protocols*, 9(1), pp.51–63.

Tietze, K. et al., 2006. Differences in innate immune responses upon stimulation with gram-positive and gram-negative bacteria. *Journal of Periodontal Research*, 41(5), pp.447–454.

Tomás, A. et al., 2015. Functional genomic screen identifies klebsiella pneumoniae factors implicated in blocking nuclear factor κB (NF-κB) signaling. *Journal of Biological Chemistry*, 290(27), pp.16678–16697.

Tomhave, E.D. et al., 1994. Cross-desensitization of receptors for peptide chemoattractants. Characterization of a new form of leukocyte regulation. *The Journal of Immunology*, 153(7), pp.3267–3275.

Trebino, C.E. et al., 2005. Redirection of eicosanoid metabolism in mPGES-1-deficient macrophages. *Journal of Biological Chemistry*, 280(17), pp.16579–16585.

Tsai, Y.K. et al., 2011. Klebsiella pneumoniae Outer membrane porins OmpK35 and OmpK36 play roles in both antimicrobial resistance and virulence. *Antimicrobial Agents and Chemotherapy*, 55(4), pp.1485–1493.

Uzodi, A.S., Lohse, C.M. & Banerjee, R., 2017. Risk Factors For and Outcomes of Multidrug-Resistant Escherichia coli Infections in Children. *Infectious Diseases and Therapy*, 6(2), pp.245–257.

Vigo, E. et al., 2005. In-vitro anti-inflammatory activity of *Pinus sylvestris* and *Plantago lanceolata* extracts: effect on inducible NOS, COX-1, COX-2 and their products in J774A.1 murine macrophages. *Journal of Pharmacy and Pharmacology*, 57(3), pp.383–391.

Wada, M. et al., 2007. Enzymes and receptors of prostaglandin pathways with arachidonic acid-derived versus eicosapentaenoic acid-derived substrates and products. *Journal of Biological Chemistry*, 282(31), pp.22254–22266.

Waksman, Y. et al., 1990. Granulocyte-macrophage colony-stimulating factor enhances cationic antimicrobial protein synthesis by human neutrophils. *Journal of immunology (Baltimore, Md. : 1950)*, 144(9), pp.3437–3443.

Wang, J. et al., 2012. Monocytic microparticles activate endothelial cells in an IL-1b dependent manner. *Blood*, 118(8), pp.2366–2374.

Wang, M. et al., 2006. Deletion of microsomal prostaglandin E synthase-1 augments prostacyclin and retards atherogenesis. *Proceedings of the National Academy of Sciences*, 103(39), pp.14507–14512.

Wang, Q. et al., 2016. A spectral and morphologic method for white blood cell classification. *Optics and Laser Technology*, 84, pp.144–148.

Welser-Alves, J., 2015. The Advantages and Difficulties of Working with Primary Cells. *ASBC*. Available at: https://www.ascb.org/newsletter/2015-november-newsletter/advantages-difficulties-working-primary-cells/.

Werber, M., 1986. Paul Ehrlich - Histological Staining, Immunology, Chemotherapy. *Archives of Neurology*, 43(6), p.544.

West, M.A. et al., 2007. Whole Blood Leukocyte Mitogen Activated Protein Kinases Activation Differentiates Intensive Care Unit Patients With Systemic Inflammatory Response Syndrome and Sepsis. , 62(4), pp.26–28.

Whicher, J., Bienvenu, J. & Monneret, G., 2001. Procalcitonin as an acute phase marker. *Annals of Clinical Biochemistry*, 38(5), pp.483–493.

WHO, 2000. *WHO report on infectious disease: overcoming antimicrobial resistance*, Geneva,.

Wilson, L., Butcher, C.J. & Kellie, S., 1993. Calcium ionophore A23 187 induces interleukin-8 gene expression and protein secretion in human monocytic cells. *Federation of European Biochemical Societies*, 325(3), pp.295–298.

Wolk, D.M. et al., 2009. Rapid detection of Staphylococcus aureus and methicillin-resistant S. aureus (MRSA) in wound specimens and blood cultures: Multicenter preclinical evaluation of the cepheid Xpert MRSA/SA skin and soft tissue and blood culture assays. *Journal of Clinical Microbiology*, 47(3), pp.823–826.

World Health Oranisation, 2014. WHO, Antimicrobial resistance: Global report on surveillance 2014.

Wuest, S.J.A. et al., 2012. Expression and regulation of 12/15-lipoxygenases in human primary macrophages. *Atherosclerosis*, 225(1), pp.121–127.

Yeung, A.T.Y., Bains, M. & Hancock, R.E.W., 2011. The sensor kinase CbrA is a global regulator that modulates metabolism, virulence, and antibiotic resistance in

Pseudomonas aeruginosa. *Journal of Bacteriology*, 193(4), pp.918–931.

Zhang, Q. et al., 2010. Circulating mitochondrial DAMPs cause inflammatory responses to injury. *Nature*, 464(7285), pp.104–7.

Zhang, Y. et al., 2016. High Prevalence of Hypervirulent Klebsiella pneumoniae Infection in China : Geographic Distribution , Clinical Characteristics , and. , 60(10), pp.6115–6120.

Zigmond, S.H., Sullivan, S.J. & Lauffenburger, D.A., 1982. Kinetic analysis of chemotactic peptide receptor modulation. *J Cell Biol*, 92(1), pp.34–43.

Appendix

Appendix I. Duterated internal standards used. Multiple reaction monitoring (MRM), deionising potential (DP). Collision energy (CE). Retention time (RT).

No.	Internal standard	MRM Transitions		DP	CE	RT (min)
		Q1	Q3			
1	Arachidonic Acid-d8	311.5	311.501	-65	-10	16.9
2	Linoleic Acid-d4	283.4	283.501	-65	-10	17.06
3	13(S)-HODE-d4	299.5	198.1	-60	-25	13.22
4	5(S)-HETE-d8	327.2	116.1	-55	-19	14.32
5	12(S)-HETE-d8	327.2	184.2	-60	-20	14.02
6	15(S)-HETE-d8	327.2	226.2	-65	-22	13.55
7	20-HETE-d6	325.2	281.2	-70	-21	12.6
8	Leukotriene B4-d4	339.2	197.2	-65	-21	10.17
9	Resolvin D1-d5	380.2	141.1	-75	-18	7.41
10	Prostaglandin E2-d4	355.2	275.301	-60	-23	6.16
11	Prostaglandin D2-d4	355.2	275.3	-55	-23	6.58
12	Prostaglandin F2α-d4	357.5	313.2	-80	-24	5.86
13	Thromboxane B2-d4	373.3	173.2	-55	-22	4.79
14	11-dehydro Thromboxane B2-d4	371.5	309.2	-55	-21	6.21
15	11(12)-EET (EpETrE) -d11	331.2	167.1	-65	-18	15.09

Appendix II. Eicosanoid standard curve formation.

Product name	c Stock (ug/mL)	c Stock (nM)	SM4000	SM3000	SM2000	SM1000	SM600	SM300	SM100	SM30	SM10	SM3	SM1	SM0.3	SM0.1	SM0.03	SM0.01
solutions used	-		stock G1	stock G1	stock G1	G1 (0.025 mL) + G2, G3 and G4 (0.25 mL each)	SM1000	SM600	SM1000	SM300	SM100	SM30	SM10	SM3	SM1	SM0.3	SM0.1
Volume (mL)			0.1	0.075	0.55	3.525	3	2.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Volume IS			9.9	9.925	9.45	6.475	2	2.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5
Final volume	-		10	10	10	10	5	5	5	5	5	5	5	5	5	5	5
			4002.4	3001.8	2001.2	1000.6											
Lauric Acid	80	400244	4	3	2	1	600.37	300.18	100.06	30.02	10.01	3.00	1.00	0.30	0.10	0.03	0.01
Myristic Acid	91	398367	3984	2988	1992	996	597.55	298.78	99.59	29.88	9.96	2.99	1.00	0.30	0.10	0.03	0.01
Palmitic Acid	105	407556	4076	3057	2038	1019	611.33	305.67	101.89	30.57	10.19	3.06	1.02	0.31	0.10	0.03	0.01
Palmitoleic Acid	100	394006	3940	2955	1970	985	591.01	295.50	98.50	29.55	9.85	2.96	0.99	0.30	0.10	0.03	0.01
Stearic Acid	114	402417	4024	3018	2012	1006	603.63	301.81	100.60	30.18	10.06	3.02	1.01	0.30	0.10	0.03	0.01
Arachidic Acid	125	398411	3984	2988	1992	996	597.62	298.81	99.60	29.88	9.96	2.99	1.00	0.30	0.10	0.03	0.01
Oleic Acid	113	398285	3983	2987	1991	996	597.43	298.71	99.57	29.87	9.96	2.99	1.00	0.30	0.10	0.03	0.01
Stearidonic Acid	110	397974	3980	2985	1990	995	596.96	298.48	99.49	29.85	9.95	2.98	0.99	0.30	0.10	0.03	0.01
Linoleic Acid	113	401147	4011	3009	2006	1003	601.72	300.86	100.29	30.09	10.03	3.01	1.00	0.30	0.10	0.03	0.01
α-Linolenic Acid	113	404051	4041	3030	2020	1010	606.08	303.04	101.01	30.30	10.10	3.03	1.01	0.30	0.10	0.03	0.01
Dihomo-γ-Linolenic Acid	125	407844	4078	3059	2039	1020	611.77	305.88	101.96	30.59	10.20	3.06	1.02	0.31	0.10	0.03	0.01
Arachidonic Acid	125	410549	4105	3079	2053	1026	615.82	307.91	102.64	30.79	10.26	3.08	1.03	0.31	0.10	0.03	0.01
Docosahexaenoic Acid	138	418585	4186	3139	2093	1046	627.88	313.94	104.65	31.39	10.46	3.14	1.05	0.31	0.10	0.03	0.01
Eicosapentaenoic Acid	120	396758	3968	2976	1984	992	595.14	297.57	99.19	29.76	9.92	2.98	0.99	0.30	0.10	0.03	0.01
(±)9-HODE	12	40472			2024	1012	607.08	303.54	101.18	30.35	10.12	3.04	1.01	0.30	0.10	0.03	0.01
(±)13-HODE	12	40472			2024	1012	607.08	303.54	101.18	30.35	10.12	3.04	1.01	0.30	0.10	0.03	0.01
9(S)-HOTrE	12	40761			2038	1019	611.41	305.71	101.90	30.57	10.19	3.06	1.02	0.31	0.10	0.03	0.01
13(S)-HOTrE	12	40761			2038	1019	611.41	305.71	101.90	30.57	10.19	3.06	1.02	0.31	0.10	0.03	0.01
5(S)-HETrE	13	40310			2016	1008	604.65	302.33	100.78	30.23	10.08	3.02	1.01	0.30	0.10	0.03	0.01
15(S)-HETrE	13	40310			2016	1008	604.65	302.33	100.78	30.23	10.08	3.02	1.01	0.30	0.10	0.03	0.01
(±)4-HDHA	14	40639			2032	1016	609.58	304.79	101.60	30.48	10.16	3.05	1.02	0.30	0.10	0.03	0.01
(±)5-HETE	13	40562			2028	1014	608.42	304.21	101.40	30.42	10.14	3.04	1.01	0.30	0.10	0.03	0.01
(±)12-HETE	13	40562			2028	1014	608.42	304.21	101.40	30.42	10.14	3.04	1.01	0.30	0.10	0.03	0.01
(±)15-HETE	13	40562			2028	1014	608.42	304.21	101.40	30.42	10.14	3.04	1.01	0.30	0.10	0.03	0.01
(±)7-HDHA	14	40058				1001	600.87	300.44	100.15	30.04	10.01	3.00	1.00	0.30	0.10	0.03	0.01
(±)8-HDHA	14	40058				1001	600.87	300.44	100.15	30.04	10.01	3.00	1.00	0.30	0.10	0.03	0.01
(±)10-HDHA	14	40058				1001	600.87	300.44	100.15	30.04	10.01	3.00	1.00	0.30	0.10	0.03	0.01
(±)11-HDHA	14	40058				1001	600.87	300.44	100.15	30.04	10.01	3.00	1.00	0.30	0.10	0.03	0.01
(±)13-HDHA	14	40058				1001	600.87	300.44	100.15	30.04	10.01	3.00	1.00	0.30	0.10	0.03	0.01
14(S)-HDHA	14	40058				1001	600.87	300.44	100.15	30.04	10.01	3.00	1.00	0.30	0.10	0.03	0.01
(±)16-HDHA	14	40058				1001	600.87	300.44	100.15	30.04	10.01	3.00	1.00	0.30	0.10	0.03	0.01
(±)17-HDHA	14	40058				1001	600.87	300.44	100.15	30.04	10.01	3.00	1.00	0.30	0.10	0.03	0.01
(±)20-HDHA	14	40058				1001	600.87	300.44	100.15	30.04	10.01	3.00	1.00	0.30	0.10	0.03	0.01
(±)8-HETE	13	39938				998	599.06	299.53	99.84	29.95	9.98	3.00	1.00	0.30	0.10	0.03	0.01
(±)9-HETE	13	39938				998	599.06	299.53	99.84	29.95	9.98	3.00	1.00	0.30	0.10	0.03	0.01
(±)11-HETE	13	39938				998	599.06	299.53	99.84	29.95	9.98	3.00	1.00	0.30	0.10	0.03	0.01
20-HETE	13	39938				998	599.06	299.53	99.84	29.95	9.98	3.00	1.00	0.30	0.10	0.03	0.01
(±)5-HEPE	13	40188				1005	602.83	301.41	100.47	30.14	10.05	3.01	1.00	0.30	0.10	0.03	0.01
(±)8-HEPE	13	40188				1005	602.83	301.41	100.47	30.14	10.05	3.01	1.00	0.30	0.10	0.03	0.01
(±)9-HEPE	13	40188				1005	602.83	301.41	100.47	30.14	10.05	3.01	1.00	0.30	0.10	0.03	0.01
(±)11-HEPE	13	40188				1005	602.83	301.41	100.47	30.14	10.05	3.01	1.00	0.30	0.10	0.03	0.01
(±)12-HEPE	13	40188				1005	602.83	301.41	100.47	30.14	10.05	3.01	1.00	0.30	0.10	0.03	0.01
(±)15-HEPE	13	40188				1005	602.83	301.41	100.47	30.14	10.05	3.01	1.00	0.30	0.10	0.03	0.01

Appendix II. Continued

Product name	c Stock (ug/mL)	c Stock (nM)	SM4000	SM3000	SM2000	SM1000	SM600	SM300	SM100	SM30	SM10	SM3	SM1	SM0.3	SM0.1	SM0.03	SM0.01
solutions used	-	-	stock G1	stock G1	stock G1 G2.1	G1 (0.025 mL) + G2, G3 and G4 (0.25 mL each)	SM1000	SM600	SM1000	SM300	SM100	SM30	SM10	SM3	SM1	SM0.3	SM0.1
Volume (mL)			0.1	0.075	0.55	3.525	3	2.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Volume IS			9.9	9.925	9.45	6.475	2	2.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5
Final volume	-	-	10	10	10	10	5	5	5	5	5	5	5	5	5	5	5
Lauric Acid	80	400244	4002.44	3001.83	2001.22	1000.61	600.37	300.18	100.06	30.02	10.01	3.00	1.00	0.30	0.10	0.03	0.01
(±)18-HEPE	13	40188				1005	602.83	301.41	100.47	30.14	10.05	3.01	1.00	0.30	0.10	0.03	0.01
Resolvin E1	14	39943				999	599.14	299.57	99.86	29.96	9.99	3.00	1.00	0.30	0.10	0.03	0.01
Resolvin D1	15	39841				996	597.61	298.80	99.60	29.88	9.96	2.99	1.00	0.30	0.10	0.03	0.01
Resolvin D2	15	39841				996	597.61	298.80	99.60	29.88	9.96	2.99	1.00	0.30	0.10	0.03	0.01
Resolvin D3	15	39841				996	597.61	298.80	99.60	29.88	9.96	2.99	1.00	0.30	0.10	0.03	0.01
Resolvin D5	15	41609				1040	624.13	312.07	104.02	31.21	10.40	3.12	1.04	0.31	0.10	0.03	0.01
Leukotriene B3	14	39882				997	598.23	299.11	99.70	29.91	9.97	2.99	1.00	0.30	0.10	0.03	0.01
Leukotriene B4	14	40119				1003	601.78	300.89	100.30	30.09	10.03	3.01	1.00	0.30	0.10	0.03	0.01
20-carboxy Leukotriene B4	15	40246				1006	603.68	301.84	100.61	30.18	10.06	3.02	1.01	0.30	0.10	0.03	0.01
20-hydroxy Leukotriene B4	14	39716				993	595.74	297.87	99.29	29.79	9.93	2.98	0.99	0.30	0.10	0.03	0.01
6-trans Leukotriene B4	13	39376				984	590.64	295.32	98.44	29.53	9.84	2.95	0.98	0.30	0.10	0.03	0.01
Lipoxin A4	14	39716				993	595.74	297.87	99.29	29.79	9.93	2.98	0.99	0.30	0.10	0.03	0.01
Maresin 1	15	40222				1006	603.33	301.66	100.55	30.17	10.06	3.02	1.01	0.30	0.10	0.03	0.01
7(S),17(S)-dihydroxy-8(E),10(Z),13(Z),15(E),19(Z)-Docosapentaenoic Acid	15	40000				1000	600.00	300.00	100.00	30.00	10.00	3.00	1.00	0.30	0.10	0.03	0.01
9-OxoODE	12	39912				998	598.68	299.34	99.78	29.93	9.98	2.99	1.00	0.30	0.10	0.03	0.01
13-OxoODE	12	39912				998	598.68	299.34	99.78	29.93	9.98	2.99	1.00	0.30	0.10	0.03	0.01
5-OxoETE	13	40031				1001	600.47	300.24	100.08	30.02	10.01	3.00	1.00	0.30	0.10	0.03	0.01
12-OxoETE	13	40031				1001	600.47	300.24	100.08	30.02	10.01	3.00	1.00	0.30	0.10	0.03	0.01
15-OxoETE	13	40031				1001	600.47	300.24	100.08	30.02	10.01	3.00	1.00	0.30	0.10	0.03	0.01
(±)9,10-DiHOME	13	39746				994	596.18	298.09	99.36	29.81	9.94	2.98	0.99	0.30	0.10	0.03	0.01
(±)12,13-DiHOME	13	39746				994	596.18	298.09	99.36	29.81	9.94	2.98	0.99	0.30	0.10	0.03	0.01
(±)5,6-DHET	14	39882				997	598.23	299.11	99.70	29.91	9.97	2.99	1.00	0.30	0.10	0.03	0.01
(±)8,9-DHET	14	39882				997	598.23	299.11	99.70	29.91	9.97	2.99	1.00	0.30	0.10	0.03	0.01
(±)11,12-DHET	14	39882				997	598.23	299.11	99.70	29.91	9.97	2.99	1.00	0.30	0.10	0.03	0.01
(±)14,15-DHET	14	39882				997	598.23	299.11	99.70	29.91	9.97	2.99	1.00	0.30	0.10	0.03	0.01
5,6-DiHETE	14	40119				1003	601.78	300.89	100.30	30.09	10.03	3.01	1.00	0.30	0.10	0.03	0.01
5(S),15(S)-DiHETE	14	40119				1003	601.78	300.89	100.30	30.09	10.03	3.01	1.00	0.30	0.10	0.03	0.01
8(S),15(S)-DiHETE	14	40119				1003	601.78	300.89	100.30	30.09	10.03	3.01	1.00	0.30	0.10	0.03	0.01
(±)14,15-DiHETE	14	40119				1003	601.78	300.89	100.30	30.09	10.03	3.01	1.00	0.30	0.10	0.03	0.01
(±)17,18-DiHETE	14	40119				1003	601.78	300.89	100.30	30.09	10.03	3.01	1.00	0.30	0.10	0.03	0.01
9(10)-EpOME	12	39629				991	594.44	297.22	99.07	29.72	9.91	2.97	0.99	0.30	0.10	0.03	0.01
12(13)-EpOME	12	39629				991	594.44	297.22	99.07	29.72	9.91	2.97	0.99	0.30	0.10	0.03	0.01
5(6)-EET	13	39782				995	596.72	298.36	99.45	29.84	9.95	2.98	0.99	0.30	0.10	0.03	0.01
8(9)-EET	13	39782				995	596.72	298.36	99.45	29.84	9.95	2.98	0.99	0.30	0.10	0.03	0.01

Appendix II. Continued

Product name	c Stock (ug/mL)	c Stock (nM)	SM4000	SM3000	SM2000	SM1000 G1 (0.025 mL) + G2, G3 and G4 (0.25 mL each)	SM600	SM300	SM100	SM30	SM10	SM3	SM1	SM0.3	SM0.1	SM0.03	SM0.01
solutions used	-	-	stock G1	stock G1	stock G1 G2.1		SM1000	SM600	SM1000	SM300	SM100	SM30	SM10	SM3	SM1	SM0.3	SM0.1
Volume (mL)			0.1	0.075	0.55	3.525	3	2.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Volume IS			9.9	9.925	9.45	6.475	2	2.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5
Final volume	-	-	10	10	10	10	5	5	5	5	5	5	5	5	5	5	5
11(12)-EET	13	39782				995	596.72	298.36	99.45	29.84	9.95	2.98	0.99	0.30	0.10	0.03	0.01
14(15)-EET	13	39782				995	596.72	298.36	99.45	29.84	9.95	2.98	0.99	0.30	0.10	0.03	0.01
8(9)-EpETE	13	40031				1001	600.47	300.24	100.08	30.02	10.01	3.00	1.00	0.30	0.10	0.03	0.01
11(12)-EpETE	13	40031				1001	600.47	300.24	100.08	30.02	10.01	3.00	1.00	0.30	0.10	0.03	0.01
14(15)-EpETE	13	40031				1001	600.47	300.24	100.08	30.02	10.01	3.00	1.00	0.30	0.10	0.03	0.01
17(18)-EpETE	13	40031				1001	600.47	300.24	100.08	30.02	10.01	3.00	1.00	0.30	0.10	0.03	0.01
7(8)-EpDPA	14	39913				998	598.69	299.35	99.78	29.93	9.98	2.99	1.00	0.30	0.10	0.03	0.01
10(11)-EpDPA	14	39913				998	598.69	299.35	99.78	29.93	9.98	2.99	1.00	0.30	0.10	0.03	0.01
13(14)-EpDPA	14	39913				998	598.69	299.35	99.78	29.93	9.98	2.99	1.00	0.30	0.10	0.03	0.01
16(17)-EpDPA	14	39913				998	598.69	299.35	99.78	29.93	9.98	2.99	1.00	0.30	0.10	0.03	0.01
19(20)-EpDPA	14	39913				998	598.69	299.35	99.78	29.93	9.98	2.99	1.00	0.30	0.10	0.03	0.01
Prostaglandin D1	14	40298				1007	604.47	302.24	100.75	30.22	10.07	3.02	1.01	0.30	0.10	0.03	0.01
Prostaglandin D2	14	40527				1013	607.90	303.95	101.32	30.40	10.13	3.04	1.01	0.30	0.10	0.03	0.01
Prostaglandin D3	14	40758				1019	611.37	305.69	101.90	30.57	10.19	3.06	1.02	0.31	0.10	0.03	0.01
Prostaglandin E1	14	40298				1007	604.47	302.24	100.75	30.22	10.07	3.02	1.01	0.30	0.10	0.03	0.01
Prostaglandin E2	14	40527				1013	607.90	303.95	101.32	30.40	10.13	3.04	1.01	0.30	0.10	0.03	0.01
Prostaglandin E3	14	40758				1019	611.37	305.69	101.90	30.57	10.19	3.06	1.02	0.31	0.10	0.03	0.01
Prostaglandin B2	13	38864				972	582.96	291.48	97.16	29.15	9.72	2.91	0.97	0.29	0.10	0.03	0.01
13,14-dihydro-15-keto Prostaglandin E2	14	39716				993	595.74	297.87	99.29	29.79	9.93	2.98	0.99	0.30	0.10	0.03	0.01
13,14-dihydro-15-keto Prostaglandin D2	14	39716				993	595.74	297.87	99.29	29.79	9.93	2.98	0.99	0.30	0.10	0.03	0.01
13,14-dihydro-15-keto Prostaglandin F2α	14	39716				993	595.74	297.87	99.29	29.79	9.93	2.98	0.99	0.30	0.10	0.03	0.01
11β-Prostaglandin E2	14	39716				993	595.74	297.87	99.29	29.79	9.93	2.98	0.99	0.30	0.10	0.03	0.01
6-keto Prostaglandin E1	15	40706				1018	610.58	305.29	101.76	30.53	10.18	3.05	1.02	0.31	0.10	0.03	0.01
8-iso Prostaglandin E2	14	39716				993	595.74	297.87	99.29	29.79	9.93	2.98	0.99	0.30	0.10	0.03	0.01
15-deoxy-Δ12,14-Prostaglandin J2	13	41087				1027	616.31	308.15	102.72	30.82	10.27	3.08	1.03	0.31	0.10	0.03	0.01
8-iso-15-keto Prostaglandin F2α	14	39492				987	592.38	296.19	98.73	29.62	9.87	2.96	0.99	0.30	0.10	0.03	0.01
6-keto Prostaglandin F2α	14	39492				987	592.38	296.19	98.73	29.62	9.87	2.96	0.99	0.30	0.10	0.03	0.01
Prostaglandin F1α	15	40486				1012	607.29	303.64	101.21	30.36	10.12	3.04	1.01	0.30	0.10	0.03	0.01
Thromboxane B2	15	40486				1012	607.29	303.64	101.21	30.36	10.12	3.04	1.01	0.30	0.10	0.03	0.01
11-dehydro Thromboxane B2	15	40706				1018	610.58	305.29	101.76	30.53	10.18	3.05	1.02	0.31	0.10	0.03	0.01

Appendix III. 110 eicosanoid and fatty acid transitions. Eicosanoids Identified in samples are highlighted. Multiple reaction monitoring (MRM), deionising potential (DP), collision energy (CE), retention time (RT), internal standard (IS).

No.	Compound Name	MRM Transitions		DP	CE	RT (min)	IS
		Q1	Q3				
1	Lauric Acid	199.3	199.301	-52	-10	14.46	Arachidonic Acid-d8
2	Myristic Acid	227.4	227.401	-54	-10	16.41	Arachidonic Acid-d8
3	Palmitic Acid	255.4	255.401	-55	-10	17.72	Linoleic Acid-d4
4	Palmitoleic Acid	253.4	253.401	-58	-10	16.74	Linoleic Acid-d4
5	Stearic Acid	283.5	283.501	-70	-10	18.8	Linoleic Acid-d4
6	Oleic Acid	281.5	281.501	-65	-10	17.9	Linoleic Acid-d4
7	Linoleic Acid	279.5	279.501	-65	-10	17.1	Arachidonic Acid-d8
8	α-Linolenic Acid	277.4	277.401	-70	-10	16.26	Linoleic Acid-d4
9	Stearidonic Acid	275.4	275.401	-75	-10	15.5	Linoleic Acid-d4
10	Arachidic Acid	311.4	311.401	-60	-10	19.94	Linoleic Acid-d4
11	Dihomo-γ-Linolenic Acid	305.5	305.501	-70	-10	17.42	Arachidonic Acid-d8
12	Arachidonic Acid	303.5	303.501	-65	-10	16.9	Arachidonic Acid-d8
13	Eicosapentaenoic Acid	301.5	301.501	-55	-10	16.17	Arachidonic Acid-d8
14	Docosahexaenoic Acid	327.2	327.201	-45	-10	16.72	Arachidonic Acid-d8
15	(±)5-HETE	319.2	115.2	-55	-19	14.4	5(S)-HETE-d8
16	(±)8-HETE	319.2	115.2	-55	-19	14.4	5(S)-HETE-d8
17	(±)9-HETE	319.2	155.201	-65	-18	14.1	5(S)-HETE-d8
18	(±)11-HETE	319.2	167.2	-50	-20	14.27	12(S)-HETE-d8
19	(±)12-HETE	319.2	167.202	-60	-19	13.91	12(S)-HETE-d8
20	(±)15-HETE	319.2	179.2	-65	-18	14.11	15(S)-HETE-d8
21	20-HETE	319.2	219.2	-55	-18	13.65	20-HETE-d6
22	(±)5-HEPE	319.2	275.1	-85	-21	12.64	5(S)-HETE-d8
23	(±)8-HEPE	317.2	115.1	-60	-20	13.17	5(S)-HETE-d8
24	(±)9-HEPE	317.2	155.2	-65	-19	12.8	5(S)-HETE-d8
25	(±)11-HEPE	317.5	167.2	-50	-18	12.99	12(S)-HETE-d8
26	(±)12-HEPE	317.2	167.201	-50	-20	12.69	12(S)-HETE-d8
27	(±)15-HEPE	317.2	179.2	-65	-18	12.91	15(S)-HETE-d8
28	(±)18-HEPE	317.2	219.2	-65	-16	12.63	15(S)-HETE-d8
29	(±)4-HDOHE	317.2	259.2	-50	-15	12.25	5(S)-HETE-d8
30	(±)7-HDOHE	343.2	101.1	-50	-17	14.66	5(S)-HETE-d8
31	(±)7-HDOHE	343.2	101.1	-50	-17	14.66	5(S)-HETE-d8
32	(±)8-HDOHE	343.2	189.2	-50	-19	14.31	5(S)-HETE-d8
33	(±)10-HDOHE	343.2	153.201	-55	-21	13.99	12(S)-HETE-d8
34	(±)11-HDOHE	343.2	121.1	-60	-18	14.14	12(S)-HETE-d8
35	(±)13-HDOHE	343.2	193.1	-55	-19	13.87	12(S)-HETE-d8
36	14(S)-HDOHE	343.2	205.2	-45	-17	13.99	15(S)-HETE-d8
37	(±)16-HDOHE	343.2	233.201	-55	-17	13.73	15(S)-HETE-d8
38	(±)17-HDOHE	343.2	201.2	-70	-15	13.79	20-HETE-d6
39	(±)20-HDOHE	343.2	241.201	-55	-17	13.47	20-HETE-d6
40	(±)9-HODE	295.2	171.1	-85	-23	13.34	13(S)-HODE-d4
41	(±)13-HODE	295.2	195.2	-85	-23	13.28	13(S)-HODE-d4
42	9(S)-HOTrE	293.2	171.2	-60	-20	12	13(S)-HODE-d4
43	13(S)-HOTrE	293.2	195.101	-70	-22	12.2	13(S)-HODE-d4
44	5(S)-HETrE	321.2	115.1	-70	-19	15.49	13(S)-HODE-d4
45	15(S)-HETrE	321.2	221.2	-70	-21	14.29	13(S)-HODE-d4

Appendix III. Continued

No.	Compound Name	MRM Transitions		DP	CE	RT (min)	IS
		Q1	Q3				
46	9-OxoODE	293.2	185.1	-85	-23	14	13(S)-HODE-d4
47	13-OxoODE	293.2	195.1	-85	-25	13.72	13(S)-HODE-d4
48	5-OxoETE	317.2	273.2	-65	-20	15.06	5(S)-HETE-d8
49	12-OxoETE	317.2	153.1	-75	-20	14.36	5(S)-HETE-d8
50	15-OxoETE	317.2	113.1	-60	-22	14	5(S)-HETE-d8
51	(±)9,10-DiHOME	313.2	201.2	-80	-29	10.9	Leukotriene B4-d4
52	(±)12,13-DiHOME	313.2	183.2	-80	-28	10.62	Leukotriene B4-d4
53	(±)5,6-DHET (DiHETrE)	337.2	145.1	-75	-24	12.64	15(S)-HETE-d8
54	(±)8,9-DHET (DiHETrE)	337.2	127.1	-70	-25	12.14	15(S)-HETE-d8
55	(±)11,12-DHET (DiHETrE)	337.2	167.1	-65	-26	11.79	15(S)-HETE-d8
56	(±)14,15-DHET (DiHETrE)	337.2	207.1	-65	-25	11.45	15(S)-HETE-d8
57	5,6-DiHETE	335.2	115.2	-60	-23	11.2	Leukotriene B4-d4
58	5(S),15(S)-DiHETE	335.3	115.2	-60	-21	9.92	Leukotriene B4-d4
59	8(S),15(S)-DiHETE	335.2	235.2	-65	-22	9.63	Leukotriene B4-d4
60	(±)14,15-DiHETE	335.301	207.2	-65	-23	10.35	Leukotriene B4-d4
61	(±)17,18-DiHETE	335.3	247.2	-65	-24	9.97	Leukotriene B4-d4
62	Resolvin E1	349.3	195.1	-65	-22	3.21	Resolvin D1-d5
63	Resolvin D1	375.5	215.1	-55	-23	7.47	Resolvin D1-d5
64	Resolvin D2	375.2	141.2	-65	-21	6.8	Resolvin D1-d5
65	Resolvin D3	375.2	147.1	-65	-24	6.49	Resolvin D1-d5
66	Resolvin D5	359.2	199.1	-65	-22	10.09	Resolvin D1-d5
67	Leukotriene B3	337.2	195.2	-65	-22	11.5	Leukotriene B4-d4
68	Leukotriene B4	335.2	195.1	-70	-23	10.22	Leukotriene B4-d4
69	20-carboxy Leukotriene B4	365.2	347.2	-80	-25	3.24	Leukotriene B4-d4
70	20-hydroxy Leukotriene B4	351.2	195.2	-80	-25	3.55	Leukotriene B4-d4
71	6-trans Leukotriene B4	335.2	195.101	-65	-23	9.89	Leukotriene B4-d4
72	Lipoxin A4	351.2	115.2	-55	-19	7.32	Resolvin D1-d5
73	Maresin 1	359.5	250.2	-60	-23	10.1	Leukotriene B4-d4
74	7(S),17(S)-dihydroxy-8(E),10(Z),13(Z),15(E),19(Z)-Docosapentaenoic Acid	361.5	263.3	-65	-20	10.38	Leukotriene B4-d4
75	9(10)-EpOME	295.3	171.1	-80	-21	14.86	11(12)-EET (EpETrE) -d11
76	12(13)-EpOME	295.3	195.2	-80	-19	14.74	11(12)-EET (EpETrE) -d11
77	5(6)-EET (EpETrE)	319.2	191.1	-60	-16	15.37	11(12)-EET (EpETrE) -d11
78	8(9)-EET (EpETrE)	319.3	167.201	-60	-15	15.15	11(12)-EET (EpETrE) -d11
79	11(12)-EET (EpETrE)	319.3	167.2	-60	-18	15.15	11(12)-EET (EpETrE) -d11
80	14(15)-EET (EpETrE)	319.2	219.3	-65	-18	14.84	11(12)-EET (EpETrE) -d11
81	8(9)-EpETE	317.2	127.2	-70	-18	14.2	11(12)-EET (EpETrE) -d11
82	11(12)-EpETE	317.2	167.2	-70	-15	14.12	11(12)-EET (EpETrE) -d11
83	14(15)-EpETE	317.2	207.2	-70	-18	14.04	11(12)-EET (EpETrE) -d11
84	17(18)-EpETE	317.2	215.2	-75	-16	13.7	11(12)-EET (EpETrE) -d11
85	7(8)-EpDPA (EpDPE)	343.2	113.1	-60	-16	15.2	11(12)-EET (EpETrE) -d11
86	10(11)-EpDPA (EpDPE)	343.2	153.2	-65	-15	15.08	11(12)-EET (EpETrE) -d11
87	13(14)-EpDPA (EpDPE)	343.2	193.2	-70	-15	15.02	11(12)-EET (EpETrE) -d11
88	16(17)-EpDPA (EpDPE)	343.2	233.2	-55	-16	14.97	11(12)-EET (EpETrE) -d11
89	19(20)-EpDPA (EpDPE)	343.2	241.2	-70	-18	14.71	11(12)-EET (EpETrE) -d11
90	PGD1	353.3	317.202	-55	-16	6.65	Prostaglandin D2-d4

Appendix III. Continued

No.	Compound Name	MRM Transitions		DP	CE	RT (min)	IS
		Q1	Q3				
91	PGD2	351.2	271.302	-50	-22	6.61	Prostaglandin D2-d4
92	PGD3	349.3	269.201	-50	-17	5.26	Prostaglandin D2-d4
93	PGE1	353.3	317.2	-60	-18	6.53	Prostaglandin E2-d4
94	PGE2	351.2	271.3	-60	-19	6.2	Prostaglandin E2-d4
95	PGE3	349.3	269.2	-60	-17	4.86	Prostaglandin E2-d4
96	PGB2	333.3	175.1	-60	-24	8.82	Prostaglandin D2-d4
97	13,14-dihydro-15-keto PGE2	351.2	235.2	-55	-19	7.33	Prostaglandin E2-d4
98	13,14-dihydro-15-keto PGD2	351.5	207.2	-50	-25	8.16	Prostaglandin D2-d4
99	13,14-dihydro-15-keto PF2α	353.501	113.001	-55	-23	7.43	Prostaglandin F2α-d4
100	11β-PGE2	351.2	271.2	-55	-23	6.38	Prostaglandin E2-d4
101	6-keto PGE1	367.2	143.1	-55	-23	3.22	Prostaglandin E2-d4
102	8-iso PGE2	351.201	271.001	-55	-21	5.94	Prostaglandin E2-d4
103	15-deoxy-Δ12,14-PGJ2	315.2	271.2	-65	-18	12.44	20-HETE-d6
104	8-iso-15-keto PGF2α	351.2	289.2	-50	-23	5.37	Prostaglandin F2α-d4
105	PGF2α	353.2	309.2	-85	-24	5.89	Prostaglandin F2α-d4
106	6-keto PGF1α	369.3	163.2	-75	-26	3.3	Prostaglandin F2α-d4
107	Thromboxane B2	369.2	169.1	-60	-22	4.83	Thromboxane B2-d4
108	11-dehydro Thromboxane B2	367.2	305.2	-60	-20	6.24	11-dehydro Thromboxane B2-d4
109	Maresin 1	359.5	250.2	-60	-23	10.1	Leukotriene B4-d4
110	DXA3	351.2	165.1	-45	-22	9.7	Thromboxane B2-d4

Appendix lV. Participant information sheet.

PARTICIPANT INFORMATION SHEET

Name of Project: Eicosanoid and cytokine responses to bacterial infection

Name of Researcher: Shayda Maleki-Toyserkani
School of Pharmacy & Pharmaceutical Sciences
Cardiff University
Redwood Building
King Edward Vll Avenue
Cardiff CF10 3NB

Part 1 – Introduction

We would like to invite you to take part in our research study. Before you decide we would like you to understand why the research is being done and what it would involve for you. One of our team will go through this information sheet with you and answer any questions you have. We suggest this should take about 10 minutes. Talk to others about the study if you wish.

You are being asked to give blood samples of up to a 100 ml (20 teaspoons of blood). We need regular blood samples, but any help will be very useful; from just once to every few weeks (a maximum of 1000 ml may be donated each year).

Part 1 tells you the purpose of this study and what will happen to you if you take part. Part 2 of this information sheet gives you more detailed information about the conduct of the study. Ask us if there is anything that is not clear.

What is the purpose of the study and why have I been invited?

The group investigates how our immune defences in the blood (such as white blood

cells) protect us against invading micro-organisms. In this study, we use your blood samples to look at blood cells (such as neutrophils, monocytes or platelets) or other factors in the plasma – these blood components will then be mixed with bacteria or viruses (or related materials) and laboratory tests will be carried out. The investigations involve microscopy, immunological assays and biochemistry to study molecules on the surface of these cells, inside the cells and molecules released by the cells. If you wish to know more, full experimental details are available from the lead researcher. If you wish to see the results of this study, please request this and the lead researcher will provide you with them when the results are published.

Do I have to take part?

No. It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form (and you may have a copy). If you are a member of staff or a student at Cardiff University, your career or degree will not be affected by choosing not to participate. If you decide to take part you are still free to withdraw at any time and without giving a reason and without your legal rights being affected. If you have a concern about any aspect of this study, you should ask to speak to the researchers who will do their best to answer your questions (Contact Shayda Maleki-Toyserkani Maleki-ToyserkaniSR@Cardiff.ac.uk). If you remain unhappy and wish to complain formally, you can do this. Details can be obtained from Cardiff Research Innovation and Enterprise Services, 029 2087 5834, 30-36 Newport Road Cardiff CF24 0DE.

If you later withdraw your consent your tissue will not be used further in this study and will be destroyed according to locally approved practices. Any tissue, or results derived from the tissue, that has already been used prior to the withdrawal of consent will continue to be used in this study.

Are there any risks?

Blood samples will only be taken by trained phlebotomists or health care professionals, thereby minimising any risks. There may be minor irritation following blood donation, but this should pass after a few hours. There is a small risk that you

will faint during blood (reported as being 2%, but this is much lower in our experience) – we ask you to sit down during donation so that there is very little risk of injury after a faint.

If the information in Part 1 has interested you and you are considering participation, please read the additional information in Part 2 before making any decision.

Part 2 – Further details

What will happen to me if I take part and what do I have to do?

You are being requested to give blood samples for experiments in the laboratory. Once you have had time to read this information sheet and had answered to your satisfaction any questions that you might have, you will be asked to sign a consent form. At a later date, Shayda or a designated member of the laboratory team will contact you to arrange a donation. Times for donating a sample will be arranged with you personally and at your convenience. We will take blood in the Redwood building and this should not take more than 20 minutes.

The volume of any single blood sample will be up to a max of 100 ml (20 teaspoons of blood). There will be a period of at least 2 weeks between requests for blood samples. A maximum of 1000 ml may be taken each year. This assumes that you are of average size and that you are not giving blood for any other reason. If you are under 65kg (10st 3lb) and/or are under 168cm (5' 6") in height, we would use the National Blood Services Blood Volume Calculator to determine how much you can donate. http://www.blood.co.uk/giving-blood/who-can-give-blood/

We request that if we have cells or material left after a planned experiment, you allow us to store this for subsequent use for this project. We also ask that we can store these samples after this study has ended, so we can repeat assays or for future projects relating to immune responses. There is a separate section of the consent form to indicate if you are happy that your tissue may be retained at the end of this study for up to 15 years for use in future research within the UK and abroad. Your tissue will not be sold. Any/all tissue will be supplied anonymously to researchers. The recipients

of the tissue will not be supplied with your name or any other identifiable information and will not be able to identify you from the tissue.

Who can take part?

We ask that you not give blood if you have consumed alcohol in the last 12 hours or if you are on any medication (you do not need to discuss what this medication might be). Please do not give blood if you have any health condition or disease, including anaemia. If you are under 65kg (10st 3lb) and are under 168cm (5' 6") in height, you might not be able to take part. You may refuse to give a blood sample at any time without giving a reason.

What about confidentiality?

All samples and data will be anonymised. To ensure you do not exceed the maximum donation volume, your name, date and volume of donation will be recorded in a password protected file accessible only to Shayda Maleki-Toyserkani and Dr Stephen Clark.

We will not be performing any tests that could reveal information about your health and we will not be looking at any validated risk factors for disease (genetic or otherwise). It is very unlikely, but possible, that we might notice that someone has a very high or very low number of platelets or white cells or red cells. If this happened, we would let you know and suggest that you contact your GP for a blood count.

What will happen to the results of the research study?

If you wish to see the results of this study, they are available on request from the lead researcher. Ultimately, results of this research will be published as articles in peer-reviewed scientific journals. If you are interested, the lead researcher will provide you with a copy of the manuscript. All results will be anonymous in any publication.

If you have understood all the information above and wish to participate in this study, you will be asked to sign a CONSENT FORM. *Thank you for considering taking part in this study and taking time to read this information sheet.*

Appendix V. Consent form

School of Pharmacy & pharmaceutical Sciences

Consent Form

Name of Project: Eicosanoid Response to Infection in Sepsis

Name of Researcher: Shayda Maleki-Toyserkani
School of Pharmacy & Pharmaceutical Sciences
Cardiff University
Redwood Building
King Edward Vll Avenue
Cardiff CF10 3NB

Part A, The Current Project

Please **Initial** Boxes

1. I confirm that I have read and understand the information sheet dated 1th June 2015 (version 6) for the above study and have had the opportunity to ask questions and these have been answered satisfactorily. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason & without my legal rights being affected. ☐
3. I understand that, if I withdraw my consent, any tissue or results derived from the tissue that has already been used will continue to be used in this study. ☐
4. I confirm that as far as I am aware, I am healthy and have no underlying conditions.
5. I agree to take part in the above study. ☐

Part B, Future Use

1. I agree that excess tissue can be stored for subsequent use for this project. ☐
2. I agree that excess tissue can be stored anonymously for up to 15 years for use in future research. ☐

You may contact me by email _____

to arrange a donation.

_____	_____	_____
Name of Participant	Date	Signature
_____	_____	_____
Name of person taking consent (If different from researcher)	Date	Signature
Shayda Maleki-Toyserkani	_____	_____
Researcher	Date	Signature