

**THE ROLE OF LPCAT IN REGULATING CELLULAR  
INFLAMMATORY RESPONSES TO TOLL-LIKE  
RECEPTOR LIGANDS**

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## **Author's Declaration**

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Signed .....J A HUGHES..... Date .....12/05/2014.....

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## Abstract

A novel lipid modifying enzyme, lysophosphatidylcholine acyltransferase (LPCAT), has been shown to regulate pro-inflammatory cytokine responses to lipopolysaccharide (LPS), a key molecule in initiating sepsis. Current research has shown that phospholipid metabolism may influence cell signalling, since that is dependent on the clustering of the receptor molecules into membrane microdomains (lipid rafts). LPCAT therefore, may be a target for novel anti-sepsis therapies.

This study aimed to determine if LPCAT is essential in the regulation of pro-inflammatory cytokine responses to Gram-positive cell bacterial components, such as peptidoglycan (PG), lipoteichoic acid (LTA) and synthetic tripalmitoylated lipopeptide Pam<sub>3</sub>CSK<sub>4</sub> in monocytes and lung epithelial cells. Inhibition of LPCAT activity consistently reduced TNF- $\alpha$ , IL-6 and IL-8 protein and mRNA levels in monocytes stimulated with all microbial ligands tested. Furthermore, cytokine production observed in monocytes primed with IFN- $\gamma$  prior to ligand stimulation was twice that seen in unprimed cells, yet still inhibition of LPCAT significantly decreased the amplified inflammatory response. Lung epithelial cells, BEAS-2B, were more immunologically responsive to Pam<sub>3</sub>CSK<sub>4</sub> where it consistently induced a high secretion of IL-8 and IL-6. However, whilst inhibition of LPCAT demonstrated a reduction in cytokine secretion, it was not a predominant as observed in monocytes indicating that LPCAT may have a lesser role in these cells.

The secondary aim was to characterise the expression levels of 5 LPCAT enzymes in diverse cell types to ascertain if there is differential expression of the LPCATs, which may help to explain the altered effects that LPCAT inhibition produces on inflammatory cytokine production between cell types. Data in this report did not demonstrate one particular LPCAT iso-form to be highly expressed by monocytes, however literature has suggested that LPCAT2 might be the enzyme to modulate the inducible phospholipid remodeling pathways in innate immune cells. Further studies on inducible LPCAT expression in human cell lines is required to support these ideas.

Previous studies have observed LPCAT to regulate translocation of TLR4 into membrane lipid raft domains and subsequent down-stream inflammatory responses. Although the translocation of TLR2 into membrane lipid rafts has been researched, its regulation by LPCAT has not, thus it was investigated in this study. Complications with experiments, however, did not allow this to be adequately assessed but, literature suggests this is the likely mechanism that LPCAT elicits its immuno-regulatory effect in response to microbial stimuli.

The present study provided evidence that LPCAT influences the complex network of cell signalling involved in microbial responses, underlying its importance in inflammatory responses and potentially offering a target for novel anti-sepsis therapies.

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## Abbreviations

AA	Arachidonic acid
APC	Activated protein C.
CD	Cluster of differentiation
CXCR	Chemokine receptor
CI-976	2,2-methyl-N-(2,4,6-trimethoxyphenyl)dodecanamide
EDTA	Ethylene diamine tetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
FCS	Foetal calf serum
GDF	Growth differentiation factor
HETP	5-Hydroxyethyl 5,3' thiophenyl pyridine
HS	Human pooled serum
Hsp	Heat shock protein
IC <sub>50</sub>	50% inhibitory concentration
IFN- $\gamma$	Interferon-gamma
IKK	inhibitor of NF- $\kappa$ B kinase
IL	Interleukin
IRAK	IL-1 receptor-associated kinase
IRF-3	Interferon regulatory factor 3
LBP	Lipopolysaccharide-binding protein
LPS	Lipopolysaccharide
LPAAT	Lysophosphatidic acid acyltransferase
LPCAT	Lysophosphatidylcholine acyltransferase
LPEAT	Lysophosphatidylethanolamine acyltransferase
LPGAT	Lysophosphatidylglycerol acyltransferase
LPSAT	Lysophosphatidylserine acyltransferase
LysoPA	Lysophosphatidic acid
LysoPC	Lysophosphatidylcholine
LysoPE	Lysophosphatidylethanolamine
LysoPG	Lysophosphatidylglycerol
LysoPI	lysophosphatidylinositol
LysoPS	Lysophosphatidylserine
LTA	Lipoteichoic acid

MAL	MyD88-adaptor-like protein
MAPK	Mitogen-activated protein kinase
MBOAT	Membrane bound O-acyltransferase
MD-2	Myeloid differentiation protein 2
MOPS	3-(N-morpholino) propane sulfonic acid
MSR	Macrophage scavenger receptor
MyD88	Myeloid differentiation protein 88
NF- $\kappa$ B	nuclear factor- $\kappa$ B
PA	Phosphatidic acid
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PG	Peptidoglycan
PS	Phosphatidylserine
PAF	Platelet activating factor
PAMP	Pathogen associated molecular pattern
Pam <sub>3</sub> CSK <sub>4</sub>	Palmitoyl-3-cysteine-serine-lysine-4
PKR	RNA-dependent protein kinase
PLA2	Phospholipase A2
qPCR	Real-time – polymerase chain reaction
SDS	Sodium Dodecyl Sulphate
TAK1	Transforming growth factor- $\beta$ -activated kinase 1
TBK1	TANK-binding kinase 1
TBS	Tris-buffered saline
TGF- $\beta$	Transforming growth factor- $\beta$
TIR	Toll-like receptor / IL-1receptor homology domain
TIRAP	TIR domain-containing adaptor protein
TLR	Toll-like receptor
TNF- $\alpha$	Tumour necrosis factor-alpha
TRAF	TNF receptor-associated factor
TRAM	TRIF-related adaptor molecule
TRIF	TIR domain-containing adaptor protein inducing IFN- $\beta$
Tollip	Toll-interacting protein

# Chapter 1

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INTRODUCTION

### 1.1 A Summary of the Innate and Adaptive Immune Systems

The human body is under constant attack from foreign invaders, such as microbes and multicellular organisms, yet encounters with these infectious agents rarely result in disease. The body has evolved a sophisticated mechanism known as immunity, to protect itself from such invaders, including disease causing microbes termed pathogens. The immune system is the collection of cells, tissues and molecules that protects the body from numerous pathogenic microbes and toxins in the environment. This defence against pathogens has been divided into two types of responses: innate immunity and adaptive immunity. The innate immune system is the first line of defence against pathogens and consists of cells and proteins that are always present and ready to mobilize and fight microbes at the site of infection. The main components of innate immunity are physical epithelial barriers, phagocytic leukocytes, dendritic cells (DCs), natural killer cells (NK) and circulating complement proteins and lysozymes (Akira *et al.*, 2006). The adaptive immune system is very dependent upon the innate immune system to receive guidance on what to respond against and what not to respond against thus is involved in the elimination of pathogens in the later phases of infection. Components of the adaptive immune system are normally dormant, however, when activated these components “adapt” to the presence of infectious agents by activating, proliferating and creating potent mechanisms for neutralizing or eliminating pathogens (Riedemann *et al.*, 2003).

The innate immune system has a major role to recognise pathogens for removal via phagocytic cells, such as monocytes, macrophages and neutrophils. For over 50 years immunologists have based views, experiments and clinical treatments on the theory that the immune system functions by discriminating between “self” and “non-self”, with leukocytes responding to any molecules they identify as foreign and no immune response triggered against self-constituents (Burnet and Fenner, 1949; Burnet, 1969; Tauber, 1994). This model has assisted the area of research well and helped to characterise pattern-recognition receptors (PRRs) present on phagocytes for the identification of microbial components, known as pathogen-associated molecular patterns (PAMPs) (Janway, 1989, 1992). However, years of detailed examination have revealed a number of inherent problems with the self-non-self theory and

questions, such as how does the immune system tolerate commensal microorganisms or why do some self-derived molecules trigger immune responses, arose?

Aside from infection, there is a growing recognition that tissue damage, leading to non-physiological cell death, can also provoke activation of the immune system. More recently, Polly Matzinger suggested an opposing theory, called the “danger theory” (Matzinger, 1994, 2001, 2002). The danger model suggests that the immune system will respond to molecules that enter the body and cause damage, activating the damaged tissues to release immune-stimulating alarm signals. These danger or alarm signals, termed danger-associated molecular patterns (DAMPs), are self-derived molecules normally found within healthy cells, but are detected in extracellular space when they are released by cells that die via an uncontrolled mode of cell death, called necrosis. Thus the model proposes that self-constituents can trigger an immune response if they are dangerous (e.g. cellular stress) and non-self constituents can be tolerated if they are not dangerous (e.g. commensal bacteria). HMGB1, a chromatin-binding protein, interleukin-1 $\alpha$  (IL-1 $\alpha$ ) and interleukin-33 (IL-33) are DAMPs known to activate the immune system (Matzinger, 2012). The scientific community has now accepted that recognition of both PAMPs and DAMPs may act synergistically to provoke more robust and effective immune responses than would occur in response to either alone (Pradeu and Cooper, 2012).

A major player in the initiation of innate immune responses is the macrophage. These cells are relatively abundant in most tissues and patrol the environment around them, looking for infectious agents through an array of pattern recognition receptors (PRRs). PRRs such as Toll-like receptors (TLRs) recognize both small, conserved structural components of pathogens (PAMPs) and self-derived DAMPs (Batista and Harwood, 2009). When activated, these receptors cause the stimulating PAMP to be endocytosed and cause multiple changes within the macrophage. Firstly the macrophage increases its ability to engulf and kill any microorganism it encounters. Secondly it begins to secrete cytokines and chemokines that have immediate effect on nearby endothelial cells lining the blood capillaries to make them more permeable. The increased vascular permeability allows plasma proteins and neutrophils that are normally restricted to the blood (due to their potentially destructive behaviour) to infiltrate the tissue at the site of infection (Harwood and Batista, 2010).

Dendritic cells (DCs) also express PRRs on their surface to recognise invading microorganisms, but unlike macrophages, DCs do not stand and fight pathogens directly. Instead DCs play a key role in awakening the adaptive immune systems as antigen presenting cells (APCs) (Janeway, 2001). Activated DCs endocytosed the PAMP, break it down into peptides and display the antigen on its surface via MHC II molecules. The APC then synthesizes additional surface molecules that will act as co-stimulators for the activation of T lymphocytes in the adaptive immune system (Kinashi, 2005).

The adaptive immune system is characterized by specificity and can develop humoral and cell-mediated responses, as well as the generation of immunological memory (Remick, 2003). The main components of adaptive immunity are B cells which mature into antibody secreting plasma cells that target antigens for destruction (humoral response) and T cells which mature into effector helper cells and cytotoxic T cells (cell-mediated response). Effective communication and coordination between innate and adaptive immunity, facilitated through multiple intermediaries and complex pathways, is vital for maximal protection against invading pathogens. Activated cytotoxic T cells (and NK cells) directly kill infected cells but helper T cells secrete cytokines that help plasma cells to mount potent antibody responses and assist macrophages in killing phagocytosed microbe (Smith-Garvin *et al.*, 2009). Thus non-lymphoid leukocytes such as macrophages participate in the elimination of microbes in both the innate and adaptive immune responses.

## 1.2 A Summary of Inflammatory Responses

Inflammation is a term given to the series of events that surround an immune response and display a number of characteristics. In the early stages of an immune response, neutrophils are the predominant cell type infiltrating the tissue. Neutrophils, like macrophages, are capable of phagocytosing invading pathogens through their PRRs, attacking and engulfing any microorganism it encounters with gusto (Van der Poll and Opal, 2008). An influx of activated macrophages, neutrophils and mast cells, further amplify the immune response through the release of cytokines, chemokines, complement fragments, histamine and prostaglandins. Release of these inflammatory mediators activate the coagulation and complement cascades, which in turn promote localised edema, increased capillary permeability, blood flow and vasodilation to the

affected area of injury and trigger the influx of more leukocytes to the site of injury to kill pathogens and remove cellular debris (Cohen, 2002). But subsequently this tissue trauma results in an elevated temperature, swelling, redness and painful area on the skin that is characteristic of an inflammatory response. The features of inflammation are the collective consequence of the actions of the inflammatory mediators that trigger vascular permeability and chemotaxis, with the extra fluid that gathers at the site of an infection contributing to the swelling seen and increasing redness of the skin tone (Carlet *et al.*, 2008).

Whilst inflammatory mediators orchestrate a complex network of immune responses designed to limit the spread of infection, they can also trigger localised tissue damage. The vasoactive amino acid histamine, released by activated mast cells, is instrumental in provoking increased permeability of blood vessels, which in turn causes the subsequent migration of neutrophils. Histamine also up-regulates platelet-activating factor and P-selectin expression on vascular endothelial cells, which instigates downstream diapedesis of neutrophils into the tissue (Van der Poll and Opal, 2008). Activation of neutrophils and macrophages release a number of cytokines, such as interleukin-1 $\beta$  (IL-1 $\beta$ ), interleukin-6 (IL-6) interleukin-8 (IL-8) and tumour necrosis factor-alpha (TNF- $\alpha$ ) that cause neutrophils to actively produce a host of cytotoxic substances, including reactive oxygen species, such as superoxide anions, hypochloride, and nitric oxide (NO) (Akira *et al.*, 2006).

Small peptide components from the complement cascade, C3a and C5a, also act directly on neutrophils and macrophages to stimulate respiratory burst during phagocytosis and the subsequent release of more reactive oxygen intermediates, including hydrogen peroxide via activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Bosmann and Ward, 2013). These factors all lead to up to damaged vascular endothelium which triggers the blood coagulation and fibrinolysis pathways, promoting tissue factor expression that enhances the production of thrombin and fibrin. However, if fibrin production is increased significantly, clots can be deposited in small blood vessels and deprive vital organs of oxygen (Glauser *et al.*, 1991; Cohen, 2002).

Although the inflammatory response is a normal, protective part of immunity that helps to prevent infection, if the duration and intensity are not effectively regulated and tissue damage is not limited, it can lead to a response that is more harmful than protective. Complement regulatory proteins, transforming growth factor- $\beta$  (TGF- $\beta$ ) and glucocorticoids, to name just a few are powerful regulators that prevent inflammation from getting out of hand. However, the case in chronic inflammatory responses such as sepsis, inflammation becomes amplified and dysregulated, where the response leads to systemic rather than localised effects.

### 1.3 Sepsis and the dysregulation of the inflammatory responses

Sepsis is a complicated clinical syndrome arising as a consequence of a predominantly cytokine-mediated hyper-inflammatory state, referred to as systemic inflammatory response syndrome (SIRS), which can lead to multiple organ failure and ultimately death (Heumann *et al.*, 1996). Microbial PAMPs such as Gram-negative bacterial lipopolysaccharide (LPS) activate macrophages, neutrophils and endothelial cells to release excessive quantities of tumour necrosis factor-alpha (TNF- $\alpha$ ), interleukin-1 (IL-1 $\beta$ ) and interleukin-6 (IL-6) (Cavaillon, 2003). Whilst release of these pro-inflammatory cytokines would normally enhance host defences by initiating multiple cascades, in sepsis, the homeostatic mechanisms controlling these inflammatory pathways become dysregulated and result in an accumulation of neutrophils in tissues, systemic inflammation and unwanted pathophysiological states at locations away from site of injury (Akira *et al.*, 2006).

A major contribution of elevated LPS-induced inflammatory responses may be the priming of Interferon-gamma (IFN- $\gamma$ ) to enhance the activation state in monocytes and macrophages (Silva & Cohen 1992; Heinzl, 1990; Doherty *et al.*, 1992). IFN- $\gamma$  has shown to sensitise macrophages to become hyper-activated upon subsequent exposure to LPS and produce excessive inflammatory mediators including TNF $\alpha$  and IL-1 $\beta$  (Figure 1.1) (Katschinski *et al.*, 1992; Billiau *et al.*, 1987). Increased plasma levels of IL-1, TNF- $\alpha$  and IFN- $\gamma$  correlate highly with rates of mortality, illustrating the detrimental effect generated by overexpression of these cytokines (Remick, 2003).

Although during normal inflammatory responses, activated neutrophils and macrophages generate substantial amounts of reactive oxygen species (ROS) which



can react with a variety of targets, in sepsis, additional amounts of ROS are released and can be damaging to cells and organs. Excessive ROS are known to induce biochemical changes that lead to an imbalance in the redox system, resulting in the formation of an oxidant state (Glauser *et al.*, 1991). There is evidence that the production of antioxidant enzymes required to regulate these pathways is impaired during sepsis, which appears to intensify SIRS and the downstream events (Bosmann and Ward, 2013). In sepsis, the coagulation pathways are also enhanced inducing increased tissue factor that leads to amplified production, but reduced removal of fibrin (Philippart and Cavaillon, 2007). The accumulation of fibrin and the disturbed homeostatic balance between procoagulant and anticoagulant mechanisms, causes the deposition of fibrin clots in small blood vessels, that in turn deprives vital organs of an adequate oxygenated blood supply (Ward, 2004).

In the later stages of sepsis there is evidence of progressive functional deterioration and dysfunction of activated phagocytes and dendritic cells (DCs), which results in degraded innate immune functions such their ability to kill phagocytized pathogens. This also results in diminished adaptive immune responses as macrophages and DCs have impaired antigen presentation to T cells. Thus the hyper-inflammatory state, together with impaired innate immune functions of phagocytes and immunosuppression is not adequate enough to contain commensal bacterial which contributes further to pathogenesis of sepsis (Bosmann and Ward, 2013).

In this manner, complement activation products together with the release of excessive pro-inflammatory cytokines by macrophages, neutrophils and dendritic cells contribute to poor myocardial contractility, impaired peripheral vascular tone, microvascular occlusion, tissue hypoperfusion and inadequate organ oxygenation and potentially leads to multiple systemic organ failure and lethality of sepsis (Figure 1.2).

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**Figure 1.1 Pleiotropic activity of Interferon gamma**

Interferon gamma (IFN- $\gamma$ ) is secreted by TH1 cells, NK cells and TC cells and acts on numerous cell types. The activation of macrophages induced by IFN- $\gamma$  plays a critical role in chronic inflammation. [Adapted from Research News, 1993, Science 259:1693] (Remick, 2003).

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**Figure 1.2 Macrophage activation and pathophysiology of sepsis**

Lipopolysaccharide (LPS) and other microbial components simultaneously activate multiple parallel cascades that contribute to the pathophysiology of sepsis. The combination of poor myocardial contractility, impaired peripheral vascular tone and microvascular occlusion leads to tissue hypoperfusion and inadequate oxygenation, and thus to organ failure. (Figure adapted from Cohen, 2002).

#### 1.4 Sepsis epidemiology and mortality rates

Currently, there are no specific therapeutic interventions to treat sepsis as no single treatment strategy has effectively managed the condition, thus it has a high mortality rate (Glauser, 2000). Severe sepsis (sepsis associated with acute organ dysfunction) is a huge and expensive medical problem throughout the world. In North America, it is estimated that there are more than 600,000 cases of severe sepsis annually, with mortality rate ranging between 30 % and 50 % (Angus and Linde-Zwirble, 2001).

Current estimates of severe sepsis in the UK are derived from adult critical-care units in the Intensive Care National Audit & Research Centre (ICNARC) Case Mix Programme (CMP) Database (National Collaborating Centre for Cancer, 2012). These indicate an increasing treated incidence of severe sepsis in critical care, rising from 50 to 70 cases per 100,000 population per year over the last decade. This now represents approximately 31,000 critical-care unit patient episodes per year. Similarly high incidence rates have been reported elsewhere (Linde-Zwirble and Angus, 2004; Padkin *et al.*, 2003). The increase in these estimates is indicative that more immunocompromised people are being kept alive rather than fatally succumbing to the infection.

In critical care units in England, Wales and Northern Ireland in 2004, hospital mortality for admissions with severe sepsis was 44.7 % and the total number of deaths was 14,000 (Harrison *et al.*, 2006) (Figure 1.3a). In 2003, Sepsis had the highest mortality rate of 36,800 deaths (Daniels, 2007), therefore claiming more lives than lung cancer, and more than breast and bowel cancer combined (Figure 1.3b).

Even these figures are likely to be an underestimate as diagnosis of sepsis can be difficult and deaths are frequently attributed to the underlying condition (for example, pneumonia) rather than to the resulting systemic inflammation (Van der Poll and Opal, 2008). The extremely high degree of mortality and morbidity associated with sepsis makes the study of the underlying contributory inflammatory mechanisms of great importance for the informed development of new therapeutic strategies.

(a) Redacted due to copyright

(b) Redacted due to copyright

**Figure 1.3 Sepsis mortality rates in the U.K.**

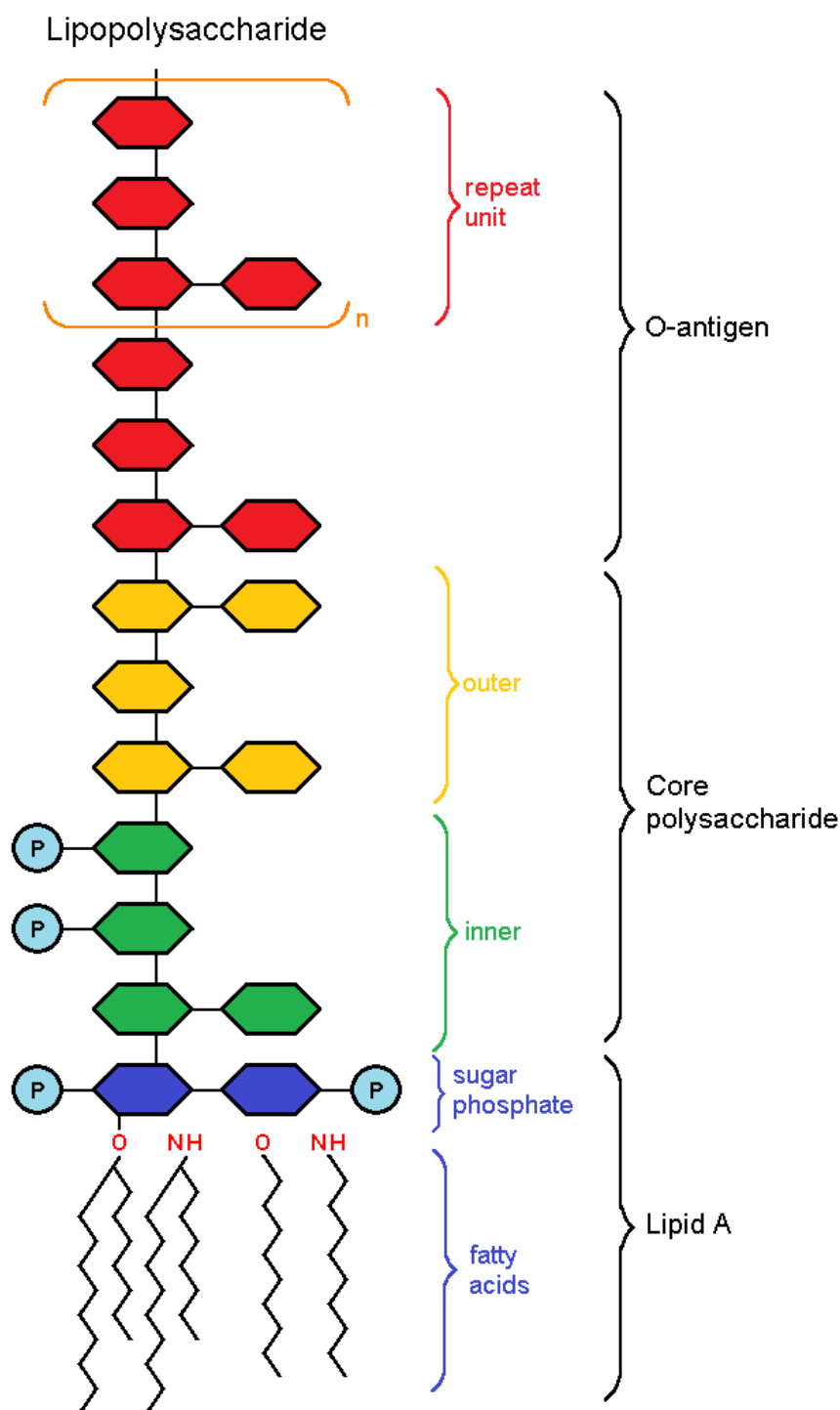
(a) Changes in outcomes for admissions with severe sepsis to the critical care unit outcomes over time, 1996 to 2004, in England, Wales and Northern Ireland hospitals (Harrison *et al.*, 2006). (b) Comparison of annual mortality rates in 2003 between severe sepsis and lung, bowel and breast cancer in the UK (Daniels, 2007).

### 1.5 Bacterial pathogen-associated molecular patterns associated with sepsis

Gram-negative bacteria are well recognised to play a significant role in the pathogenesis of sepsis because of one notorious immune-stimulatory structural component of the bacterial cell wall; lipopolysaccharide (LPS), or endotoxin. LPS is a powerful inflammatory stimulant, capable of inducing the production of inflammatory mediators in many leucocytes and thus is highly associated with induction of bacterial sepsis (Bosmann and Ward, 2013). The predominant causative agent of meningitis and severe sepsis in young adults worldwide, for example, is *Neisseria meningitides*, of which LPS is crucial to its pathogenicity (Brouwer *et al.*, 2010).

LPS is a unique and abundant glycolipid found in the Gram-negative bacteria outer membrane, classified within a group of structural components termed pathogen-associated molecular patterns (PAMPs). LPS structure is composed of three domains: a membrane-distal hydrophilic polysaccharide (specific O-antigen region) attached to a basal core oligosaccharide and a highly conserved hydrophobic lipid moiety termed lipid A that anchors the structure to the outer bacterial membrane (Figure 1.4) (Lu *et al.*, 2008). The composition of the O-antigen varies from strain to strain, characterizing specific species of bacterium, however, the lipid A anchor is highly conserved across bacterial species. The structure of lipid A is responsible for the endotoxic activity of LPS and exerts an immune stimulatory effect via toll-like receptor 4 (TLR4) signalling in diverse types of immune cells. It activates antigen presenting cells by inducing cytokine secretion, co-stimulatory molecule expression, and antigen presentation, which links innate immune response to adaptive response (Dobrovolskaia, 2002). Thus lipid A derivatives with reduced toxicity have been targets for the development of human vaccine adjuvant (Han, *et al.*, 2014).

Initially, Gram-negative bacteria were considered to be the predominant causative agents of sepsis, but the incidence of Gram-positive bacterial sepsis is now reported to be at equivalent levels (Riedemann *et al.*, 2003). The Gram-positive bacterium *Staphylococcus aureus* is one of the bacteria most commonly isolated from patients with sepsis (Wang *et al.*, 2003). Peptidoglycan (PG) and lipoteichoic acid (LTA) are two major cell walls components in Gram-positive bacteria and have been shown to stimulate inflammatory responses and like LPS are recognised by Toll-like receptors (TLRs) present on phagocytes (Seam and Suffredini, 2007; Triantafilou *et al.*, 2012).



**Figure 1.4 Diagrammatic representation of the structure of lipopolysaccharide.**

General structure of Gram-negative bacterial lipopolysaccharides (LPS). LPS also known as endotoxin, are found in the outer membrane of Gram-negative bacteria and elicit strong immune responses. LPS are large molecules consisting of a lipid and a polysaccharide composed of O-antigen, outer core and inner core joined by a covalent bond. The lipid A anchor is highly conserved across bacterial species, however composition of the O-antigen side chain varies from strain to strain, characterizing specific species of bacteria. The presence or absence of O-antigen determines whether the LPS are considered smooth or rough. Full-length O-chains would render the LPS smooth, whereas the absence or reduction of O-chains would make the LPS rough.

Gram-positive bacteria also produce membrane bound lipopeptides and some secrete exotoxins, such as staphylococcal enterotoxin B (SEB) and toxic shock syndrome toxin-1 (TSST-1) (Cohen, 2002). Such exotoxins have the properties of superantigens and have also demonstrated to induce fatal hypersensitivity to LPS (Dinges and Schlievert, 2001), thus supporting the dominant role LPS may play in all types of sepsis. Although the bacterial load and expression of virulence factors such as exotoxins can contribute to the outcome of severe infections, much of the damage inflicted during sepsis is attributable to the host's amplified inflammatory response to microbial PAMPs (Van der Poll and Opal, 2008).

### 1.6 Pattern recognition receptors (PRRs)

Pattern recognition receptors (PRRs) are a diverse group of receptors that can either be cell-associated or soluble and are divided into at least five distinct families based upon structural features; Toll-like receptors (TLRs), C-type lectin receptors (CTLRs), NOD-like receptors (NLRs), RIG-like helicase receptor (RLRs) and scavenger receptors (Akira *et al.*, 2006). Multiple receptors also exist in each class of PRRs with the result that in excess of 50 distinct PRRs may be expressed by a phagocyte at any given time, enabling the immune system to detect PAMPs and DAMPs and instigating a range of responses upon encounter with their appropriate ligands (Pradeu & Cooper 2012).

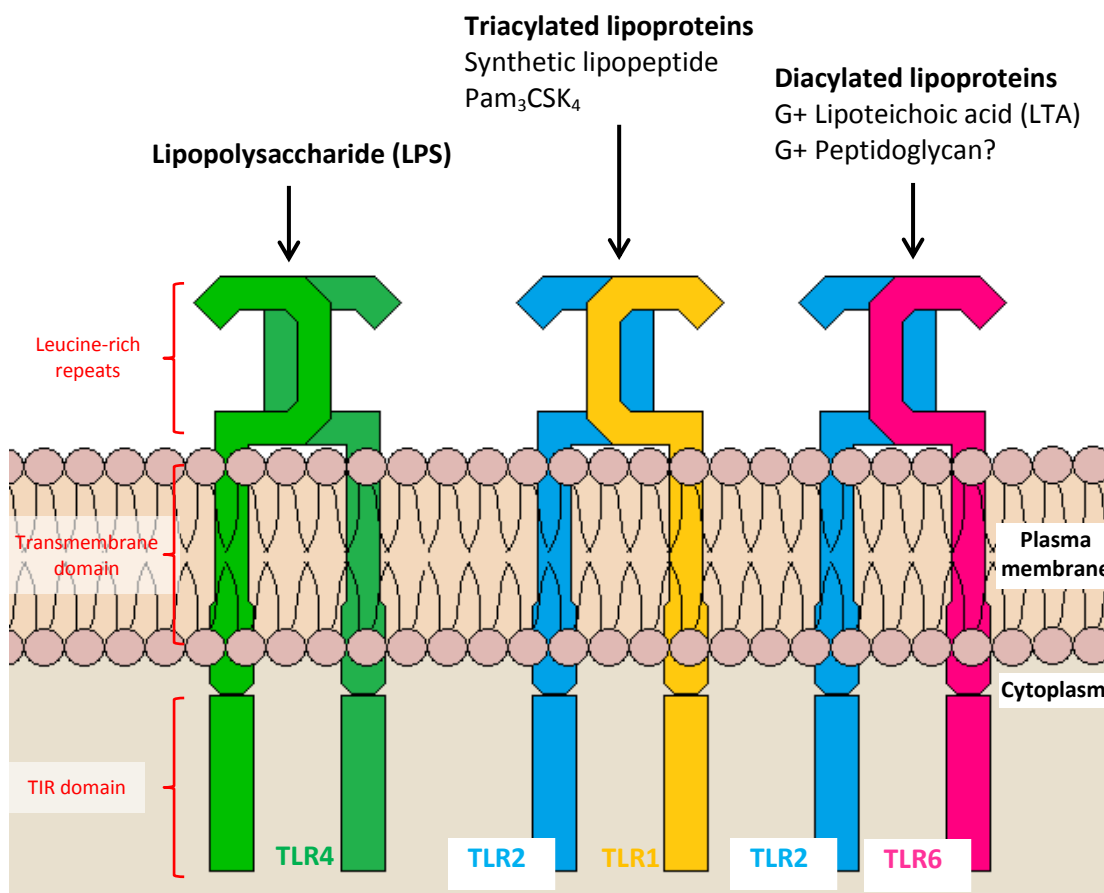
Phagocytes display CTLRs, one of which, macrophage mannose receptor (CD206), generates intracellular activation signals that facilitate macrophage phagocytosis of microorganisms, on encounter with its respective microbial PAMP (Kawai and Akira, 2010). NLRs are soluble proteins that reside in the cytoplasm of phagocytes where they act as receptors for pathogen-derived molecular patterns and typically recruit proteases or kinases, such as NF $\kappa$ B-activating kinase, upon activation (Janeway, 2002). RLRs also reside in the cytoplasm, acting as intracellular sensors for viral-derived products and are capable of activating NF $\kappa$ B in response to double-stranded RNA. Scavenger receptors represent yet another class of phagocytic receptors that recognise a variety of anionic polymers and low-density proteins (Le Roy, 2001). The role of the CD14 scavenger receptor will be described in later sections.



### 1.7 Toll-like receptors (TLRs)

The Toll-like receptors (TLRs) are a major subset of PRRs, able to detect a diverse range of PAMPs and DAMPs and are called so because of their similarity to the Toll receptor in the fruit fly. Research on TLRs began with the discovery of interleukin-1 receptor (IL-1R) (Sims *et al.*, 1988). IL-1 activates many cell types and induces many pro-inflammatory genes, thus the necessity to map its signalling pathway prompted the cloning of its receptor, IL-1R. In 1991, its homology to the *Drosophila melanogaster* fruit fly protein Toll was first reported (Gay and Keith, 1991). Subsequent research demonstrated its implication in host fly defences and proved that adult flies lacking the toll gene would succumb to fungal infections. Thus Toll had a role of sensing fungal pathogens (Lemaitre *et al.*, 1996). Importantly, at around the same time that similarities between *Drosophila* Toll and IL-1R were being identified, speculation that Toll might perform a similar role in human fungal infections was being investigated (Sims and Dower, 1994). In 1997, a mammalian Toll homologue, which was termed hToll, was cloned and studied (Medzhitov *et al.*, 1997). Soon after, five mammalian Toll homologues were described and named Toll-like receptors (TLRs) - these included hToll which was renamed as TLR4 (Rock *et al.*, 1998). To date, 10 functioning TLRs have been described in humans and 13 in mice (O'Neill *et al.*, 2013). TLRs 1, 2, 4, 5 and 6 are expressed on the cell surface but TLRs 3, 7, 8 and 9 are almost exclusively expressed in intracellular compartments such as endosomes.

The TLR family of PRRs are characterized by three domains: leucine-rich repeats (LRR), a transmembrane region and the intracellular Toll/IL-1 receptor (TIR) domain (O'Neill *et al.*, 2013). The TIR domain of TLRs is shared by the IL-1R family, which enabled its discovery, but small differences in the extracellular and intracellular regions of TLRs give rise to the specificity of recognising precise microbial products (Jin and Lee, 2008). Each TLR has demonstrated to detect a specific type of microbial PAMP and to signal the presence of infections (Iwasaki and Medzhitov, 2004). TLR4 has been definitively identified as the signalling receptor for LPS (Poltorak *et al.*, 1998; Qureshi *et al.*, 1999; Hoshino *et al.*, 1999) and TLR2 has shown to sense Gram positive bacterial lipopeptides (Jin *et al.*, 2007; Kang *et al.*, 2009). For the purpose of this study, a summary of the key PAMPs recognised by only TLR4 and TLR2 are given in Figure 1.5.



**Figure 1.5 Key bacterial cell wall PAMPs recognised by TLR 4 and TLR2**

The TLR family of PPRs are characterized by three domains: leucine-rich repeats (LRR), a transmembrane domain and the intracellular Toll/IL-1 receptor (TIR) domain. Toll-like receptors (TLR) mediate immune responses to a range of cell wall based microbial stimuli. Recognition of Gram negative (G-) LPS is via the TLR4 homodimer and is aided by two accessory proteins: CD14 and MD-2. TLR2 recognizes Gram positive (G+) bacterial ligands and functions as a heterodimer with either TLR1 or TLR6 (TLR2/TLR1 or TLR2/TLR6). Lipoteichoic acid (LTA) activates cells via the TLR2/TLR6 heterodimer, whereas Synthetic tripalmitoylated lipopeptide Palmitoyl-3-cysteine-serine-lysine-4 (Pam<sub>3</sub>CSK<sub>4</sub>) activates the cells via the TLR2/TLR1 heterodimer. Some Gram positive bacterial PG has shown to activate through TLR2, forming a heterodimer with TLR6, but these observations remain controversial as PG has also been shown to stimulate independent of TLRs through NODs. For the purpose of this study, other TLRs expressed on the cell surface (such as TLR5) or TLRs expressed in intracellular compartments (such as TLRs 3, 7, 8 and 9) are not included in the figure.

### 1.8 TLR2 bacterial PAMP recognition

TLR2 has been shown to recognize a broad range of Gram positive bacterial ligands and functions as a heterodimer with either TLR1 or TLR6 (TLR2/TLR1 or TLR2/TLR6), which appear to be involved in the discrimination of subtle changes in the lipid portion of lipoproteins (Figure 1.5.) (Takeuchi, 1999). Lipoteichoic acid (LTA) contains a diacylated moiety and activates the cells via the TLR2/TLR6 heterodimer (Jin and Lee, 2008). Synthetic tripalmitoylated lipopeptide Palmitoyl-3-cysteine-serine-lysine-4 (Pam<sub>3</sub>CSK<sub>4</sub>) contains a triacylated moiety and activates the cells via the TLR2/TLR1 heterodimer (Jin *et al.*, 2007; Triantafilou *et al.*, 2006).

TLR2 has also been reported to recognize Gram positive peptidoglycan (PG) (Tapping, 2009). In a study conducted by Ozinsky and colleagues, *S. aureus* PG was shown to induce TNF- $\alpha$  production in mouse macrophage cell line, RAW-TT10 (Ozinsky, *et al.*, 2000). TLR2 was reported to physically associate with TLR6 on encountering PG and both receptors were recruited to phagosomes, leading to the constitutive activation of nuclear factor- $\kappa$ B (NF- $\kappa$ B) to induce TNF- $\alpha$  production. Other *in vitro* models have also shown that PG induced NF- $\kappa$ B activation and cytokine production is mediated by TLR2 and TLR6 (Abrahams *et al.*, 2008; Triantafilou *et al.*, 2006) although this observation remains controversial.

Studies using PG components that have been biochemically purified from bacteria are often inconclusive due to the possibility of contaminating LTA. A report in 2004, used a range of highly purified PGs and showed that PG was not sensed through TLR2, TLR2/1 or TLR2/6 (Travossos *et al.*, 2004). Instead the report revealed that PG sensing was lost after removal of lipoteichoic acids from Gram-positive cell walls and implied that research linking PG with TLR2 recognition have relied mainly on the use of commercial *S.aureus* PG that was contaminated with LTA. In contrast, the report showed that peritoneal murine macrophages did not produce TNF- $\alpha$  or IL-6 in response to purified PGs and suggested that PG detection is more likely to occur independently of TLRs through intracellular NODs (Nod1/Nod2). Further research also indicated that PG signalling can occur via NLRs (O'Neill, 2004), thus the identification of the universal signalling receptor for most types of PG has remained elusive.

### 1.9 PAMP induced activation clusters

In addition to the involvement of TLRs, which function as central sensors for Gram-negative and Gram-positive bacterial products, other accessory molecules have found to be involved in establishing cellular activation (Hoshino *et al.*, 1999). Serum proteins, such as lipopolysaccharide-binding protein (LBP) have been observed to bind LPS or LTA and deliver it to its cellular target (Figure 1.6a) (Schumann, *et al.*, 1990; Wright, *et al.*, 1990). CD14 a glycosylphosphatidylinositol (GPI)-anchored protein, is also believed to act as a transfer molecule for both Gram-negative and Gram-positive bacteria (Passon-McDermott and O'Neill, 2004). In the case of LPS recognition, it has been further shown that a soluble accessory molecule myeloid differentiation protein 2 (MD-2), is recruited to the site of CD14–LPS ligation (Figure 1.6b) (Triantafilou, *et al.*, 2001) and possibly additional receptor components are required. Chemokine receptor 4 (CXCR4), heat shock proteins (Hsps) 70 and 90 or CD55 have been suggested to be part of this activation cluster (Triantafilou *et al.* 2004). In the case of LTA recognition, TLR2 seems to form receptor clusters, comprising of at least CD14, TLR2, TLR6, and CD36 (Figure 1.6b) (Tapping, 2009; Triantafilou and Triantafilou, 2002). Cell activation via the TLR2/TLR1 heterodimer by Pam<sub>3</sub>CSK<sub>4</sub>, however, is not CD36 dependent (Travossos *et al.*, 2004).

CD14 lacks a transmembrane domain and thus require TLRs to act as a signal transducing molecule to associate with. It is widely accepted that TLRs pre-exist as homodimers (in the case of TLR4) or heterodimers (TLR2/1 or TLR2/6) and are not induced by the ligand (Akira *et al.*, 2004; Kawai & Akira, 2011). However, expression levels of TLRs are modulated rapidly in response to pathogens, a variety of cytokines and environmental stress. Recently it had been proposed that receptor-receptor association of the extracellular TLR domains forced a series of protein conformational changes and initiated dimerization of the cytoplasmic domains as well (Motshwene *et al.*, 2009). Upon ligand engagement, TLRs are recruited into lipid bilayer microdomains termed 'lipid rafts', where they associate with lipid raft resident protein CD14 to form an activation cluster (Figure 1.6c). Binding of appropriate microbial ligand leads to clustering of receptors and triggers activation of multiple intracellular signalling cascades that lead, via NF- $\kappa$ B, to production and secretion of pro-inflammatory cytokines and IFNs (Passon-McDermott and O'Neill, 2004; Triantafilou and Triantafilou, 2004).

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### **Figure 1.6 Receptor cluster complex on cell membrane**

This schematic figure represents the events involved in detecting and responding to lipopolysaccharide (LPS) and Lipotechoic acid (LTA). (a) products released from bacteria bind to the serum protein, LPS-binding protein (LBP), and forms a complex. LBP catalyzes the transfer of LPS or LTA to membrane-bound. (b) CD14 requires the formation of trimolecular receptor cluster with the toll-like receptor homodimer (TLR4) or heterodimer (TLR2/TLR6). The accessory molecule myeloid differentiation protein 2 (MD-2) or CD36 are required to mediate a transmembrane signal in response to LPS and LTA, respectively. (c) clusters of activated receptors concentrate in lipid raft domains and their intracellular machinery clusters as well, forming a signalling platform that is crucial for the activation of downstream TLR signalling pathways. Model adapted from Triantafilou *et al.* (2011).

### 1.10 TLR2 and TLR4 Intracellular signalling Pathway

Identifying the ligand specificity and downstream pathways of each TLR dimer has been the subject of intense research for the last 15 years (Vandenbon *et al.*, 2012). Recent data suggests that multiple membrane-based and intracellular molecules interact upon activation and additional serum proteins are needed to mount an ideal response against the invading pathogen (Triantafilou *et al.*, 2012). Two main TLR signalling pathways have been identified: the myeloid differentiation factor 88 (MyD88)-dependent and the TIR domain-containing adaptor protein inducing IFN- $\beta$  (TRIF)-dependent pathways. The MyD88-dependent pathway is activated by all known TLRs except TLR3, and leads to the production of pro-inflammatory cytokines. TLR3 and TLR4 can activate the TRIF-dependent pathway, which leads to the production of type I interferons (IFNs) against viral infection (Figure 1.7).

In the case of the MyD88-dependent signalling pathway, the Toll/IL-1 receptor (TIR) domain of the TLR dimer engages directly with TIR domain-containing adaptor proteins: MyD88 and MyD88-adaptor-like protein (MAL; also known as TIRAP) found in the cell cytoplasm (Kawai & Akira, 2011). MAL in turn localizes to the plasma membrane, where it serves to bridge the interaction between MyD88 and TLR upon ligand engagement. The MyD88 then recruits IL-1R-associated kinases (IRAKs), TNF receptor-associated factor 6 (TRAF6) and the Transforming growth factor-beta-activated kinase 1 (TAK1) complex, leading to the downstream activation of nuclear factor- $\kappa$ B (NF- $\kappa$ B) and mitogen-activated protein kinases (MAPKs) (O'Neill *et al.*, 2013). A major consequence of TLR signalling is the induction of pro-inflammatory cytokines and IFN.

TLR4, which is expressed on the cell surface, initially transmits signals for the early-phase activation of NF- $\kappa$ B via MyD88-dependant pathways, but is then endocytosed and moves from the plasma membrane to endosomes, allowing the switch to TRIF-dependant signalling pathways. In phagosomes, TLR4 forms a complex with TIR domain-containing adaptor protein inducing IFN- $\beta$  (TRIF) and TRIF-related adaptor molecule (TRAM). The complex then recruits TRAF3 and the protein kinases TANK-binding kinase 1 (TBK1) and inhibitor of NF- $\kappa$ B kinase (IKKi), which catalyse the phosphorylation of interferon regulatory factor 3 (IRF3), leading to late-phase activation of NF- $\kappa$ B for the induction of type I IFN (Kawai & Akira, 2011).

**Figure 1.7 TLR trafficking and signalling pathways**

PAMP engagement induces conformational changes of TLRs expressed on the cell surface of macrophages and dendritic cells, which allow homo- or heterodimer interactions of TLRs. Heterodimers of TLR1-TLR2 and TLR2-TLR6 induce NF- $\kappa$ B activation through recruitment of MAL and MyD88 (MyD88-dependent signalling pathway). Homodimers of TLR4 can signal through the MyD88-dependent pathway (as above) or through TRIF-dependent pathway, via recruitment of adaptor proteins TRIF and TRAM. Both pathways for NF- $\kappa$ B activation is required for the induction of the signalling cascade that leads to the activation of synthesis of pro-inflammatory cytokines and effector molecules. Model adapted from Kawai & Akira (2011) and O'Neill *et al.* (2013).

### 1.11 Experimental trials for the treatment of sepsis

The clinical therapy of sepsis presents the difficult task of removing the infection while simultaneously controlling the excessive inflammatory response to infection without compromising host immunity. Whilst many key factors, including patient group, length of stay in ITU, pathogen load and antibiotic resistance, are crucial to treatment, there is no specific therapy for sepsis and it is often difficult to obtain a rapid microbial diagnosis before treatment begins. The current best available therapeutic approach is centred on supportive therapy, by such means as mechanical ventilation, administration of fluids, drainage of the source of infection, appropriate support for organ dysfunction and empirical treatment with antimicrobials (Cohen, 2002). However such treatment strategies are not always effective and thus sepsis carries a high mortality rate (Carlet *et al.*, 2008). Numerous potential targets and compounds for the treatment of sepsis have been examined within a clinical trials setting and are summarised in Figure 1.1 Table 4.1.

TLR4 represents a potential target for treatment strategies of Gram-negative mediated sepsis. Anti-TLR4 antibodies have been shown to inhibit intracellular signalling, markedly reducing cytokine production, and subsequently protecting mice from lethal endotoxic shock and *Escherichia coli* sepsis when administered as a prophylactic agent (Roger, *et al.*, 2009). TLR4 and MyD88 knockout mice were fully resistant to *E. coli*-induced septic shock, strongly supporting the concept of TLR4-targeted therapy for management of Gram-negative sepsis (Wittebole *et al.*, 2010).

More recently, a phase II trial in patients with severe sepsis has shown that Eritoran tetrasodium may be a useful contender in the treatment of sepsis (Tidswell *et al.*, 2010). Eritoran tetrasodium (E5564) is a synthetic lipopolysaccharide analogue that acts as a TLR4 antagonist and interferes with signalling responses to endotoxin. The structure of the molecule is based on the lipid A portion of a naturally occurring, weakly agonistic endotoxin found in *Rhodobacter sphaeroides*. E5564 is a potent *in vitro* antagonist of endotoxin that directly binds to the hydrophobic pocket of MD-2, competitively inhibits the lipid A component of endotoxin from binding to the same site, and thereby prevents dimerization of TLR4 and intracellular signalling. In this trial, E5564 did not appear to have an adverse effect on immune protection and consistently had a favourable (but not statistically significant) trend toward a lower



**Table 4.1 Targets in clinical trials for treatment of sepsis**

Target	Treatment for Clinical trials
Immune modulation	Glucocorticoids (inhibition of over-activation of the immune system) Intravenous IgG (improvement of host defense)
Endotoxin (LPS)	Anti-endotoxin antibodies: polyclonal human antiserum human monoclonal anti-lipid A (HA-1A) murine monoclonal anti-lipid A (E5) human monoclonal antibodies Bactericidal / permeability-increasing protein LPS elimination (hemofiltration)
TNF- $\alpha$	TNF- $\alpha$ antibodies: murine monoclonal antibodies F(ab)2 anti-TNF- $\alpha$ Soluble TNF receptors (TNF inhibition)
IL-1	IL-1 receptor antagonist
PAF	Phospholipase A2 antagonist (reduction of PAF) PAF antagonist PAF-acetylhydrolase (PAF inactivation)
Bradykinin	Bradykinin antagonist
Arachidonic acid metabolites	Prostaglandin (PG) E1 and liposomes containing PGE1 (anti-inflammatory) Thromboxane inhibitors (anti-inflammatory) Ketoconazole (thromboxane synthetase inhibition) Ibuprofen (COX inhibition)
Reactive oxygen species	N-acetylcysteine (restoration of cellular antioxidant potential) Selenium has been used to bolster selenium-dependent glutathione peroxidase, which is a scavenger for oxygen.
NO	L-NAME (NOS inhibition) L-NMMA (iNOS inhibition) Methylene blue (guanylyl cyclase inhibition) PHP (NO scavenger)
Phosphodiesterase	Pentoxifylline (phosphodiesterase inhibition, cAMP increase)
Neutrophil activity	IFN- $\gamma$ (reactivation of neutrophil immune functions) G-CSF, GM-CSF (increase of immune-competent blood cells) PGG-glucan (increase of phagocytosis and bacterial killing)
Complement system	C1 inhibition (inhibition of classical and lectin pathway activation)
Coagulation	Antithrombin III (inhibition of thrombin, factors IXa, Xa, XIa and XIIa) TFPI (inhibition of factors X and IX) APC (inactivation of factors Va and VIIIa)
TLR-4	Molecules interfering with TLR4 and TLR4-mRNA expression Molecules interfering with TLR4-related intracellular signalling

mortality rate in subjects. However, Eritoran tetrasodium treatment was only beneficial to some subsets of the study population; namely those patients presenting a high risk of death, thus limiting its application (Wittebole *et al.*, 2010).

One major problem with treatments targeting TLR4 is activation of the inflammatory system via mechanisms independent of TLR4, such as those demonstrated in meningococcal studies (Brouwer *et al.*, 2010; Prins *et al.*, 1998). *Neisseria meningitidis* homologue (NhhA), a meningococcal outer membrane protein, was found to trigger release of proinflammatory cytokines from RAW 264.7 macrophages via two distinct pathways; Interleukin-6 (IL-6) secretion was dependent on activation of TLR4 and required MyD88. In contrast, release of tumor necrosis factor (TNF) was TLR4- and MyD88-independent (Sjölinder *et al.*, 2012). Since *Neisseria meningitidis* represents the leading cause of meningitis and severe sepsis in young adults worldwide, success with treatment targeted at TLR4 would be limited.

Recombinant human activated protein C (rhAPC) is an alternative anti-sepsis treatment as it has anti-thrombotic, anti-inflammatory and anti-fibrinolysis properties. In previous studies, rhAPC has produced dose-dependent reductions in the levels of markers of coagulation and inflammation in patients with severe sepsis (Bernard *et al.*, 2001). A 6.1 % decrease in mortality was noted in the rhAPC group compared with the placebo group. Based on these data, in 2008, the International Surviving Sepsis Campaign Guidelines Committee reviewed the available literature on rhAPC trials. It was concluded that rhAPC was of benefit to those patients with severe sepsis and a high risk of death, most of whom had multiple organ failure. Subsequently, in 2009, the British Committee for Standards in Haematology (BCSH) guideline recommended the use of APC in patients with severe sepsis.

Results, however, consistently fail to show benefits for the subgroup of patients at lower risk of death and consistently showed increases in serious bleeding (Dellinger *et al.*, 2008). The BCSH therefore concluded that there was likely to be no benefit in treating patients at a low risk of death and also recommended against the use of rhAPC in children. Consequently, with the increasing uncertainty surrounding efficacy and concerns over the bleeding risk, rhAPC was recently withdrawn from the

market worldwide pending the results of a phase II trial focusing on the use of rhAPC in combination with low-dose corticosteroids (Thachil *et al.*, 2012).

An alternative concept is the use of drugs that increase activated protein C, a natural anticoagulant which breaks down blood clots, which may lead to improve survival rates (Fisher and Yan, 2000; Looney and Matthay, 2001). Activated protein C has been shown to inhibit TNF amplification in response to endotoxin in several animal models and *in vitro*, noticeably reducing the rate of death due to severe sepsis (Bernard *et al.*, 2001). Initial data suggests this to be a promising approach which continues to be assessed in clinical trials (Esmon, 2002).

Long courses of low-dose corticosteroids (LDC) have also shown to improve systemic symptoms of sepsis and reduce the time on vasopressor treatment (Beale *et al.*, 2010). As with other clinical trials, however, a debate remains on how best to characterise the patient population that is most likely to benefit from the treatment, the optimum dose and duration. Thus the use of rhAPC, E5564 and LDC remain of limited application.

Despite the crucial role for TNF- $\alpha$  and IL-1 in mediating sepsis, studies have demonstrated only limited success with treatments for sepsis based on antibodies directed at these pro-inflammatory cytokines (Glauser *et al.*, 1991; Tracey *et al.*, 1987). So far blocking the actions of pro-inflammatory cytokines has only proven successful if antibodies to TNF or IL-1 are given at specific time points before bacterial challenge (Hinshaw *et al.*, 1990; Bagby *et al.*, 1991; McIntyre, 1991). However, both TNF and IL-1 can also mediate anti-inflammatory responses that may protect against tissue injury. TNF, for example, up-regulates the cytolytic activity of lymphocytes, complement receptors, oxidative burst and stimulates the proliferation of B and T cells, all of which participate in the host's defence against infection (Wakabayashi, 1991). Hence, the use of anti-TNF/IL-1 in the treatment of human sepsis may in fact interfere with the cytokine concentrations that are required for an appropriate control of infection and worsen the very infection responsible for sepsis (Ozinsky *et al.*, 2000).

An alternative strategy to cytokine control is the use of antibodies to block endotoxins (Natanson *et al.*, 1994; Ziegler *et al.*, 1991). Synthetic antibodies to the specific O-oligosaccharide side chain of LPS have complement-dependent bactericidal activity; however the O-side chain of LPS is structurally and antigenically diverse between different Gram-negative bacterial genera, species and strains. An antibody to a specific O-side chain would therefore only be likely to be able to target one bacterial species, hugely limiting its potential clinical application. This problem has led to investigation of antibodies directed at the core glycolipid or lipid A structures of endotoxin, which are more highly conserved and retain structural similarities amongst common Gram-negative bacterial pathogens. Antibodies directed at lipid A or the core glycoprotein could, in theory, provide cross-protection against a wide and diverse range of Gram-negative bacteria. A murine anti-lipid A monoclonal antibody, E5, was tested in a clinical trial and gave no significant benefit to patients with Gram-negative septic shock (Greenman *et al.*, 1991). Those treated that entered the trial with the infection but not in shock, conversely, had statistically significant lower mortality (Greenman, *et al.*, 1991), though this has not been replicated among all trials performed with E5 (Bone *et al.*, 1995). As yet studies blocking specific components of pathogenic bacteria have proved inconclusive meaning that alternative approaches must also be explored.

Though many trials have been performed for the treatment for sepsis, all have arisen from work in animal models and have only achieved limited success. Differences between animal and human TLRs also exist implying the response to a bacterial stimulus may differ between species (Passon McDermott and O'Neill, 2004). Perhaps the lack of success in clinical trials to date may be partly attributed to the differences between animal experiments and the disease process in humans. Sepsis results from a complex interplay between bacterial and host factors. The regulation of inflammatory mediators is known to be crucial in preventing a detrimental outcome. This, and the lack of effective treatment options necessitates further elucidation of the underlying inflammatory mechanism in order that novel targets, compounds and strategies might be identified. Understanding the role of membrane-bound PRRs and their recruitment into receptor-signalling complexes by lipid rafts represents one such novel approach for the development of therapeutic options.

### 1.12 Membrane structure and dynamics

Cells of all organisms are enclosed by a plasma membrane, which for over 40 years, scientists have based views and experiments on the fluid mosaic membrane model of Singer and Nicolson (1972). According to the Singer-Nicolson fluid mosaic model, biological membranes can be considered as a two-dimensional liquid in which lipid and protein molecules diffuse more or less easily. Although the lipid bilayers that form the basis of the membranes do indeed form two-dimensional liquids by themselves, the plasma membrane also contains a large quantity of proteins, which provide more structure (Munro, 2003). In recent years, new models of the structure of the plasma membrane of mammalian cells has helped understand membrane dynamics and function and has suggested that the fluid mosaic model is greatly oversimplified (Olsson and Sundler, 2006). It has been demonstrated that the plasma membrane consists of lipids, cholesterol and proteins with lateral heterogeneities, patches and microdomains, often called lipid or membrane rafts. Lipid rafts are enriched in cholesterol and sphingolipids and appear as highly ordered, detergent-insoluble microdomains and are proposed to represent sites of cellular signalling (Koseki *et al.*, 2007).

### 1.13 The role of membrane regulation in inflammatory response

Accumulating evidence suggests that an important feature in LPS recognition is the clustering of the receptor molecules within lipid rafts (Munro, 2003; Triantafilou and Triantafilou, 2004). LPS has been observed to cause the translocation of several signalling molecules into lipid rafts including; translocation of TLR4 into human A549 lung epithelial cell (Abate, *et al.*, 2010) and human monocytic MM6 cell lipid rafts (Triantafilou, *et al.*, 2002), and translocation of CD-14 and MAP kinases into macrophage cell RAW264.7 lipid rafts (Olsson and Sundler, 2006).

Translocation into lipid rafts enhances LPS-mediated signalling by allowing co-localisation of receptors which co-operate to provide a signalling event. CD14, hsp70 and hsp90, are found to localise in lipid rafts constitutively, whereas TLR4 and CXCR4 are recruited into the lipid rafts only after LPS binding (Triantafilou, *et al.*, 2002). Signalling molecules, such as MyD88, are also recruited into lipid rafts following microbial stimulation (Triantafilou, *et al.*, 2011). To allow recruitment of molecules into lipid rafts, modifications to the plasma membrane phospholipid

microenvironment through incorporation of unsaturated fatty acids must occur (Darmani *et al.*, 1993).

#### 1.14 Lipid metabolism and membrane phospholipid re-modelling

Phospholipids are the major component of the plasma membrane. Cellular membranes are comprised of several different classes of phospholipids which have numerous structural and functional roles. An important phospholipid component of many membranes is phosphatidylcholine (PC) (Figure 1.8) (Choy and Arthur, 1989). The distribution of fatty acids within phospholipids results from remodelling of newly synthesized PC rather than *de novo* biosynthesis (Arthur and Choy, 1984).

Phospholipids are first formed by the *de novo* pathway (Kennedy pathway) and subsequently undergo modification in the remodelling pathway. The pathway for the remodelling of PC was first identified by Lands (1960) and involves the deacylation of PC to a lyso-PC and its subsequent re-acylation back to PC with a different acyl chain composition. The Lands Cycle, that is the acylation and deacylation of membrane phospholipids, provides a mechanism for the incorporation of unsaturated fatty acids, mainly arachidonic acid, into different phospholipids to provide a range of lipid mediators and to generate a mature membrane with asymmetry and diversity (Yamashita *et al.*, 1997).

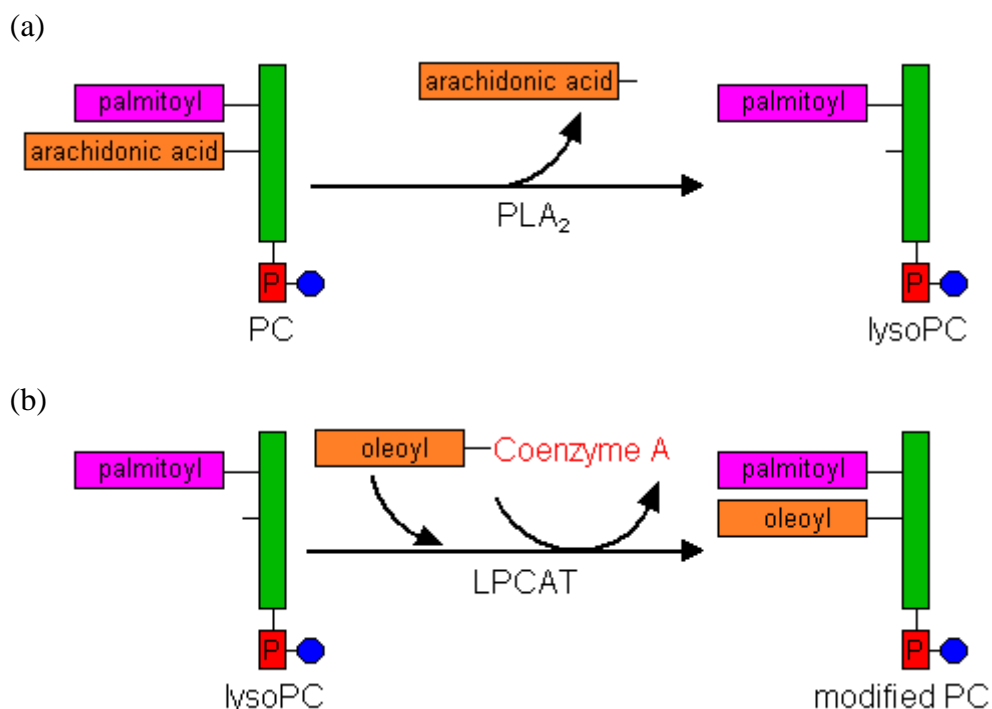
Fatty acids are cleaved from phospholipids by the action of phospholipase A2 and re-incorporated by acyltransferases (Lands, 2000) (Figure 1.9). Studies have revealed that arachidonic-acid is first incorporated into phospholipids containing a 1-acyl linkage by Coenzyme A (CoA)-dependent enzymes (Lands 1960; Chilton *et al.*, 1996). Arachidonic acid is subsequently transferred by CoA-independent transacylases from 1-acyl linked phospholipids to 1-alkyl and 1-alk-1-enyl lysophospholipids to form 1-alkyl and 1-alk-1-enyl-2-arachidonyl, which are important for the synthesis of platelet activating factor (PAF). Reacylation of lysoPC to PC is catalysed by the action of lysophosphatidylcholine acyltransferase (LPCAT). Thus LPCAT plays a significant role in the remodelling of membrane PC.

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**Figure 1.8 Basic structure of Phosphatidylcholine**

The basic structure of phosphatidylcholine (PC) comprising of a saturated fatty acyl chain at the sn-1 position (palmitoyl) and an unsaturated fatty acyl chain at the sn-2 position (oleoyl). Saturated fatty acids have no double bonds between the individual carbon atoms of the fatty acid chain, unsaturated fatty acids contain carbon-carbon double bonds or triple bonds. Adapted from Jackson and Parton (2004).



**Figure 1.9** LPCAT modulates PC acyl composition via substitution of unsaturated fatty acids

Land's cycle: (a) Phospholipase A<sub>2</sub> (PLA<sub>2</sub>) cleaves the fatty acyl chain at the sn-2 position, releasing a free fatty acid such as arachidonic acid. (b) Lysophosphatidylcholine (lysoPC) is then reacylated by Coenzyme A dependent lysophosphatidylcholine acyltransferase (LPCAT), which incorporates an unsaturated fatty acid (donor acyl-CoA) such as oleoyl, to yield a remodeled phosphatidylcholine (PC).

**Table 4.2** Summary of saturated and unsaturated fatty acids

Type	Carbon:double bond ratio	Systematic name	Common name
Saturated	2:0	Ethanoic acid	Acetic acid
	12:0	dodecanoic acid	lauric acid
	14:0	tetradecanoic acid	myristic acid
	16:0	hexadecanoic acid	palmitic acid
	18:0	octadecanoic acid	stearic acid
Unsaturated	18:1	<i>cis</i> -9-octadecenoic acid	oleic acid
	18:2	<i>cis</i> -6-octadecadienoic acid	linoleic acid
	18:3	<i>cis</i> -6-octadecatrienoic acid	linolenic acid
	20:4	<i>cis</i> -6-eicosatetraenoic acid	arachidonic acid
	22:6	<i>cis</i> -3-docosahexaenoic acid	cervonic acid



### 1.15 Evidence of enzymes with LPCAT-like activity

The LPCAT enzyme was first described in rat liver microsomes (Lands, 1960) and has since been identified in a diversity of species ranging from bacteria (Proulx and Van Deenen, 1966), plants (Devor *et al.*, 1971), insects (Heckman *et al.*, 1977) and fish (Holub *et al.*, 1976) to mammals (Choy and Arthur, 1989). The current literature supports the identification of 5 human acyltransferases with LPCAT activity; i.e. they have all been shown to incorporate an acyl-CoA into lysoPC, reacylating it to PC (Shindou *et al.*, 2009). This includes several enzymes from the 1-acylglycerol-3-phosphate O-acyltransferase (AGPAT) and membrane bound O-acyltransferases (MBOAT) families that have been reported to reacylate lysoPC to PC. LPCAT1, LPCAT2 and LPEAT2 are members of the AGPAT family, whereas LPCAT3 and LPCAT4 are members of the MBOAT family. These enzymes are characterized in terms of their substrate specificity, donor fatty acid preference and lysoPC acceptor (Table 4.2 and Figure 1.10). Research has demonstrated sequence homology between mouse, rat and human LPCATs and expression patterns in a variety of tissue and cultured cell-lines, indicating these genes are conserved across several species (Shindou *et al.*, 2008).

#### 1.15.1 LPCAT1

LPCAT1, formerly AGPAT7 and AYTL2, was discovered independently by two groups in 2006 and was observed to be highly expressed in the lung, especially in alveolar type II cells, responsible of the secretion of surfactant components (Chen *et al.*, 2006; Nakanishi *et al.*, 2006). Pulmonary surfactant is a lipoprotein complex that coats the surface of alveoli, reducing surface tension and preventing alveolar collapse. Deficiencies and/or dysfunction of the surfactant system are known to contribute to the pathogenesis of several pulmonary diseases and can lead to severe respiratory failure (Nakanishi *et al.*, 2006). Thus the production of surfactant lipids is highly regulated. The phospholipid content in pulmonary surfactant is thought to be predominantly synthesized by LPCAT1, suggesting a critical role for this enzyme within respiratory physiology. In fact when *Lpcat1* mRNA levels were reduced in newborn mice it could be directly correlated with low PC content, LPCAT1 activity, and survival (Bridges *et al.*, 2010).

**Figure 1.10 Characterisation of lysophospholipid acyltransferases (LPLATs)**

(a) Phylogenetic tree of mouse lysophospholipid acyltransferases (LPLATs). (b) Summary of functionally identified LPLATs and their preference for donor fatty acyl chain (defined in ) incorporation into the sn-2 position of phospholipids such as phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidic acid (PA), phosphatidylglycerol (PG) and phosphatidylinositol (PI). Darker colors indicate higher enzymatic activity for acyl-CoAs. Additional LPLATs that may exist are indicated with question marks. Reproduced from Hishikawa *et al.* (2008) © the National Academy of Sciences.

LPCAT1 is principally involved in catalysing disaturated-PC and disaturated-PG synthesis, specifically utilising linoleic acid (18:2)-CoA or linolenic acid (18:3)-CoA as donor fatty acids *in vitro* (Nakanishi *et al.*, 2006). Disaturated phospholipids, mainly dipalmitoyl- phosphatidylcholine (DPPC), are major components of pulmonary surfactant. Studies found that 55-75 % of DPPC in alveolar type II cells was formed by the remodelling pathway (Chen *et al.*, 2006; Nakanishi *et al.*, 2006).

In 2008 Soupene *et al.* (2008) reported the characterization of the product of the Ayt12 gene in adult human red blood cells (RBCs). Mammalian RBCs lack *de novo* lipid synthesis but maintain membrane composition by rapid turnover of acyl moieties at the sn-2 position of phospholipids. The authors suggested that AYTL2 is the reacylating enzyme for lysoPC in the RBC membrane and identified it to have the function of a LPCAT protein. As more sequences became available, multiple sequence alignment of AYTL2 and LPCAT1 revealed that they were in fact identical (Harayama *et al.*, 2009).

Interestingly, Agarwal *et al.*, (2007) reported that human AGPAT9/LPCAT1 had a similar cDNA sequence to the mouse LPCAT1 reported by Chen *et al.* (2006) and Nakanishi *et al.* (2006). Notably, Agarwal, *et al.* (2007) did not observe any LPCAT activity when LPCAT1 was overexpressed in Chinese hamster ovary (CHO) cells. Instead of favoring lysoPC as a substrate in the *in vitro* studies, LPCAT1 exhibited a preference for lysophosphatidic acid (lysoPA), which could argue for LPCAT1's role in lung physiology. Conversely, when hLPCAT1 was overexpressed in human embryonic kidney cells (HEK293), ample LPCAT activity was obtained (Zhao *et al.*, 2008). This discrepancy may have resulted from a problem related to the location of the epitope tag in the plasmid construction used to overexpress LPCAT1 in CHO cells as suggested by Mansilla *et al.* (2009). It may however, also be possible that each LPCAT performs a unique function within different cell types, either maintaining cellular membrane structure and /or producing specific phospholipid molecules (Harayama *et al.*, 2009).

Recent findings implicate a role for LPCAT1 within colorectal cancer (CRC). A common characteristic in cancer cells and solid tumours has revealed to be the elevation of PC. This elevation has been observed in almost every single cancer type

studied by NMR spectroscopy and is employed as an endogenous biomarker of cancer (Ackerstaff *et al.*, 2003). When 168 colorectal adenocarcinomas were examined, LPCAT1 mRNA was shown to be upregulated 2.5-fold in CRC tissues as compared to normal mucosa (Mansilla *et al.*, 2009). The hLPCAT1 protein in a colon cancer cell-line (SW480) was found to predominately have LPCAT, rather than AGPAT, enzyme activity. hLPCAT1 activity was associated with an increased reacylation of lysoPC and correlated with a significantly increased the growth rate of the cancer cells. By altering the specific lipid profile, LPCAT1 contributes to the total choline metabolite accumulation found in CRC - a process that may consequentially influence potential therapies due to the membrane structure affecting drug delivery (Mansilla *et al.*, 2009). During the progression of human mammary epithelial cells to a malignant phenotype, an increase in PC and total choline containing compounds has been observed, as well as altered ratios of glycerophosphocholine (GPC) to PC (Glunde *et al.*, 2006).

#### 1.15.2 LPCAT2

AGPAT11 cDNA has recently been identified as LPCAT2, lyso-platelet-activating factor acyltransferase (Lyso-PAF-AT), previously named acyltransferase-like 1 (AYTL1) (Shindou *et al.*, 2007). As the names would suggest, this enzyme utilises the same precursor (lyso-PAF) to synthesise both PAF and PC in inflammatory cells, although the mechanism(s) as to how the two activities are differentially regulated remains unknown.

Protein sequence alignments have revealed hLPCAT2 to be 58 % homologous to hLPCAT1, with 41.5 % identical amino acids (Agarwal and Garg, 2010). These isoforms are more similar than any other two human ATs within the AGPAT family and share comparable expression patterns for most human tissue types. Like LPCAT1, LPCAT2 is largely expressed in the lung, spleen and leukocytes, except LPCAT2 is expressed one or two fold less. Since the overexpression of hLPCAT1 protein has been detected in CRC tissue (Mansilla *et al.*, 2009), an investigation into the expression of LPCAT2 in human tumour tissues has been reported (Agarwal and Garg, 2010). Like LPCAT1, AGPAT11/LPCAT2 expression is significantly increased in colorectal, breast and cervical cancer tissue compared to normal tissues in these anatomical sites. Expression of LPCAT2 was found to be reflective of the

degree of tumour tissue present. In normal colon tissue LPCAT2 is naturally expressed, but in CRC tissue expression is amplified considerably.

Agarwal and Garg (2010) also determined LPCAT2's acyl-CoA and lysophospholipid specificity for AGPAT activity. LPCAT2 appears to have a broad preference for lysophosphatidic acid (lysoPA) -containing saturated fatty acids. Lysophosphatidylserine (lysoPS) was the only other lysophospholipid for which LPCAT2 retained some activity. This group did not publish their lysoPC data and stated only that LPCAT activity did not reach statistical significance, in contrast to other groups who have shown LPCAT activity with this cDNA (Shindou *et al.*, 2007; Harayama *et al.*, 2008).

Mouse LPCAT2 shows 48.2 % amino acid sequence similarity to mouse LPCAT1 and 88.4 % sequence homology to human LPCAT2, therefore, *in vitro* and *in vivo* studies are frequently performed using murine models (Shindou *et al.*, 2007; Harayama *et al.*, 2008). In mouse peritoneal macrophages treated with LPS, the LPCAT2 mRNA was upregulated (Shindou *et al.*, 2005). However only the lyso-PAF-AT activity of LPCAT2 was activated; the LPCAT activity of LPCAT2 was not shown to be elevated. When hLPCAT2 siRNA was transfected into HEK293 cells, not only did it considerably reduce the mRNA level of hLPCAT2 by 70-80 %, but also significantly decreased lyso-PAF-AT activity by 50-60 % (Shindou *et al.*, 2007). Thus hLPCAT2 appears to be the principal enzyme for PAF production in HEK293 cells providing an inducible remodelling pathway for PAF synthesis.

### 1.15.3 LPCAT3 / MBOAT5

LPCAT3, a member of the MBOAT family, has been found abundantly in liver, pancreas and adipose tissue and is thought to be primarily responsible for hepatic LPCAT activity (Shindou *et al.*, 2008). The latter is shown by LPCAT3 siRNA transfection into human hepatoma Huh7 cells, where a 90 % reduction in LPCAT activity was observed relative to the control (Zhao *et al.*, 2008). This suggests that LPCAT3 is the major enzyme contributing to LPCAT activity in hepatocytes *in vivo*. When hLPCAT3 was overexpressed in HEK293 cells, it displayed LPCAT activity equivalent to that of LPCAT1 (Zhao *et al.*, 2008) and in these cells, LPCAT3

exhibited substrate specificity only for lysoPC and lysoPS (Hishikawa *et al.*, 2008; Zhao *et al.*, 2008; Matsuda *et al.*, 2008).

In 2008, Kazachkov *et al.* (2008) concluded that hLPCAT3 expressed in a yeast strain exhibited a dramatic increase in LPCAT activity compared to the control. mLPCAT3 expressed in CHO-K1 cells provided further evidence that LPCAT3 preferred mono- or polyunsaturated fatty acyl-CoAs and played a major role in LPCAT activity in these cells (Hishikawa *et al.*, 2008). It was also noted that LPCAT3 expression was not influenced by innate immune agonists, confirming that it contributes to membrane biogenesis in a constitutive manner (Gijón *et al.*, 2008; Jain *et al.*, 2009). LPCAT3 has been shown to be widely distributed in human tissues further indicating that this is a principal enzyme in the regulation of the remodelling pathway.

#### 1.15.4 LPCAT4 / MBOAT2

MBOAT2, also known as LPCAT4, has shown both LPCAT and LPEAT activities in mice (Gijón *et al.*, 2008). mLPCAT4 mRNA is highly expressed in epididymis, brain, testis and ovary tissue and has demonstrated a clear preference for lysoPC or lysoPE. mLPCAT4 also exhibited higher LPCAT activity with 1-acyl-lysoPC than 1-O-alkyl-lysoPC or 1-O-alkenyl-lysoPC as acceptors, suggesting it may recognise differences between the ester and ether bond at the sn-2 position of lysoPC. Localisation of this enzyme was also examined and found to mainly reside in the endoplasmic reticulum, rather than the Golgi or mitochondria in CHO-K1 cells (Hishikawa *et al.*, 2008). Toll-like receptor 3, 4, or 9 agonists did not induce mRNA expression of mLPCAT4 in macrophages, indicating that like LPCAT3, mLPCAT4 may play a constitutive role in membrane biogenesis, as opposed to an inducible remodelling pathway in response to external stimuli (Hishikawa *et al.*, 2008).

#### 1.15.5 LPEAT2

LPEAT2, also known as acyltransferase-like 3 (AYTL3) and AGPAT7, shares a high degree of protein sequence homology with both LPCAT1 and LPCAT2 (Ye *et al.*, 2005) and is predominantly expressed in brain and inflammatory cells (Cao *et al.*, 2008). Similarly to both these LPCATs, LPEAT2 shows high LPEAT and moderate LPCAT and LPGAT activity, with preference toward C18:1 and C16:0-CoAs. The mouse ortholog of LPEAT2 was found to share 93 % amino acid identity with the

human gene (Cao *et al.*, 2008). mLPEAT2 mRNA was detected in erythroleukemic mouse Friend cells (as was LPCAT1 and 2), but was not detected in reticulocytes or adult RBCs unlike LPCAT1 (Soupene *et al.*, 2008). In contrast to LPCAT1 and LPCAT2, mRNA expression levels were very low in both mouse and human liver and lung tissues.

Whilst some research suggests dominant LPEAT activity and a small increase in LPCAT activity when mLPEAT2 was overexpressed in *E.coli* (Cao *et al.*, 2008), other group's have found only weak LPCAT and undetectable LPEAT activity (Soupene *et al.*, 2008). This apparent contrast may be the result of intrinsic differences between *E.coli* and mammalian expression systems. However, when LPEAT2 was depleted by siRNA in HEK293 cells, only LPEAT activity significantly decreased. Neither LPGAT nor LPCAT activity was affected in these cells, suggesting LPEAT2 mainly contributes to phosphatidylethanolamine (PE) formation in these cells. Since LPEAT2 is also primarily expressed in brain tissue, it may suggest a possible role for LPEAT2 in modulating brain PE composition.

#### 1.16 LPCAT's role in cell signalling

An alteration to fatty acid or acyl composition of the plasma membrane, facilitated by LPCAT, subsequently modifies not only membrane structure but also its function (Shindou *et al.*, 2008). It is suggested that by altering the plasma membrane properties to become more fluid, LPCAT enzymes allow the membrane to modulate movement of proteins for the assembly of receptor complexes (Schmid *et al.*, 2003). In previous studies, (Neville *et al.*, 2005; Jackson *et al.*, 2008) LPCAT has shown to alter the phospholipid composition of the plasma membrane in monocytes and macrophages and findings also confirmed that lysophospholipid metabolism facilitates TLR4 membrane translocation in A549 epithelial cells, after LPS challenge (Abate *et al.*, 2010). LPCAT could therefore potentially be involved in the activation of TLR4 in response to LPS and hence may play a role in the control of subsequent inflammatory cytokine signalling events associated with sepsis. However it is not clear which cytokine signalling processes are regulated in this way and the role of LPCAT during inflammatory responses to other microbial stimuli, such as TLR2 ligands, is not clearly understood.

Further studies have shown that inflammatory cytokine interferon (IFN)- $\gamma$  can directly up-regulate the activity of LPCAT in monocytes (Neville *et al.*, 2003). Furthermore, IFN- $\gamma$  primes monocytes for subsequent challenge with LPS through, a process that involves changes in the plasma membrane fatty acid composition, generating increased inflammatory responses to LPS (Schmid *et al.*, 2003). The control of the consequential amplified release of cytokines by IFN- $\gamma$  primed monocytes is important in the pathogenesis of sepsis, but how LPCAT may regulate these inflammatory responses to microbial stimuli, are poorly understood. Under such conditions the inhibition of LPCAT represents a potential method to better understand these processes.

### 1.17 Inhibitors of LPCAT

In 2003, Schmid and colleagues identified inhibitors for LPCAT by high-throughput screening. A promising candidate that emerged from this study is 5 hydroxyethyl 5,3' thiophenyl pyridine (HETP), a noncompetitive specific inhibitor of CoA-dependent LPCAT which has since been synthesized by a method adapted from Yamada *et al.* (2005). In the cell systems used, HETP was found to have an IC<sub>50</sub> of 10  $\mu$ M for the inhibition of LPCAT and demonstrated a subsequent reduction in the production of two key inflammatory cytokines TNF- $\alpha$  and IL-6, in LPS-stimulated monocytes (Schmid *et al.*, 2003).

In 2004, Chambers and Brown identified a novel acyl-CoA cholesterol acyltransferase inhibitor, 2,2-methyl-N-(2,4,6-trimethoxyphenyl) dodecanamide (CI-976). Studies utilising this inhibitor demonstrate that CI-976 inhibits multiple membrane trafficking steps, including ones found in the endocytic and secretory pathways, and it has been suggested to non-specifically inhibit LPCAT (Brown *et al.*, 2008). Both LPCAT inhibitors were investigated during this study.



### 1.18 Rationale and aims of the present study

LPCAT describes the activity of enzymes which catalyse the reacylation of lysoPC to PC in the plasma membrane. This activity serves to alter membrane properties and modulate the movement of proteins for assembly of receptor complexes and may therefore be important in the regulation of inflammatory responses and hence the pathogenesis of sepsis. As such it would be beneficial to study the immuno-regulatory properties of LPCAT in response to diverse microbial stimuli, in monocytes and airway epithelial cells that regularly come into contact with pathogenic components.

Previous studies have indicated that LPCAT plays a crucial role in monocyte inflammatory responses to TLR4 ligand, LPS (Schmid *et al.*, 2003; Abate *et al.*, 2010). Furthermore, TLR2 associated PAMPs arising from Gram-positive bacterial cell components, including peptidoglycan and lipoteichoic acid, have shown to be of similar importance in the induction of bacterial sepsis (Seam and Suffredini, 2007). Studies of LTA-induced inflammatory responses have shown that the TLR heterodimer TLR2/TLR6 translocates into the lipid raft domain of monocytes upon encounter with LTA and initiates the downstream signalling cascades, producing pro-inflammatory cytokine TNF- $\alpha$  (Triantafilou *et al.*, 2004). However the effect of LPCAT in LTA-induced inflammatory responses has not yet been studied.

This study aims to determine if LPCAT has a key role in the regulation of TLR2 ligand induced inflammatory responses and consequently Gram-positive bacterial sepsis and to identify if a correlation between the expression of specific LPCAT isoforms and the inflammatory response regulatory mechanisms exists in inflammatory cells to elucidate if LPCAT could be a potential target in the treatment of sepsis.

### 1.18.1 Objectives

The objectives of this study were:

- To use a specific LPCAT inhibitor (HETP) to demonstrate the role of LPCAT in the production of an array inflammatory cytokines, TNF- $\alpha$ , IL6 and IL8, with TLR2 ligands LTA, PG and Pam<sub>3</sub>CSK<sub>4</sub> in monocytic cell line, MM6, primary blood derived monocytes and epithelial cells, BEAS-2B.
- To determine if IFN- $\gamma$  primes monocytes for subsequent challenge with TLR2 ligands LTA, PG and Pam<sub>3</sub>CSK<sub>4</sub> whilst using the specific HETP to investigate the role of LPCAT in IFN- $\gamma$  priming.
- To ascertain if LPCAT2 is the major remodelling enzyme involved in inflammatory pathways through detecting the intrinsic mRNA expression level of 5 LPCATs in monocytic (MM6) and epithelial (BEAS-2B) cell lines.
- To determine how LPCAT activity is regulated during an inflammatory immune response, by examining LPCAT mRNA and protein expression and LPCAT activity before and after IFN- $\gamma$  stimulation.
- To determine if LPCAT is involved in TLR2 translocation into the lipid rafts of MM6 cells during stimulation with TLR2 ligands LTA and Pam<sub>3</sub>CSK<sub>4</sub>.

### 1.19 Hypothesis:

The hypothesis of this study was that inhibition of LPCAT with HETP will suppress secretion of pro-inflammatory cytokines, TNF- $\alpha$ , IL-6 and IL-8, in response to LPS, LTA, PG and Pam<sub>3</sub>CSK<sub>4</sub> stimulation, as previously demonstrated with LPS stimulation in the MM6 cell line and primary blood monocytes (Schmid *et al.*, 2003; Jackson and Parton, 2004). Thus it was also predicted that LPCAT will play a crucial role in the translocation of TLR2 into the lipid raft domain of monocytes upon stimulation, as previously seen with TLR4 (Jackson *et al.*, 2008; Abate *et al.*, 2010). As lung tissues are delicate and have designated functions to perform, they cannot allow themselves to be overly infiltrated with immune cells that might destroy them or their function in the process of fighting infection, it was anticipated that inflammatory responses to LPS, LTA, PG and Pam<sub>3</sub>CSK<sub>4</sub> in lung epithelial cells, BEAS-2B, would be less than seen in monocytes.

It was expected that LPCAT would play a role in IFN- $\gamma$  priming of monocytes for subsequent challenge with TLR2 ligands, due to an up-regulation in LPCAT activity, generating an amplified production of inflammatory cytokines as seen with LPS stimulation (Schmid *et al.*, 2003). It was hypothesized that LPCAT isoforms are unique to particular cell types and expression levels of individual LPCATs will reflect this. Additionally, it was anticipated that one LPCAT, most likely LPCAT2, (Shindou *et al.*, 2007) will be predominately expressed in monocytes, where inflammatory responses are initiated and that LPCAT mRNA and protein expression will be amplified after IFN- $\gamma$  stimulation in monocytes as seen with LPCAT activity (Neville *et al.*, 2003).

# Chapter 2

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MATERIALS AND METHODS

## 2.1 Reagents

All reagents were obtained from Sigma (Poole, UK), unless stated otherwise. Palmitoyl-3-cysteine-serine-lysine-4 (Pam<sub>3</sub>CSK<sub>4</sub>) hydrochloride was supplied by Merck Chemicals Ltd. (Nottingham UK). ELISA DuoSet kits were obtained from R&D Systems (Oxford, UK). Precast gels were from Invitrogen (Paisly, UK), antibodies for Western blotting were from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and antibodies for flow cytometry were from e-Bioscience (San Diego, CA, USA). ECL Plus detection kit was from Amersham Biosciences (Buckinghamshire, UK).

## 2.2 Cell Lines

All cell lines were seeded at a density recommended by the respective supplier and grown at 37°C in humidified air and 5 % CO<sub>2</sub>.

### 2.2.1 MonoMac6 cells

A human acute monocytic leukemia cell line, established from the peripheral blood of a 64-year-old man, obtained from German Cell Collection (DSMZ, Braunschweig, Germany). MonoMac6 cells (MM6) were maintained in RPMI-1640 medium, supplemented with 10 % (v/v) foetal bovine serum, 2 mM L-glutamine, 1 mM Sodium pyruvate, 1 % (v/v) non-essential amino acids and 1 % (v/v) penicillin/streptomycin.

### 2.2.2 A549 cells

A human lung carcinoma cell line, established from an explanted lung tumour which was removed from a 58-year-old Caucasian man, obtained from American Type Culture Collection (Manassas, VA, USA). A549 cells were maintained in RPMI-1640 medium, supplemented with only 5 % (v/v) foetal bovine serum (or without if serum free), 2 mM L-glutamine and 1 % (v/v) penicillin/streptomycin.

### 2.2.3 BEAS-2B cells

A transformed human bronchial epithelial cell line, obtained from autopsy of non-cancerous individuals, infected with an adenovirus 12-SV40 virus hybrid (Ad12SV40) and cloned. The cells were a kind donation from Dr Amanda Tonks (Cardiff University, Cardiff, UK). BEAS-2B cells were maintained in F-12 Kaighn's

modification medium, supplemented with 5 % (v/v) foetal calf serum (or without if serum free) and 1 % (v/v) penicillin/ streptomycin.

#### 2.2.4 HEPG2

A human liver epithelial cell line established from a hepatocellular carcinoma of a 15 year old adolescent male. HEP G2 cells were maintained in EMEM, supplemented with 10 % (v/v) foetal bovine serum, 2 mM L-glutamine, 1 % (v/v) non-essential amino acids and 1 % (v/v) penicillin/ streptomycin.

#### 2.2.5 HEK 293

A human embryonic kidney cell line transformed with adenovirus 5 DNA in the early 70s (Graham *et al.*, 1977). HEK 293 cells were maintained in EMEM, supplemented with 10 % (v/v) foetal bovine serum and 1 % (v/v) penicillin/ streptomycin.

#### 2.2.6 RAW 264.7

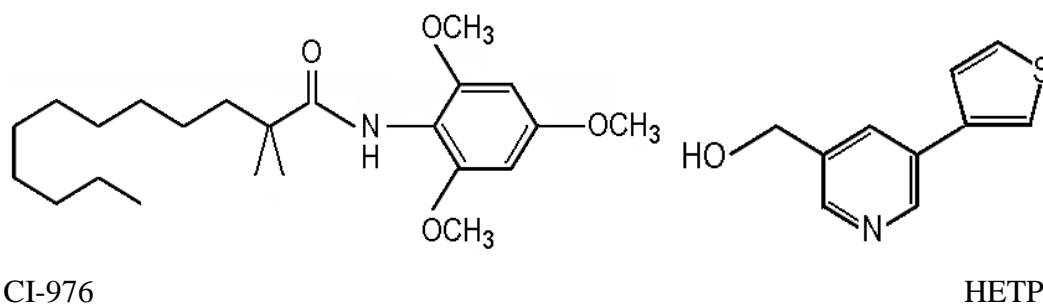
A murine macrophage cell line, established from a tumour induced by Abelson murine leukemia virus. RAW 264.7 cells were maintained in 4.5 g/L glucose DMEM, supplemented with 10 % (v/v) foetal bovine serum, 1 % (v/v) non-essential amino acids and 1 % (v/v) penicillin/ streptomycin.

### 2.3 Inhibitors of LPCAT Activity

Initially a range of inhibitor concentrations were tested to determine the appropriate concentration to use for a significant reduction in cytokine secretion, without affecting cell viability. HETP: 5-Hydroxyethyl 5,3'thiophenyl pyridine (HETP), a non-competitive, specific inhibitor of CoA-dependent LPCAT, was synthesized by a collaborator Simon Jones, (University of Sheffield, UK), using a method adapted from Yamada *et al.* (2005).

To analyse the dose response of HETP, a range of concentrations (0-100  $\mu$ M) was added to cells for 30 minutes prior to co-culture with or without LPS. In experiments conducted after this preliminary test, cells were routinely treated with 0  $\mu$ M or 50  $\mu$ M HETP for 30 minutes prior to ligand stimulation.

Another inhibitor, 2,2-methyl-N-(2,4,6-trimethoxyphenyl) dodecanamide (CI-976): a novel acyl-CoA cholesterol acyltransferase inhibitor (GlaxoSmithKline pharmaceuticals, Essex, UK), was also tested for its ability to inhibit LPCAT. CI-976 was added to cells, ranging from 0 to 50  $\mu\text{M}$  for 30 minutes prior to co-culture with or without LPS. However, cell viability data demonstrated that even at low concentrations (10  $\mu\text{M}$ ) this inhibitor was toxic to cells, so CI-976 was not used in further experiments.



#### 2.4 Cell Stimulants

Experiments to determine the inhibitor concentration were stimulated with 100 ng/ml *Escherichia coli* 0111:B4 lipopolysaccharide (LPS). As well as TLR4 ligand, LPS, further experiments used TLR2 ligands; 10  $\mu\text{g/ml}$  *Staphylococcus aureus* lipoteichoic acid (LTA), 10  $\mu\text{g/ml}$  *S. aureus* peptidoglycan (PG) (all from Sigma, Poole, UK) and 1.0  $\mu\text{g/ml}$  N-palmitoyl-S-dipalmitoylglyceryl (Pam<sub>3</sub>) Cys-Ser-(Lys)<sub>4</sub>trihydrochloride (Pam<sub>3</sub>CSK<sub>4</sub>) (a synthetic lipohexapeptide analog of the immunologically active portion of bacterial lipoprotein) (Merck Chemicals Ltd. Nottingham UK). Recombinant human Interferon gamma (IFN- $\gamma$ ) (R&D Systems, Oxford, UK) was used at 10  $\mu\text{g/ml}$  (250 units/ml) to prime cells before stimulation, and enhance inflammatory responses, as previously shown with LPS (Neville *et al.*, 2005).

## 2.5 Assessment of LPCAT inhibition on inflammatory responses to TLR ligands by ELISA

### 2.5.1 Co-culture of Cells for Cytokine secretion analysis by ELISA

For standard analysis of cytokine production by ELISA, cells were cultured in 1 ml of media in 12 well plates. Selected cells were then incubated with or without IFN- $\gamma$  for 16 hours. The cells were then treated with or without HETP for 30 minutes prior to co-culture with or without LPS, LTA, PG or Pam<sub>3</sub>CSK<sub>4</sub>. The negative controls were cell alone. One plate was co-cultured for 6 hours (to analyse TNF- $\alpha$  concentrations) and the other for 24 hours (to analyse IL-6 and IL-8 concentrations), then 1 ml samples were collected, centrifuged (2000 rpm, 5 minutes) and two 300  $\mu$ l samples of supernatant were collected, for later detection of cytokine production, and stored at -80°C.

### 2.5.2 Detection of Cytokine secretion by ELISA

The cell supernatants collected from the 6 hour culture were probed for human TNF- $\alpha$ /TNFSF1A and the cell supernatants collected from the 24 hour culture were probed for human IL-6 and CXCL8/IL-8 using DuoSet ELISA kits (R&D Systems, Oxford, UK) and KPL Sureblue TMB Microwell Peroxidase Substrate (Insight Biotechnology Ltd, Wembley, UK). A set of seven 2-fold serial dilutions of the standard provided in the kit, was generated for each assay. Each standard, control, and sample in the assay was tested and applied in triplicate to the 96 well plate and the method continued according to the manufacturer's protocol. The plate was analysed using an Anthos II Optical Absorbance microplate reader set to 450 nm, with correction wavelength 540 nm. The optical density data was analysed with a linearized plot of log of the cytokine concentrations versus the log of the optical density. If the optical density value of sample fell outside of the standard plot, samples were diluted accordingly and the assay repeated.



## 2.6 Assessment of LPCAT inhibition on inflammatory responses to TLR ligands by qPCR

### 2.6.1 Co-culture of Cells for Cytokine Analysis by qPCR

For standard analysis of mRNA expression by qPCR, cells ( $3 \times 10^6$ ) were set up 3 ml of media in 6 well plates. Selected cells were incubated with or without IFN- $\gamma$  for 16 hours, or directly treated with or without HETP for 30 minutes (post IFN- $\gamma$  incubation) prior to co-culture with or without LPS, LTA, PG or Pam<sub>3</sub>CSK<sub>4</sub>. The negative controls were cell alone. The plate was co-cultured for 4 hours and then the cells were collected and centrifuged (5 minutes, 2000 rpm). Total RNA isolation was achieved using an acid guanidinium thiocyanate-phenol-chloroform extraction method.

### 2.6.2 Total RNA isolation

Briefly, 500  $\mu$ L of denaturing solution was added directly to the cell pellet and allowed to stand for 15 minutes. The following was then sequentially added to cell lysates: 50  $\mu$ L of 2 M sodium acetate, pH 4, mixed thoroughly; 500  $\mu$ L water saturated phenol, mixed thoroughly; 100  $\mu$ L of chloroform: isoamyl alcohol (49:1) and shake vigorously by hand for 10 seconds. The samples were then cooled on ice for 15 minutes and centrifuged for 20 minutes at  $15000 \times g$  at 4°C. The upper aqueous phase, which contains mostly RNA, was transferred to a new RNase- and DNase-free eppendorf tube, containing 500  $\mu$ L of isopropanol to precipitate the RNA. Samples were left to incubate overnight at -20°C and then centrifuged for 20 minutes at  $15000 \times g$  at 4°C. The gel-like precipitate, which is RNA, was kept and the supernatant discarded. The RNA pellet was dissolved with 300  $\mu$ L of denaturing solution and 300  $\mu$ L of isopropanol. The sample was incubated for 30 minutes at -20°C and then centrifuged for 10 minutes at  $15000 \times g$  at 4°C. The supernatant was discarded and the RNA pellet resuspended with 500  $\mu$ L of 75 % ethanol. The samples were vortex for 10 seconds and incubated for 15 minutes at room temperature to dissolve residual guanidinium thiocyanate. Once again, the samples were centrifuged for 10 minutes at  $15000 \times g$  at 4°C, supernatant discarded and the RNA pellet left to air-dry for 5 minutes at room temperature. Finally, the RNA was dissolved in RNase- and DNase-free water and incubated for 15 minutes at 60°C to ensure complete solubilisation. The RNA was then stored at -80°C for reverse transcription.

### 2.6.3 RNA quantification and integrity

The RNA was quantified using a Nanodrop 1000 Spectrophotometer (Thermo Fisher Scientific, Loughborough, UK) and the RNA integrity was calculated using an Agilent RNA 6000 Nano kit and Agilent 2100 bioanalyser (Agilent Technologies Ltd, Stockport, UK). RNA with an integrity number (RIN) less than 8 were not used and the majority of samples had a RIN of 9 or 10.

### 2.6.4 Reverse Transcription

Reverse transcription was carried out according to manufacturer's protocol using Superscript II Reverse Transcriptase and Oligo (dT)12-18 (Invitrogen, Paisley, UK). Briefly, 2 µg of total RNA and 0.1 ng of RuBisCo mRNA (plant mRNA spike used as an internal control) was added to 1 µl of dNTP mix (10mM each) and made up to 13 µl with sterile water. This mixture was heated to 65°C for 5 minutes in a PTC-200 Peltier Thermal Cycler (MJ Research), to denature RNA tertiary structure and then chilled on ice. Next 4 µl of 5× first strand buffer and 2 µl of DTT (0.1M) was added and the mixture heated to 42°C for 2 minutes. Finally, 1 µl of Superscript II RT was added, mixed and incubated at 42°C for 1 hour 30 minutes. The reaction was then inactivated by heating at 70°C for 15 minutes. For internal control RuBisCO mRNA sequence see appendix 4.

### 2.6.5 qPCR

Real time PCR was performed in triplicate using SYBR GreenER qPCR SuperMix (Invitrogen, Paisley, UK) for the iCycler (Bio-Rad, Hertfordshire, UK) according to manufacturer's protocols. Briefly, 10 µl of SYBR GreenER, 400 nM of forward and reverse primers and 5 µl of cDNA (diluted between 1:50-1:100 from RT to achieve approximately 30 µg per reaction) was used in each reaction in a total volume of 20 µl. For specific primer sequences see appendix 4.

### 2.6.6 Agarose gel for electrophoresis of PCR products

A 2 % agarose gel was made with TRIS Borate-EDTA buffer (TBE) and 0.5 µg/ml ethidium bromide. The gel was set aside to solidify for at least 30 minutes before removing the comb. qPCR samples were mixed with loading buffer before adding to the wells and DNA Hyperladder V (Bioline Ltd, London, UK) was loaded to the

outer well to assist base-pair separation. The agarose gel was run in TBE at 120V for 1 hour.

## 2.7 Assessment of human primary monocyte responses to TLR ligands

### 2.7.1 Isolation of Peripheral Blood Mononuclear Cells

Briefly, 50 ml of human donor blood was diluted with an equal volume of sterile PBS. The blood sample was then overlaid on equal volume of LymphoPrep (Axis-shield, Cambridgeshire, UK) and centrifuged at 2,900 rpm for 30 minutes at room temperature with no brake. The buffy coat layer was carefully removed and placed into clean tubes with PBS for cell washing (2,400 rpm, 15 minutes). The PBMC pellets were then combined and washed in sterile water (1,300 rpm, 10 minutes). The PBMCs were resuspended in 20 ml of  $\times 1$  red blood cell lysis buffer and left at room temperature for 10 minutes. The cells were then washed twice in PBS (1,300 rpm, 10 minutes) and prepared for culture or monocyte isolation.

### 2.7.2 Isolating an Enriched Monocyte Population

Enrichment of human monocytes from donor PBMCs, involved the trialling of three methods following manufacturer's instruction; the first followed the adherent method as used by Schmid *et al.* (2003), the second required separation of monocytes from a leukocyte-rich plasma by flotation through a discontinuous iodixanol gradient using OptiPrep (Axis-shield, Cambridgeshire, UK) (Graziani-Bowering *et al.*, 1997) and the third comprised of an indirect magnetic labelling system (MACS monocyte isolation kit II; Miltenyi Biotec Ltd, Surrey, UK) which was subsequently use in additional experiments.

### 2.7.3 Adherence method by Schmid *et al.* (2003)

Following isolation of PBMCs, cells were washed twice with NaCl/Pi RPMI 1640 medium without additives to give a cell density of  $5 \times 10^6$ /ml. To each well of a 24-well plate, 400  $\mu$ l of the cell suspension was added and incubated at 37°C for 2 h. The monolayer was washed thrice with warm RPMI 1640 medium. 1 ml fresh complete RPMI 1640 medium (with all additives detailed above) was added and the cells incubated at 37°C.

#### 2.7.4 OptiPrep™ (Axis-Sheild)

This method separated monocytes from a leukocyte-rich plasma by flotation through discontinuous iodixanol gradient (Axis-Sheild Cell Application Sheet C09). Briefly, two solutions of 1.068 g/ml and 1.084 g/ml were prepared by mixing OptiPrep™ and a solution containing 1.0 % (w/v) NaCl, 1 mM EDTA, 10 mM Hepes-NaOH, pH 7.4 and 0.5 % (w/v) bovine serum albumin. After isolation of PBMCs, cells were mixed with OptiPrep™ (10 ml + 4 ml, respectively) and overlaid with 7.5 ml of the 1.084 g/ml solution and 20.0 ml of the 1.068 g/ml solution. Finally a small volume (approx. 0.5 ml) of Hepes-buffered saline was layered on top. The discontinuous gradient was centrifuged at 600-800×g in a swinging-bucket rotor for 20-25 min at 20° C.

#### 2.7.5 MACS monocyte isolation kit II (Axis-shield)

The indirect magnetic labelling system isolated untouched monocytes and was used according to the manufacturer's guidelines. In summary, PBMCs were resuspended in 30 µl of PBS buffer/10<sup>7</sup> cells, 10 µl of FcR blocking reagent/10<sup>7</sup> cells and 10 µl /10<sup>7</sup> cells of biotin conjugated antibodies against CD3, Cd7, Cd16, CD19, Cd56, Cd123 and CD235a and cells were incubated for 10 minutes at 4°C. 30 µl PBS buffer/10<sup>7</sup> cells and 20 µl of anti-biotin microbeads/10<sup>7</sup> cells were subsequently added and incubated for an additional 15 minutes at 4°C. Following washing with PBS buffer, cells were resuspended in 500 µl /10<sup>8</sup> cells and applied to a 3 ml LS MACS separator column, where unlabelled cells passed through and effluent was collected. This cell suspension represented an enriched population of monocytes.

#### 2.7.6 Assessment of enriched cell population purity by flow cytometry

PE Mouse anti-human CD14 antibody (BD Bioscience, Oxford, UK) was used to assess the percentage of monocytes in PBMCs before isolation and to check the purity of the enriched monocyte population by flow cytometry. Cells were blocked with BSA for 10 minutes at RT prior to staining with conjugated CD14-PE antibody at 1 µg /10<sup>6</sup> cells for 40 minutes. For the iso-type control, cells from each treatment were incubated with PE secondary antibody for 40 minutes. All cell samples and treatments were then analysed using Accuri C6 Flow Cytometer. Cell debris and dead cells are excluded from the analysis based on scatter signals and fluorescence.

## 2.8 Analysis of LPCAT and TLR2 protein expression by western blot

### 2.8.1 Co-culture of cells for analysis by western blot

For standard analysis of protein expression by western blot, cells ( $6 \times 10^6$ ) were set up 3 ml of supplemented RPMI media in 6 well plates. Selected cells were incubated with or without IFN- $\gamma$  for 18 hours, or directly treated with or without HETP for 30 minutes (post IFN- $\gamma$  incubation) prior to co-culture with or without LPS, LTA, PG or Pam<sub>3</sub>CSK<sub>4</sub>. The plate was co-cultured for 1 hour and then the cells were collected and centrifuged (5 minutes, 2000 rpm). The negative controls were cell alone.

### 2.8.2 Preparation of total cell lysate

Following incubation, cells were harvested, washed twice with ice cold PBS and centrifuged at 1500 rpm, for 5 minutes at 4°C. Cells were lysed in 300  $\mu$ l of ice cold Triton X extraction buffer for 1 hour at 4°C and sonicated on ice to homogenize the lysate in 4 $\times$ 11 second bursts and reduce sample viscosity. Complete lysis of cells was checked using light microscopy, before centrifuging the samples at 2,500 $\times$ g at 4°C for 5 minutes to remove cell debris and retain protein in the supernatant.

### 2.8.3 Quantification of Total Protein

Protein quantification of cell lysate was determined using Pierce BCA microplate Protein assay kit (Thermo Fisher Scientific, Loughborough, UK). This method combines the well-known reduction of Cu<sup>+2</sup> to Cu<sup>+1</sup> by protein in an alkaline medium (the biuret reaction) with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu<sup>+1</sup>) using a unique reagent containing bicinchoninic acid.

### 2.8.4 Western blot method

Equal volumes of each lipid fractions or 20  $\mu$ g of total cell lysate were mixed with the same volume of  $\times$ 2 LDS sample buffer and heated at 95°C for 5 minutes before being loaded onto Criterion XT Precast (4-12 %) Bis-Trisgels (Bio-Rad, Hertfordshire, UK). Biotinylated Protein ladder (New England Biolabs, Hertfordshire, UK) and see blue plus 2 pre-stained standard (Invitrogen, Paisley, UK) were loaded at either end of the gel to aid identification of proteins and assess the progress of the separation. The gels were run in a Criterion Electrophoresis Tankat 100V for 1.5 hours in the presence of XT-MOPS running buffer.

The gel was then transferred onto Hybond-C Extra nitrocellulose membrane (Amersham via GE Healthcare, Buckinghamshire, UK) using a Criterion blotter at 50V for 1 hour at 4°C in the presence of transfer buffer. After transfer the membrane was washed with TBS-T (two 5 minute washes) and left for 1 hour in blocking solution (5 % low fat dried milk dissolved in TBS-T), to block non specific binding sites, then washed with TBS-T (three 5 minute washes). Membranes were probed with the appropriate dilution of primary antibody in blocking solution overnight at 4°C, including; IMGENEX monoclonal mouse anti-human TLR2 and anti-human CD71 (transferrin receptor) (Cambridge Bioscience, Cambridge, UK), mouse anti-human RNF40 polyclonal antibody (Abnova distributed by Novus Biologicals Ltd, Cambridge, UK), rabbit anti-human polyclonal LPCAT1 and LPCAT2 (ProteinTech, Manchester, UK), mouse anti-human monoclonal GAPDH (0411) antibody (Santa Cruz, CA, USA).

Overnight incubation was followed by three 5 minute washes in TBS-T. Membranes were then incubated for 1 hour with HRP-conjugated anti-biotin antibody to detect biotinylated protein markers, and the appropriate dilution of HRP-conjugated secondary antibody in blocking buffer (either anti-mouse or anti-rabbit IgG) (Cell Signalling Technology, supplied by New England Biolabs, Hertfordshire, UK). After extensive washing with TBS/T to remove excess antibody, the antigen was visualised using Amersham ECL Plus Western Blotting Detection Reagents (GE Healthcare, Buckinghamshire, UK) according to the manufacturer's instructions. Re-probing of membranes was achieved by stripping the membrane in 1M sodium hydroxide for 7 minutes, followed by extensive washing with PBS and TBS/T and repeating the above process from the blocking step. The electrophoresis gel was also fixed and stained with a Brilliant Blue G-Colloidal mix to confirm successful transfer of proteins.

## 2.9 Determining TLR translocation into lipid raft domains

### 2.9.1 Preparation of Total Cell Lysate

MonoMac-6 cells ( $3 \times 10^6$ ) were incubated with or without IFN- $\gamma$  for 18 hours and then treated with or without HETP for 30 minutes prior to co-culture with or without LPS, LTA, PG or Pam<sub>3</sub>CSK<sub>4</sub>. The negative controls were cell alone. The plate was co-cultured for 45 minutes and then the cells were collected and centrifuged (2000 rpm, 5 minutes). Cells were lysed in 300  $\mu$ l of ice-cold extraction buffer containing 1 % Triton X-100 and 1 tablet of Complete Mini EDTA-free protease inhibitor cocktail (Roche, Hertfordshire, UK), for 1 hour on ice. The cell lysate was sonicated for four 11 second bursts to homogenize the lysate, shear DNA and reduce sample viscosity. Complete lysis was checked using light microscopy. Lysates were centrifuged at 2000 rpm for 5 minutes to remove the nuclei and cell debris and the supernatant transferred and stored at -80°C for later use.

### 2.9.2 Isolation of Lipid Fractions

The lipid raft domains were isolated from cells as described by Triantafilou *et al.* (2004) with slight modifications. MonoMac-6 cells ( $1 \times 10^7$ ) were incubated with or without IFN- $\gamma$  for 18 hours and then treated with or without HETP for 30 minutes prior to co-culture with or without LPS, LTA, PG or Pam<sub>3</sub>CSK<sub>4</sub>. The negative controls were cell alone. The plate was co-cultured for 45 minutes and then the cells were collected and centrifuged (2000 rpm, 5 minutes). Cells were lysed in 500  $\mu$ l of MES buffered saline (MBS) containing 1 % Triton X-100 (a detergent that facilitates the separation of soluble and insoluble fractions) and 1 tablet of Complete Mini EDTA-free protease inhibitor cocktail (Roche, Hertfordshire, UK), to inhibit proteolytic activity, for 1 hour on ice. The cell lysate was sonicated for four 11 second bursts to homogenize the lysate and reduce sample viscosity. The cell lysate was then mixed with an equal volume of 90 % sucrose in MBS and placed at the bottom of a centrifuge tube. The sample was overlaid with 5.5 ml of 30 % sucrose and 4.5 ml of 5 % sucrose in MBS and centrifuged at  $100,000 \times g$  for 16 hours (4°C) using SW40Ti rotor on Beckman Coulter Optima L-100XP Ultracentrifuge. This allowed the membrane microdomains, rich in sphingolipids and cholesterol, to be separated out based on their insolubility in Triton X-100 and by low buoyant density in sucrose gradients. Twelve membrane fractions (1 ml each) were then gently removed from the top of the gradient and n-Octyl- $\beta$ -D-glucopyranoside (Sigma,

Poole, UK) added to each fraction (60  $\mu\text{M}$  final concentration) to solubilise the lipid fractions. These were stored at  $-80^{\circ}\text{C}$  for later use.

### 2.9.3 Assessment of lipid raft isolation by Dot Blot

To determine which fractions contained the lipid raft, samples (5  $\mu\text{l}$ ) from each of the twelve fractions were taken and left to dry on nitrocellulose membrane for 1 hour. The membrane was then probed for GM1 (a ganglioside receptor prevalent in lipid rafts) with 1:1000 dilution of HRP-conjugated cholera-toxin  $\beta$ -subunit (which specifically binds to GM1) (Sigma, Poole, UK) for 1 hour in blocking buffer (5 % low fat dried milk dissolved in TBS, 0.1 % Tween 20). The membrane was washed with 15 ml washing buffer, TBS +0.1 % Tween 20 (TBS-T), four 5 minute washes and two 2 minute washes in water, to remove any excess antibody. The antigen was then visualised using Amersham ECL Plus Western Blotting Detection Reagents (GE Healthcare, Buckinghamshire, UK) according to the manufacturer's instructions.

### 2.9.4 Protein concentration

Concentrating protein from cell lysate fractions following lipid raft isolation, involved the trialling of three methods; methanol/chloroform precipitation (Wessel and Flugge, 1984), acetone precipitation (Pierce Biotechnical Inc, USA) and phenol/ether precipitation (Sauvé *et al.*, 1995). Following extensive experimental optimisation and analysis, it was evaluated that the phenol/ether precipitation method was the most proficient at recovering protein from the samples.

### 2.9.5 Methanol/chloroform precipitation

The original method by methanol/chloroform precipitation Wessel and Flugge (1984) was optimised to incorporate a large volume of sample. Briefly, 700  $\mu\text{l}$  of methanol was added to 300  $\mu\text{l}$  of sample and mixed before the addition of 400  $\mu\text{l}$  of chloroform. Samples were mixed well by vortexing, then 200  $\mu\text{l}$  of de-ionised water was added to assist phase separation. Samples were centrifuged at  $13,000\times g$  for 15 minutes at  $4^{\circ}\text{C}$ . The top aqueous phase was removed and discarded, whilst the organic phase was dissolved in 800  $\mu\text{l}$  methanol. Samples were centrifuged at  $13,000\times g$  for 30 minutes at  $4^{\circ}\text{C}$  and the supernatant was removed, subsequently leaving the protein pellet to air dry before being resuspended in SDS buffer.



### 2.9.6 Acetone precipitation

Modifications to the original protocol by Pierce Biotechnical Inc. included an additional precipitation cycle necessary to completely remove the interfering sucrose as follows; chilled acetone (-20°C) was added at four times the sample volume and incubate for 60 minutes at -20°C. Samples were centrifuged at 13,000×g for 15 minutes at 4°C before removal of the supernatant. A second precipitation cycle was repeated with samples incubating with acetone at -20°C for an additional 20 minutes. The acetone was allowed to evaporate from the uncapped sample at room temperature for 30 minutes prior to dissolving the protein pellet in SDS buffer.

### 2.9.7 Phenol/ether precipitation

The use of the rapid method for concentration of protein based on extraction with phenol and ether, as described by Sauvé *et al.* (1995), was used in subsequent experiments. In summary, an equal volume of phenol for each sample was added and mixed thoroughly by vortexing. Samples were centrifuged at 13,000×g for 5 minutes at 4°C before removal of the upper phase. Two times the volume of ether was added to the phenol phase, mixed and centrifuged as above. The upper phase was again discarded and the previous step repeated with the addition of twice the volume of ether. The lower aqueous phase was dried by vacuum centrifugation prior to solubilising the protein pellet in SDS buffer.

## 2.10 Data analysis

Independent experiments were performed on separate dates with distinctive cells or progressive cell passage numbers. Statistical comparisons among groups were analysed using a two-tailed, paired *t*-test. Minitab software Version 15 (Minitab Inc., UK) was used for all analyses. The paired *t*-test compares the mean of the two sets of data and produces a probability, or *P* value, that the difference between the two sets of data is likely to be due to chance. Paired *t*-test results were interpreted depending on whether the *P* value was small or large. If  $P > 0.05$ , then it was more than likely that the two sets of data were the same and that any differences were due to chance. If  $P < 0.05$ , then it was more than likely that the two sets of data were significantly different and that the differences were due to treatment. Assumptions of the *t*-test are that both sets of data follow normal distribution, which was confirmed with a Kolmogorov–Smirnov normality test.

### 2.10.1 ELISA data analysis

Cell supernatants were tested for the presence of human TNF- $\alpha$ , IL-6 and IL-8 using DuoSet ELISA kits (R&D Systems, Oxford, UK). Each standard, control and sample was tested in triplicate and the average optical density of the triplicate readings was used to calculate the resulting concentration. The average optical density for the zero standards was then subtracted from each sample. A seven point standard curve using 2-fold serial dilutions of the standard was generated for each set of samples assayed. Then a linearized plot of the log of the cytokine concentrations versus the log of the optical density of the standards was produced. The best fit line was determined by regression analysis and used to calculate cytokine concentrations from the optical density data. Where samples were diluted, the concentration read from the linearized plot was multiplied by the dilution factor.

Statistical comparisons among groups were determined by calculating a *P* value. For assessment of multiple concentration comparisons of pharmacological inhibitors a one-way analysis of variance (ANOVA) was performed. For all other ELISA data analysis, a two-tailed, paired *t*-test was used to compare treatments. Data from cells stimulated with a microbial ligand were paired with the data from cells, in the same independent experiment, also treated with the inhibitor, HETP.

### 2.10.2 qPCR data analysis

Real-time PCR data for assessment of cytokine expression were analysed using  $2^{-\Delta\Delta CT}$  and relative gene expression methods (Livak and Schmittgen, 2001) by normalizing data to the housekeeping gene, GAPDH, and then calculating the relative fold difference against untreated cells as a reference. For analysis of endogenous LPCAT expression in different cell types, all cells were untreated, therefore expression levels were relative to each other. The data are presented as the mean fold change in mRNA expression, normalized to GAPDH and relative to MM6 cells for comparison. Statistical comparisons among groups were analysed using a two-tailed, paired *t*-test.

### 2.10.3 Western blot data analysis

Protein expression of LPCAT1 and LPCAT2 in different cells was determined via western blot. Protein band intensity was measured using ImageJ software and used as a qualitative measure of protein expression (Abramoff *et al.*, 2004).

### 2.10.4 Flow cytometry data

Human blood monocytes extracted using the monocyte indirect magnetic labelling system were analysed by flow cytometry using Accuri C6 Flow Cytometer. Cell debris and dead cells are excluded (gated) from the analysis based on scatter signals and fluorescence and 10, 000 events recorded.

# Chapter 3

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RESULTS AND DISCUSSION

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THE INFLUENCE OF LPCAT REGULATION ON TLR2-  
INDUCED CYTOKINE RESPONSES

### 3.1 INTRODUCTION

LPCAT enzymes are potential targets for the regulation of inflammatory responses. Previous studies have examined the effect of a specific inhibitor for LPCAT on LPS induced cytokine production in human monocytic cells; the inhibitor 5 hydroxyethyl 5,3' thiophenyl pyridine (HETP or YM 50201) was shown to reduce TNF- $\alpha$  secretion induced by LPS in MonoMac6 (MM6) cells (Schmid *et al.*, 2003) and subsequent investigation observed that this inflammatory response was largely mediated by signalling through TLR4 (Jackson *et al.*, 2008). The work presented in this chapter details experiments that assess the effect of LPCAT inhibitors on TLR2 mediated responses to determine if LPCAT has a key role in the regulation of Gram-positive bacterial induced inflammatory responses in monocytes. This will ascertain whether the inhibitory effects of HETP observed in LPS induced cells are either general to surface TLRs or associated with TLR4 specifically.

The lungs are in constant contact with the external world of commensals and pathogens and therefore they are organs that are always under some condition of damage and danger. Lung tissues are delicate and have designated functions to perform, thus they cannot allow themselves to be overly infiltrated with immune cells that might destroy them or their function in the process of fighting infection. In this chapter, the effect of LPCAT inhibitors on TLR2 mediated responses in lung epithelial cells will be assessed to establish if the inflammatory responses are as sensitive as those observed in monocytes.

Gram positive bacterial components, LTA, PG and Pam<sub>3</sub>CSK<sub>4</sub> were chosen to stimulate TLR2 responses because of the diversity of signalling pathways they provide; LTA through TLR2/TL6, Pam<sub>3</sub>CSK<sub>4</sub> through TLR2/TLR1 and PG possibly through the TLR2/TL6 heterodimer or intracellular NODs (Nod1/Nod2) (Travassos *et al.*, 2004; O'Neill *et al.*, 2013).

### 3.2 AIMS

The key aim of this chapter was to assess the effect of LPCAT inhibition on TLR2 and TLR4 ligand-mediated inflammatory responses in human monocyte and lung epithelial cells.

### 3.3 METHODS

Following Schmid *et al.* (2003), experiments in this study used MM6 cells, which are phenotypically and functionally similar to mature monocytes, to investigate the role of LPCAT in inflammatory signalling processes. Unless specified otherwise, “cells” refers to the MM6 cell line.

#### 3.3.1 Toxicity assessment for LPCAT inhibitors

A toxicity assay was performed in parallel to dose response assays in order to assess the effectiveness of the LPCAT inhibitors and ensure that selected inhibitors exhibited no cell toxicity. MM6 cells ( $5 \times 10^5$ ) were co-cultured with a specified concentration of either HETP (15-100  $\mu\text{M}$ ) or CI-976 (10-50  $\mu\text{M}$ ) alone or 30 minutes prior to stimulation with 100 ng/ml *E. coli* LPS and incubated for 24 hours. After culture, the cells were gently resuspended and a cell viability count performed using the Typan blue dye exclusion method.

#### 3.3.2 Determination of IC<sub>50</sub>

Under identical conditions and in parallel to the toxicity assay, the effect of LPCAT inhibitors on cytokine secretion were assessed by dose response experiments. After 6 or 24 hours culture, cell supernatants were collected and tested for the presence of TNF- $\alpha$ , IL-6 and IL-8 by ELISA. The concentrations of secreted cytokines were analysed to determine the respective IC<sub>50</sub> for each inhibitor and inhibitor concentrations for further work were selected on the basis of these results and cell toxicity assays.

#### 3.3.3 Assessment of the effects of HETP on inflammatory responses to TLR ligands

Cultures of MM6 cells ( $5 \times 10^5$ ) were incubated in the presence or absence of 50  $\mu\text{M}$  HETP, 30 minutes prior to stimulation with either 100 ng/ml *E. coli* O111:B4 lipopolysaccharide (LPS), 10  $\mu\text{g/ml}$  *S. aureus* lipoteichoic acid (LTA), 10  $\mu\text{g/ml}$  *S. aureus* peptidoglycan (PG) or 1.0  $\mu\text{g/ml}$  Pam<sub>3</sub>CSK<sub>4</sub> and subsequently co-cultured for 6 or 24 hours. The concentrations of three cytokines central to inflammation, TNF- $\alpha$ , IL-6 and IL-8, were then determined using specific ELISAs.

### 3.3.4 Assessment of human primary monocyte responses to TLR4 and TLR2 ligands and the effects of HETP

To confirm that MM6 cells were a good model for human monocytes, human primary monocyte cells were obtained from healthy volunteer PBMCs using three isolation methods and the purity of each enriched monocyte population was assessed by staining with anti-human CD14 antibody (BD Bioscience, Oxford, UK) and flow cytometry.

Monocytes were purified by several methods including the adherence method as used by Schmid *et al.* (2003), a floatation method using OptiPrep discontinuous gradients (Axis-shield, Cambridgeshire, UK) (Graziani-Bowering *et al.*, 1997) and a monocyte indirect magnetic labelling system (MACS monocyte isolation kit II; Miltenyi Biotec Ltd, Surrey, UK). However the first two methods were not considered to be viable options due to low recovery, therefore the magnetic labelling system was used in further experiments.

All experiments were completed in the presence of human serum, since it is well documented that serum increases responsiveness of monocytes to LPS due to the availability of soluble CD14, a key factor in the inflammatory responses (Kreutz *et al.*, 1997; Adib-Conquy *et al.*, 2002; Schmid *et al.*, 2003). Peripheral blood monocytes were incubated under the same conditions as the experiments with MM6 cells (section 3.3.3) to confirm that the results observed with MM6 cells were representative of human monocytes.

### 3.3.5 Efficacy of HETP to inhibit inflammatory cytokine mRNA expression

qPCR was used to determine the influence of HETP on cytokine mRNA to establish if cytokine inhibition at a protein level was caused by changes at the transcriptional level. MM6 cells ( $2.2 \times 10^6$ ) were treated for 4 hours in the presence of a TLR ligand or with prior inhibition by addition of HETP (50  $\mu$ M), before total RNA isolation by acid guanidinium thiocyanate, phenol, chloroform extraction. Using qPCR, the mRNA of inflammatory cytokines, TNF- $\alpha$ , IL-6, IL-8, were normalized with housekeeping gene, GAPDH, and the fold difference calculated using untreated cells as a reference ( $2^{-\Delta\Delta CT}$  method).

### 3.3.6 Determination of interactions between IFN priming of cells and HETP, and the effect on cellular responses to TLR4 and TLR2 ligands

Experiments were performed to examine the effect of IFN- $\gamma$  priming on monocyte cytokine secretion in response to TLR4 and TLR2 ligands and the potential of HETP to down-regulate cytokine secretion. MM6 cells ( $5 \times 10^5$ ) were pre-incubated with 250 U/ml IFN- $\gamma$  for 16 hours, as was optimal for LPCAT activity in previous studies (Neville *et al.*, 2005), prior to treatment with HETP (50  $\mu$ M) and TLR ligand stimulation, then co-cultured for 6 or 24 hours. As described previously (section 3.3.3), concentrations of inflammatory cytokine secretion were determined by ELISA.

### 3.3.7 Assessment of the effects of HETP on inflammatory responses to TLR4 and TLR2 ligands in broncho-epithelial cells

BEAS-2B cells were maintained in media supplemented with 5 % (v/v) foetal calf serum, however, these cells were initially unresponsive to microbial stimulants under these culture conditions. Further experimental investigation found that lung epithelial cells required human serum components to respond to LPS-induced activation, supporting the findings of Schulz *et al.* (2002).

BEAS-2B cells ( $1 \times 10^5$ ) were cultured in 12 well plates for 2 days to reach confluency, before changing to serum free media overnight. For the cytokine experiments, fresh media supplemented with 2 % pooled human serum (HS), required for a low concentration LPS induced inflammatory response, was added. BEAS-2B cells were treated with or without HETP prior to stimulation with TLR4 and TLR2 ligands, as with previous experiments and concentrations of inflammatory cytokines were determined by ELISA (section 3.3.3).



### 3.4 RESULTS

#### 3.4.1 Pharmacological LPCAT inhibitors show differences in cellular toxicity

Viability experiments were performed using a range of inhibitor concentrations, either in the presence or absence of LPS. With the specific LPCAT inhibitor, HETP, cell viability was over 80 % using a concentration up to 50  $\mu\text{M}$  in the presence of LPS (Figure 1.1 Table 4.1). However, cell viability significantly deteriorated with 100  $\mu\text{M}$  HETP, demonstrating that higher concentrations of this inhibitor were toxic to the cells. Conversely cell viability was significantly impaired using even low concentrations (10  $\mu\text{M}$ ) of the non-specific inhibitor, CI-976. This reduction in viability is likely a result of CI-976 inhibiting multiple membrane trafficking steps, including those required for cells to survive (Brown *et al.*, 2008).

#### 3.4.2 Pharmacological inhibitors of LPCAT reduce cytokine secretion in a dose-dependent manner

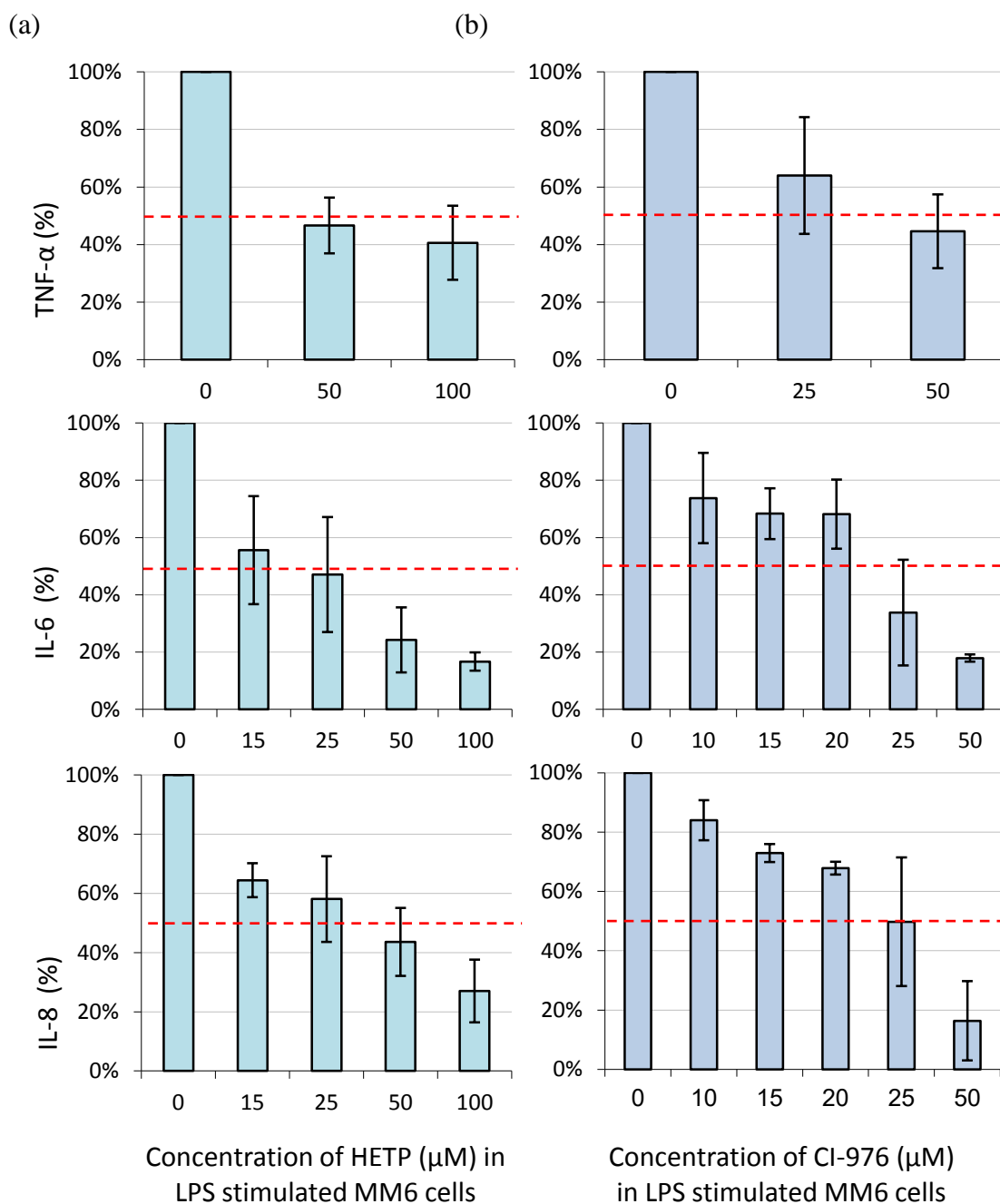
Alongside the toxicity assay, TNF- $\alpha$ , IL-6 and IL-8 secretions were measured using ELISA to determine the  $\text{IC}_{50}$  for each inhibitor. Figure 3.1 shows that although both LPCAT inhibitors reduce individual cytokine secretion in a dose dependant manner, 50 % inhibition across all three cytokines can be achieved using 50  $\mu\text{M}$  HETP and 25  $\mu\text{M}$  CI-976. However, at this concentration, CI-976 was shown to be toxic to the cells and thus this inhibitor was excluded from further studies. Future experiments, therefore, were completed using the specific LPCAT chemical inhibitor, HETP, at a concentration of 50  $\mu\text{M}$  to maintain significant cytokine inhibition and cell viability over 80 %. Statistical analysis of control cells co-cultured with 50  $\mu\text{M}$  HETP was found not to significantly ( $P>0.1$ ) effect cytokine secretion when compared to endogenous cytokine levels secreted by MM6 cells (Figure 3.2).

HETP ( $\mu\text{M}$ )	concentration	MM6 cell viability (%)	
		Without LPS	With LPS
0		94.7 $\pm$ 2.3	84.6 $\pm$ 5.1
25		92.4 $\pm$ 2.1	93.0 $\pm$ 1.8
50		86.7 $\pm$ 3.3	83.0 $\pm$ 4.3
100		75.4 $\pm$ 4.6	62.7 $\pm$ 8.6

CI-976 ( $\mu\text{M}$ )	concentration	MM6 cell viability (%)	
		Without LPS	With LPS
0		94.7 $\pm$ 2.3	84.6 $\pm$ 5.1
10		81.6 $\pm$ 7.9	76.2 $\pm$ 4.6
25		77.9 $\pm$ 5.3	56.3 $\pm$ 10.2
50		23.2 $\pm$ 12.4	0.00

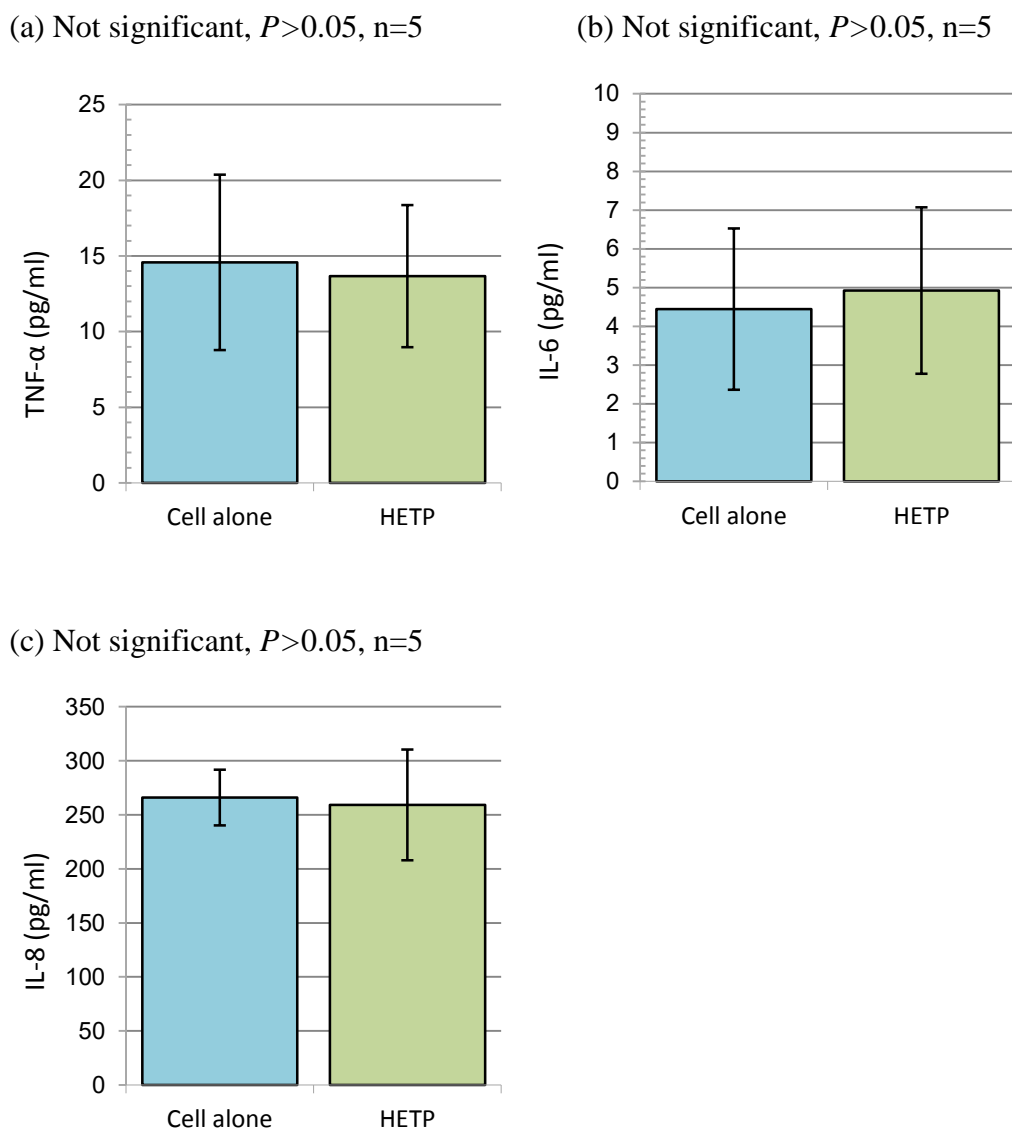
**Table 4.1 The effect of CI-976 and HETP concentration on MM6 cell viability.**

MM6 cells were cultured with a specified concentration of either HETP (25-100  $\mu\text{M}$ ) or CI-976 (10-50  $\mu\text{M}$ ) for 24 h alone or with 100 ng/ml *E. coli* LPS. After culture the cells were gently suspended and a cell viability count performed using the Typan blue dye exclusion method. Results are a mean percentage  $\pm$  S.D. of viable cells from 3 independent experiments.



**Figure 3.1 The effect of pharmacological LPCAT inhibitors on TNF- $\alpha$ , IL-6 and IL-8 secretion in LPS stimulated MM6 cells.**

MM6 cells were treated for 6 or 24 h with 100 ng/ml *E. coli* LPS alone or together with LPCAT inhibitor, HETP (15-100  $\mu$ M) or CI-976 (10-50  $\mu$ M). After culture cell, supernatants were collected and tested for the presence of each cytokine by ELISA. Results are a percentage of the value obtained with cells stimulated with LPS and are the mean  $\pm$  S.E.M of 5 independent experiments. The red dashed line represents IC<sub>50</sub> of HETP (a) and CI-976 (b). These data were analysed for significance by ANOVA (see 2.10.1 for details).



**Figure 3.2 Control cells - LPCAT inhibitor, HETP, has no significant effect on TNF- $\alpha$ , IL-6 and IL-8 secretion in MM6 cells.**

The supernatants from MM6 cells alone or after incubation with LPCAT inhibitor, HETP (50  $\mu\text{M}$ ) for 30 minutes were collected and tested for the presence of (a) TNF- $\alpha$ , (b) IL-6 or (c) IL-8 by ELISA. Results represent the intrinsic level of cytokine secretion by control cells and are the mean cytokine concentration  $\pm$  S.E.M of 5 independent experiments. These data were analysed for significance by paired  $t$ -test.

### 3.4.3 MM6 cells treated with HETP have reduced inflammatory response on stimulation with TLR2 ligands

As observed previously, HETP demonstrated an ability to significantly reduce secretion of inflammatory cytokines TNF- $\alpha$ , IL-6 and IL-8 in cells stimulated with TLR4 ligand LPS, suggesting that LPCAT has a role in TLR4 initiated cytokine responses. In order to determine if a similar effect could be reproduced in cells stimulated with TLR2 ligands, experiments were conducted with PG, LTA and the synthetic tripalmitoylated lipopeptide, Pam<sub>3</sub>CSK<sub>4</sub>. Concentrations of inflammatory cytokine secretion were determined as previously by ELISA.

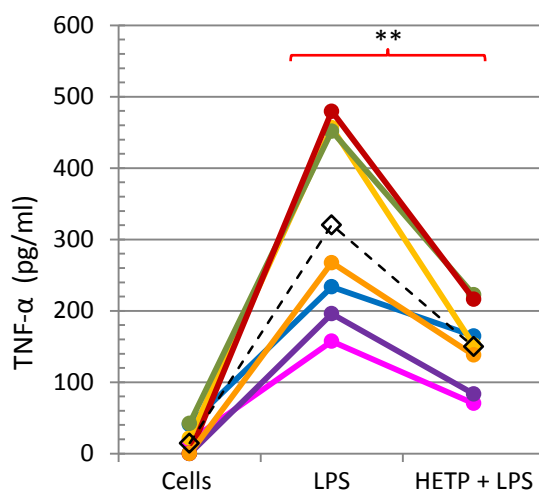
*E. coli* LPS was employed as a positive control for cytokine stimulation and induced a mean of 320 pg/ml TNF- $\alpha$  in MM6 cells (Figure 3.3a). A significant reduction in the concentration of TNF- $\alpha$  produced by these cells was seen with pre-incubation of HETP (50  $\mu$ M) to a mean of 150 pg/ml, inhibiting TNF- $\alpha$  secretion by 53 % ( $P < 0.01$ ).

In cells stimulated with LTA, the LPCAT inhibitor reduced TNF- $\alpha$  secretion by 22 % to a mean concentration of 109 pg/ml (Figure 3.3b). Although this was to a lesser extent when compared to LPS stimulated cells, the inhibition was still significant ( $p = 0.04$ ). LTA alone induced TNF- $\alpha$  secretion to a mean of 140 pg/ml, approximately half the concentration as seen with LPS stimulation. This may indicate that LTA is not as proficient as LPS in inducing TNF- $\alpha$  secretion in MM6 cells.

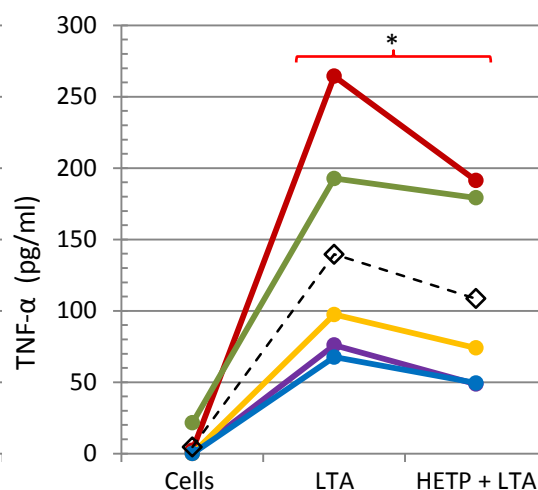
Pre-incubation with HETP in PG stimulated MM6 cells also displayed significant cytokine inhibition; a 51 % reduction ( $p = 0.02$ ) of secreted TNF- $\alpha$  (264 pg/ml) was observed (Figure 3.3c). Interestingly, stimulation with TLR2 ligand PG alone produced higher concentrations of TNF- $\alpha$  compared to LPS; inducing a mean of 533 pg/ml in these cells.

In spite of the minimal TNF- $\alpha$  concentration that Pam<sub>3</sub>CSK<sub>4</sub> alone induced (58 pg/ml), the largest suppression of TNF- $\alpha$  secretion by HETP was seen in cells stimulated with this ligand (Figure 3.3d). Results displayed a significant 54 % reduction ( $p = 0.03$ ) of secreted TNF- $\alpha$  to a mean of 27 pg/ml.

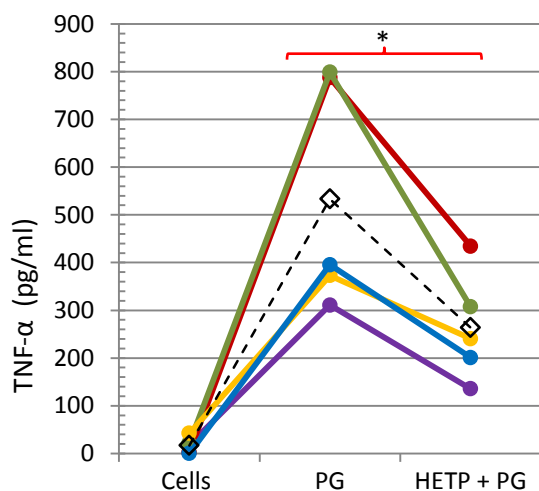
(a) \*\*  $P < 0.01$ ,  $n = 7$



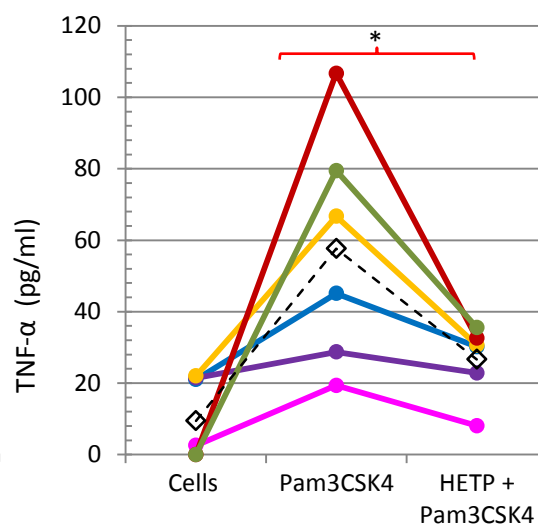
(b) \*  $P < 0.05$ ,  $n = 5$



(c) \*  $P < 0.05$ ,  $n = 5$



(d) \*  $P < 0.05$ ,  $n = 6$



Key:

—●— Exp 1   —●— Exp 2   —●— Exp 3   —●— Exp 4   —●— Exp 5   —●— Exp 6   —●— Exp 7   - -◇- - Mean

**Figure 3.3 The effect of HETP on TNF- $\alpha$  secretion in MM6 cell stimulated with LPS, LTA, PG or Pam<sub>3</sub>CSK<sub>4</sub>.**

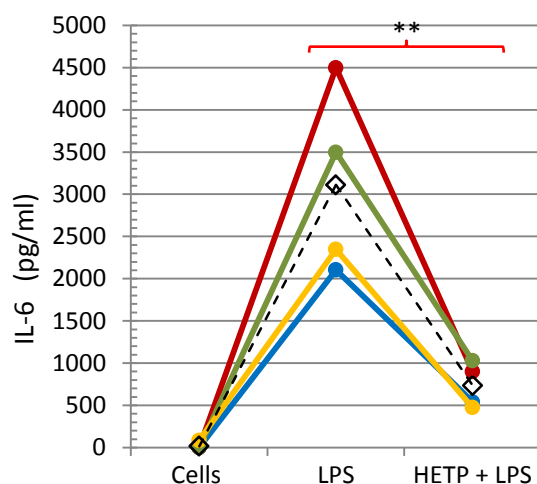
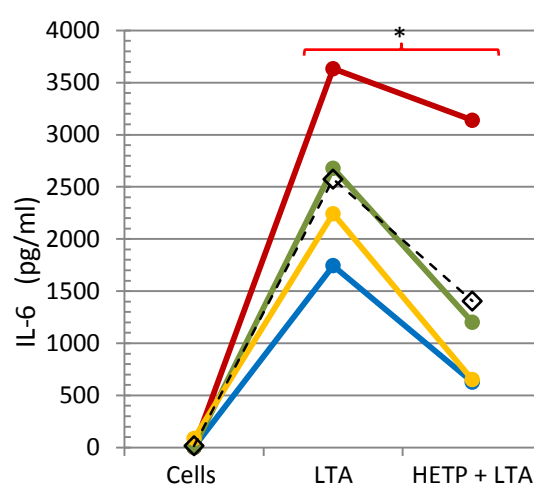
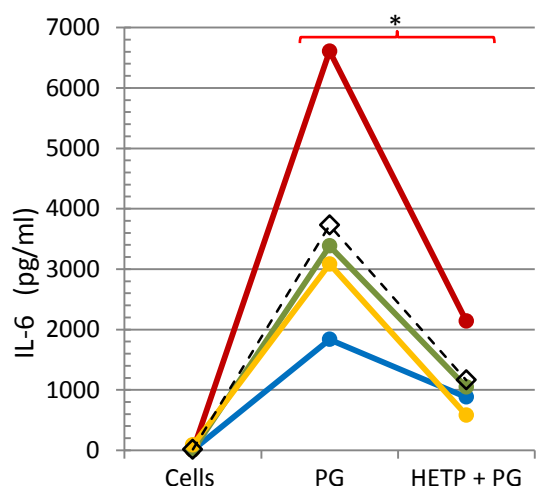
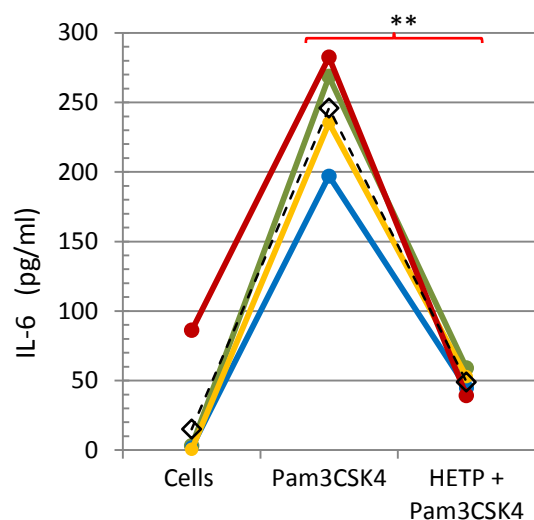
MM6 cells were treated for 6 hours with (a) 100 ng/ml LPS, (b) 10  $\mu$ g/ml LTA, (c) 10  $\mu$ g/ml PG or (d) 1.0  $\mu$ g/ml Pam<sub>3</sub>CSK<sub>4</sub> alone or with prior inhibition by addition of HETP (50  $\mu$ M). After culture cell supernatants were collected and tested for the presence of TNF- $\alpha$  by ELISA. Results represent the TNF- $\alpha$  concentration from at least 5 independent experiments and the mean TNF- $\alpha$  concentration. Control cells with HETP only were also run alongside experiments (Figure 3.2). These data were analysed for significance by paired *t*-test.

The next inflammatory cytokine studied, IL-6, expressed a similar trend to TNF- $\alpha$ . LPS induced a mean of 3.1 ng/ml in MM6 cells (Figure 3.4a) and IL-6 secretion was significantly suppressed with pre-incubation of HETP to a mean of 736 pg/ml; equivalent to 76 % inhibition ( $p=0.01$ ). LTA induced strong IL-6 secretion (Figure 3.4b), comparable in concentration to the LPS positive control stimulus (2.6 ng/ml). This IL-6 secretion was significantly inhibited by 45 % ( $p=0.02$ ) when cells were stimulated with LTA in the presence of the LPCAT inhibitor (1.4 ng/ml).

Similarly, PG induced high levels of IL-6, displaying a mean of 3.8 ng/ml (Figure 3.4c). Pre-incubation with HETP in PG stimulated MM6 cells also displayed significant cytokine inhibition; a 69 % reduction ( $p=0.04$ ) of secreted IL-6 to 1.1 ng/ml was observed. Comparable to TNF- $\alpha$  concentrations, Pam<sub>3</sub>CSK<sub>4</sub> alone induced minimal mean concentrations of IL-6 (246 pg/ml) in these cells. However, in the presence of HETP (Figure 3.4d) this IL-6 secretion was strongly inhibited by 80 % ( $P<0.01$ ) to a mean of 49 pg/ml.

Notably, IL-8 data followed a similar trend to that seen with both TNF- $\alpha$  and IL-6. LPS induced a high concentration of IL-8 (8.0 ng/ml) (Figure 3.5a) and in the presence of HETP this cytokine secretion was suppressed by 49 %, to a mean of 4.0 ng/ml ( $p=0.05$ ) in MM6 cells. LTA induced an equivalent inflammatory response to LPS stimulated cells, secreting a mean concentration of 7.8 ng/ml IL-8 (Figure 3.5b). LPCAT inhibitor HETP, was shown to significantly reduce ( $P<0.01$ ) IL-8 secretion by 41 % to 4.6 ng/ml in MM6 cells.

Compared with LPS and the other TLR2 ligands, PG consistently induced the highest secretion of all the inflammatory cytokines studied, with a mean of 11.5 ng/ml IL-8 being secreted by MM6 cells (Figure 3.5c). As seen previously, a significant 42 % reduction ( $P<0.01$ ) in IL-8 was observed with pre-incubation of HETP, demonstrating that LPCAT plays a key role in PG induced inflammatory responses.

(a) \*\*  $P < 0.01$ ,  $n = 4$ (b) \*  $P < 0.05$ ,  $n = 4$ (c) \*  $P < 0.05$ ,  $n = 4$ (d) \*\*  $P < 0.01$ ,  $n = 4$ 

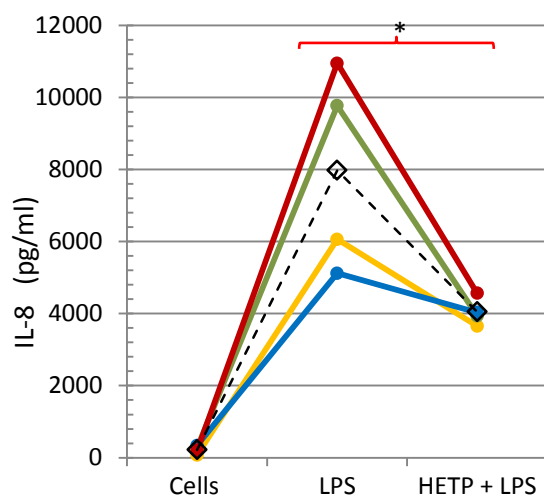
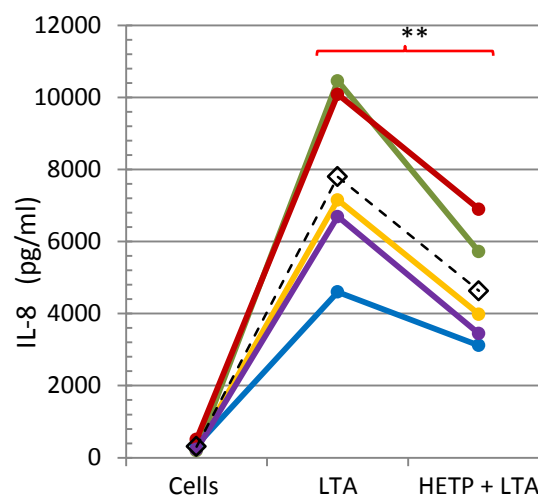
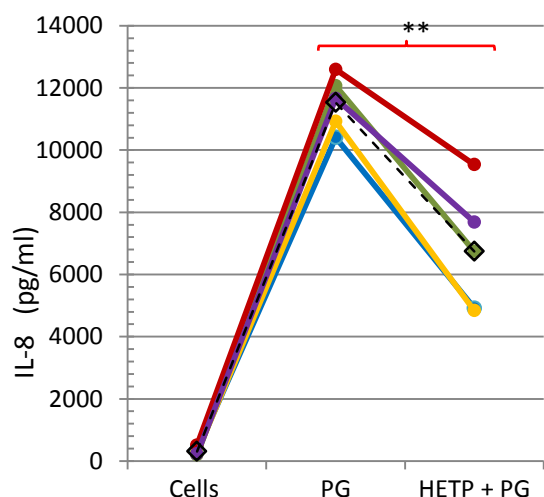
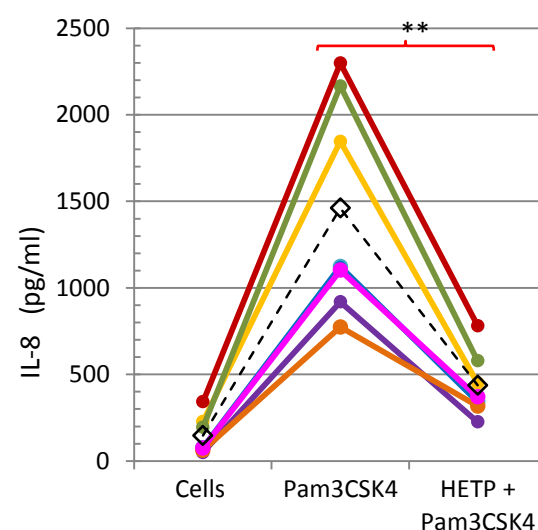
Key:

● Exp 1   
 ● Exp 2   
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 ● Exp 4   
 -◇- Mean

**Figure 3.4 The effect of HETP on IL-6 secretion in MM6 cell stimulated with LPS, LTA, PG or Pam<sub>3</sub>CSK<sub>4</sub>.**

MM6 cells were treated for 24 hours with (a) 100 ng/ml LPS, (b) 10  $\mu$ g/ml LTA, (c) 10  $\mu$ g/ml PG or (d) 1.0  $\mu$ g/ml Pam<sub>3</sub>CSK<sub>4</sub> alone or with prior inhibition by addition of HETP (50  $\mu$ M). After culture cell supernatants were collected and tested for the presence of IL-6 by ELISA. Results represent the IL-6 concentration from 4 independent experiments and the mean IL-6 concentration. Control cells with HETP only were also run alongside experiments (Figure 3.2). These data were analysed for significance by paired *t*-test.



(a) \*  $P < 0.05$ ,  $n = 4$ (b) \*\*  $P < 0.01$ ,  $n = 5$ (c) \*\*  $P < 0.01$ ,  $n = 5$ (d) \*\*  $P < 0.01$ ,  $n = 7$ 

Key:

—●— Exp 1   
 —●— Exp 2   
 —●— Exp 3   
 —●— Exp 4   
 —●— Exp 5   
 —●— Exp 6   
 —●— Exp 7   
 - -◇- - Mean

**Figure 3.5 The effect of HETP on IL-8 secretion in MM6 cell stimulated with LPS, LTA, PG or Pam<sub>3</sub>CSK<sub>4</sub>.**

MM6 cells were treated for 24 hours with (a) 100 ng/ml LPS, (b) 10 µg/ml LTA, (c) 10 µg/ml PG or (d) 1.0 µg/ml Pam<sub>3</sub>CSK<sub>4</sub> alone or with prior inhibition by addition of HETP (50 µM). After culture cell supernatants were collected and tested for the presence of IL-8 by ELISA. Results represent the IL-8 concentration from at least 4 independent experiments and the mean IL-8 concentration. Control cells with HETP only were also run alongside experiments (Figure 3.2). These data were analysed for significance by paired *t*-test.

IL-8 concentrations in Pam<sub>3</sub>CSK<sub>4</sub> stimulated MM6 cells followed the same trend as observed with TNF- $\alpha$  and IL-6. Although, in comparison to the other ligands, Pam<sub>3</sub>CSK<sub>4</sub> induced a low concentration of IL-8 (1.5 ng/ml), a significant 70 % reduction of IL-8 ( $P < 0.01$ ) was seen in HETP treated cells (Figure 3.5d). Under these conditions, Pam<sub>3</sub>CSK<sub>4</sub> stimulated MM6 cells consistently displayed the greatest cytokine reduction with the LPCAT inhibitor compared to the cytokine inhibition observed with the other stimulants.

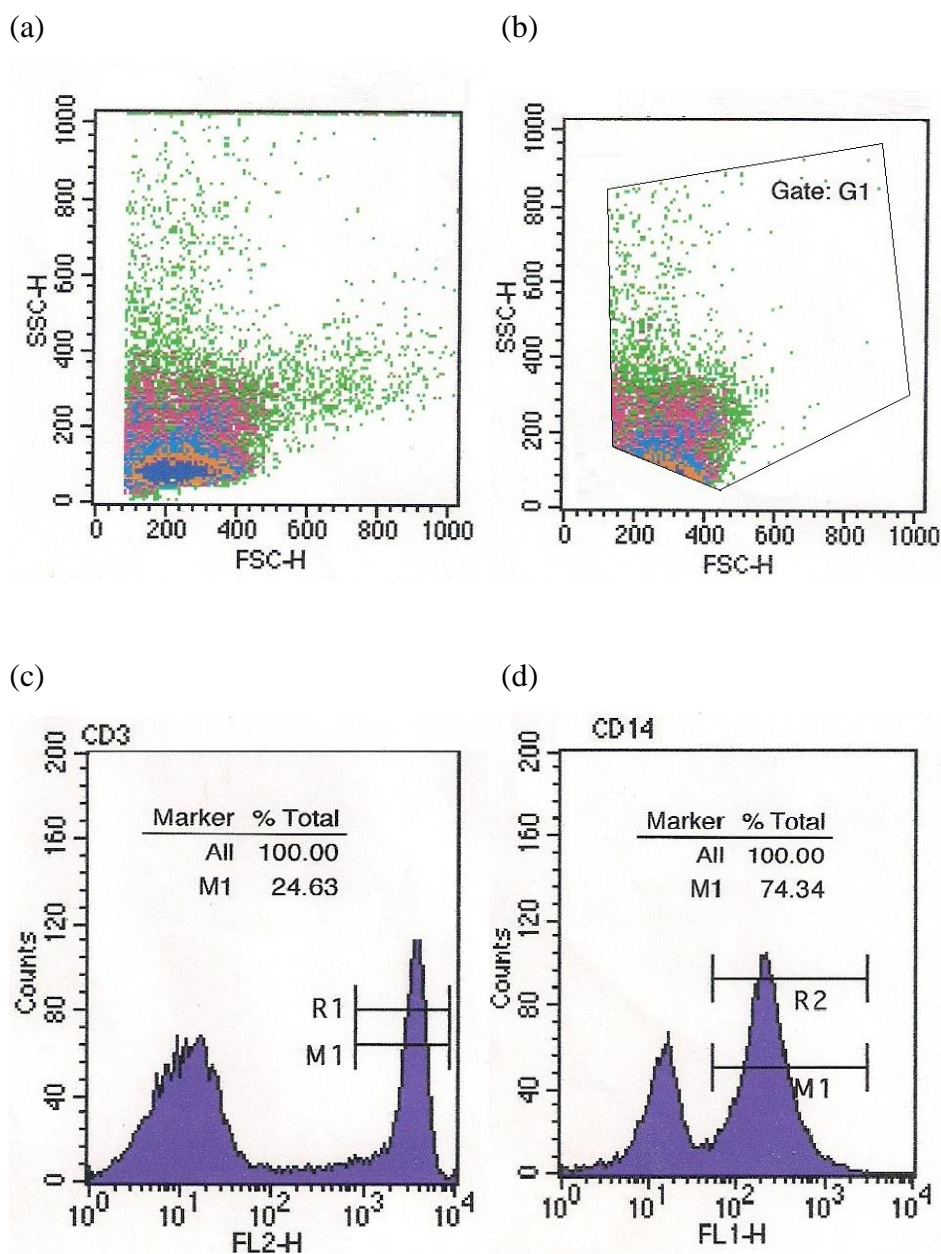
In summary, HETP has shown to significantly inhibit cytokine secretion in the MM6 cell line, irrespective to the size of the inflammatory response, when cells are simulated with LPS, LTA, PG or Pam<sub>3</sub>CSK<sub>4</sub>, thus suggesting that LPCAT has a role in regulating normal inflammatory responses.

#### 3.4.4 Pre-incubation with HETP reduces cytokine secretion in primary human monocytes

To confirm that MM6 cells were a good model for human monocytes, human primary monocyte cells were obtained from healthy volunteer PBMCs. The enriched monocyte population produced by magnetic labelling system was assessed by staining with anti-human CD14 antibody (BD Bioscience, Oxford, UK) and flow cytometry and was consistently >74 % CD14+ and <24 % CD3+ T cells (Figure 3.6). Subsequently, experiments were performed on the enriched CD14+ culture to confirm that the results observed with MM6 cells were representative of human monocytes and thus the peripheral blood monocytes were incubated under the same conditions as the experiments with MM6 cells.

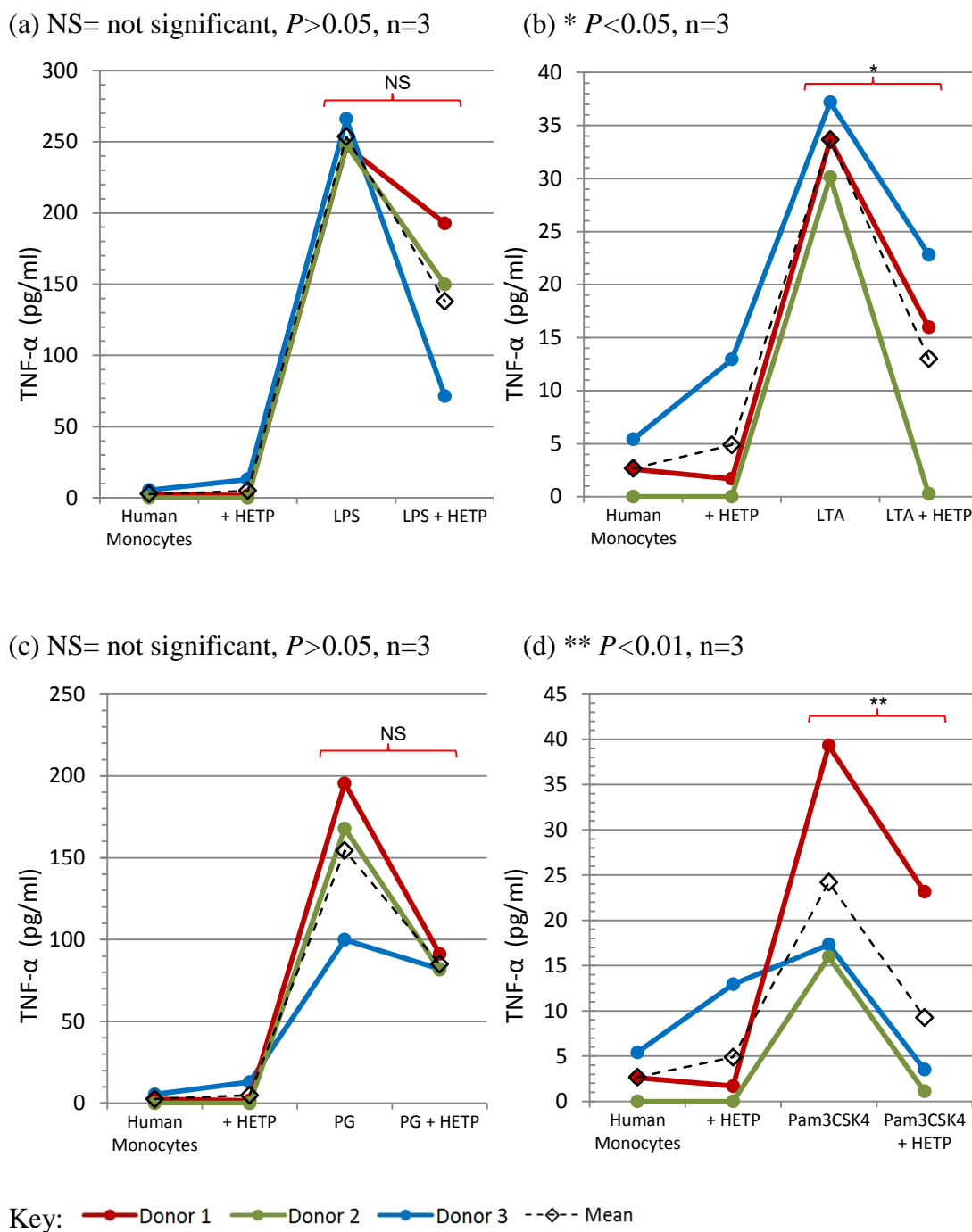
Results are expressed as cytokine concentration from 3 healthy donors obtained as previously by ELISA and stimulated with the corresponding microbial ligand. Actual levels of TNF- $\alpha$  secretion from donor monocytes challenged with LPS were comparable to that observed in MM6 cells: a mean of 253 pg/ml compared to 320 pg/ml TNF- $\alpha$  (Figure 3.7a). However, when compared to MM6 cells, donor monocytes produced 2 fold lower concentrations of TNF- $\alpha$  with Pam<sub>3</sub>CSK<sub>4</sub> stimulation (24 pg/ml), 3 fold lower with PG stimulation (154 pg/ml) and 4 fold lower with LTA stimulation (34 pg/ml), which may indicate that human blood monocytes are not as proficient at secreting this inflammatory cytokine (Figure 3.7).

Despite the lower production of TNF- $\alpha$  from LTA and Pam<sub>3</sub>CSK<sub>4</sub> induced donor cells, pre-incubation with HETP significantly suppressed TNF- $\alpha$  secretion by 61 % ( $P < 0.05$ ) and 62 % ( $P < 0.01$ ), respectively. Similarly, as seen with LPS-induced MM6 cells, the LPCAT inhibitor reduced TNF- $\alpha$  secretion by 46 % to a mean 138 pg/ml and by 45 % in PG stimulated donor cells to a mean of 85 pg/ml, although the neither reduction was statistically significant ( $P > 0.10$ ).



**Figure 3.6 CD3<sup>+</sup> and CD14<sup>+</sup> cell population percentages of enriched donor cells**

Human blood monocytes were extracted using the monocyte indirect magnetic labeling system and assessed by flow cytometry, collecting 10,000 events per sample. (a) Unlabelled enriched monocyte population control cells. (b) Cell debris and dead cells are excluded (gated) from further the analysis based on scatter signals and fluorescence (c) Conjugated CD3 (c) and CD14 (d) antibodies were used to assess the (gated) enriched monocyte population.



**Figure 3.7 The effect of HETP on TNF- $\alpha$  secretion in human blood monocytes stimulated with LPS, LTA, PG or Pam<sub>3</sub>CSK<sub>4</sub>**

Human blood monocytes were extracted using the monocyte indirect magnetic labeling system and the enriched monocyte population were treated for 6 hours with 100 ng/ml LPS, 10  $\mu$ g/ml LTA, 10  $\mu$ g/ml PG or 1.0  $\mu$ g/ml Pam<sub>3</sub>CSK<sub>4</sub> alone or with prior inhibition by addition of HETP (50  $\mu$ M). After culture cell supernatants were collected and tested for the presence of TNF- $\alpha$  by ELISA. Results represent the TNF- $\alpha$  concentration from 3 healthy donors and the mean TNF- $\alpha$  concentration. These data were analysed for significance by paired  $t$ -test.

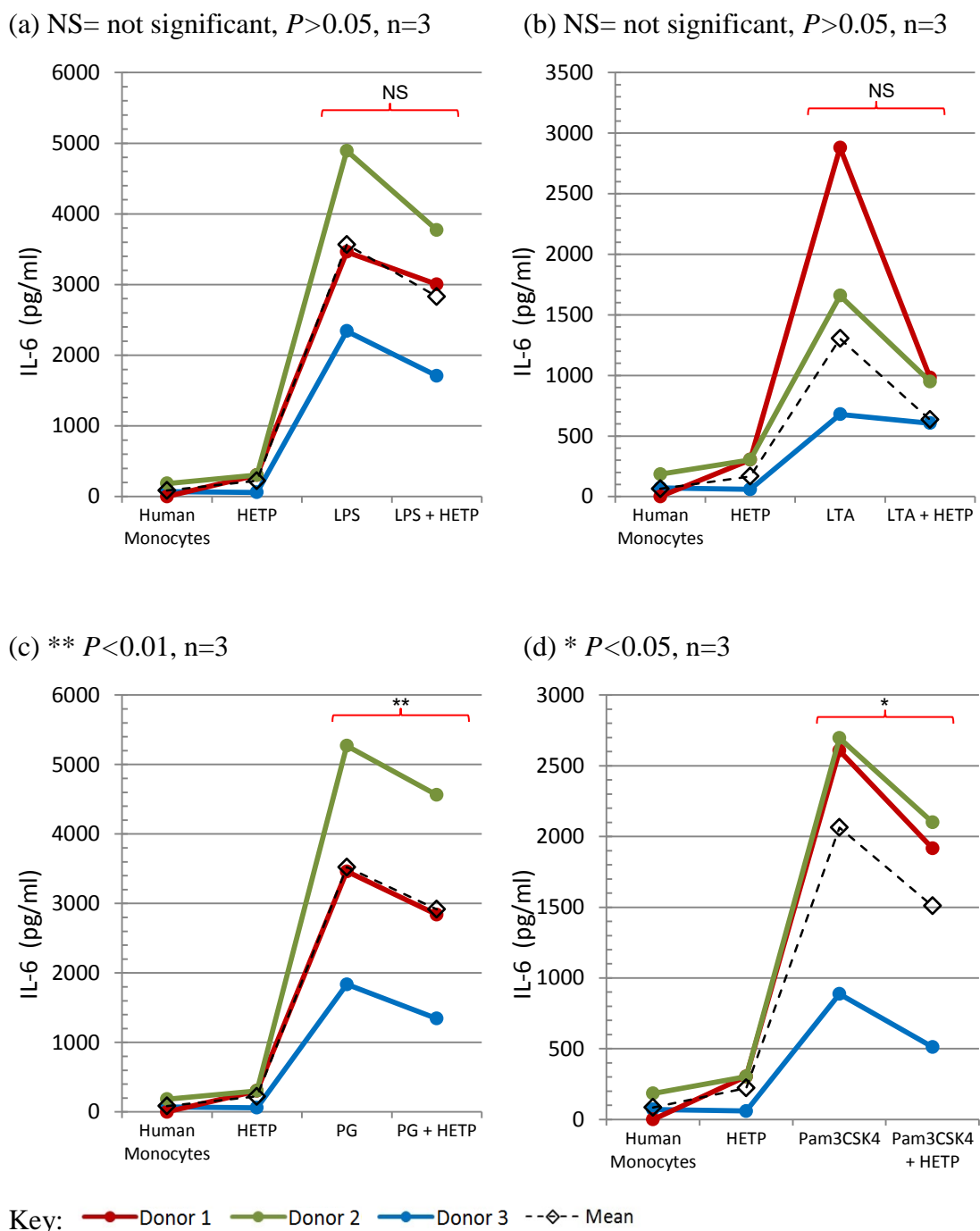
The next inflammatory cytokine studied, IL-6, showed equivalent concentrations to those seen with MM6 cells stimulated with LPS, LTA or PG. LPS induced a mean of 3.6 ng/ml, LTA induced a mean of 1.7 ng/ml and PG induced a mean of 3.5 ng/ml in donor monocytes (Figure 3.8a-c). Unexpectedly, Pam<sub>3</sub>CSK<sub>4</sub> induced 8 fold higher IL-6 concentrations, a mean of 2.0 ng/ml (Figure 3.8d), in these donor cells in contrast to the MM6 cell line (246 pg/ml). In comparison to MM6 cells, the efficacy of HETP to inhibit IL-6 secretion upon stimulation was considerably decreased in donor monocytes. The LPCAT inhibitor reduced the secreted IL-6 concentration by 21 % (p=0.07) to a mean of 2.8 ng/ml when challenged with LPS and by 51 % (p=0.24) to a mean of 846 pg/ml when challenged with LTA. However these data were not found to be statistically significant by paired *t*-test thus differences in the concentrations observed with HETP are more likely to be random variability in the data than actual inhibition.

In contrast, treatment with HETP in donor monocytes stimulated by PG reduced IL-6 concentration by 17 % (p=0.01) to a mean of 2.9 ng/ml and with Pam<sub>3</sub>CSK<sub>4</sub> stimulation, IL-6 levels reduced by 27 % (p=0.03) to a mean of 1.5 ng/ml. Whilst the cytokine reduction with these stimulants was not as profound as seen in the MM6 cells (a reduction of 69 % and 80 %, respectively), the inhibitions are statistically significant demonstrating that the LPCAT inhibitor has some effect on IL-6 production in human monocytes.

In donor monocytes stimulated with LPS alone an increased concentration of IL-8 (18.0 ng/ml) was observed (Figure 3.9a); over twice that seen in MM6 cells. A similar trend was seen with the other microbial stimulants, where LTA and PG both induced 2.5 fold higher concentrations of IL-8 (19.1 ng/ml and 18.6 ng/ml, respectively), but most significantly in Pam<sub>3</sub>CSK<sub>4</sub> induced over 10 fold higher (16.2 ng/ml) than MM6 cells. This suggests that human monocytes are prolific IL-8 secreting cells and that perhaps this cytokine induction pathway may be more sensitive to microbial stimuli than the other inflammatory cytokines. Due to these large increases in IL-8 concentrations, the effectiveness of HETP to inhibit cytokine production was impeded. In the presence of HETP, the stimulant induced IL-8 secretion by donor monocyte cells could only be inhibited to a small extent, observing a reduction of 9 % to a mean of 16.3 ng/ml with LPS challenge, 13 % to a

mean of 16.6 ng/ml with LTA challenge, 13 % to a mean of 15.9 ng/ml with PG challenge and 8 % to a mean of 14.0 ng/ml with Pam<sub>3</sub>CSK<sub>4</sub>. However, these reductions in IL-8 secretion were found to be statistically insignificant by paired *t*-test ( $P>0.08$ ) thus likely not to be due to the LPCAT inhibitor.

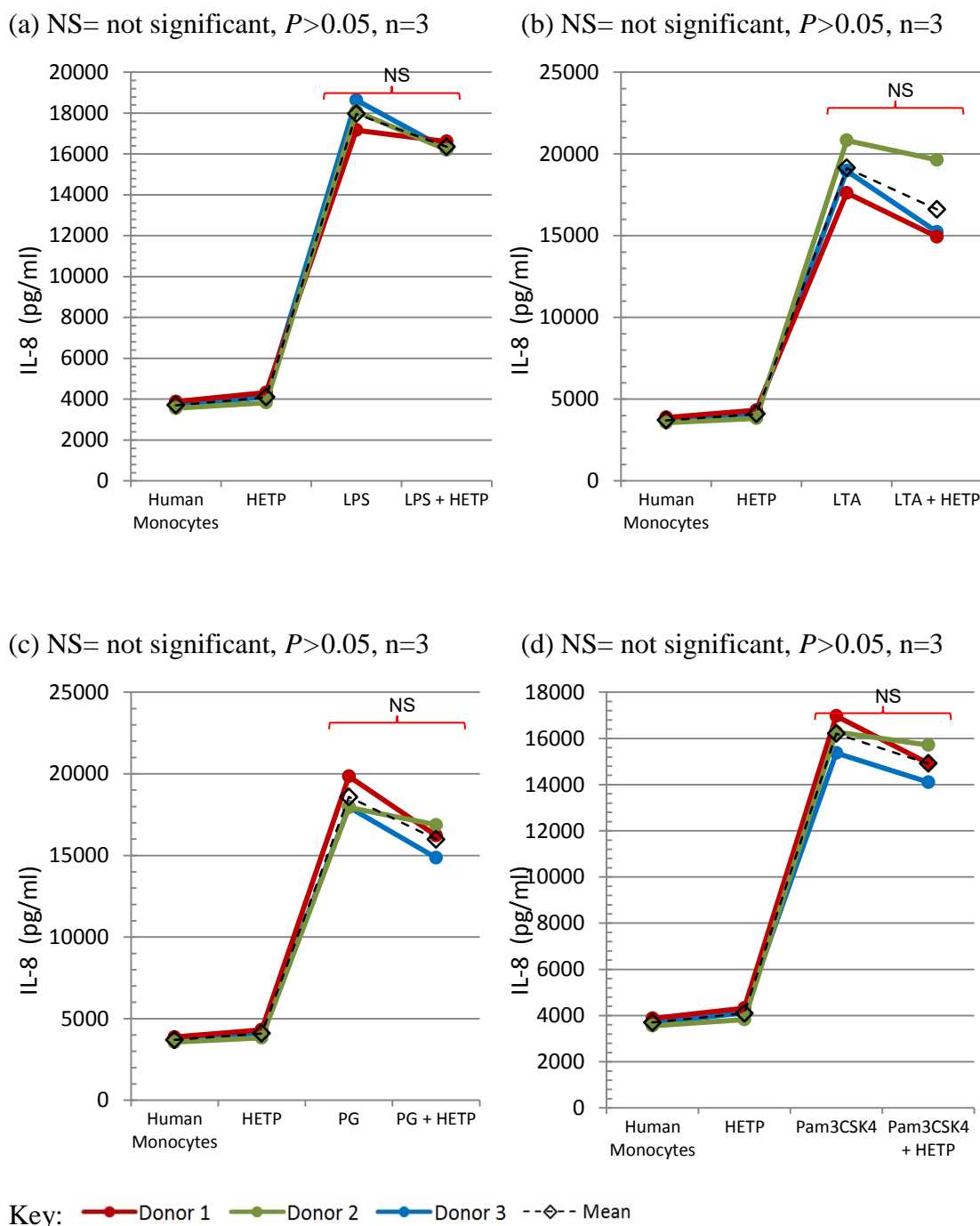
Overall, in comparison to MM6 cells, human donor monocytes secreted TNF- $\alpha$  at lower levels, IL-6 at equivalent levels and IL-8 at higher levels when induced with either TLR4 stimulant LPS or the TLR2 stimulants studied. A noteworthy observation in human monocytes was the amplified secretion of both IL-6 and IL-8 with Pam<sub>3</sub>CSK<sub>4</sub> stimulation, suggesting that this synthetic tripalmitoylated lipopeptide is an effective inflammatory cytokine inducer in these cells. However, unlike MM6 cells, the efficacy of HETP to inhibit secretion was impeded because of the vast IL-8 concentrations secreted. Conversely, in most cases TNF- $\alpha$  and IL-6 inhibition was seen with HETP treatment, although it was not always statistically significant suggesting natural variability between the donor cells.



**Figure 3.8** The effect of HETP on IL-6 secretion in human blood monocytes stimulated with LPS, LTA, PG or Pam<sub>3</sub>CSK<sub>4</sub>

Human blood monocytes were extracted using the monocyte indirect magnetic labeling system and the enriched monocyte population were treated for 24 hours with 100 ng/ml LPS, 10 μg/ml LTA, 10 μg/ml PG or 1.0 μg/ml Pam<sub>3</sub>CSK<sub>4</sub> alone or with prior inhibition by addition of HETP (50 μM). After culture cell supernatants were collected and tested for the presence of IL-6 by ELISA. Results represent the IL-6 concentration from 3 healthy donors and the mean IL-6 concentration. These data were analysed for significance by paired *t*-test.





**Figure 3.9 The effect of HETP on IL-8 secretion in human blood monocytes stimulated with LPS, LTA, PG or Pam<sub>3</sub>CSK<sub>4</sub>**

Human blood monocytes were extracted using the monocyte indirect magnetic labeling system and the enriched monocyte population were treated for 24 hours with 100 ng/ml LPS, 10 µg/ml LTA, 10 µg/ml PG or 1.0 µg/ml Pam<sub>3</sub>CSK<sub>4</sub> alone or with prior inhibition by addition of HETP (50 µM). After culture cell supernatants were collected and tested for the presence of IL-8 by ELISA. Results represent the IL-8 concentration from 3 healthy donors and the mean IL-8 concentration. These data were analysed for significance by paired *t*-test.

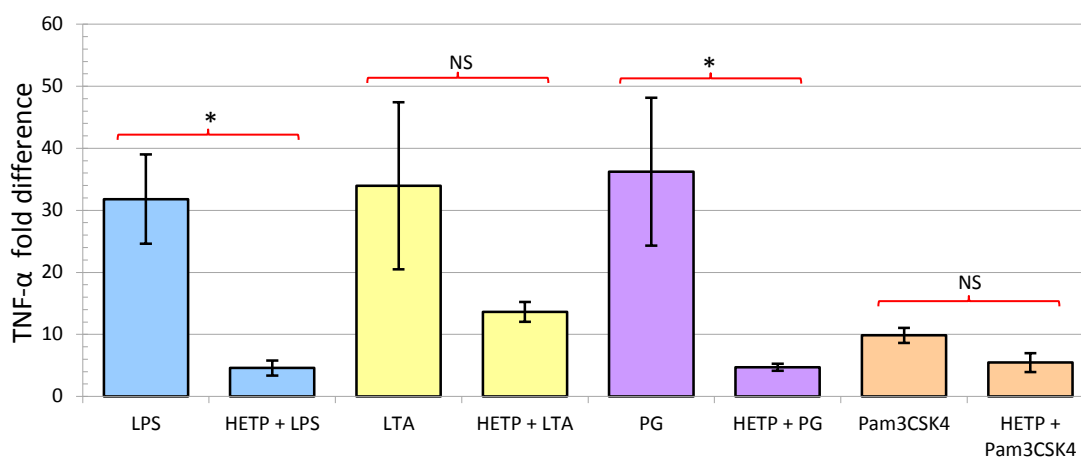
### 3.4.5 HETP inhibits inflammatory cytokine mRNA expression

HETP was shown to significantly reduce secretion of inflammatory cytokines TNF- $\alpha$ , IL-6 and IL-8 in cells stimulated with both TLR4 and TLR2 ligands. In order to establish if this was caused by changes at the transcriptional level, qPCR was used to determine the influence of HETP on cytokine mRNA. Real time PCR of MM6 cell RNA was completed under similar experimental conditions to those used in the inflammatory cytokine ELISA detection experiments, but with a shorter incubation time of 4 hours, as per studies by Jackson *et al.* (2008).

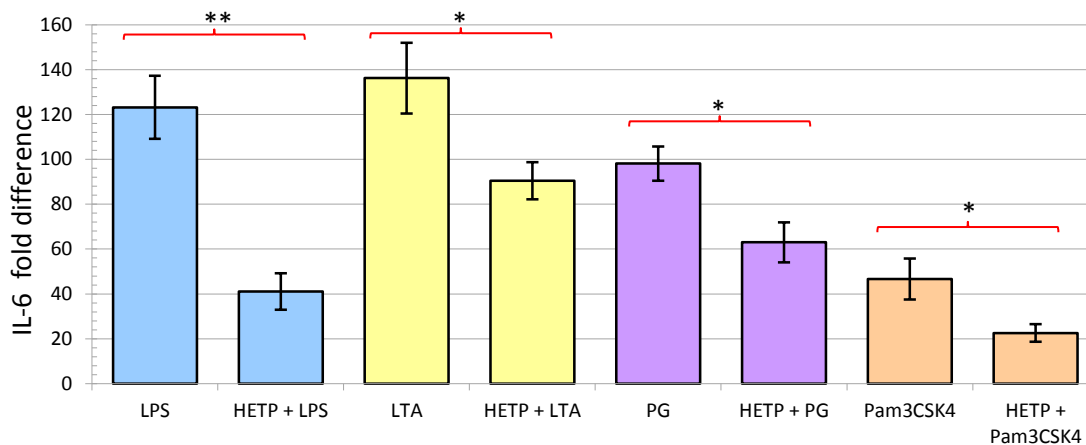
Data was normalized using the housekeeping gene, GAPDH, and the relative difference calculated using untreated cells as a reference. Levels of TNF- $\alpha$  mRNA expression were comparable between LPS, LTA and PG stimulated cells (Figure 3.10a) whilst the previous trend for Pam<sub>3</sub>CSK<sub>4</sub> to induce a lower TNF- $\alpha$  concentration was apparent at the mRNA level. Results show significantly reduced mRNA in cells stimulated with LPS and PG in the presence of HETP reaching approximately 85 % decrease in mRNA fold expression in both cases ( $P < 0.05$ ). IL-6 mRNA expression was considerably higher than observed with TNF- $\alpha$  and IL-8 mRNA with all microbial stimulants; 3 to 4 times greater, suggesting that IL-6 has a central role in inflammatory responses (Figure 3.10b). HETP significantly inhibited induction of IL-6 mRNA expression, irrespective of the microbial stimulant. IL-8 mRNA expression followed a similar trend to IL-6, although to a lesser degree (Figure 3.10c). LTA stimulated the highest IL-8 mRNA expression, which was considerably inhibited, by 85 % ( $P < 0.01$ ), in the presence of HETP.

Overall, qPCR results show that LPS produced the most consistent mRNA inhibition across all cytokines in the presence of HETP. As with results obtained by ELISA, Pam<sub>3</sub>CSK<sub>4</sub>, induced the weakest mRNA expression. Interestingly, it was LTA which stimulated a highest IL-6 and IL-8 mRNA expression, and not PG, in contrast to ELISA results. These data suggest that, whilst LPCAT appears to regulate inflammatory cytokine mRNA expression in MM6 cells, there may be further regulatory mechanisms such as post-transcriptional modifications with cytokine mRNA that would account for the differences observed between mRNA expression and protein secretion, particularly with LTA and PG stimulated cells.

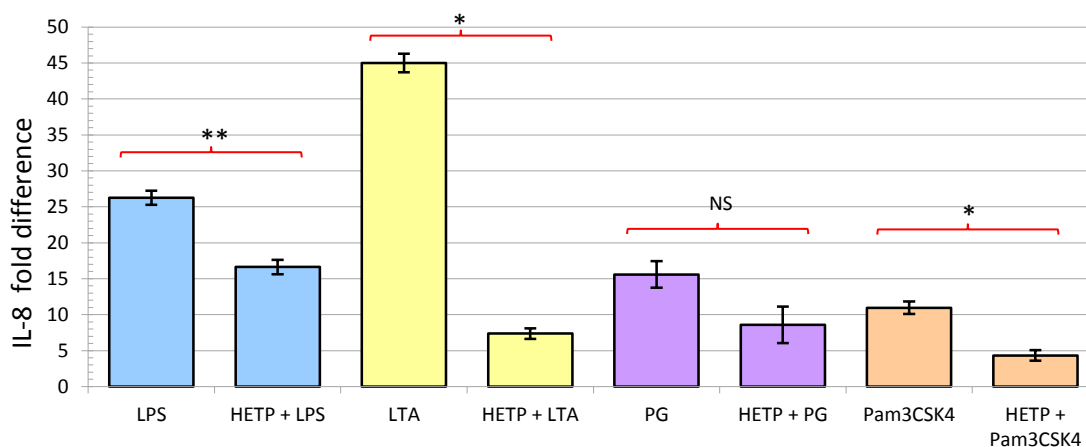
(a) \*  $P < 0.05$ , NS= not significant, n=4



(b) \*\*  $P < 0.01$ , \*  $P < 0.05$ , NS= not significant, n=4



(c) \*\*  $P < 0.01$ , \*  $P < 0.05$ , NS= not significant, n=3



**Figure 3.10 The effect of HETP on cytokine mRNA expression in MM6 cells.**

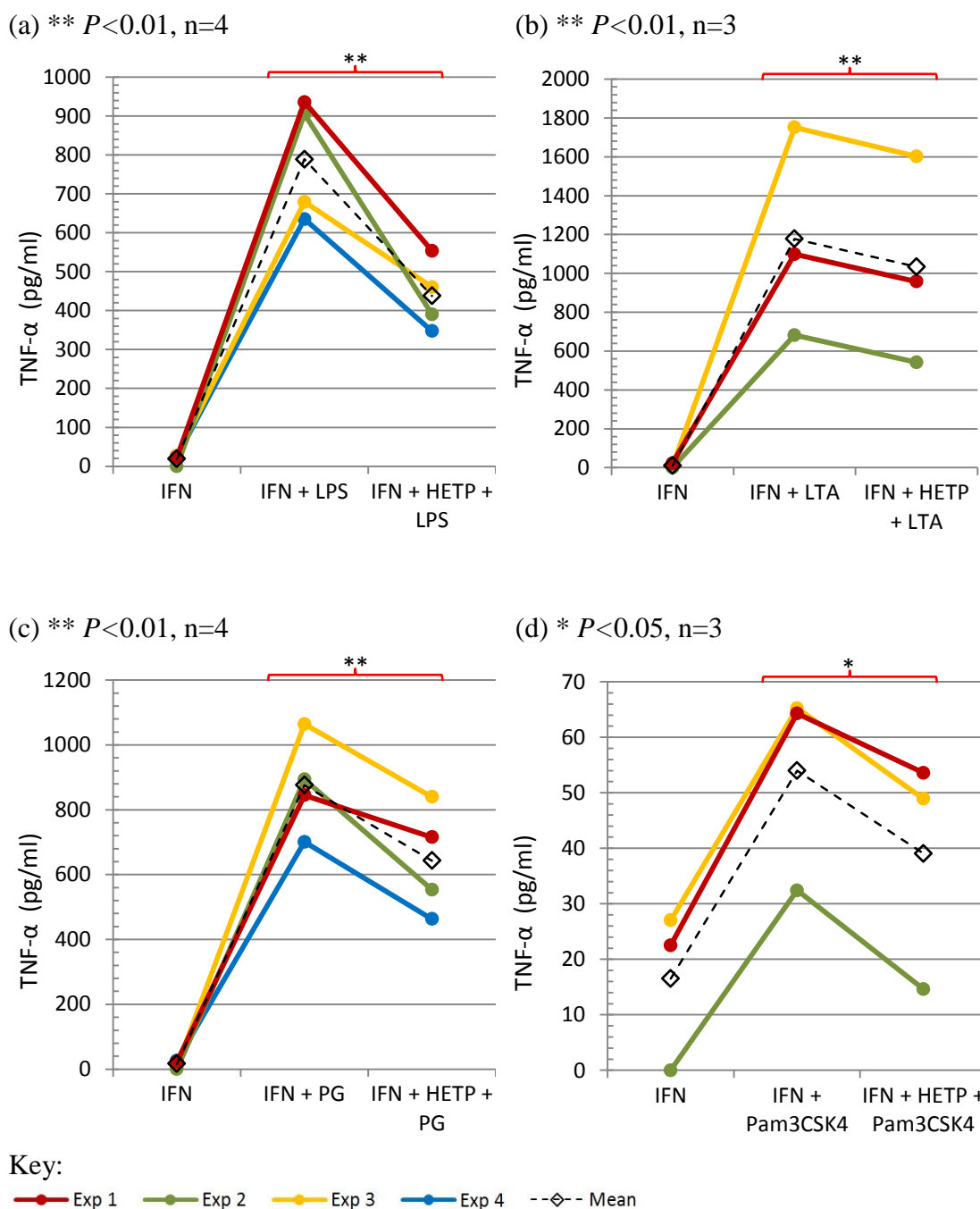
MM6 cells were treated for 4 hours in the presence of LPS (100 ng/ml), LTA (10  $\mu$ g/ml), PG (10  $\mu$ g/ml) or Pam<sub>3</sub>CSK<sub>4</sub> (1.0  $\mu$ g/ml) alone or with prior inhibition by addition of HETP (50  $\mu$ M), before total RNA extraction. Using qPCR, the mRNA of inflammatory cytokines, TNF- $\alpha$  (a), IL-6 (b), IL-8 (c), were normalized with housekeeping gene, GAPDH, and the fold difference calculated using untreated cells as a reference ( $2^{-\Delta\Delta CT}$  method). The results represent the mean  $\pm$  S.E.M of 3 independent experiments and analysed by paired  $t$ -test.

### 3.4.6 IFN- $\gamma$ primed MM6 cells are inhibited by HETP

In previous studies, (Jackson *et al.*, 1992; Darmani *et al.*, 1993) IFN- $\gamma$  was shown to significantly up-regulate monocyte LPCAT activity thus significantly modifying the phospholipid composition of monocyte plasma membranes. In 2003, Schmid and colleagues primed MM6 cells with IFN- $\gamma$  prior to stimulation with LPS and demonstrated that IFN- $\gamma$  amplified the release of inflammatory cytokine TNF- $\alpha$ . Moreover, Schmid found that LPCAT inhibitor, HETP, could decrease cytokine release to a greater degree in IFN- $\gamma$  primed monocytes than in unprimed cells (Schmid *et al.*, 2003). These results suggest that whilst IFN- $\gamma$  primes monocytes for subsequent challenge with LPS, LPCAT may regulate the consequential release of cytokines associated with sepsis. However it is not clear if other inflammatory cytokines are also regulated in the same way or if a similar mechanism exists for monocytes challenged with TLR2 ligands.

As per Schmid *et al.* (2003), in this study MM6 cells were co-cultured with 250 U/ml IFN- $\gamma$  for 16 hours prior to treatment with HETP and stimulation with LPS, LTA, PG or Pam<sub>3</sub>CSK<sub>4</sub>. This examined the effect of IFN- $\gamma$  on monocyte cytokine secretion in response to TLR2 ligands and the potential of HETP to down-regulate cytokine secretion, as determined by ELISA. Results are expressed as cytokine concentration from independent experiments and are analysed by paired *t*-test for significance.

Results show that addition of IFN- $\gamma$  alone did not elicit an inflammatory response, but rather primed the monocytes to respond to microbial stimuli, in most instances leading to increased cytokine production. LPS induced a mean of 789 pg/ml TNF- $\alpha$  in MM6 cells (Figure 3.11a), over twice that previously seen in unprimed cells. With pre-incubation of HETP, a significant reduction in TNF- $\alpha$  secreted was observed, with a mean inhibition of 44 % ( $P < 0.01$ ). This suggests that, although priming cells with IFN- $\gamma$  extensively increases the concentration of cytokine secreted, the cell inflammatory processes could still be considerably inhibited by HETP. LTA induced the highest TNF- $\alpha$  secretion in IFN- $\gamma$  primed MM6 cells to a mean concentration of 1.2 ng/ml (Figure 3.11b), nearly 10 fold higher than unprimed cells. LPCAT inhibitor HETP reduced TNF- $\alpha$  secretion to a lesser extent when compared to the LPS positive control stimulus, by a mean of 12 %, but the reduction was still significant ( $P < 0.01$ ).



**Figure 3.11 The effect of HETP on TNF- $\alpha$  secretion in IFN- $\gamma$  primed MM6 cell stimulated with LPS, LTA, PG or Pam<sub>3</sub>CSK<sub>4</sub>**

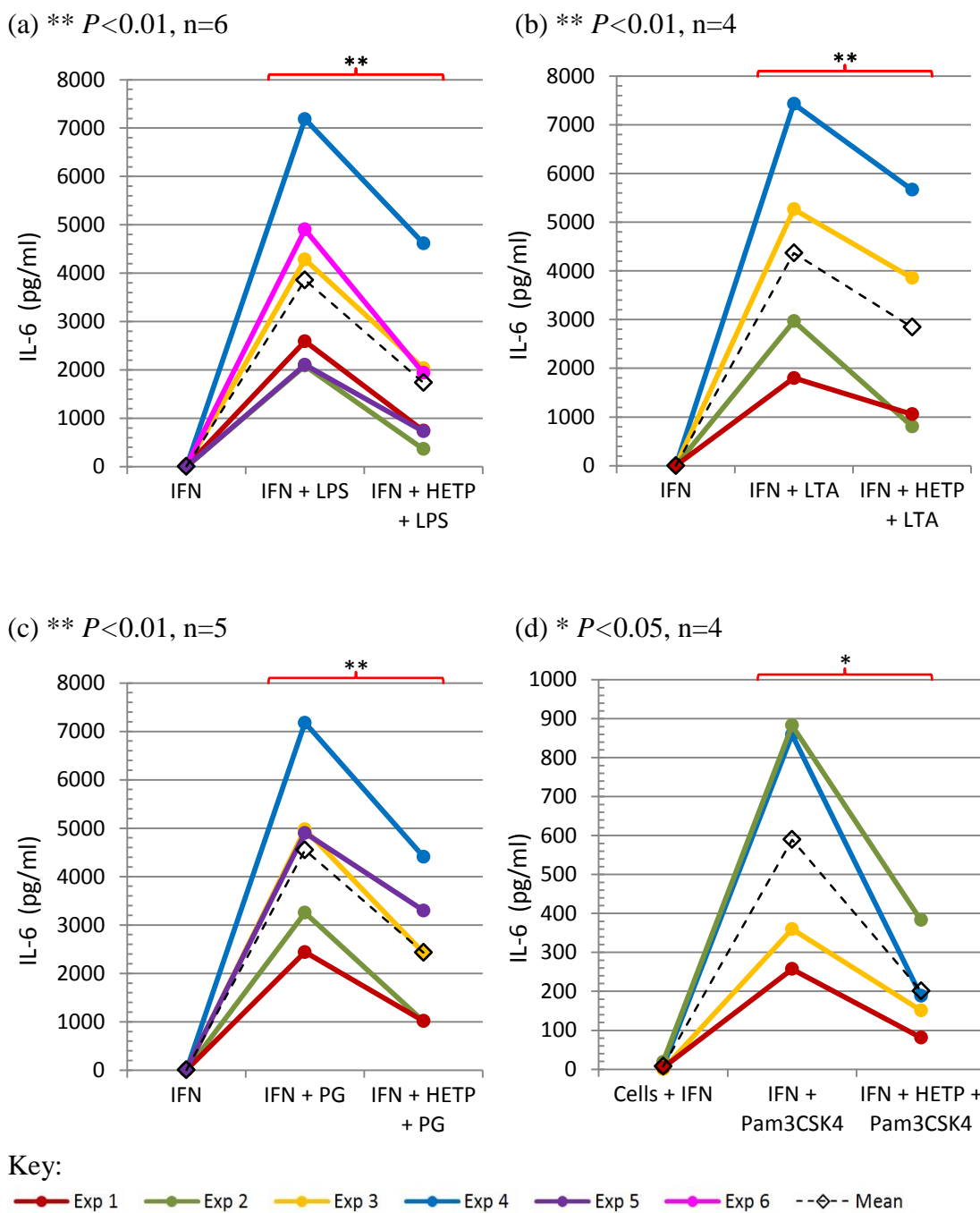
MM6 cells were primed with IFN- $\gamma$  for 16 hours prior to treatment with (a) 100 ng/ml LPS, (b) 10  $\mu$ g/ml LTA, (c) 10  $\mu$ g/ml PG or (d) 1.0  $\mu$ g/ml Pam<sub>3</sub>CSK<sub>4</sub> for 6 hours alone or with prior inhibition by addition of HETP (50  $\mu$ M). After culture cell supernatants were collected and tested for the presence of TNF- $\alpha$  by ELISA. Results represent the TNF- $\alpha$  concentration from at least 3 independent experiments and the mean TNF- $\alpha$  concentration. These data were analysed for significance by paired *t*-test.

Stimulation with PG induced comparable concentrations of TNF- $\alpha$  compared to LPS, with a mean of 876 pg/ml in primed cells (Figure 3.11c). In the presence of HETP, significant cytokine inhibition was observed ( $P < 0.01$ ), but this was noticeably less compared to the unprimed PG stimulated cells; a mean of 27 % suppression in contrast to 50 %, respectively. Interestingly, in IFN- $\gamma$  primed cells stimulated with Pam<sub>3</sub>CSK<sub>4</sub>, TNF- $\alpha$  secretion (Figure 3.11d) was equivalent to that observed in unprimed cells; a mean of 54 pg/ml. This may suggest that IFN- $\gamma$  plays a lesser role in the regulation of TNF- $\alpha$  production with this microbial stimulant. However, HETP significantly reduced TNF- $\alpha$  secretions by a mean of 28 % ( $P < 0.05$ ) confirming the role of LPCAT in this inflammatory process.

The next inflammatory cytokine studied, IL-6, was highly secreted by IFN- $\gamma$  primed MM6 cells in response to the microbial stimulants, but most noticeably with LTA and Pam<sub>3</sub>CSK<sub>4</sub> where nearly double IL-6 (a mean of 4.4 ng/ml and 590 pg/ml, respectively) was produced (Figure 3.12a-d) compared to that observed for unprimed cells. IL6 concentrations were significantly reduced in the presence of LPCAT inhibitor, regardless of the stimulant, with the most prominent inhibition occurring for Pam<sub>3</sub>CSK<sub>4</sub> stimulated cells (a mean inhibition of 66 %,  $P < 0.05$ ) (Figure 3.12d).

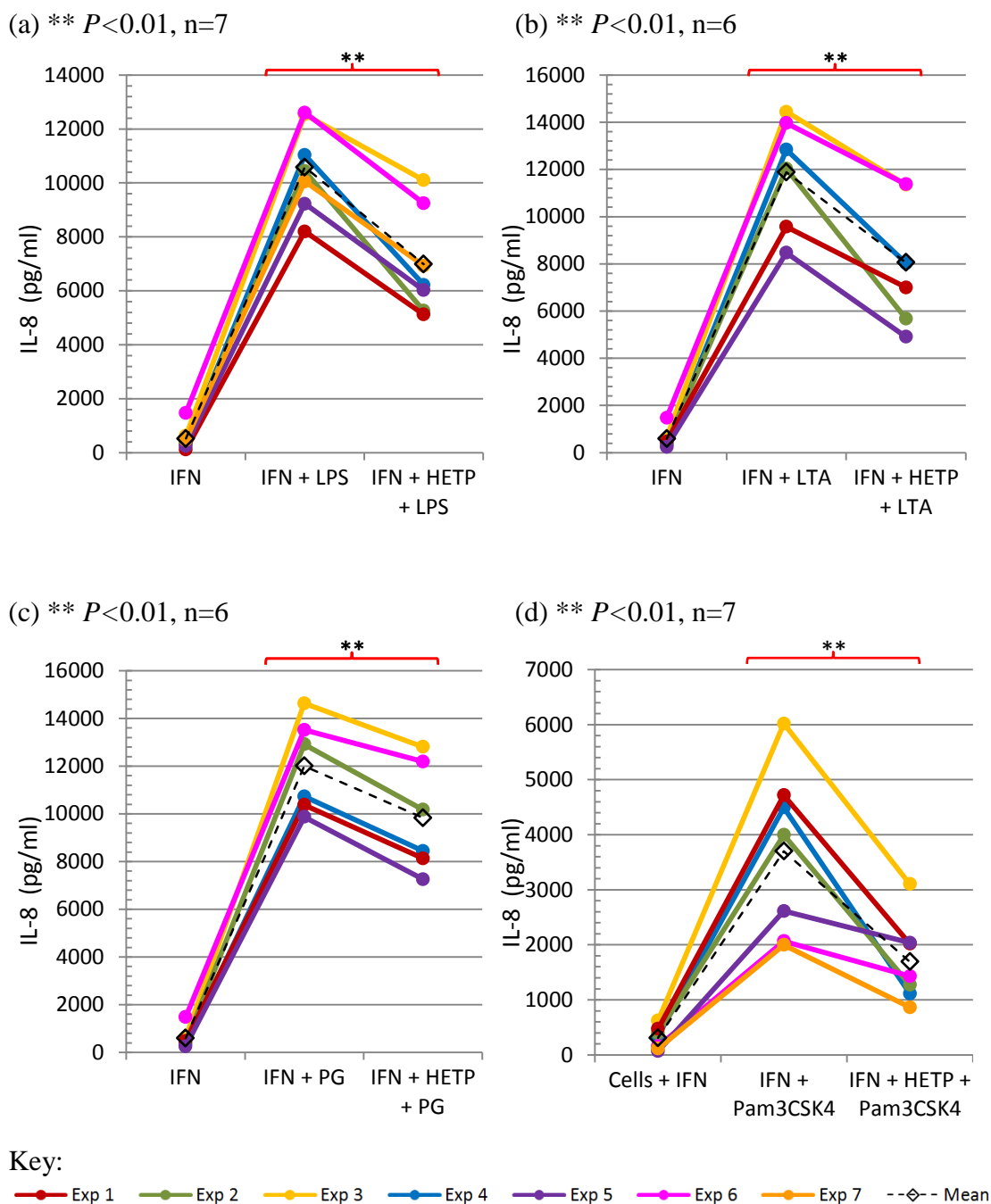
A similar trend to IL-6 was observed in IL-8 secretion by IFN- $\gamma$  primed MM6 cells, with Pam<sub>3</sub>CSK<sub>4</sub> and LTA inducing the highest inflammatory responses (Figure 3.13a-d). Pam<sub>3</sub>CSK<sub>4</sub> induced a mean of 3.7 ng/ml (Figure 3.13d) and LTA 11.9 ng/ml (Figure 3.13c), twice that seen with unprimed cells. IFN- $\gamma$  primed cells stimulated with PG induced comparable levels of IL-8 seen with unprimed cells, however, LPCAT inhibition of cytokine secretion was still statistically significant (a mean inhibition of 18 %,  $P < 0.01$ ). The level of cytokine was considerably suppressed with pre-incubation of HETP across the stimulants, with Pam<sub>3</sub>CSK<sub>4</sub> induced IFN- $\gamma$  primed cells being largely inhibited by a mean of 54 % ( $P < 0.01$ ).

These results demonstrate that, although priming cells with IFN- $\gamma$  increases the concentration of cytokine secreted in most instances, the cell inflammatory processes could still be consistently inhibited by HETP. This suggests that, even where IFN- $\gamma$  promotes the monocytes to secrete an exaggerated level of inflammatory cytokines, LPCAT has an important role in regulation and subsequent release.



**Figure 3.12 The effect of HETP on IL-6 secretion in IFN- $\gamma$  primed MM6 cell stimulated with LPS, LTA, PG or Pam<sub>3</sub>CSK<sub>4</sub>**

MM6 cells were primed with IFN- $\gamma$  for 16 hours prior to treatment with (a) 100 ng/ml LPS, (b) 10  $\mu$ g/ml LTA, (c) 10  $\mu$ g/ml PG or (d) 1.0  $\mu$ g/ml Pam<sub>3</sub>CSK<sub>4</sub> for 24 hours alone or with prior inhibition by addition of HETP (50  $\mu$ M). After culture cell supernatants were collected and tested for the presence of IL-6 by ELISA. Results represent the IL-6 concentration from at least 4 independent experiments and the mean IL-6 concentration. These data were analysed for significance by paired *t*-test.



**Figure 3.13 The effect of HETP on IL-8 secretion in IFN- $\gamma$  primed MM6 cell stimulated with LPS, LTA, PG or Pam<sub>3</sub>CSK<sub>4</sub>**

MM6 cells were primed with IFN- $\gamma$  for 16 hours prior to treatment with (a) 100 ng/ml LPS, (b) 10  $\mu$ g/ml LTA, (c) 10  $\mu$ g/ml PG or (d) 1.0  $\mu$ g/ml Pam<sub>3</sub>CSK<sub>4</sub> for 24 hours alone or with prior inhibition by addition of HETP (50  $\mu$ M). After culture cell supernatants were collected and tested for the presence of IL-8 by ELISA. Results represent the IL-8 concentration from at least 6 independent experiments and the mean IL-8 concentration. These data were analysed for significance by paired *t*-test.

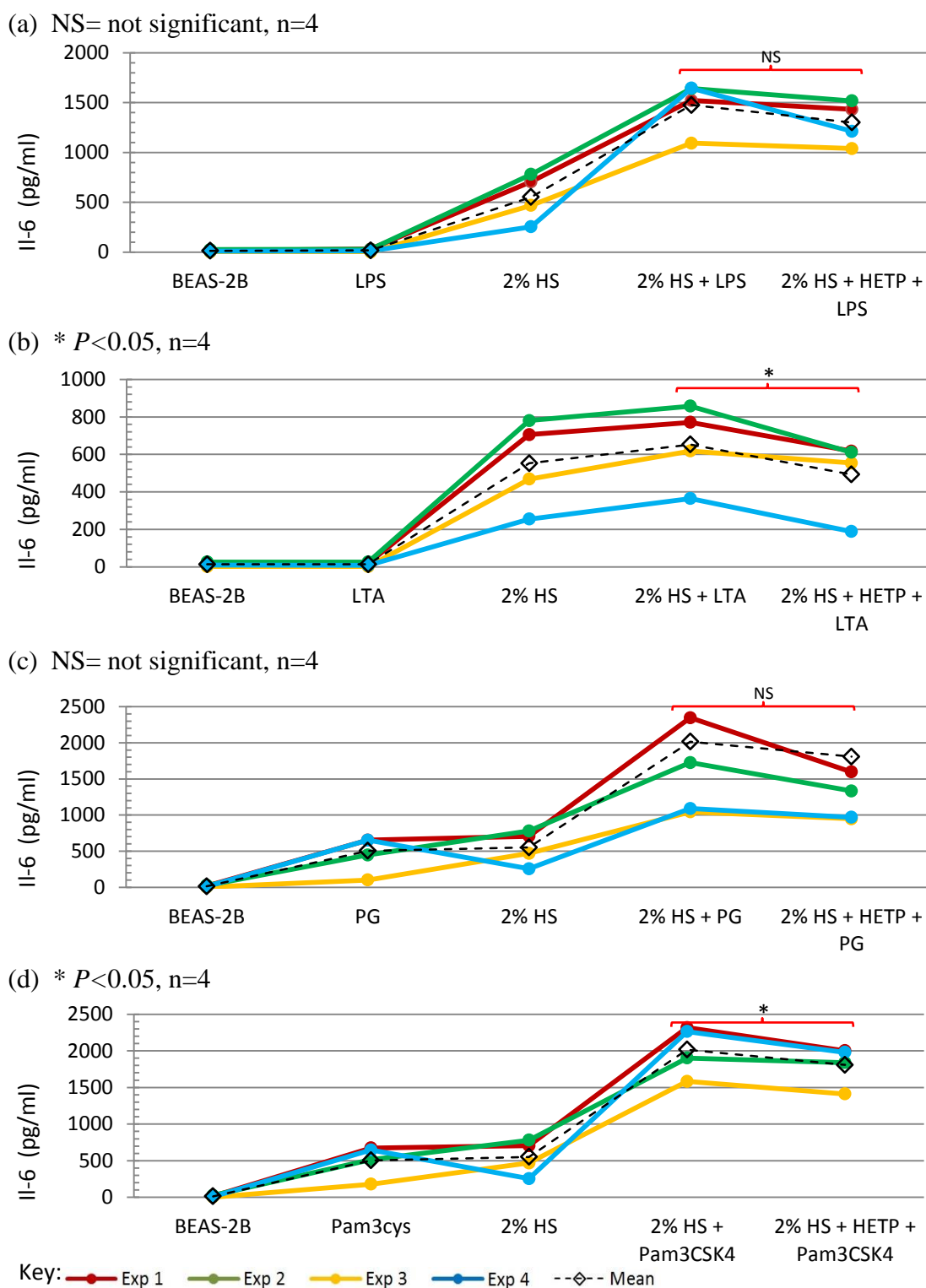


### 3.4.7 The effect of LPCAT inhibition in BEAS-2B cells on inflammatory responses to TLR ligands

Human airways regularly come into contact with pathogenic components making respiratory epithelium an important environmental interface. Although at different levels, like monocytes, epithelial cells express TLR4 on their surface, enabling LPS to induce an inflammatory response. Human broncho-epithelial cell line (BEAS-2B) display TLR1-6 expression demonstrating that they have the receptors required for a response to a range of microbial ligands and have previously been used to study LPCAT initiated synthesis of surfactant protein DPPC (Schulz *et al.*, 2002). In this study BEAS-2B cells were used to investigate if LPCAT has a role in regulating inflammatory responses in broncho-epithelial cells.

Initial results showed that BEAS-2B basal cytokine expression in serum free conditions were negligible, although PG and (synthetic lipopeptide) Pam<sub>3</sub>CSK<sub>4</sub> induced minimal concentrations of IL-6 and IL-8 (Figure 3.14 & Figure 3.15). Cytokine release in BEAS-2B cells was substantially activated by the presence of 2 % pooled human serum (HS) when compared to serum free concentrations.

LPS induced a mean of 1.5 ng/ml IL-6 in BEAS-2B cells (Figure 3.14a) and IL-6 secretion was only slightly suppressed with pre-incubation of HETP to a mean of 1.2 ng/ml; equivalent to 12 % inhibition, however this was not statistically significant ( $P>0.05$ ). In contrast, LTA induced lower levels of IL-6, displaying a mean of 653 pg/ml (Figure 3.14b). Pre-incubation with HETP in LTA stimulated BEAS-2B cells displayed significant cytokine inhibition; a 25 % reduction ( $p=0.02$ ) of secreted IL-6 to 493 pg/ml, less than that induced by the presence of HS, was observed. Comparable in concentration to LPS, PG induced IL-6 secretion (Figure 3.14c) to a mean of 1.5 ng/ml. This IL-6 secretion was not significantly inhibited in the presence of the LPCAT inhibitor ( $P>0.05$ ), but secretion was suppressed by an average of 29 %. Pam<sub>3</sub>CSK<sub>4</sub> induced strong concentrations of IL-6 (2.0 ng/ml) and in the presence of HETP (Figure 3.14d) IL-6 secretion was significantly inhibited by 10 % ( $p=0.04$ ) to a mean of 1.8 ng/ml.



**Figure 3.14 The effect of HETP on IL-6 secretion in BEAS-2B cells stimulated with LPS, LTA, PG or Pam<sub>3</sub>CSK<sub>4</sub>**

BEAS-2B cells were incubated alone or with 2 % pooled human serum (HS) and treated for 24 hours with (a) 100 ng/ml LPS, (b) 10 µg/ml LTA, (c) 10 µg/ml PG or (d) 1.0 µg/ml Pam<sub>3</sub>CSK<sub>4</sub> alone or with prior inhibition by addition of HETP (50 µM). After culture cell supernatants were collected and tested for the presence of IL-6 by ELISA. Results represent the IL-6 concentration from 4 independent experiments and the mean IL-6 concentration. Data were analysed for significance by paired *t*-test.

As anticipated, IL-8 data followed a similar trend to that seen with IL-6 in BEAS-2B cells. LPS induced a high concentration of IL-8 (3.1 ng/ml) (Figure 3.15a) and in the presence of HETP this cytokine secretion was suppressed, although not significantly ( $P>0.05$ ), by 17 % to a mean of 2.6 ng/ml.

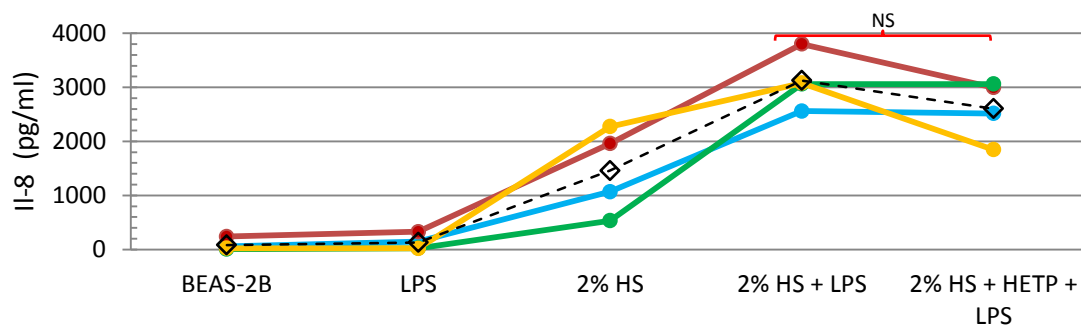
As observed for IL-6 secretions in BEAS-2B cells, LTA induced a lower inflammatory response than LPS stimulated cells, secreting a mean concentration of 2.1 ng/ml IL-8 (Figure 3.15b). However, most noticeably, LPCAT inhibitor HETP was shown to significantly reduce ( $p=0.02$ ) IL-8 secretion by 54 % in BEAS-2B cells, below that induced by HS alone. PG induced a mean of 3.5 ng/ml IL-8 in BEAS-2B cells (Figure 3.15c) and pre-incubation with HETP displayed significant cytokine inhibition; a 15 % reduction ( $P<0.01$ ) of secreted IL-8 to 2.9 ng/ml.

BEAS-2B cells were more immunologically responsive to Pam<sub>3</sub>CSK<sub>4</sub>, where it consistently induced the highest secretion of inflammatory cytokine, with a mean of 3.8 ng/ml IL-8 (Figure 3.15d). As seen previously with IL-6, a small reduction (13 %) in IL-8 was observed when cells were pre-incubated with HETP.

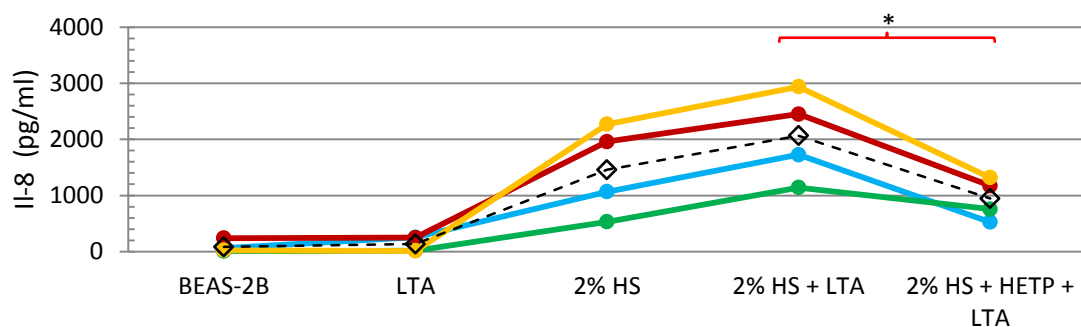
Under these conditions, Pam<sub>3</sub>CSK<sub>4</sub> stimulated BEAS-2B cells to consistently secrete the largest cytokine concentrations, indicating that perhaps this synthetic lipopeptide is a more prolific stimulant in lung epithelial cells than the other TLR ligands. This is in contrast to observations in the monocyte (MM6) cell line, where PG induced the highest levels of cytokine secretion. These data may suggest that Pam<sub>3</sub>CSK<sub>4</sub> is a better immunological stimulus for inflammatory mediated responses by lung epithelial cells.

Inhibition of LPCAT demonstrated a reduction in cytokine secretion by BEAS-2B cells; however in some cases this was not statistically significant, highlighting that LPCAT's involvement in these inflammatory mechanisms, is not as predominant as in monocyte cells.

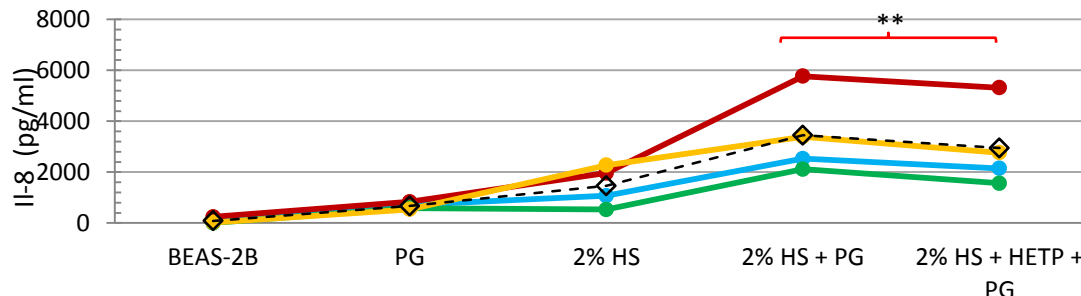
(a) NS= not significant, n=4



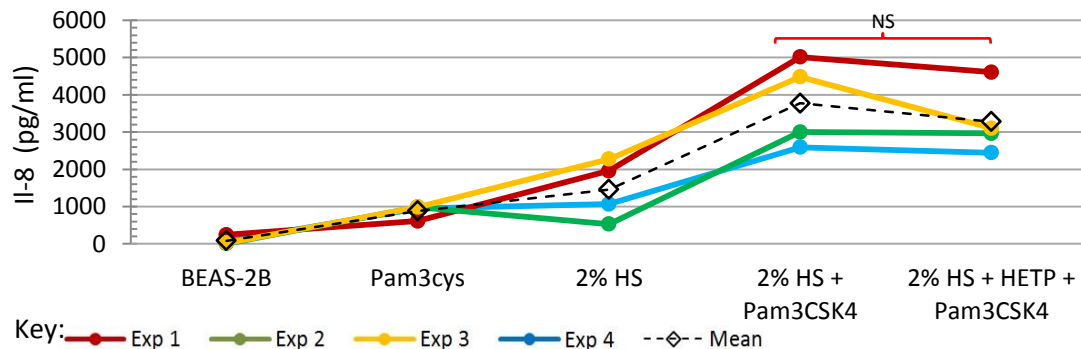
(b) \*  $P < 0.05$ , n=4



(c) \*\*  $P < 0.01$ , n=4



(d) NS= not significant, n=4



**Figure 3.15 The effect of HETP on IL-8 secretion in BEAS-2B cells stimulated with LPS, LTA, PG or Pam<sub>3</sub>CSK<sub>4</sub>**

BEAS-2B cells were incubated alone or with 2 % pooled human serum (HS) and treated for 24 hours with (a) 100 ng/ml LPS, (b) 10 µg/ml LTA, (c) 10 µg/ml PG or (d) 1.0 µg/ml Pam3CSK4 alone or with prior inhibition by addition of HETP (50 µM). After culture cell supernatants were collected and tested for the presence of IL-8 by ELISA. Results represent the IL-8 concentration from 4 independent experiments and mean IL-8 concentration. Data were analysed for significance by paired *t*-test.

### 3.5 DISCUSSION

In this study, a specific inhibitor to LPCAT, 5 hydroxyethyl 5,3' thiophenyl pyridine (HETP), was shown to reduce inflammatory cytokine secretion induced not only by LPS, as observed by Schmid *et al.* (2003), but also by LTA, PG and Pam<sub>3</sub>CSK<sub>4</sub>. This perhaps suggests that LPCAT enzymes are potential targets in the regulation of inflammatory responses.

Schmid *et al.* (2003) evaluated chemical inhibition of LPCAT and found that only 20 µM of HETP was required for an IC<sub>50</sub> of TNF-α in LPS-stimulated MM6 cells; in this report an increased concentration of 50 µM HETP was required to reach levels of inhibition, which may be due to batch-to-batch variation that occurs in its production.

The finding that HETP significantly inhibited TNF-α, IL-6 and IL-8 cytokine secretion in MM6 cells stimulated with TLR2 ligands adds weight to the theory that LPCAT may be involved in TLR2 activation and signalling. On treatment with HETP, a reduction in all cytokines secreted was observed (≥50 %), and was significant for all conditions (*P* value <0.05 or <0.01). Furthermore, qPCR data added evidence that LPCAT inhibition occurred at the mRNA level, subsequently affecting the level of TNF-α, IL-6 and IL-8 cytokine mRNA expression in MM6 cells. This strongly suggests that LPCAT controls cytokine secretion by altering transcription of the gene or mRNA stability, rather than affecting translational or post-translational events.

In agreement with findings in this report, Jackson *et al.* (2008) found LPS to be more potent than Pam<sub>3</sub>CSK<sub>4</sub> in stimulating inflammatory cytokine secretion in MM6 cells, supporting the evidence that MM6 cells are not as responsive to this synthetic lipopeptide compared to the other TLR ligands studied here. However, irrespective to the size of the inflammatory response, MM6 cells stimulated with Pam<sub>3</sub>CSK<sub>4</sub> consistently displayed the greatest cytokine reduction with the LPCAT inhibitor, suggesting that this inflammatory mechanism is most noticeably regulated by LPCAT.

To confirm that MM6 cells are representative of human monocytes, the same experimental conditions were used on an enriched population of peripheral blood

monocytes. In agreement with results obtained from MM6 cells, LPS was found to be the most potent mediator to stimulate inflammatory cytokine secretion in this population of donor monocytes. Kreutz *et al.* (1997) found that Salmonella LPS and (50 ng/ml) Pam<sub>3</sub>-Cys-Ala-Gly (500 ng/ml) greatly induced cytokine responses in monocytes isolated from human PBMCs, reporting IL-6 and IL-8 concentrations on average 10 times higher than levels seen in the present study.

Schmid *et al.* (2003) also studied the TNF- $\alpha$  and IL-6 secretion in isolated peripheral blood monocytes challenged with LPS. Schmid found that LPS induced double the TNF secretion in donor monocytes to that seen in the present study. In contrast, LPS stimulation of donor monocytes in the present study induced 70 fold more IL-6 compared to the concentrations Schmid reported. The variance between reported data may be due to basal differences in cytokine secretion between donor PBMCs, differences in experimental isolations of peripheral blood monocytes and source of the stimulants and stimulant concentrations used.

A noteworthy observation in the enriched population of human monocytes was the amplified (10 fold) secretion of both IL-6 and IL-8 with Pam<sub>3</sub>CSK<sub>4</sub> stimulation compared to the MM6 cell line, suggesting that this synthetic tripalmitoylated lipopeptide is a prolific inflammatory cytokine inducer in donor monocytes. The culture of donor monocytes in the presence of human serum may have some influence on this observation, as Pam<sub>3</sub>CSK<sub>4</sub> is not dependent on co-receptor CD36, but an increased concentration of CD36 (as is likely to be in human serum) has been shown to increase monocytes responsiveness (Jin *et al.*, 2007; Triantafilou *et al.*, 2006). This also suggests that the MM6 cell line is less sensitised to Pam<sub>3</sub>CSK<sub>4</sub>-induced inflammatory responses which may be linked to the stimulants signalling pathway via a TLR2/TLR1 heterodimer.

Although large discrepancies can be observed between the concentration of cytokines secreted by donor monocytes by Schmid and the present study, the inhibitory effects of HETP on TNF- $\alpha$  and IL-6 upon LPS stimulation were comparable. The findings in this report indicate that LPCAT has a crucial role in regulating the release of inflammatory cytokines in enriched populations of donor peripheral blood

monocytes, supporting the theory that the MM6 cell line provides model cells to conduct this study.

An important contribution to the pathogenesis of LPS-induced septic shock is the priming of monocytes/macrophages with IFN- $\gamma$ , leading to an increased inflammatory responsiveness to LPS. IFN- $\gamma$  has previously been shown to induce hypersensitivity to LPS by up regulating monocyte LPCAT activity (Jackson and Parton, 2004), inducing increased plasma membrane fluidity through altering phospholipid composition (Darmani *et al.*, 1993) and by increasing expression of TLR4 and LPS binding sites (Remick, 2003). It was, therefore, of interest to study the effect of IFN- $\gamma$  primed MM6 cells on the secretion of inflammatory cytokines.

In agreement with other studies (Neville *et al.*, 1997; Schmid *et al.*, 2003) IFN- $\gamma$  was found not to elicit an inflammatory response alone, but rather primed the monocytes to respond to microbial stimuli, leading to increased production of inflammatory cytokines. On average the size of the inflammatory response in IFN- $\gamma$  primed monocytes was twice that seen in unprimed cells, with LTA and Pam<sub>3</sub>CSK<sub>4</sub> stimulation providing the most consistent up-regulation. Schmid *et al.* (2003) found that the secretion of TNF- $\alpha$  and IL-6 was five times greater in MM6 cells primed with IFN- $\gamma$  than unprimed on stimulation with LPS, in contrast to the present study.

Inhibition of LPCAT activity, with HETP, also significantly blocked the secretion of TNF- $\alpha$ , IL-6 and IL-8 in not only LPS stimulated IFN- $\gamma$  primed MM6 cells (as previously reported by Schmid), but also LTA, PG and Pam<sub>3</sub>CSK<sub>4</sub> stimulated, primed MM6 cells. When LPCAT activity was inhibited, a significant ( $P$  value <0.01) reduction in the cytokines secreted was seen, however, the reduction was not as sizeable as Schmid reported (90 % inhibition).

In short, these findings indicate that LPCAT plays a crucial role in regulating the release of inflammatory cytokines upon microbial stimulated, in both unprimed and IFN- $\gamma$  primed monocytes, suggesting that LPCAT is involved in the activation of monocytes possibly at the level of the TLR or subsequent signalling pathways.

Although monocytes and macrophages are important producers of cytokines involved in the pathophysiology of bacterial sepsis, lung epithelium also provides an important

interface between the environment and host and may play a role in immune regulation. Lung epithelium are constantly exposed to environmental microbial PAMPs, so mechanisms to limit activation of uncontrolled inflammatory responses to inhaled bacteria must exist. Mayer *et al.* (2007) found BEAS-2B cells expressed lower levels of TLR2 than MM6 cells and were missing expression of co-receptor CD36, which restricts TLR2 signalling pathways. Thus the differences between bronchial epithelial and monocytic immune responses and the influence LPCAT may have on these responses were of interest to study.

In comparison to monocytes, BEAS-2B cells secreted a lower concentration of inflammatory cytokines, though LPCAT inhibition was still able to impede cytokine secretion. Inhibition by LPCAT was not as substantial as for the monocyte cell line, varying from 10 to 20 %, but was found to be significant in some cases. Interestingly, Pam<sub>3</sub>CSK<sub>4</sub> stimulated BEAS-2B cells to secrete IL-6 concentrations 8 fold higher than seen in MM6 cells and comparable to that observed in the enriched populations of donor monocytes. These data may indicate that the synthetic lipopeptide is a more prolific stimulant and that LPCAT may therefore also have an important role in regulating inflammatory responses in epithelial cells, though perhaps to a lower extent than seen in monocytes. Unfortunately, no other studies on lung epithelial cells stimulated with TLR2 ligands are available to compare these results with, as lung epithelial cells are principally used to investigate environmental particle inflammatory effects (Verath *et al.* 2008).

Interestingly, Schulz *et al.* (2002) discovered that, in the presence of a low concentration of LPS (10 ng/ml), BEAS-2B cells do not respond as efficiently to LPS if serum is substituted with sCD14 and suggested that BEAS-2B cells require an alternative serum signalling molecule other than CD14 in order to elicit an inflammatory response to LPS (Schulz *et al.*, 2002). This information may help to explain the increased cytokine secretion on incubation with HS, which appears to be dependent on the presence and concentration of these factors. Literature methods are widely variable for the best culture of BEAS-2B cells and the current conditions may not be optimal for the assay.



Recent studies have characterized the expression of LPCAT like acyltransferases in diverse cell types and it has been suggested different iso-forms of LPCAT exist (Shindou and Shimizu, 2009; Agarwal *et al.*, 2007; Hishikawa *et al.*, 2008; Chen *et al.*, 2006; Shindou *et al.*, 2007; Zhao *et al.*, 2008; Cao *et al.*, 2008). An important difference between lung epithelial cells and monocytes inflammatory responses could be as a result of contrasting LPCAT isoform expression levels which may regulate these mechanisms. It is now of interest to explore the endogenous LPCAT expression and the effect of TLR ligands on LPCAT expression in monocytes and bronchial epithelial cell, as determined in the next chapter.

# Chapter 4

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RESULTS AND DISCUSSION

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EXPRESSION LEVELS OF  
LYSOPHOSPHATIDYLCHOLINE ACYLTRANSFERASES  
IN DIFFERENT CELL TYPES

#### 4.1 INTRODUCTION

Current literature describes the identification of 5 LPCATs that have been shown to incorporate acyl-CoA into lysoPC, reacylating it to PC (Shindou *et al.*, 2007; Nakanishi *et al.*, 2006; Harayama *et al.*, 2009; Chen *et al.*, 2006; Agarwal *et al.*, 2007). The literature describes the LPCATs in terms of preference for the donor fatty acid and the lysoPC acceptor, and has determined mRNA expression levels in a variety of mouse, rat and human tissues or cell lines. However, very little research about the inflammatory role of these LPCAT-like enzymes has been published. LPCAT1 is thought to be most notably expressed in lung tissue, where it plays a role in producing DPPC (Agarwal *et al.*, 2010) whilst LPCAT2 is reported to play a constitutive role in inducible PC remodelling pathways in response to external stimuli in mouse peritoneal macrophages (Shindou *et al.* 2005). To consolidate findings that LPCAT2 is the major remodelling enzyme involved in inflammatory pathways, the expression levels of LPCAT2 were investigated in part of the study.

#### 4.2 AIMS

Monocytic and lung epithelial cell lines were examined to ascertain if they have differential expression of the LPCATs between cell types, which may help to explain the altered inhibitory effects that HETP treatment produces on inflammatory cytokine expression in different cell types. A further aim was to investigate whether LPCAT expression can be induced by TLR4 or TLR2 ligands and if a correlation exists between increased inflammatory cytokine secretion and LPCAT expression. Additionally LPCAT mRNA and protein levels were examined to determine how IFN- $\gamma$  stimulation alters expression.

#### 4.3 METHODS

qPCR and western blot analysis were used to determine the endogenous mRNA and protein expression of the LPCATs in a panel of different cell types. LPCAT mRNA and protein expression levels were also further examined following treatment with TLR ligands.

##### 4.3.1 Relative quantification of LPCAT mRNA expression

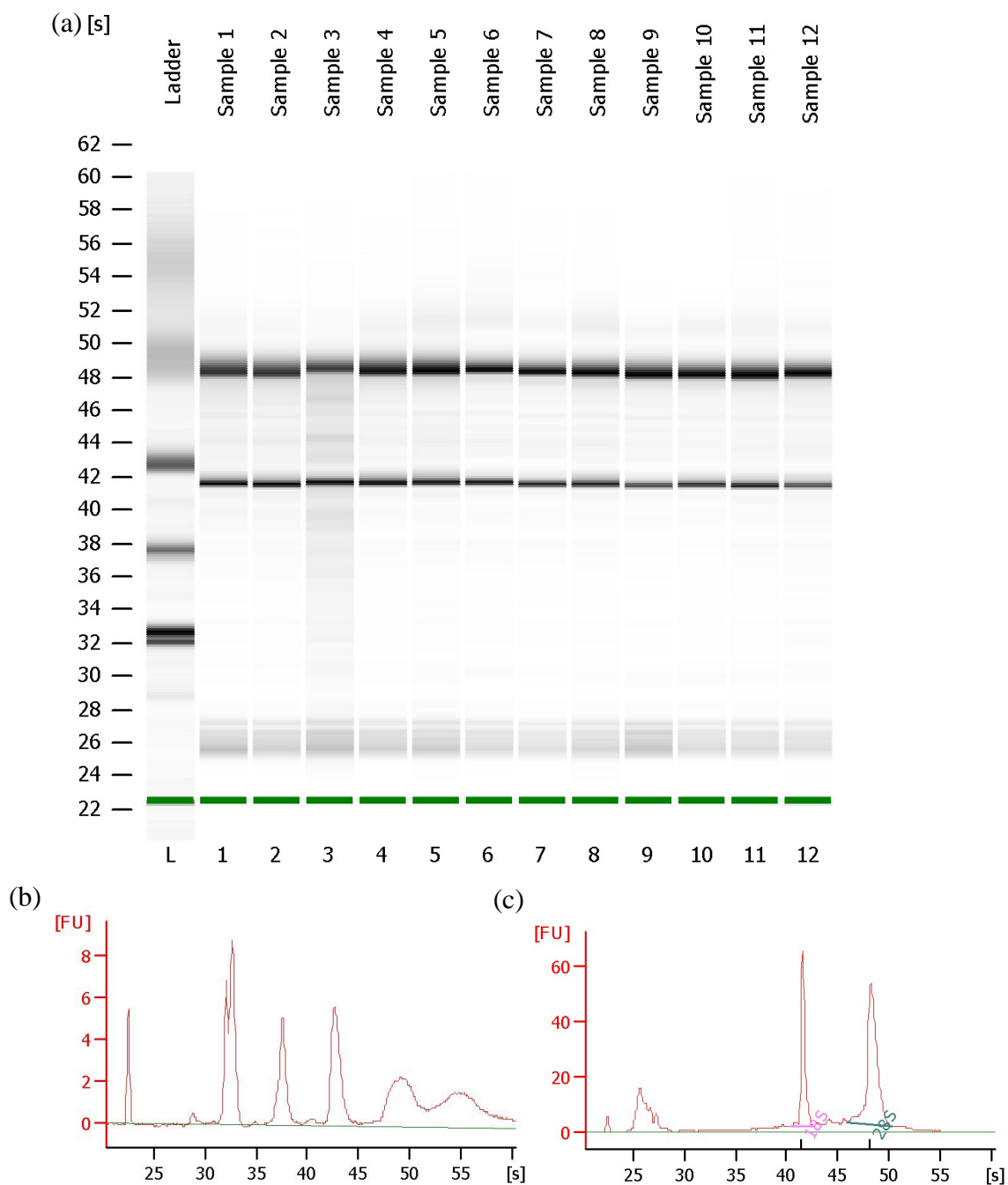
Following total RNA extraction from MM6, BEAS-2B, A549, HepG2 and Hek293cells ( $3 \times 10^6$ ) with an acid guanidinium thiocyanate-phenol-chloroform

extraction method, RNA was quantified and the integrity checked using an Agilent RNA 6000 Nano kit (Agilent Technologies Ltd, Stockport, UK). The Agilent bioanalyser assesses the intactness of RNA by showing a detailed picture of the size distribution of RNA fragments. RNA degradation is a gradual process, where a decrease in the 18S and 28S ribosomal band ratio occurs and the bioanalyser uses software algorithms to produce the RNA Integrity Number (RIN). Samples with significantly degraded RNA, with an integrity number (RIN) less than 8, were discarded. The results from a processed Agilent chip can be viewed in Figure 4.1 and is representative of the data analysed.

The RNA samples were spiked prior to reverse transcription with 0.1ng of *Senecio vulgaris* ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCO) small subunit mRNA. This plant cDNA was then used as an internal control to validate the reverse transcription process and GAPDH was the assumed house-keeping gene for all cells tested. First strand cDNA was synthesized from 2 µg total RNA and real time PCR completed in triple technical replicates using SYBR GreenER qPCR SuperMix (Invitrogen, Paisley, UK) for the iCycler (Bio-Rad, Hertfordshire, UK) according to manufacturer's protocols.

Three sets of primers for each of the 5 LPCATs mentioned in the introduction were designed using Primer-BLAST Primer design software (NCBI database), the information from the reviewed literature and BLAST database searches to ensure each primer's specificity (Figure 1.1 Table 4.1).

Real-time PCR results were analysed using  $2^{-\Delta\Delta CT}$  and relative gene expression methods (Livak and Schmittgen, 2001). Relative quantification relates the PCR signal of the target transcript to that of another sample, such as an untreated control. This report set out to analyse the endogenous expression of different cell types. All cells were untreated, therefore, expression levels were relative to each other. The data are presented as the mean fold change in mRNA expression, normalized to GAPDH and relative to MM6 cells for comparison.



### Figure 4.1 Representative Agilent chip

RNA integrity was calculated using an Agilent RNA 6000 Nano kit and Agilent 2100 bioanalyser (Agilent Technologies Ltd, Stockport, UK). (a) is the results from a processed chip which is representative of the qPCR data analysed. (b) is an electropherogram of the ladder – RNA Area: 83.9, RNA Concentration: 150 ng/ $\mu$ L. (c) is an electropherogram of Sample 1 –RNA Area: 364.9, RNA Concentration: 652 ng/ $\mu$ L, rRNA Ratio [28s / 18s]: 1.7, RNA Integrity Number (RIN): 9.4.

**LPCAT1** (AYTL2: AGPAT9)

Chromosome 5	Gene ID: 79888		mRNA sequence: NM_024830.3		
PRIMER	5' to 3' Forward primer		3' to 5' Reverse Primer		Product length
Primer 1 sequence	LA1F	<a href="#">CCAGCCTGTGGTTTTACGAT</a>	LA1R	<a href="#">TCCTCCTCAGAAGGGCTGTA</a>	156 bp
Primer 2 sequence	LB1F	<a href="#">AGATATTCGAGGGAAGGGA</a>	LB1R	<a href="#">AAGTCGCCACGAAGAGAAAA</a>	168 bp
Primer 3 sequence	LC1F	<a href="#">GGTGTGAACTCAAGGGCCTA</a>	LC1R	<a href="#">TATCCAACCTCGGACTGGAG</a>	159 bp

**LPCAT2** (LysoPAFAT: AYTL1: AGPAT11)

Chromosome 16	Gene ID: 54947		mRNA sequence: NM_017839.4		
PRIMER	5' to 3' Forward primer		3' to 5' Reverse Primer		Product length
Primer 1 sequence	LD1F	<a href="#">TGTGCAACCAGTTTTGGTGT</a>	LD1R	<a href="#">TGGAATCCTGGAATGAAGG</a>	184 bp
Primer 2 sequence	LE1F	<a href="#">GAAATAGCCAAGGGGACTC</a>	LE1R	<a href="#">TGCTTTTCAGGGCTGACTTT</a>	182 bp
Primer 3 sequence	LF1F	<a href="#">ACATTGCCTATGGAAGCTGG</a>	LF1R	<a href="#">GGCGAATTCTCAATTCAA</a>	150 bp

**LPCAT3** (MBOAT5)

Chromosome 12	Gene ID: 10162		mRNA sequence: NM_005768.5		
PRIMER	5' to 3' Forward primer		3' to 5' Reverse Primer		Product length
Primer 1 sequence	LG1F	<a href="#">GCCACATCTTCTTCTGAGC</a>	LG1R	<a href="#">GCTGTGAAAAGGGAGACGAG</a>	154 bp
Primer 2 sequence	LH1F	<a href="#">CTCTCGTTGCTATTCCTGGC</a>	LH1R	<a href="#">GAGCCAGTGGATGGTCTGTT</a>	198 bp

**LPCAT4** (MBOAT2)

Chromosome 2	Gene ID: 129642		mRNA sequence: NM_138799.2		
PRIMER	5' to 3' Forward primer		3' to 5' Reverse Primer		Product length
Primer 1 sequence	LL1F	<a href="#">ATGGGCCATTTTTACCACAA</a>	LL1R	<a href="#">AGGAGGTACAATCTGGGGCT</a>	153 bp
Primer 2 sequence	LM1F	<a href="#">CAGGCCACTTTGCTCTTAC</a>	LM1R	<a href="#">AACAAGGACAGCCACAAAC</a>	169 bp
Primer 3 sequence	LN1F	<a href="#">GTTTGTGGGCTGTCCTTGT</a>	LN1R	<a href="#">AGCTAGCGTCCATGCAAAAT</a>	171 bp

**LPEAT2** (AGPAT7: AYTL3)

Chromosome 15	Gene ID: 254531		mRNA sequence: NM_153613.2		
PRIMER	5' to 3' Forward primer		3' to 5' Reverse Primer		Product length
Primer 1 sequence	LI1F	<a href="#">ATTGGAGCCCTTCTTCGATT</a>	LI1R	<a href="#">AGCAAAGCCTTCTTGTGGA</a>	167 bp
Primer 2 sequence	LJ1F	<a href="#">CCGGTCTTAGTGAGGAGCAG</a>	LJ1R	<a href="#">TGGGGTCAAAGAAAGTGGAG</a>	198 bp
Primer 3 sequence	LK1F	<a href="#">TCTGGGAACTGGGAAAAGTG</a>	LK1R	<a href="#">CTGCTGGAAGTAGCCAAAGG</a>	167 bp

**Table 4.1** Primer sets of LPCAT-like acyltransferases.

LPCAT primer RNA sequences. Details on how the primer sequence span across each LPCAT mRNA and how they compare to sequences used in literature, can be found in Appendix 1.

#### 4.3.2 Protein expression profile of LPCAT1 and LPCAT2

Western blot analysis was used to examine the endogenous protein expression of hLPCAT1 and hLPCAT2 in MM6, A549, BEAS-2B, HepG2, Hek293 and mouse Raw26.7 cell lines. Equal volumes and concentrations of protein (20 µg of protein per lane) were loaded and 5 µl of biotinylated protein ladder was loaded in lane 1 to aid identification of proteins on nitrocellulose membranes. Commercially designed anti-human polyclonal LPCAT1 and LPCAT2 antibodies (ProteinTech, Manchester, UK) were used to detect protein expression of hLPCAT1 and hLPCAT2.

#### 4.3.3 Effect of IFN- $\gamma$ and LPS on LPCAT mRNA expression

Cells ( $3 \times 10^6$ ) were incubated either with IFN- $\gamma$  (250 units/ml) or *Escherichia coli* O111:B4 LPS (100 ng/ml) for 18 or 4 hours, respectively. Following total RNA extraction, first strand cDNA was synthesized and qPCR completed in triple technical replicates as described previously (section 4.3.1). Results were analysed using  $2^{-\Delta\Delta CT}$  and are presented as the fold change in mRNA expression normalized to GAPDH and relative to the corresponding untreated cell.

#### 4.3.4 Effect of TLR4 and TLR2 ligand stimulation on LPCAT1 and LPCAT2 protein expression

Western blot analysis was used to examine the effect of TLR4 and TLR2 ligands, and the influence of HETP, on LPCAT protein expression in MM6 cells. Cultures of MM6 cells ( $6 \times 10^6$ ) were incubated for 30 minutes in the presence or absence of 50µM HETP, prior to stimulation with LPS (100 ng/ml), LTA (10 µg/ml) PG (10 µg/ml) or Pam<sub>3</sub>CSK<sub>4</sub> (1.0 µg/ml) and further co-culture for 24 hours. As for previous experiments, equal volumes and concentrations of protein were loaded. Membranes were then striped and re-probed with hGADPH antibody to aid densitometry analysis.

#### 4.3.5 Effect of IFN- $\gamma$ on LPCAT1 and LPCAT2 protein expression

MM6 cells ( $6 \times 10^6$ ) were co-cultured with IFN- $\gamma$  (250 units/ml) for 18 hours prior to incubation with HETP and treatment with TLR4 and TLR2 ligands. As with previous experiments, western blot analysis was used to determine the effect of IFN- $\gamma$  on LPCAT1 and LPCAT2 expression.

## 4.4 RESULTS

### 4.4.1 LPCAT mRNA expression varies among cell types

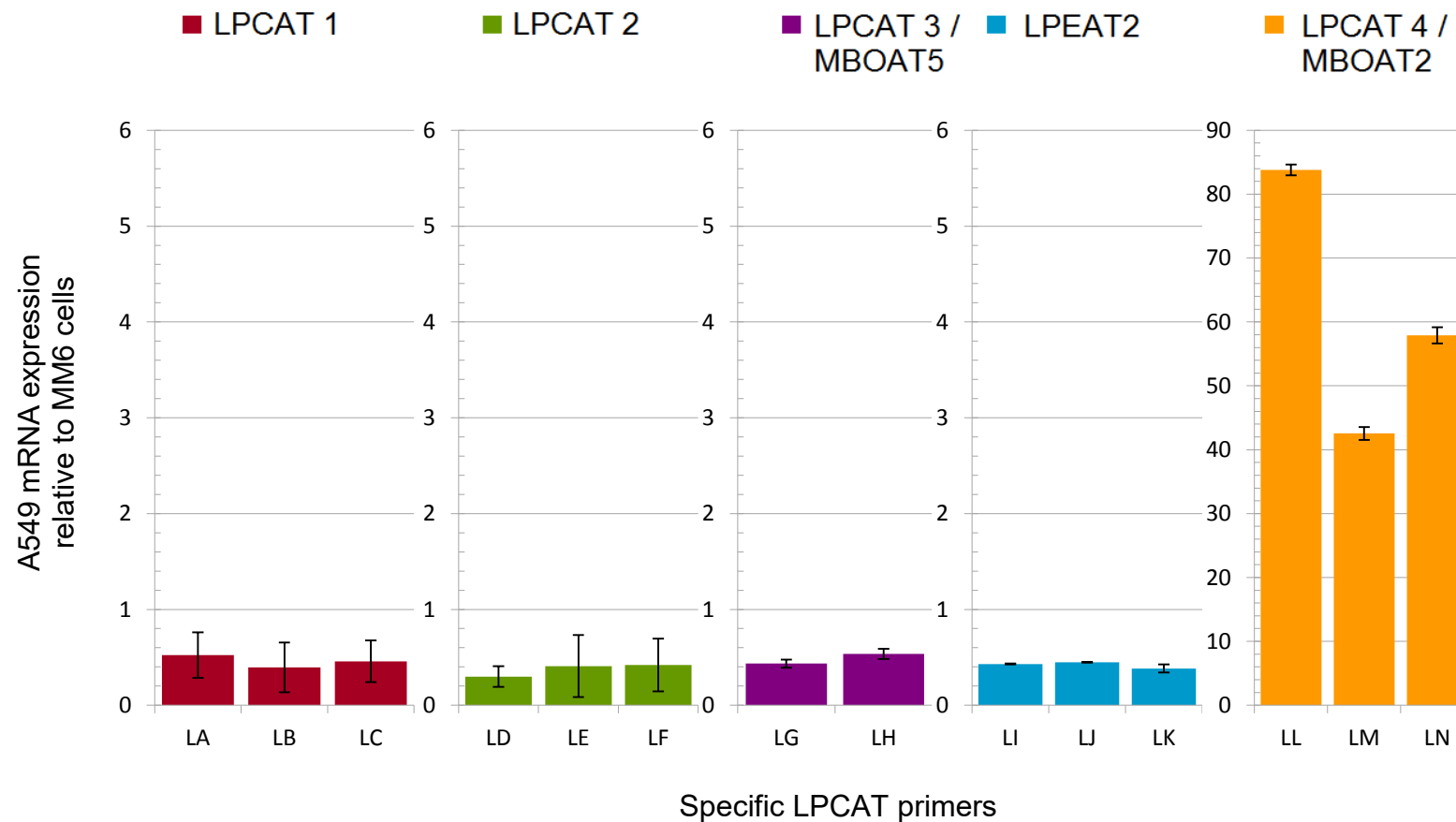
Figure 4.2 shows the expression of LPCAT1, LPCAT2, LPCAT3, LPEAT2 and LPCAT4 in lung epithelial A549 cells relative to MM6 cells. The results suggest that A549 cells express LPCAT1, 2, 3 and LPEAT2 mRNA at equivalent levels to MM6 cells. Conversely A549 cells were observed to have significantly more LPCAT4 expression (85 fold) than MM6 cells, which did not appear to express any detectable LPCAT4.

LPCAT expression in BEAS-2B cells followed a similar trend to that seen with A549 cells (Figure 4.3); BEAS-2B cells expressed comparable mRNA levels of LPCAT1, LPCAT2, LPCAT3 and LPEAT2 to MM6 cells, but like A549 cells, highly expressed LPCAT4, indicating that LPCAT4 is likely to be of importance in lung epithelial cells regulatory pathways.

The human embryonic kidney cell line Hek293 has been reported to be the cell of choice to analyse LPCAT1 (Chen *et al.*, 2006) and LPCAT3 over-expression (Zhao *et al.*, 2008) as well as siRNA knockdown of LPCAT2 (Shindou *et al.*, 2007) and of LPEAT2 (Cao *et al.*, 2008). Liver tissue and hepatocyte cell lines have also displayed high expression of LPCATs therefore offering a reliable means for the investigation of expression levels (Agarwal *et al.*, 2007; Hishikawa *et al.*, 2008). Thus the mRNA from both HepG2 and Hek293, were analysed by the same method to provide a positive comparison for the qPCR experiments.

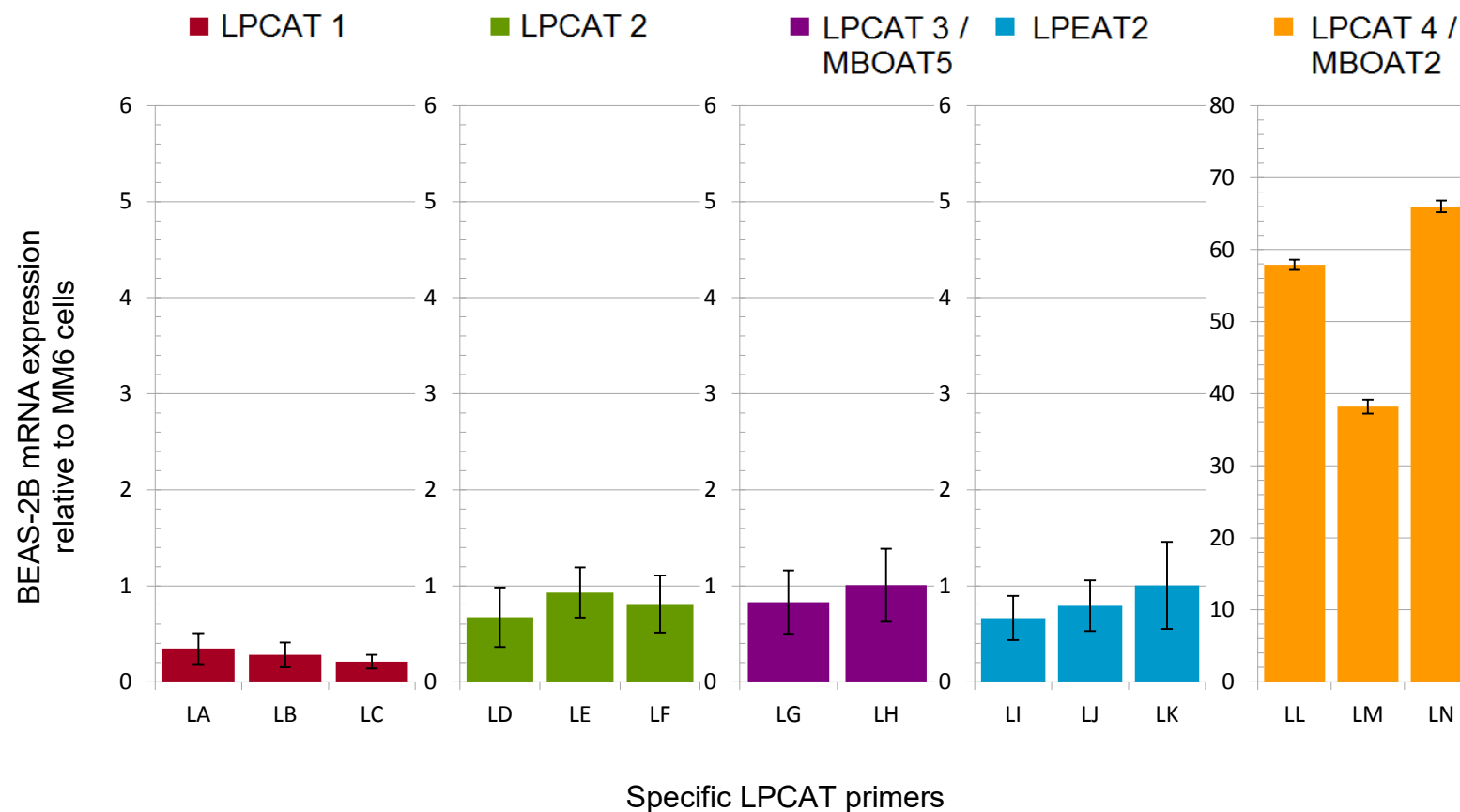
Relative expression of LPCAT1 and LPEAT2 in HepG2 cells were of comparable levels to MM6 cells (Figure 4.4), but LPCAT2 and LPCAT3 appear to be elevated, at 2.5 and 1.5 fold higher than MM6 cells (respectively). However, in contrast to the lung epithelial cells, expression of LPCAT4 in HepG2 was comparable to MM6; below the limit of detection.





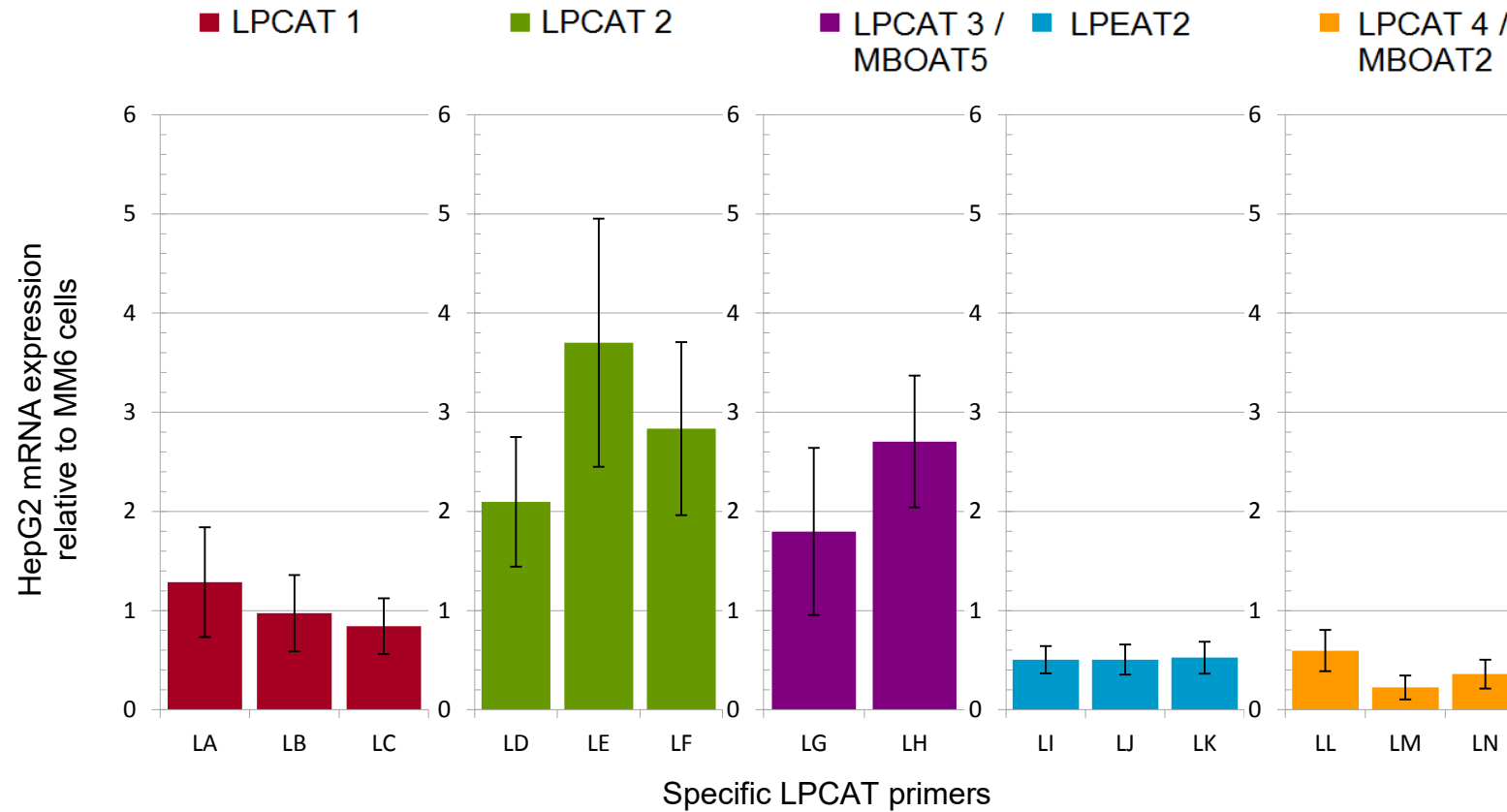
**Figure 4.2** Intrinsic expression of LPCAT-like acyltransferases in A549 cells

Intrinsic LPCAT expression of A549 cells, presented as the mean mRNA expression normalized to GAPDH and relative to MM6 cells. Results are the mean +/- S.E.M of 3 independent experiments.



**Figure 4.3** Intrinsic expression of LPCAT-like acyltransferases in BEAS-2B cells

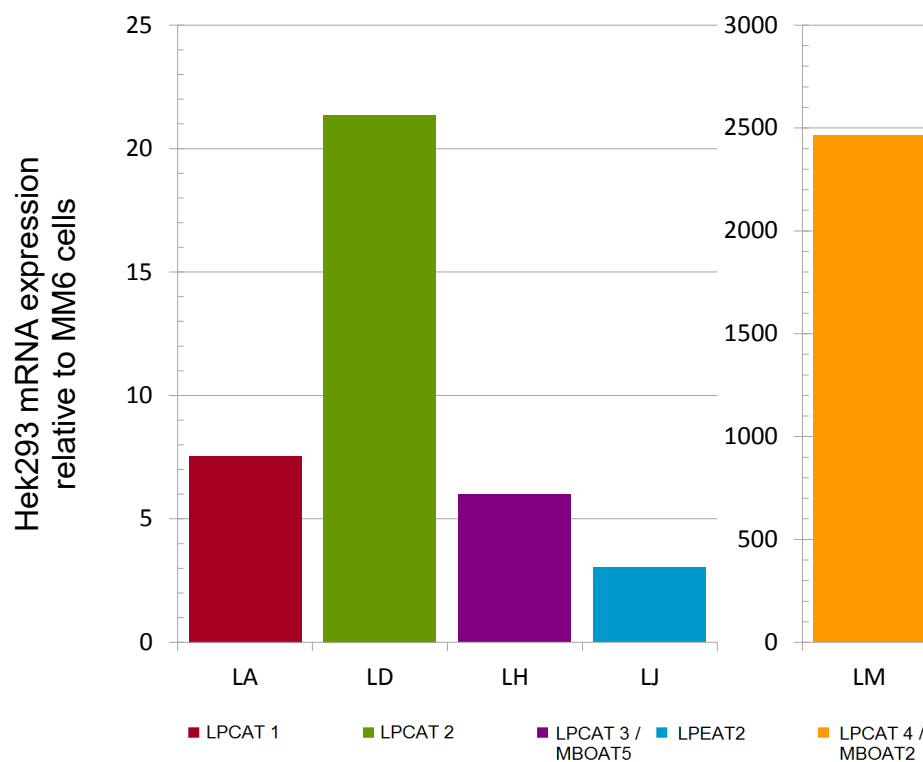
Intrinsic LPCAT expression of BEAS-2B cells, presented as the mean mRNA expression normalized to GAPDH and relative to MM6 cells. Results are the mean +/- S.E.M of 3 independent experiments.



**Figure 4.4** Intrinsic expression of LPCAT-like acyltransferases in HepG2 cells

Intrinsic LPCAT expression of HepG2 cells, presented as the mean mRNA expression normalized to GAPDH and relative to MM6 cells. Results are the mean +/- S.E.M of 3 independent experiments.

Since Hek293 cells are widely used to study LPCAT, it was not surprising to observe that the preliminary data for the intrinsic LPCAT expression in Hek293 cells was the highest across the cells studied (Figure 4.5). Expression of all the LPCAT genes were substantially increased in comparison to MM6 expression; most notably LPCAT2 expression (21 fold more) and LPCAT4 (over 2000 fold more). However, although the expression level of LPCAT4 was significantly increased, it is worth noting that results are displayed relative to MM6 (not actual quantities) and MM6 cells did not appear to express any LPCAT4 which may account for the sizeable difference. Initial results may suggest that LPCAT4 is crucial to Hek293 cell functions, although data was observed from one sample and further replicates are required in order to consolidate these findings.



**Figure 4.5 Intrinsic expression of LPCAT-like acyltransferases in Hek293 cells**

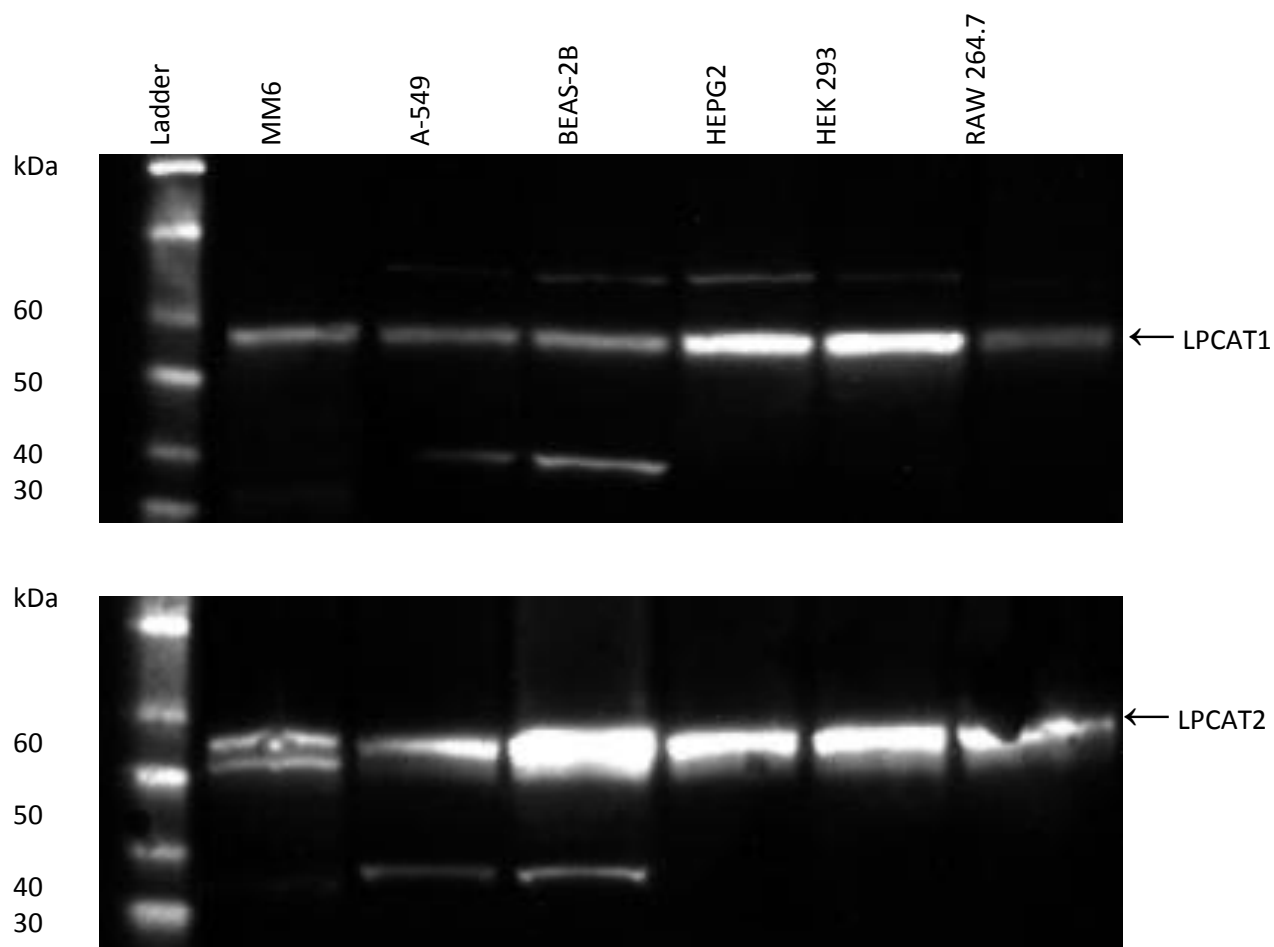
Preliminary data for intrinsic LPCAT expression of Hek293 cells, presented as mRNA expression normalized to GAPDH and relative to MM6 cells. Results are preliminary from a single sample of mRNA, with qPCR.

#### 4.4.2 Endogenous LPCAT1 and LPCAT2 protein expression is highest in BEAS-2B, HepG2 and Hek293 cells

Since LPCAT1 and LPCAT2 mRNA was detected in all the cell lines studied, the LPCAT protein level in these cells was examined. Protein bands at the corresponding molecular mass to hLPCAT1 and hLPCAT2 were identified in each cell line (Figure 4.6). Strong LPCAT1 bands appeared in protein from the metabolically active cells, HepG2 and Hek293. However, bronchial epithelial cell protein was shown to have stronger LPCAT2 signals than both HepG2 and Hek293 cells. MM6 cells on the other hand, display a low intensity of both LPCAT1 and LPCAT2.

Noticeably, the anti-human LPCAT2 antibody appears to have identified two bands of slightly different molecular mass in the MM6 cell protein. Whilst it must be noted that the antibodies are polyclonal, this second band may suggest that an isoform of LPCAT2 protein exists as suggested by NCBI database / UniProtKB (GenBank accession number Q7L5N7) and have been found in mice (Shindou *et al.*, 2007; Soupene *et al.*, 2008) or alternatively that the antibody is detecting one of the other LPCATs. This may also explain the large band observed in BEAS-2B cells when probed with anti-human LPCAT2 antibody. In 2009, Harayama *et al.* showed that antiserum against mouse LPCAT1 could also detect human LPCAT1, supporting the evidence of high amino acid homology that exists between species. On this basis, murine macrophage, RAW 264.7 cell lysate was also examined with anti-human LPCAT1 and LPCAT2 antibodies. The antibodies not only had cross-reactivity with the mouse cell lysate, but showed a strong signal with anti-human LPCAT2.

Although the protein levels analysed by western blot were not quantified by comparison to a known concentration of LPCAT protein, the results show some similarities to the qPCR data. HepG2 and Hek293 expressed the highest levels of LPCAT1 and LPCAT2 mRNA (relative to MM6) which is also implied in the western blots. However, BEAS-2B cell protein appears to have the highest LPCAT2 protein signal, in contrast to the qPCR data. Without standards and loading controls, however, these observations must be considered with caution.



**Figure 4.6** Expression profile of LPCAT1 and LPCAT2 in various cell lines.

The endogenous expression of hLPCAT1 and hLPCAT2 in MM6, A549, BEAS-2B, HepG2, Hek293 and mouse cell line Raw26.7 as determined by western blot. 20  $\mu$ g of protein cell lysate was loaded per lane and probed using anti-hLPCAT1 and anti-hLPCAT2 antibodies. Arrowhead indicates bands of expected molecular mass ( $\approx$ 59kDa) for LPCAT1 and ( $\approx$ 53kDa) for LPCAT2. Results are representative of 2 identical experiments

#### 4.4.3 LPS and IFN- $\gamma$ do not significantly affect the mRNA expression levels of LPCATs

LPCAT regulates the increased inflammatory cytokine production from IFN- $\gamma$  primed and microbial induced cells, as described in Chapter 3. IFN- $\gamma$  has also been observed to up-regulate LPCAT activity and significantly modify the phospholipid composition of monocytes (Schmid *et al.*, 2003). Shindou and colleagues (2005) observed LPCAT2 activity to double in LPS induced mouse peritoneal macrophages, confirming the crucial role that LPCAT plays in regulating inflammatory responses. It was therefore of interest to study the effects of IFN- $\gamma$  and LPS on LPCAT mRNA expression in MM6 and BEAS-2B cells.

Following qPCR, results were analysed using  $2^{-\Delta\Delta CT}$  and are presented as the fold change in mRNA expression normalized to GAPDH and relative to the corresponding untreated cell. No significant changes in mRNA expression of LPCAT 1, 2, 3, 4 or LPEAT2 (Table 4.2) were observed with LPS stimulation in MM6 or BEAS-2B cells. Preliminary experiments with IFN- $\gamma$  treatment (Table 4.3) also found that neither MM6 nor BEAS-2B mRNA displayed any significant changes in expression of the LPCATs. This is suggestive that changes in the two sets of data are due to random variability and not influenced by treatment, however, this data should be viewed cautiously as only one IFN- $\gamma$  experiment was completed and the characteristic of qPCR is to study one specific time point in the experiment, which perhaps need optimising.



(a) MM6 mean expression change with LPS treatment			(b) BEAS-2B mean expression change with LPS treatment		
Primer	Mean 2- $\Delta\Delta$ CT	S.E	Primer	Mean 2- $\Delta\Delta$ CT	S.E
LPCAT 1 (LA)	0.5	0.0	LPCAT 1 (LA)	1.2	0.1
LPCAT 2 (LD)	0.5	0.1	LPCAT 2 (LD)	1.7	0.0
LPCAT 3 (LH)	0.8	0.1	LPCAT 3 (LH)	1.0	0.0
LPEAT 2 (LJ)	0.3	0.0	LPEAT 2 (LJ)	1.0	0.1
LPCAT 4 (LM)	0.8	0.2	LPCAT 4 (LM)	1.5	0.1

**Table 4.2 The effect of LPS stimulation on LPCAT mRNA expression in MM6 and BEAS-2B cells.**

LPCAT mRNA expression of MM6 (a) and BEAS-2B (b) cells treated with LPS, normalized to GAPDH and relative to untreated cells, presented as the mean fold change in mRNA expression. One primer set for each LPCAT was selected and displays the representative expression. Results are the mean  $\pm$  SEM of 3 independent experiments.

(a) MM6 expression change with IFN- $\gamma$ treatment		(b) BEAS-2B expression change with IFN- $\gamma$ treatment	
Primer	2- $\Delta\Delta$ CT	Primer	2- $\Delta\Delta$ CT
LPCAT 1 (LA)	1.2	LPCAT 1 (LA)	0.7
LPCAT 2 (LD)	0.8	LPCAT 2 (LD)	1.0
LPCAT 3 (LH)	0.9	LPCAT 3 (LH)	1.4
LPEAT 2 (LJ)	1.0	LPEAT 2 (LJ)	1.0
LPCAT 4 (LM)	0.5	LPCAT 4 (LM)	1.2

**Table 4.3 The effect of IFN- $\gamma$  treatment on LPCAT mRNA expression in MM6 and BEAS-2B cells.**

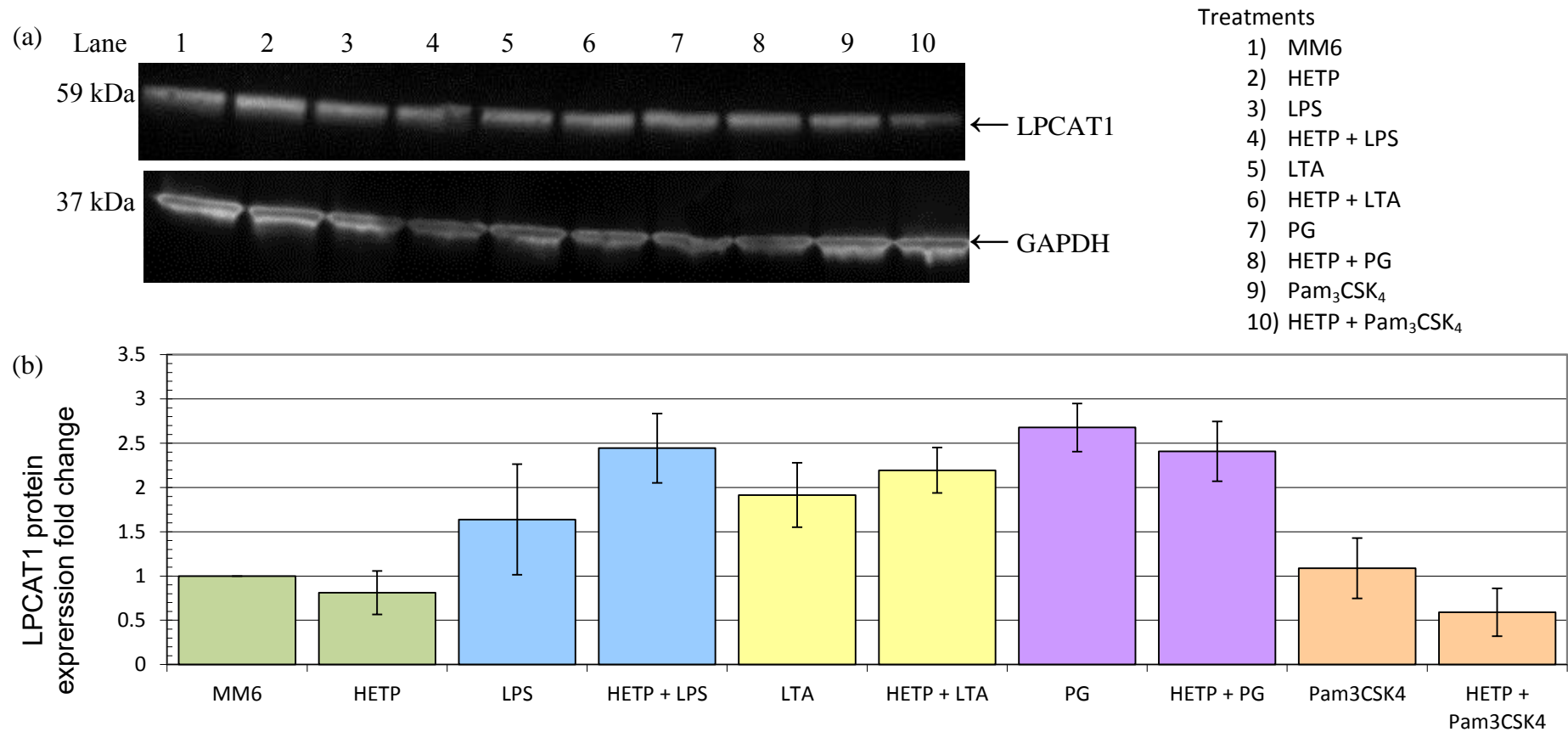
LPCAT mRNA expression of MM6 (a) and BEAS-2B (b) cells treated with IFN- $\gamma$ , normalized to GAPDH and relative to untreated cells, presented as the fold change in mRNA expression. The same primer set for each LPCAT (as above) was selected and displays the representative expression. Results are preliminary data of one experiment.

#### 4.4.4 LPCAT protein is up-regulated with PG stimulation

Whilst LPCAT mRNA expression appears unaffected by interactions with TLR4 ligand LPS and IFN- $\gamma$ , it was of interest to study their effects on LPCAT protein expression. Western blot analysis was used to show the protein expression of LPCAT1 and LPCAT2 in MM6 cells stimulated with TLR4 and TLR2 ligands, and to assess its reduction in expression after treatment with HETP. LPCAT band intensity was measured using ImageJ software and used as a qualitative measure of protein expression (Abramoff *et al.*, 2004).

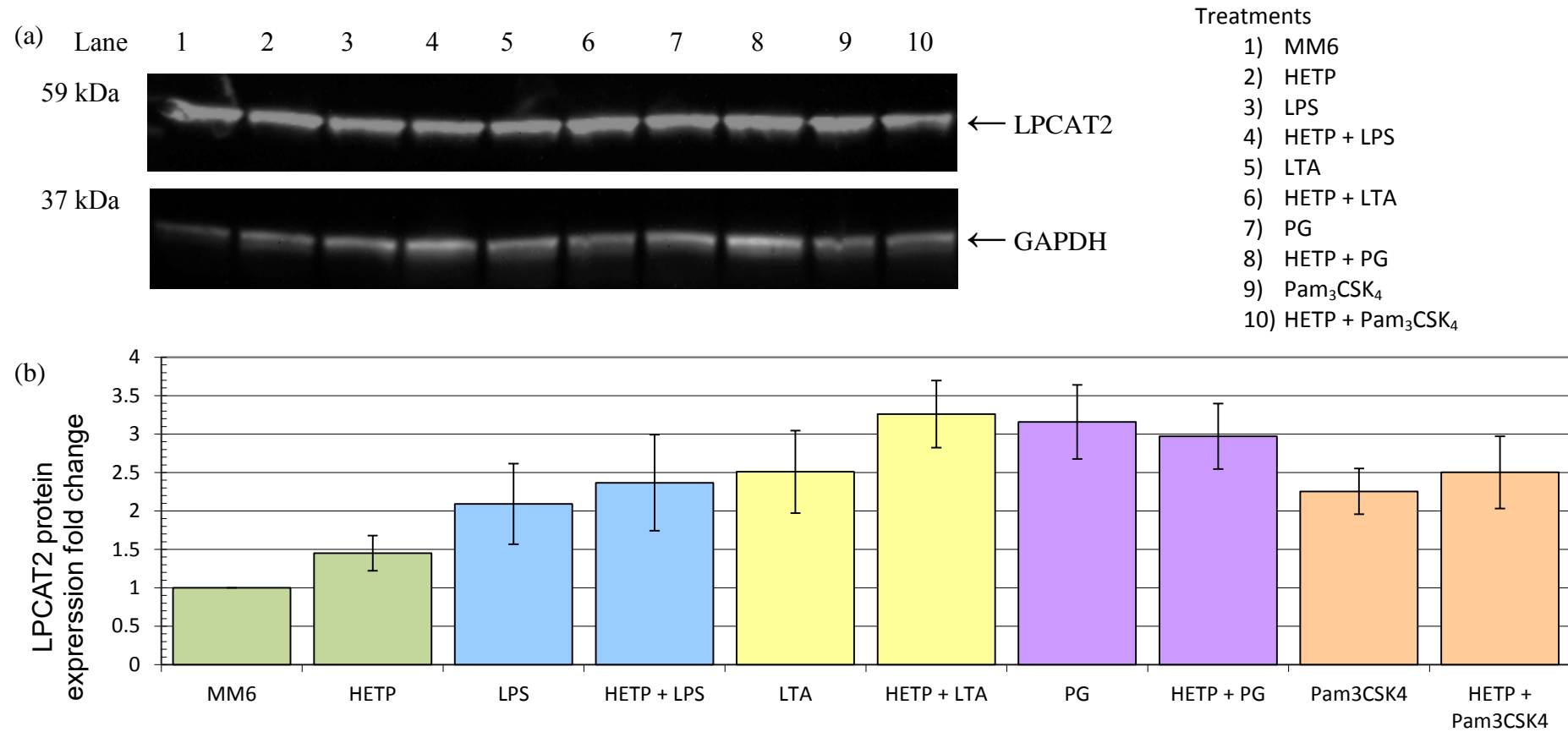
Detection using hLPCAT1 antibody revealed a protein band at approximately 59kDa in all the untreated and treated cell lysates which corresponds to the expected weight of LPCAT1 (Figure 4.7a). ImageJ analysis suggests that stimulation with LPS, LTA or Pam<sub>3</sub>CSK<sub>4</sub> did not significantly affect expression of the 59kDa band (Figure 4.7b). However PG treatment resulted in a small, but significant ( $P < 0.05$ ), 2.5 fold increase in expression of LPCAT1.

Similar findings were found using hLPCAT2 antibody. In all the untreated and treated cell lysates, a protein band at approximately 53kDa was identified which corresponds to the expected weight of LPCAT2 (Figure 4.8**Error! Reference source not found.**a). Comparable to results seen with LPCAT1 antibody, PG treatment resulted in a small, but significant ( $P < 0.05$ ), 3 fold increase in expression of LPCAT2, whereas stimulation with LPS, LTA or Pam<sub>3</sub>CSK<sub>4</sub> did not significantly affect expression (Figure 4.8b). It was noted that in all experiments no reduction in LPCAT1 or LPCAT2 expression was observed in cells treated with HETP.



**Figure 4.7 Effect of TLR ligands on LPCAT1 protein expression in MM6 cells**

MM6 cells ( $3 \times 10^6$ ) were incubated in the presence or absence of 50  $\mu$ M HETP, 30 minutes prior to stimulation with LPS (100 ng/mL), LTA (10  $\mu$ g/mL) PG (10  $\mu$ g/mL) or Pam<sub>3</sub>CSK<sub>4</sub> (1.0  $\mu$ g/mL) for 24 hours. An equal volume and concentration (20  $\mu$ g) of cell lysate was loaded per lane. (a) Representative western blot probed with hLPCAT1 antibody. Membranes were stripped and re-probed with GAPDH. (b) Densitometry data of western blots probed with LPCAT1 antibody. Results are the mean  $\pm$  S.E.M of 3 independent experiments, normalized to GAPDH and relative to the untreated control.



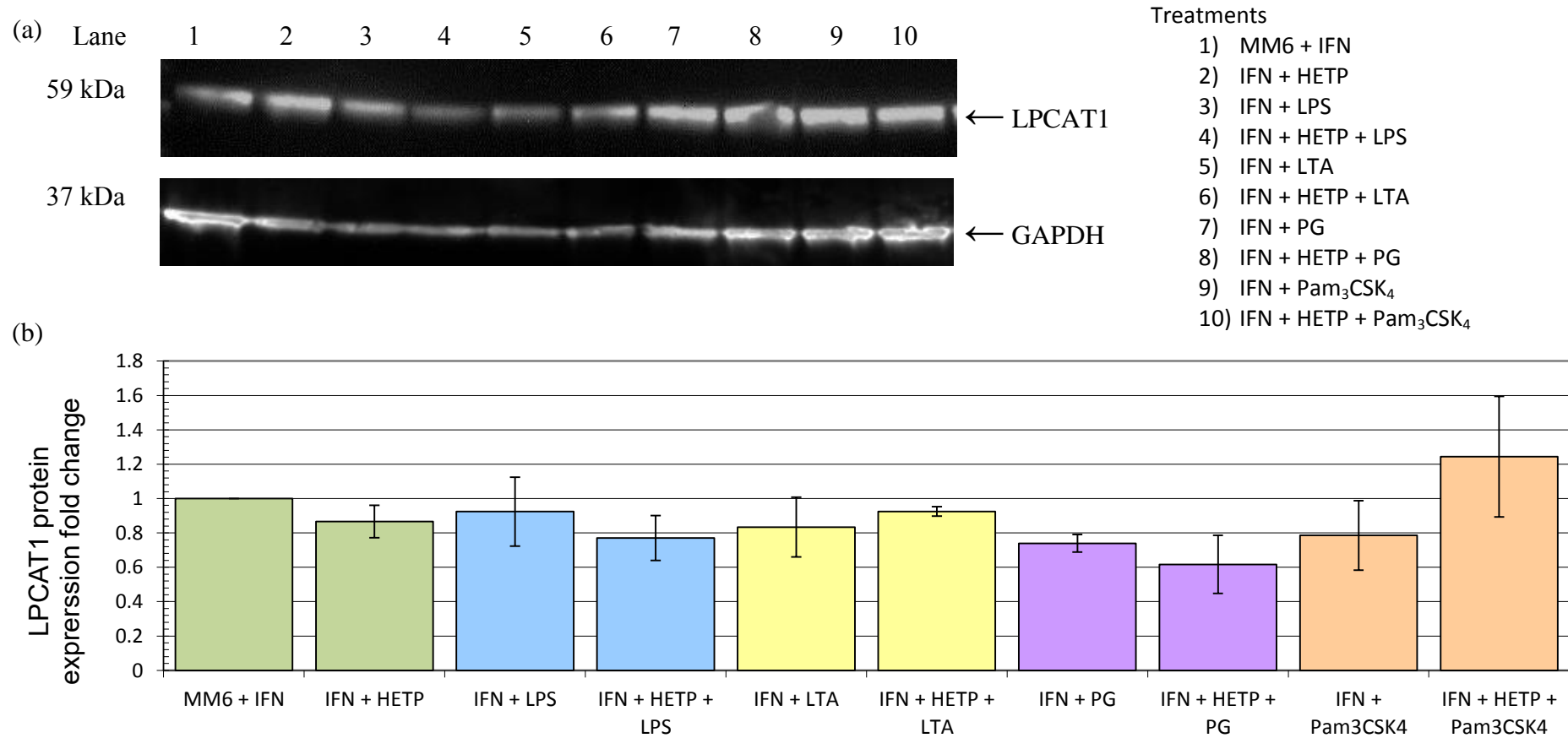
**Figure 4.8 Effect of TLR ligands on LPCAT2 protein expression in MM6 cells**

MM6 cells ( $3 \times 10^6$ ) were incubated in the presence or absence of 50  $\mu$ M HETP, 30 minutes prior to stimulation with LPS (100 ng/mL), LTA (10  $\mu$ g/mL) PG (10  $\mu$ g/mL) or Pam<sub>3</sub>CSK<sub>4</sub> (1.0  $\mu$ g/mL) for 24 hours. An equal volume and concentration (20  $\mu$ g) of cell lysate was loaded per lane. (a) Representative western blot probed with hLPCAT2 antibody. Membranes were stripped and re-probed with GAPDH. (b) Densitometry data of western blots probed with LPCAT2 antibody. Results are the mean  $\pm$  S.E.M of 3 independent experiments, normalized to GAPDH and relative to the untreated control.

#### 4.4.5 IFN- $\gamma$ does not significantly affect LPCAT protein expression

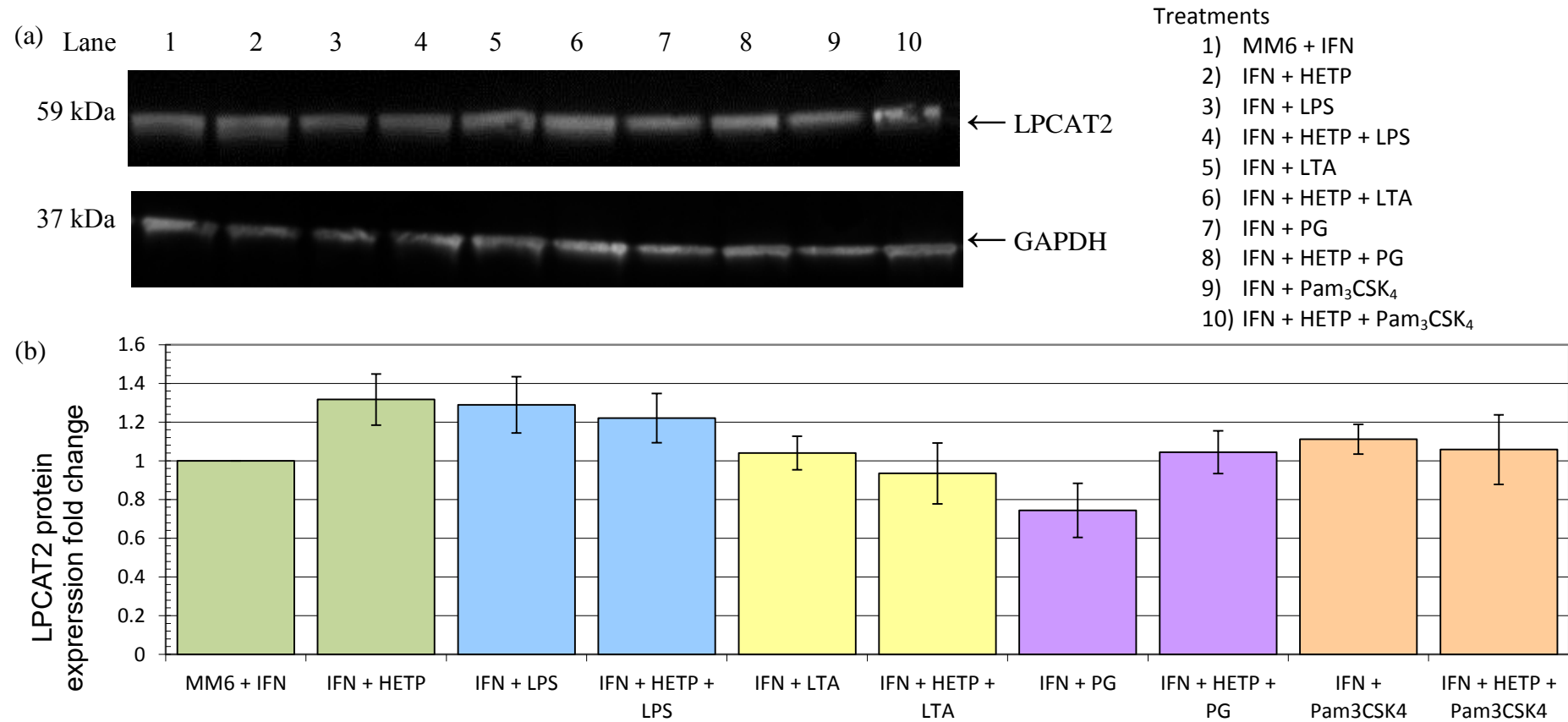
It was investigated if there was a correlation between increased inflammatory cytokine secretion in cells treated with IFN- $\gamma$  (as seen in chapter 3) and the expression levels of LPCAT. Preliminary results suggest that IFN- $\gamma$  does not induce a significant change in LPCAT mRNA expression, but it was of interest to investigate if IFN- $\gamma$  alters expression at the protein level.

As for previous experiments, ImageJ software was used to quantify protein LPCAT expression by measuring band intensity of western blots. Figure 4.9 and **Error! Reference source not found.** Figure 4.10 identified protein bands in all lanes to the corresponding molecular weights of LPCAT1 and LPCAT2. The protein bands detected in cell lysates treated with IFN- $\gamma$  and/or TLR ligands revealed intensities equivalent (within 1 fold) to untreated MM6 cell lysate.



**Figure 4.9 Effect of IFN- $\gamma$  on LPCAT1 protein expression in MM6 cells**

MM6 cells ( $3 \times 10^6$ ) were pre- incubated with IFN- $\gamma$  for 18 hours prior to inhibition with of  $50 \mu\text{M}$  HETP for 30 minutes and stimulation with LPS ( $100 \text{ ng/mL}$ ), LTA ( $10 \mu\text{g/mL}$ ) PG ( $10 \mu\text{g/mL}$ ) or Pam<sub>3</sub>CSK<sub>4</sub> ( $1.0 \mu\text{g/mL}$ ) for 24 hours. An equal volume and concentration ( $20 \mu\text{g}$ ) of cell lysate was loaded per lane. (a) Representative western blot probed with hLPCAT1 antibody. Membranes were stripped and re-probed with GAPDH. (b) Densitometry data of western blots probed with LPCAT1 antibody. Results are the mean  $\pm$  S.E.M of 3 independent experiments, normalized to GAPDH and relative to the untreated control.



**Figure 4.10** Effect of IFN- $\gamma$  on LPCAT2 protein expression in MM6 cells

MM6 cells ( $3 \times 10^6$ ) were pre-incubated with IFN- $\gamma$  for 18 hours prior to inhibition with 50  $\mu$ M HETP for 30 minutes and stimulation with LPS (100 ng/mL), LTA (10  $\mu$ g/mL) PG (10  $\mu$ g/mL) or Pam<sub>3</sub>CSK<sub>4</sub> (1.0  $\mu$ g/mL) for 24 hours. An equal volume and concentration (20  $\mu$ g) of cell lysate was loaded per lane. (a) Representative western blot probed with hLPCAT2 antibody. Membranes were stripped and re-probed with GAPDH. (b) Densitometry data of western blots probed with LPCAT2 antibody. Results are the mean  $\pm$  S.E.M of 3 independent experiments, normalized to GAPDH and relative to the untreated control.

## 4.5 DISCUSSION

### 4.5.1 Expression of LPCAT is ubiquitous, but LPCAT4 is highly in lung epithelial cells.

Many groups have published findings on the expression levels of acyltransferases with LPCAT activity in human and mouse tissue panels; Nakanishi *et al.* (2006) reported the highest mLPCAT1 expression in mouse lung tissue. Harayama *et al.* (2009) also observed hLPCAT1 expression to be highest in the lung, then spleen and leukocytes confirming these findings. Furthermore, rat alveolar type II cells were isolated and found to be enriched with LPCAT1, where surfactant proteins are secreted (Chen *et al.*, 2006). In agreement with these studies, Agarwal and co-workers (2007) showed the expression of hAGPAT9/hLPCAT1 to be highly expressed in lung tissue. In comparison, leukocytes expressed approximately 3 fold less and liver tissue expressed 40 fold less than lung tissue (Agarwal *et al.*, 2007). Furthermore, alveolar type II cells were found to express the majority of LPCAT2 observed in total lung tissue.

Literature would therefore suggest that hLPCAT1 is most notably expressed in lung tissue, where it plays a role in producing DPPC, with leukocytes expressing a little less (Nakanishi *et al.*, 2006). Results from the present study show comparable mRNA expression of LPCAT1 between lung epithelial and monocytic cell lines, supporting this observation. The liver hepatocyte cell line HEPG2 was also found to have similar expression of LPCAT1 in contrast to the significantly reduced expression observed by Agarwal *et al.* (2007) in primary liver tissue. This discrepancy could be due to HepG2 being a transformed cell line, possibly expressing more LPCAT1 mRNA than primary hepatocytes.

LPCAT2 shows significant amino acid sequence homology to LPCAT1 (Shindou *et al.*, 2007) and it may therefore not be surprising that LPCAT2 has been reported to also have the highest expression in lung tissue and leukocytes (Agarwal *et al.*, 2010). In fact Shindou *et al.* (2007) found the highest expression level of mLPCAT2 in resident macrophages (230 arbitrary units) and a low level seen lung tissue (12 arbitrary units), suggesting that LPCAT2 has an important role to play in mouse macrophages. Shindou *et al.* (2005) reported LPCAT2 to play a constitutive role in inducible PC remodelling pathways in response to external stimuli in mouse



peritoneal macrophages. Thus initial thoughts are that LPCAT2 is the major remodelling enzyme involved in inflammatory pathways.

However, a discrepancy is observed in the literature as this is inconsistent to the findings of Agarwal *et al.* (2010) who reported that leukocyte expression of hLPCAT2 was identical to expression in lung tissue. Results from the current study revealed that hLPCAT2 mRNA expression is alike in lung and monocyte cell lines in agreement with the observations made by Agarwal *et al.* (2010). Although mouse LPCAT2 was reported to have 88.4 % amino acid sequence homology to human LPCAT2 (Shindou *et al.*, 2007), perhaps the discrepancy seen in the literature highlights the difference between the role of mouse LPCAT2 and human LPCAT2 in macrophages. As shown with LPCAT2 siRNA, human LPCAT2 actually appears to be the principal enzyme for PAF production in HEK293 cells (Shindou *et al.*, 2007) which supports the high expression levels found in the present study.

Mouse LPCAT3 (MBOAT5) has been detected ubiquitously amongst metabolically active tissue, with abundant expression levels in testis, liver and kidney tissues, compared to very low expression in both lungs and macrophages (Hishikawa *et al.*, 2008). Zhao, *et al.* (2008) supported these findings, observing high mLPCAT3 expression in liver and pancreas tissue, suggesting it to be primarily responsible for hepatic LPCAT activity, but very low expression in lung tissue. However, expression levels of human LPCAT3/MBOAT5, is currently unreported in the literature. The present study found the mRNA levels of LPCAT3 comparable between A-549, BEAS-2B and MM6 cell lines. In contrast, HEPG2 mRNA expression of LPCAT3 was observed to be 1.5 fold more than LPCAT3 expression in monocytes, similar to the increased level observed in liver tissue as published, indicating LPCAT3 as a principal enzyme in the regulation of the remodelling pathway.

Contrasting to LPCAT3, LPEAT2 has been reported virtually undetectable in human lung and liver tissues, and was primarily expressed in both human and mouse brain tissue (Cao *et al.*, 2008; Ye *et al.*, 2005). As reported here, very low mRNA expression levels of LPEAT2 were seen across the panel of cell lines tested, suggesting that LPEAT2 has greater importance in modulating brain phospholipid composition, than in lungs, liver or monocytes.

Finally LPCAT4 (MBOAT2) mRNA was observed to have a remarkably high expression in both lung epithelial cell lines (Figure 4.2 and Figure 4.3) in the present study. In contrast, low expression of mLPCAT4 was detected in lung tissue by Hishikawa *et al.* (2008) who revealed that mLPCAT4 is predominantly expressed in mouse epididymis, brain and testis tissue, playing a constitutive role in membrane biogenesis. It may be that this variation exists because no LPCAT4 mRNA was detected in monocytes, to which the expression level of LPCAT4 in A-549 and BEAS-2B cells was relative. However, even the equivalent CT values display an enlarged expression of LPCAT4 in these lung cells (data not shown).

It is possible that each LPCAT performs a unique function within different cell types in mice and humans, either maintaining cellular membrane structure and /or producing specific phospholipid signalling molecules. As such LPCAT expression observed in mice may not be representative of human expression. Nevertheless, data from the present study indicates that LPCAT4 may have a function in regulating the phospholipid remodelling pathway in lung epithelial cells.

#### 4.5.2 Expression of LPCAT is unaffected by stimulation and priming

The present study has demonstrated that inhibition of LPCAT down-regulates inflammatory cytokine production in monocytes and epithelial cells. Jackson *et al.* (2008) discovered that this observation was due the inhibition of LPCAT preventing translocation of TLR4 into membrane lipid raft domains. These observations demonstrated a regulatory mechanism that can facilitate innate immune responses to microbial derived stimuli, but the specific LPCAT responsible remains unknown.

In 2005 Shindou and colleagues reported the existence of an LPS-inducible remodelling pathway in response to external stimuli, where LPS induced LPCAT2 activity doubled in mouse peritoneal macrophages. Initially, this indicated that LPCAT2 might be the candidate enzyme to modulate the phospholipid remodelling pathway in innate immune cells, but also that it regulates the production of specific signalling molecules in response to microbial molecular patterns, such as those seen in sepsis.

It was believed that LPCAT2 could be the LPCAT responsible for regulating inflammatory responses in monocytes and thus it was hypothesised that LPCAT2 could be highly expressed in these immune cells. However, endogenous mRNA expression of LPCAT2 was found to be no more than observed in lung epithelial cells. So LPS-induced LPCAT2 expression was then investigated. Conflicting to these ideas, data in this report suggests that LPS does not significantly change the expression of LPCAT2 or any of these LPCAT-like ATs in monocytes or alveolar epithelial cell lines. However, although PCR data should be viewed with caution as only one experiment was completed. Additionally, western blot analysis of MM6 cell lysate demonstrated no protein expression alterations with LPS treatment or any TLR2 ligand. Furthermore, prior treatment with LPCAT inhibitor, HETP, did not reduce LPCAT1 or LPCAT2 protein expression

IFN- $\gamma$ , found to increased inflammatory responsiveness to LPS, is an important factor in the development of LPS-induced biological activity and its presence is linked to the mortality of several forms of endotoxic shock (Car *et al.*, 1994). Schmid *et al.* (2003) observed IFN- $\gamma$  to up-regulate LPCAT activity and significantly modify the phospholipid composition of monocytes. In addition to this activity, it was of interest to ascertain if IFN- $\gamma$  can increase the expression of LPCAT in monocytes. However, IFN- $\gamma$  did not significantly change the mRNA expression of any monocytic, nor protein expression of LPCAT1 or LPCAT2, which was relative to the constitutive expression.

Nevertheless, qPCR and western blot analysis only looks at a snapshot in the timeline of mRNA and protein expression and this report looked at expression levels after 18 hours of incubation with IFN- $\gamma$ , which in hindsight could have been too late to observe any increased expression. The same conclusion could be reached with TLR ligand induced LPCAT expression which was observed at 4 hours post stimulation for PCR and 24 hours for western blot analysis. It would be advantageous to study different incubation time points to demonstrate what effect this has on mRNA and protein expression.

Further investigation could reveal that timely post-transcriptional modifications occur and allow for increased production in LPCAT protein, or more likely that modulation of LPCAT's activity rather than expression is influenced by TLR ligands and IFN- $\gamma$ , as Schmid *et al.* (2003) observed. This may also help to reveal why the LPCAT inhibitor HETP did not affect LPCAT expression levels and perhaps suggests that HETP impedes LPCAT enzyme activity.

# Chapter 5

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RESULTS AND DISCUSSION

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TOLL-LIKE RECEPTOR 2 MEMBRANE  
TRANSLOCATION IN INFLAMMATORY RESPONSES

## 5.1 INTRODUCTION

An accumulating body of evidence exists suggesting that TLR4 and TLR2 are not constitutively found in membrane lipid rafts, but are recruited into them only after microbial stimulation (Abate *et al.*, 2010; Jackson *et al.*, 2008; Triantafilou *et al.*, 2004). To date the receptor molecules that cluster within lipid rafts have been characterized and demonstrated to show that translocation of these molecules provide a focused signalling event (Triantafilou *et al.*, 2004). Furthermore, LPCAT enzymes have been shown to modify the plasma membrane phospholipid microenvironment facilitating the recruitment of receptor proteins into lipid rafts for the assembly of receptor complexes (Darmani *et al.*, 1993; Schmid *et al.*, 2003).

In 2008, research by Jackson *et al.* confirmed that membrane lysophospholipid metabolism facilitates TLR4 translocation and thus is involved in the regulation of inflammatory signalling responses. This discovery was achieved through blocking LPCAT activity with the specific LPCAT inhibitor, HETP, which demonstrated the subsequent inhibition of TLR4 translocation into the lipid raft domain of MM6 cell membranes following stimulation with LPS (Jackson *et al.*, 2008). Consequently, the receptor complex was prevented from assembling and a significant reduction in inflammatory cytokine secretion was observed.

In addition, Abate *et al.* (2010) examined the involvement of TLR4 translocation into lipid rafts in the induction of chemokine release by lung epithelial cells A549 cells by disrupting the raft microdomains with mycostatin. The disruption of the lipid rafts caused a dose-dependent inhibition of LPS-mediated IL-8 production, which suggested that raft microdomains are important in LPS signalling in lung epithelial cells. In a manner analogous to TLR4, TLR2 forms activation clusters with TLR1 or TLR6 depending on the microbial ligand it is exposed to; diacylated lipoproteins, such as LTA, require TLR2/6 heterodimers for activation, whereas triacylated lipoproteins, such as Pam<sub>3</sub>CSK<sub>4</sub>, induce activation of the innate immune system mainly through TLR2/TLR1 heterodimers (Triantafilou *et al.*, 2006). These clusters trigger signalling from the cell surface, activating an inflammatory response in a lipid-raft dependent pathway (Triantafilou *et al.*, 2004).

## 5.2 AIMS

The aim of this study was to assess whether LPCAT inhibition prevents TLR2 translocation into the membrane lipid raft, thus preventing the subsequent formation of the receptor signalling complex.

## 5.3 METHODS

### MM6 cell stimulation with TLR ligands

MM6 cells ( $1 \times 10^7$ ) were co-cultured with or without 50  $\mu$ M HETP for 30 minutes prior to co-culture in the presence or absence of LTA (10  $\mu$ g/ml), PG (10  $\mu$ g/ml) or Pam<sub>3</sub>CSK<sub>4</sub> (1.0  $\mu$ g/ml) and further incubated for 45 minutes, as recommended by Triantafidou *et al.* (2006) and Jackson *et al.* (2008). Cells were lysed in a Triton X extraction buffer for 1 hour at 4°C and sonicated on ice to homogenize the lysate in 4×11 second bursts and reduce sample viscosity. Complete lysis of cells was checked using light microscopy.

### 5.3.1 Total lipid isolation

Since membrane lipid rafts are enriched in cholesterol, sphingolipids and are detergent-insoluble, they can be separated from other plasma membrane components based upon their insolubility in Triton X-100 and by low buoyant density in sucrose gradients. To determine if inhibition of LPCAT affects translocation of TLR2 into the lipid raft domain of MM6 cell membranes in response to a microbial stimulant, total lipid fractions were isolated from cell lysate using sucrose density gradient ultracentrifugation as described by Triantafidou *et al.* (2002). A total of twelve 1 ml membrane fractions (1-12) were then recovered from the gradient and taken forward for analysis.

### 5.3.2 Assessment of lipid separation by dot blot analysis

To determine which fractions contained the lipid raft, samples from each of the twelve fractions were probed for GM1, a ganglioside receptor prevalent in lipid rafts, with HRP-conjugated cholera-toxin  $\beta$ -subunit that specifically binds to GM1. The antigen was then visualised using Amersham ECL Plus Western Blotting Detection Reagents (GE Healthcare, Buckinghamshire, UK) in accordance with the manufacturer's instructions.

### 5.3.3 Determining TLR translocation by western blot

Western blot analysis was used to examine the location of TLR2 within the lipid raft fractions after microbial ligand stimulation. Equal volumes of protein (20  $\mu$ l of sample per lane) were loaded and 5  $\mu$ l of biotinylated protein ladder was loaded in lane 1 to aid identification of proteins on nitrocellulose membranes. Commercially designed hTLR2 antibody (Cambridge Bioscience, Cambridge, UK) was used to detect protein expression of TLR2.



## 5.4 RESULTS

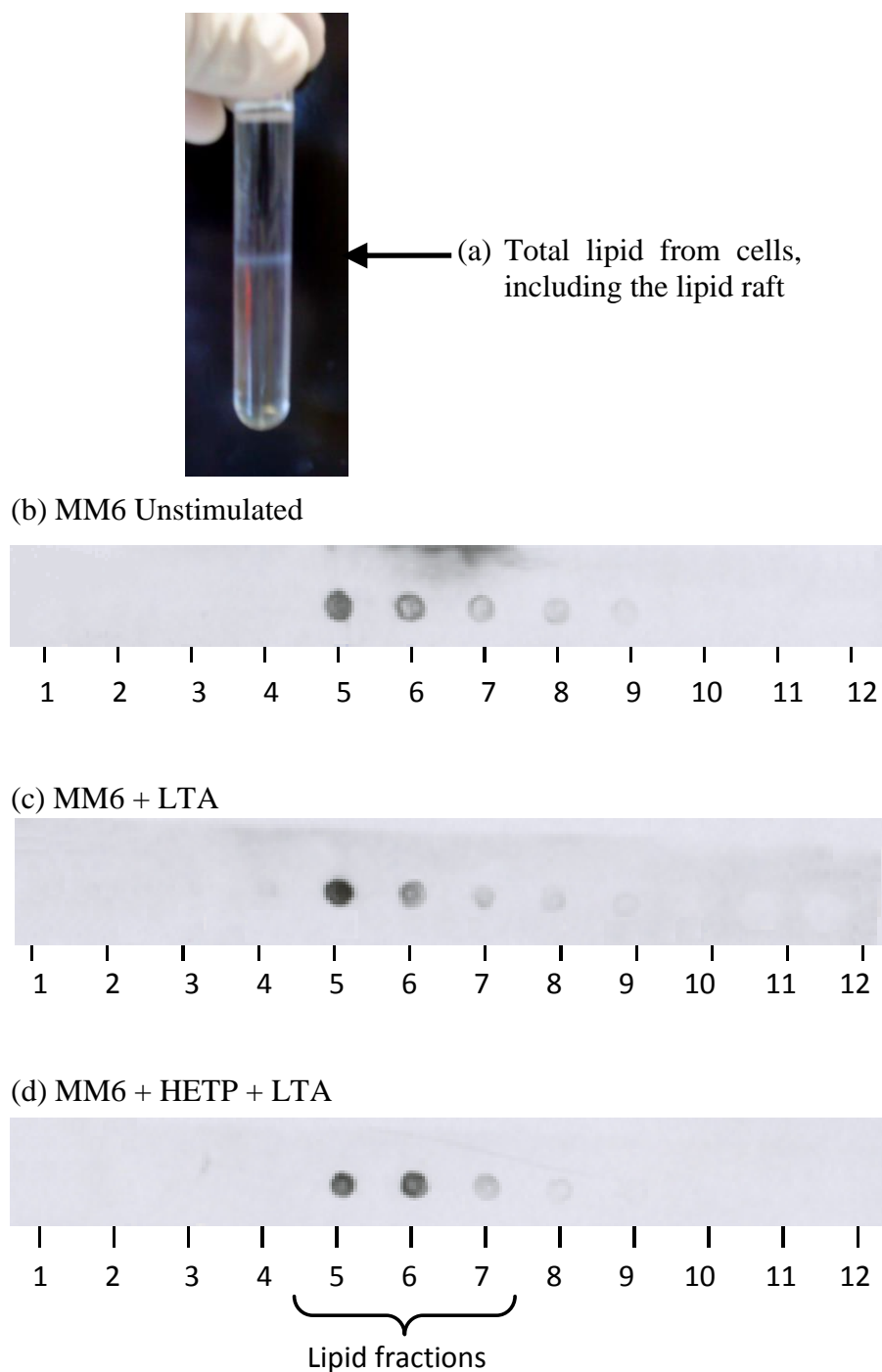
### 5.4.1 Assessment of lipid raft separation by dot blot analysis

The dot blots in Figure 5.1 show the isolation of the total lipid fraction by sucrose density gradient ultracentrifugation (Figure 5.1a). GM1, a ganglioside receptor prevalent in lipid rafts, was used as a probe to identify those fractions containing lipid rafts. GM1 was detected in membrane fractions 5, 6 and 7 from unstimulated MM6 total cell lysate, indicating the presence of lipid rafts (Figure 5.1b). MM6 cells stimulated with TLR2 ligand LTA (Figure 5.1c), and with prior treatment with HETP (Figure 5.1d) were also probed with GM1 and dot blots verify that the membrane lipid rafts remained in fractions 5, 6 and 7.

### 5.4.2 Determining TLR2 translocation by Western blot analysis

Western blot analysis was used to identify the location of TLR2 in the fractions of untreated MM6 cells in order to assess TLR2 translocation upon stimulation with TLR2 ligands and to determine the degree of inhibition of TLR2 translocation by HETP. Initially, 20  $\mu$ l of each membrane fraction were applied onto the Bis-Tris gel (4-12 %). However, after probing the nitrocellulose membrane with hTLR2 antibody, sample protein concentrations from neat fractions were insufficient to visualise protein bands. Whilst significant protein concentration can be seen on western blots by Triantafidou *et al.* (2002) who also loaded equivalent portions of membrane fractions, Jackson *et al.* (2008) however, concentrated proteins in each fraction prior to western blot. Thus future experiments following this trial followed this model of concentrating proteins by methanol:chloroform precipitation as described by Wessel and Flugge (1984).

Despite optimising the ratios of methanol and chloroform used for protein concentration by Wessel and Flugge (1984), concentrating the proteins from membrane fractions proved more challenging than expected. One specific issue was that sucrose was concentrated along with the protein in the fractions, which ultimately interfered with protein concentration assays. Problems were also encountered with the limitation of micro-centrifuge tube volumes so alternative protein concentration methods were investigated.



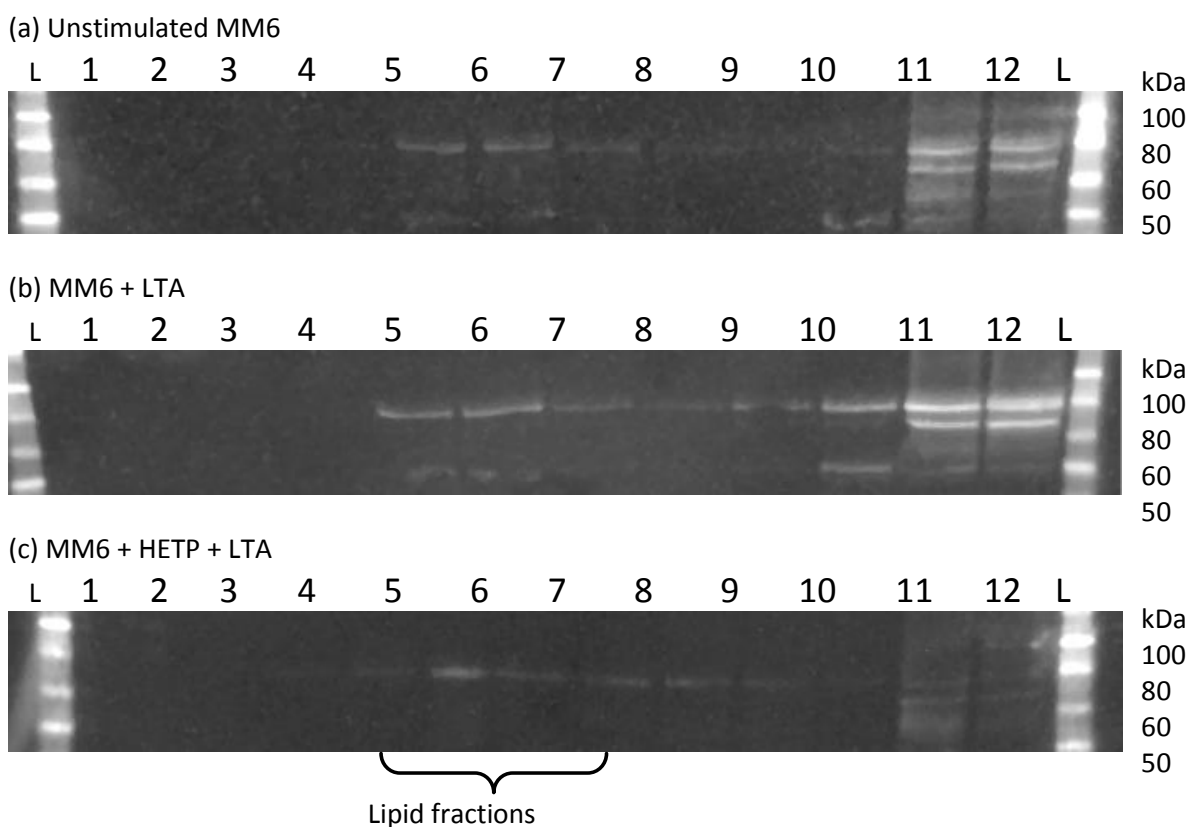
**Figure 5.1** Dot Blot of lipid (5-7) and non-lipid (1-4 and 8-12) fractions.

Plasma membrane fractions were obtained through total cell lysate sucrose density gradient ultracentrifugation. (a) twelve 1 ml fractions were removed and probed for GM1, a lipid raft specific protein. The presence of GM1 was detected with HRP-conjugated cholera-toxin and visualised using ECL procedure (Amersham). GM1 was found in fractions 5, 6 and 7 in (b) unstimulated MM6 cells, (c) LTA stimulated MM6 cells and (d) HETP and LTA treated MM6 cells, indicating that these fractions contain the total lipid from the cells, including the lipid rafts. These dot blots are representative of all total lipid isolations performed and all additional experiments revealed the lipids in the same fractions, 5-7.

To this end, several other protein concentration methods were investigated, including trichloroacetate (TCA- Pierce Technical resources) and phenol/ether (Sauvé *et al.*, 1995) precipitation. Whilst these methods achieved high protein concentrations and recovery from standard cell lysate, difficulties were still experienced with proteins suspended in sucrose solutions; including increased sample viscosity, difficulty re-solubilising the pellet or substance interference with protein quantification assays. It was therefore of interest to investigate dialysing or desalting methods to remove the undesirable sucrose from lipid samples. However, while both methods served to reduce sucrose content, neither concentrated the protein.

After extensively investigating a variety of dialysing and desalting methods and protein concentration and quantification assays, the phenol/ether method concentration method offered the most potential to eradicate the problematic sucrose. This method was scaled up, optimised and used thereafter to concentrate proteins directly from each 1 ml membrane fraction. The resulting pellet was resuspended using  $\times 2$  LDS sample buffer compatible with protein quantification assays, supplemented with 2 % SDS to aid re-solubilisation. Protein concentrations were determined using Pierce BCA microplate Protein assay kit (Thermo Fisher Scientific, Loughborough, UK) and equal volumes and concentrations (20  $\mu$ g) of each lipid fractions were then used for western blot analysis.

Probing using hTLR2 antibody revealed a protein band at approximately 80 kDa, 10 kDa lower than the expected molecular weight of TLR2 in the non-lipid fractions (11 and 12) of both untreated and treated cell lysates (Figure 5.2). As these fractions were the last to be removed from the sucrose density gradient, they likely contained residual cell lysate; therefore the presence of TLR2 in these fractions was not unexpected. However, western blot analysis also identified the presence of TLR2 at low concentration in the lipid (fractions 5-7), in both unstimulated and treated MM6 cells. Even after protein concentration, the TLR2 protein was expressed at a significantly lower amount in the lipid than seen in the reports by both Triantafidou *et al.* (2002) and Jackson *et al.* (2008).



(d) Percentage of total protein band intensity in membrane fractions

	Non-lipid	Lipid	Non-lipid, cell lysate fragments
Treatment	Fractions 1-4	Fractions 5-7	Fractions 8-12
Untreated	1.5 %	11.4 %	87.1 %
LTA	0.6 %	11.4 %	88.0 %
HETP + LTA	6.0 %	27.8 %	66.2 %

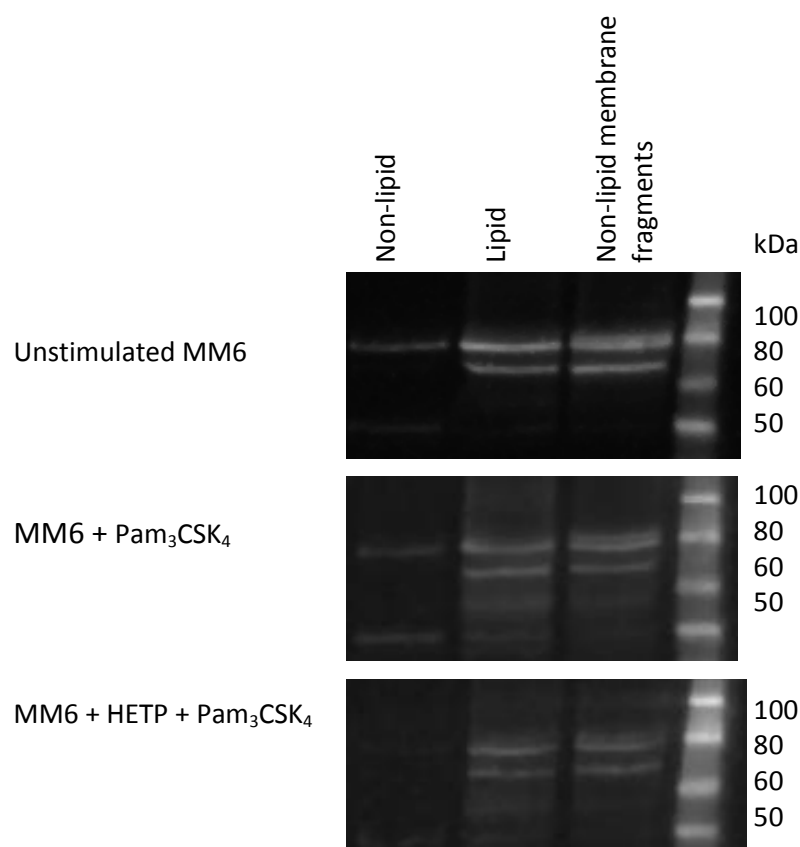
### Figure 5.2 Expression of TLR2 in monocyte plasma membrane fractions.

1 ml plasma membrane fractions were obtained through total cell lysate sucrose density gradient ultracentrifugation and concentrated. An equal concentration (20  $\mu\text{g}$ ) and volume of concentrated protein was loaded per lane. The nitrocellulose membrane was probed with hTLR2 antibody for expected protein bands of 90 kDa. This is a representative western blot analysis of TLR2 expression in (a) unstimulated, (b) LTA treated (10  $\mu\text{g}/\text{ml}$ ) and (c) HETP (50  $\mu\text{M}$ ) + LTA (10  $\mu\text{g}/\text{ml}$ ) treated cell membrane fractions. Similar results were found in two other identical experiments.

Protein band intensities were analysed using ImageJ software. The intensity of the 80 kDa band was measured in each fraction and displayed as a percentage of the total band intensity on each western blot. (d) Results determine protein band intensity found in total lipid fractions (including lipid rafts) or non-lipid fractions in unstimulated, LTA and HETP + LTA treated cells.

To further assess whether TLR2 was being translocated into the lipid fractions after stimulation with LTA, the 80 kDa band intensity was measured using ImageJ software and used as a qualitative measure of protein expression. Analysis of western blot images using ImageJ calculated that stimulation with LTA did not significantly affect expression of the 80 kDa band in the lipid fractions (Figure 5.2d); in fact 11.4 % of protein was found in the lipid fractions before and after stimulation with LTA. However a small increase in the percentage of protein expressed in lipid fractions was identified with HETP treatment. Although it is worth noting that the overall protein intensity in this western blot, Figure 5.3c, was over 30 % less than observed in blot (a) and (b), which was reflected in the images.

As TLR2 protein was expressed at a significantly lower amount in the lipid fractions than seen in the reports by both Triantafilou *et al.* (2002) and Jackson *et al.* (2008), an attempt to increase the sensitivity of protein detection by western blot was made. An alternative method to assess the cell lysate fractions, involved pooling the concentrated lipid fractions together, prior to loading equal volumes onto Bis-Tris gels. This gave the potential for any protein bands present in the lipid fraction to be identified. Furthermore, MM6 cells were stimulated with 10 µg/ml Pam<sub>3</sub>CSK<sub>4</sub>, a concentration 10 fold higher than used in previous assays, to investigate whether the TLR2/TLR1 ligand exhibited a greater capacity to influence translocation into the lipid raft domain. However, results indicate (Figure 5.3) that these modifications made no significant difference to the expression of the 80 kDa band in the lipid fractions. The protein band intensity identified in Pam<sub>3</sub>CSK<sub>4</sub> stimulated cell lysate fractions were in fact comparable to both untreated cells and cells treated with HETP prior to stimulation. This may indicate that TLR2 translocation is not evident, although the protein concentration process may have masked slight but significant changes in expression.



(b) Percentage of total protein band intensity in membrane fractions

	Non-lipid	Lipid	Non-lipid, cell lysate fragments
Treatment	Fractions 1-4	Fractions 5-7	Fractions 8-12
Untreated	13.8	38.6	47.6
Pam <sub>3</sub> CSK <sub>4</sub>	10.2	45.7	44.1
HETP + Pam <sub>3</sub> CSK <sub>4</sub>	10.7	44.2	45.1

**Figure 5.3** Western blot analysis of pooled, concentrated plasma membrane fractions

1 ml plasma membrane fractions were obtained through total cell lysate sucrose density gradient ultracentrifugation and concentrated. An equal volume of concentrated lipid protein was loaded. Fractions were pooled into non-lipid (fractions 1-4), lipid (fractions 5-7) and non-lipid membrane fragments (fractions 8-12). The nitrocellulose membrane was probed with hTLR2 antibody. (a) a western blot analysis of TLR2 expression in unstimulated, Pam<sub>3</sub>CSK<sub>4</sub> (10 µg/ml) treated and HETP (50 µM) + LTA (10 µg/ml) treated cell membrane fractions.

(b) Protein band intensities from lipid fraction isolation western blots probed with TLR2 antibody were analysed using ImageJ software. The intensity of the 80 kDa band was measured in each fraction and displayed as a percentage of the total band intensity on each western blot.

## 5.5 DISCUSSION

### 5.5.1 Assessment of TLR2 translocation

In order to gain an insight into LPCAT's function in TLR2 translocation as a response to microbial challenge, an attempt was made to study the protein expression in plasma membrane lipid raft fractions. Despite considerable efforts to identify detectable levels of TLR2 protein and to visualise TLR2 translocation via western blot analysis, the effect of LPCAT inhibitor, HETP, was unable to be adequately assessed.

Total lipid isolation by sucrose density gradient ultracentrifugation was repeatedly successful, with GM1 being consistently detected membrane fractions 5, 6 and 7. However, neat protein from each fraction was at a low concentration and no protein could be detected when probed with TLR2 antibody. Whilst the methods of Triantafyllou and colleagues (2004) had been followed as precisely as possible, further analysis revealed some discrepancies; for example, in 2002, where they studied TLR4 translocation, they state to have only used  $1 \times 10^6$  MM6 cells, whereas in 2004, they processed  $1 \times 10^8$  from buffy coats to investigate TLR2 translocation from  $12 \times 1$  ml fractions. The present study utilised  $1 \times 10^7$  MM6 cells for cell lysate before lipid raft isolation following methods described by Jackson *et al.* (2008) and Abate *et al.* (2010).

However, despite the differences in cell density which would have ultimately affected the resultant protein concentration, Jackson *et al.* (2008) concentrated lipid fractions by methanol precipitation as described by Wessel and Flugge (1984) before application to western blot. In the first approach this method was applied to membrane fractions, but it quickly became apparent that the concentration of sucrose was also increasing in the samples. Whilst there was no apparent reason for the accumulation of sucrose, one unavoidable technical difference was the volume limitations associated with the ultracentrifuge used in this study. Jackson *et al.* (2008) used the same density of MM6 cells, but a smaller total volume of 2.1 ml before ultracentrifugation. Unfortunately the smallest capacity that the current ultracentrifuge could contain was 12 ml; over 5 times larger volume, resulting in the use of a significantly greater volume of sucrose.

Whilst similar volumes were used by Triantafilou and colleagues (2004) and Olsson and Sundler (2006), in this study it led to the exploration of an assortment of protein concentration methods, with the aim to increase the assay sensitivity to TLR2 protein. Ultimately, the phenol/ether precipitation method (Sauvé *et al.*, 1995) was used to increase protein concentration before western blot analysis since it removed the unwanted sucrose and did not interfere with downstream quantification assays.

In spite of each lysate fraction undergoing protein concentration, western blot analysis of plasma membrane fractions in this report, revealed that TLR2 was present in low concentration in the non-lipid (fractions 8-12) of unstimulated MM6 cells, a finding that contradicts data reported in previous studies (Triantafilou *et al.*, 2002; Triantafilou *et al.*, 2004; Triantafilou *et al.*, 2006). Similarly, TLR2 was also identified in the lipid fractions (5-7) of unstimulated cells, which is inconsistent with reports by Triantafilou and colleagues (2002) where TLR2 was only detected in lipid raft fractions after TLR2 ligand stimulation. Whilst it must not be ignored that there is a possibility the unstimulated MM6 cells were already stimulated, perhaps by a low level infection, this anomaly is most likely due to the broad concentration methods. Though each protein fraction needed to be concentrated for TLR2 detection by western blot, the consequence may have been that minor changes in protein expression were undetectable.

Despite the presence of TLR2 in the lipid fractions of untreated cells, stimulation of MM6 cells with LTA and Pam<sub>3</sub>CSK<sub>4</sub> was examined to determine if the concentration of TLR2 increased, as a result of membrane translocation. Unfortunately there was no significant change in TLR2 expression observed in all experiments. In the present study an incubation time of 45 minutes with either TLR2 stimulant was used as recommended by Triantafilou *et al.* (2004). However, dissimilarity between methods occurred; Triantafilou *et al.* (2004) stimulated human monocytes with 100mg/ml LTA, in contrast to the 10 µg/ml LTA used in the present study. This variation may account for the absence of elevated levels of TLR2 in the lipid fractions, suggesting that the detection of TLR2 translocation is dependent on the concentration of the stimuli. Thus these experiments warrant further investigation and optimisation to achieve give an insight into the effects of LPCAT inhibition on translocation.



# Chapter 6

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FINAL DISCUSSION AND CONCLUSIONS

## 6.1 SUMMARY

Understanding the pathophysiology of sepsis is vital for successful therapeutic intervention. Recently a lysophospholipid metabolizing enzyme LPCAT (lysophosphatidylcholine acyltransferase) has been identified to play a significant role in regulating the inflammatory mediator expression of monocytes in response to LPS. It has previously been shown that phospholipid metabolism may influence cell activation and responses, since the clustering of the receptor molecules into membrane microdomains (lipid rafts) is required to trigger inflammatory responses. LPCAT therefore, may be a target for novel anti-sepsis therapies; thus its expression and activation in different cell types and the molecular mechanisms of its immune-regulatory activity are being studied here.

The aim of this study was to further investigate the effects of LPCAT phospholipid regulation on the expression of inflammatory mediators, in response to other bacterial stimuli in both monocytic and epithelial cells; including evaluating the influence of LPCAT to activate other receptor molecules into lipid rafts, and to characterise the expression of reported LPCATs in the same cell types. To firstly address the aims of this study, the immuno-regulatory properties of LPCAT were investigated in monocytes stimulated with TLR2 ligands. Subsequently a suitable in-vitro model was developed using HETP, a specific inhibitor to LPCAT, and measured inflammatory mediator expression from monocytes in response to microbial stimuli.

The results of this study demonstrated that inhibition of LPCAT significantly reduced ( $\geq 50$  %) the secretion of pro-inflammatory cytokines (TNF- $\alpha$ , IL-6 and IL-8) in response to a variety of TLR2 ligands. This was supported by evidence that cytokine reduction also occurred at the mRNA level, strongly suggesting that LPCAT controls cytokine secretion by altering transcription of the gene or mRNA stability and demonstrating that inflammatory response in monocytes are noticeably regulated by LPCAT. The findings in this report also indicate that LPCAT has a crucial role in regulating the release of inflammatory cytokines in enriched populations of donor peripheral blood monocytes, supporting the use of the monocytic cell line as a model for this investigation.

Neville *et al.* (1997) and Schmid *et al.* (2003) reported that IFN- $\gamma$  increased monocytes inflammatory responsiveness to LPS and that this was as a result of increased phospholipid remodelling by LPCAT. The present study further investigated the role of LPCAT on inflammatory responses by IFN- $\gamma$  primed monocytes via LPCAT inhibition prior to stimulation. A significant reduction in secreted cytokines was observed in response to all TLR2 ligands, indicating that LPCAT also plays a crucial role in the activation of primed monocytes, possibly at the level of the TLR or subsequent signalling pathways.

In this report differences between LPCAT regulation of bronchial epithelial and monocytic immune responses were studied. BEAS-2B cells were observed to secrete a lower concentration of inflammatory cytokines, in comparison to monocytes, although LPCAT inhibition still resulted in a reduced cytokine secretion, but to a lower extent than seen in monocytes. Whilst this may indicate that LPCAT also has a role in regulating inflammatory responses in epithelial cells, it should be noted the function of LPCAT within this cell type may predominately be for the production specific phospholipid molecules.

Recent studies have characterized the expression of LPCAT like acyltransferases in diverse cell types and animal tissue, suggesting that different iso-forms of LPCAT exist with alternative functions (Shindou *et al.*, 2007; Nakanishi *et al.*, 2006; Harayama *et al.*, 2009; Chen *et al.*, 2006; Agarwal *et al.*, 2007). This study looked at the expression levels of these 5 enzymes within monocytic, lung epithelial and hepatocyte cell lines and aimed to evaluate the correlation between LPCAT isoform expression levels and the inflammatory response regulatory mechanisms in diverse cell types.

Literature suggests that LPCAT1 is most notably expressed in lung tissue, in particular alveolar type II cells, where it plays a role in producing DPPC, a crucial surfactant protein (Nakanishi *et al.*, 2006; Chen *et al.*, 2006). Results from the present study however, did not observe increased LPCAT1 mRNA or protein expression in lung epithelial cells as might be expected. LPCAT2 has been reported to play a constitutive role in inducible PC remodelling pathways in response to external stimuli in mouse peritoneal macrophages (Shindou *et al.*, 2007). Whilst this suggests that

LPCAT2 might be the candidate enzyme to modulate the phospholipid remodelling pathway in innate immune cells, results from this study revealed that hLPCAT2 mRNA and protein expression was not increased in monocytes, but comparable across cell lines, and neither LPS nor IFN- $\gamma$  significantly changed the expression of LPCAT2. Results from the present study did however confirm that human LPCAT2 is highly expressed in HEK293 cells, where it appears to be the principal enzyme for PAF production (Shindou *et al.*, 2007).

In agreement with Hishikawa *et al.* (2008), LPCAT3 was detected with abundant mRNA expression levels in liver cells (HEPG2), suggesting it to be primarily responsible for hepatic LPCAT activity (Zhao, *et al.*, 2008). In contrast, very low mRNA expression levels of LPEAT2 were seen across the panel of cell lines tested, supporting other studies that observed it to be virtually undetectable in human lung and liver tissues, and who reported LPEAT2 to have a greater importance in modulating both human and mouse brain phospholipid composition (Cao *et al.*, 2008; Ye *et al.*, 2005). LPCAT4 mRNA was observed to have a remarkably high expression in both lung epithelial cell lines; dissimilar to a previous study that reported low expression of mLPCAT4 in lung tissue (Hishikawa *et al.*, 2008) and who discovered that mLPCAT4 plays a constitutive role in mouse epididymis, brain and testis tissue membrane biogenesis.

Literature supports the belief that each LPCAT performs a unique function within different cell types, either maintaining cellular membrane structure and /or producing specific phospholipids as precursors to other inflammatory mediators. As such LPCAT expression observed in mice may not be representative of human expression. Whilst data in this report did not demonstrate one particular LPCAT iso-form to be highly expressed by monocytes, further investigation is need on LPCAT expression in human cell lines or tissues to consolidate the evidence that LPCAT2 might be the enzyme to modulate the inducible phospholipid remodelling pathways in innate immune cells.

Jackson *et al.* (2008) observed that inhibition of LPCAT prevented translocation of TLR4 into membrane lipid raft domains, subsequently reducing down-stream inflammatory responses. This is the suggested mechanism that LPCAT elicits its

immuno-regulatory effect in response to microbial stimuli. Although the translocation of TLR2 into membrane lipid rafts has been examined (Triantafilou *et al.*, 2004) and other raft-localised proteins identified (Olsson and Sundler, 2005), this study aimed to assess whether LPCAT inhibition could prevent TLR2 translocation, and thus prevent the subsequent formation of the receptor signalling complex. To achieve this aim, well characterized proteomic approaches were used however successful translocation of TLR2 was not achieved and so the effect of LPCAT inhibitor, HETP, was unable to be adequately assessed. Nonetheless this is the most likely mechanisms for LPCAT to down-regulate TLR2 induced inflammatory cytokine production in monocytes and epithelial cells as demonstrated by data in this report.

The present study provided sufficient evidence that inhibiting LPCAT affects the complex network of cell signalling involved in microbial responses, underlying the importance of LPCAT, and potentially offering a target for novel anti-sepsis therapies. When considering LPCAT as a target for therapeutic intervention however, it must be taken into account that LPCAT is also essential for membrane biogenesis in many cell types and is fundamental for cell viability. Although the present study has underlined the importance of the enzyme in microbial induced cellular responses, inhibiting LPCAT may hence not be beneficial for the treatment of sepsis. Novel anti-sepsis treatment would become more feasible if tissue or cells specific isoforms of LPCAT were targeted, with the aim to reduce LPCAT activity rather than completely block it. With its central role in monocyte signalling, LPCAT would be investigated as a potential marker for patient susceptibility to sepsis. Further characterisation of LPCAT is thus required before contemplating it as a target for sepsis treatment, however this study has consolidated evidence that LPCAT as an important regulator of immunological responses.

## 6.2 FUTURE WORK

The mechanism by which HETP inhibits LPCAT activity is unknown. Whilst the down-stream effects of LPCAT inhibition can be observed, HETP did not affect LPCAT mRNA or protein expression levels and this perhaps suggests that HETP impedes LPCAT enzyme activity.

Enzyme activity assays, similar to those used by Schmid *et al.* (2003) and Neville *et al.* (2005), could be used to determine if LPCAT enzyme activity is inhibited by HETP. LPCAT enzyme activity is assessed in a radio-assay where a known amount of radiolabelled substrate is converted to labelled product; i.e. LPCAT incorporates a radioactive fatty acid into phosphatidylcholine. Preliminary enzyme assays were completed during this study, but were unsuccessful.

To further consolidate evidence that inhibition of LPCAT down-regulates monocytic inflammatory responses, an LPCAT mRNA knockdown cell line was investigated to study the effects on inflammatory responses. Using small RNA interference technology, (siRNA) assays were extensively performed in a murine macrophage cell line (RAW 264.7) over a six month period during this study but were unsuccessful. Whilst complete knockout of LPCAT mRNA will almost certainly be lethal, another attempt to control attenuation of LPCAT mRNA levels should be attempted.

If LPCAT knockdown cells are successful, there is the potential to reinvestigate all of the immune-regulatory effects seen in this study with HETP. Stimulation with TLR4 and TLR2 ligands in knockdown cells would support LPCAT's role in both signalling pathways. The potential down-regulation of TLR translocation into lipid rafts could also be investigated.

Another attempt to optimise the lipid raft isolation assays should be made in future studies to repeat the findings of Jackson *et al.* (2008) and Abate *et al.* (2010), with TLR2 stimulants. TLR1 and TLR6 have also been shown to translocate into lipid rafts upon stimulation (Triantafilou *et al.*, 2004), thus it would be interesting to determine if LPCAT is involved. These findings would support the importance of LPCAT in regulating inflammatory responses, as found in this study. If lipid raft

assays are successful, future studies could investigate the location of LPCAT within the plasma membrane before and after stimulus.

Mitogen-activated protein kinase (MAPKs) inflammatory signalling pathways such as p38 and p44/42 MAPK , can be activated in response to stimuli. Activation of MAPK is through phosphorylation, which subsequently activates transcription factors required to elicit production of cytokines. Preliminary data from this study suggests that LPCAT may regulate p38 phosphorylation when MM6 cells are stimulated with LPS. However further flow cytometry experimental replicates are required to clarify this potential relationship.

To determine if LPCAT modulates inflammatory pathways other than those induced by TLR2 and TLR4 stimulants, an alternative ligand could be used to stimulate epithelial cells. Polyinosinic-polycytidylic acid (poly(I-C)) is a synthetic dsRNA analog, previously demonstrated to rely on TLR3 for recognition (Guillot *et al.*, 2005). TLR3 is expressed in epithelial cells but does not seem present in monocytes or lymphocytes. Poly(I-C) has previously been shown to stimulate BEAS-2B cells to secrete high levels of IL-8, so this would be a good model to demonstrate the effect of LPCAT inhibition on TLR3 activation pathways.

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APPENDIX 1 – qPCR primer sequences

mRNA sequences below are displayed with the primers designed in this study and primers used in literature spanning across the mRNA.

**LPCAT1** (AYTL2: AGPAT9)

Chromosome 5

Gene ID: 79888

mRNA sequence NM\_024830.3

Primers used in this study:

**Primer 1 sequence:** LA1F and LA1R. Product length = 156 bp

**Primer 2 sequence:** LB1F and LB1R. Product length = 168 bp

**Primer 3 sequence:** LC1F and LC1R. Product length = 159 bp

Primers used in literature:

hLPCAT1 primer sequence; product length= 154 bp. Harayama *et al.*, 2009.

hAGPAT9 primer sequence; product length= 100 bp. Agarwal *et al.*, 2007.

GCCCGCTCCAGCCGCCGCGCATCCTCGGCCCGCGCCCCGAGACCCGCGCC  
 CAGCTAGCCCCGGCCCCGCTCGGCGCCCCAGGCAGCTCGGCTGCGCTCGC  
 CGCGGGACGGCGCGGCCATGAGGCTGCGGGGATGCGGACCCCGGGCCGC  
 CCCTGCCTCCAGCGCAGGGGCCAGCGACGCTCGGCTGCTGGCGCCCCCGG  
 GGCGGAACCCCTTCGTGCACGAGCTGCGCCTCAGCGCCCTGCAGAAGGCC  
 CAGGTGGCCCTCATGACACTGACGCTCTTCCCGGTCCGGCTCCTGGTTG  
CCGCTGCCATGATGCTGCTGGCCTGGCCCCTCGCACTTGTCGCATCCCTGG  
GCTCTGCGGAGAAGGAACCCGAGCAGCCCCGGCCCTGTGGAGGAAGGT  
TGTGGACTTCCTGCTGAAGGCCATCATGCGCACCATGTGGTTCGCCGGC  
 GGCTTCCACCGGGTGGCCGTGAAGGGGCGGCAGGCGCTGCCACCGAGG  
 CGGCCATCCTCACGCTCGCGCCTCACTCGTCCTACTTCGACGCCATCCCTG  
 TGACCATGACGATGTCCTCCATCGTGATGAAGGCAGAGAGCAGAGACATC  
 CCGATCTGGGGAACTCTGATCCAGTATATACGGCCTGTGTTTCGTGTCCCG  
 GTCAGACCAGGATTCTCGCAGGAAAACAGTAGAAGAAATCAAGAGACGG  
 GCGCAGTCCAACGGAAAGTGGCCACAGATAATGATTTTTCCAGAAGGAA

CTTGTACAAACAGGACCTGCCTAATTACCTTCAAACCTGGTGCATTCATCC  
CTGGAGCGCCCGT **CCAGCCTGTGGTTTTACGAT**ATCCAAATAAACTGGA  
**CACCATCACATGGACGTGGCAAGGACCTGGAGCGCTGGAAATCCTGTGGC**  
**TCACGCTGTGTCAGTTTCACAACCAAGTGGAAATCGAGTTCCTTCCTGTGT**  
**ACAGCCCTTCTGAGGAGGA**GAAGAGGAACCCCGCGCTGTATGCCAGCA  
ACGTGCGGCGAGTCATGGCCGAGGCCTTGGGTGTCTCCGTGACTGACTAC  
ACGTTCGAGGACTGCCAGCTGGCCCTGGCGGAAGGACAGCTCCGTCTCCC  
CGCTGACACTTGCCTTTTAGAATTTGCCAGGCTCGTGCGGGGCCTCGGGCT  
AAAACCAGAAAAGCTTGAAAAAGATCTGGACAGATACTCAGAAAGAGCC  
AGGATGAAGGGAGGAGAGAAGATAGGTATTGCGGAGTTTGCCGCCTCCC  
TGGAAGTCCCCGTTTCTGACTTGCTGGAAGACATGTTTTCACTGTTTCGACG  
AGAGCGGCAGCGGCGAGGTGGACCTGCGAGAGTGTGTGGTTGCCCTGTCT  
GTCGTCTGCCGGCCGGCCCGGACCCTGGACACCATCCAGCTGGCTTTCAA  
GATGTACGGAGCGCAAGAGGACGGCAGCGTCGGCGAAGGTGACCTGTCC  
TGCATCCTCAAGACGGCCCTGGGGGTGGCAGAGCTCACCGTGACCGACCT  
ATTCCGAGCCATTGACCAAGAGGAGAAGGGGAAGATCACAT **TCGCTGAC**  
**TTCCACAGGTTTG**CAGAAATGTACCCTGCCTTCGCAGAGGAATACCTGT  
**ACCCGGATCAGACACATTTCG****AAAGCTGTGCAGAGACCTCACC**TGCGCC  
AATCCCAAACGGCTTCTGTGCCGATTTACAGCCCGGAAAACCTCAGACGCTG  
GGCGGAAGCCTGTTTCGCAAGAAGCTGGATTAGGACCCAGGGTTGCGGAG  
AGACGCGGCCCTCCCGCGTGGACATCACCGCCATGAGCCTCTTTGCGAG  
TGACCTCTGGGCTCCGCTCCTCACTCCTGCTGTACAGGCACTGTCTTCAGC  
CCGAGTTCCAGGGGCCTCGGGGGCTGTTTGTATCTTGTTCCTTTGTGAAGT  
GTGTTGCAGAACCGACGCTTACTGTGCGAGAATCGGAGGGCGCGCACGC  
GGATCCCCCGCCTGGCCTGGACCCCGTGGGGTCAGGTTCCCTGCCGGGCG  
GGGGGCACCGGTGCCGCCCCGTGTTCTCCACGGGGCCCTGGTTTCGAGT  
CTCTGTACAGCCTCTTCCGGCGGCAGCGTGCACCGGGCGGGCCTCCGTG  
CACACTCAGCACACGCCTGCCACACAGCGTGCCTTGCGTGTCACTCTGG  
CACGAAACCTGTCTGCCTCTGTGGATCCACAGCCTGGCAGAGCCGAGCCG  
TCACCTGATTTTTTCAGTGTTTCTACCTGTGTGCTGGAGCTCATGAGTATTTT  
ATAAACTCCATTTAGGTACTTCAGGAAACATGCAGCATTTTTTAAAAAAT  
GAAAATTGTTTTTCTACTTCATTTTTTCTTTTAGAGTCAAAGGATATTTATT  
TATAGGCCTTTTTTTTTTTAATATAGAATCTGAGGCTGTTTGGGCTTTGACT  
TAAATTTCCATCAGGCCTCTCTCCAGCAGGTAATCCCTCTCCTTCCGCTGG

GTCCCCTGGGGAGGTGTGAACTCAAGGGCCTAGCCCCAAAACACTTTTT  
CTGCTTTTCTTAATCCTTTTCCAGTCCCCTCTTTTTTTATAAACGTTGGCAG  
TTTGATGTTTCTGTTTCGGCATAACGTAATCCATTTCACTGTAGCCTAAAC  
TCCAGTCCGAGGTTGGATATTGTTCAAATGAGCAGGGCCCAGCTGGAA  
GCGCAAGGCAGCCGCGCCGTGCCGCTCCTCCCTTGCCCTCAGGCCAGGT  
CCCTGCTGGAAGCGGCTGCATCTTCTGTGACCCCTGGTTTCCATGGTGAC  
TGCGTACACGACCCGAGTATGGCTGACCTTCTGCAGAGAGAGGA  
GCCGCAGTCTTTTGCTTGTGGAAGGAGACGCTGGGGCTGTGCGGTGCGGAG  
GGTGATGAGGATGTCTGGTGACAGCCGTGCGGACACCACTCCTCTCTGCA  
GCACTGCCTCCCAGCGCCAGGGTCGCGGGCACATCCCCTGAGAGCGGG  
GGTCCCTGCCCCATCTTAGAGTCAAAGGCAGAGGGGCTTCCAGGCCCTGGA  
TGGGGTATTTTGGTGTCACCTGAAGTCCCTCTGACATCACCTTGTTTCATC  
ATTTTTATGACAGAATTAGAAACCCATCCTTCAAGCACAATAATCATCA  
CAGACTTGAGTTTGCTTCTAAAGCAAAGGCTCCGGGTTTGTGGGAAAA  
TTTTTTGATTTCTGAAATGAATTGATTTTTATATTTGGGGCATCTCTATAG  
AAAGTGACCACCAAGGCCAGTAAGTACGGGAAAAAATGTTTACTAACTTC  
CTCAGAGATTCGTGATACGCGTTTCTCCACTGACAGACATTTAAAAACAA  
CCTTCAGCTCCGTTTCAATCAATCACCTCGACTTGTTTTTTAGCATGGACA  
CTGCCAGCAGGACAGACAGGGATGGAGTAAACCGAAGTCAATTCAGGG  
CTCTTGCGTGTTGGACACAGAAGAAATCCTAGTGCAGCCTTTGGTAGCT  
AACAGTCACTGATTTTATAATTGGAGAATGCGTAAAGATTCATTTTTCAA  
GGAGAAGAGCCTGCAAATGGCCAATGAAGGAGGTAAATAAACTAAGATA  
TTCCGAGGGAAGGGACCCAGGCCACCTCCCTTCCGCAGGTCTGCAGATG  
AAGGGTTTTTTGAATGAAATGCCACTGTGCATTTTCAGAAAAAAAATCT  
CTGATAAACAGACTTTGAATGGATGTTTGTTCCTCCTGATTCTCTTTTTCTC  
TTCGTGCGACTTAGAGTTGGCGGATATTCGGAAGTGTGAATGTACATA  
GCGTTGAGTTAAACCCCTTGTGTGTGAGACAGGACGCAGCGGGCCCCTGG  
TGGCCTGGGGGCCAGACCCGTGGGCAGGTGGGGCATGGGCCCTGGCCTG  
CGGGGACCTGCTGGGGTGTGAGGGCAGAGGGAGGGTTGCCATGAAGGAA  
CTTGGGATTTTCAATGGAATAAGTAAAACATAAAGTCTATACTTGGGAAA  
AA

**LPCAT2** (LysoPAFAT: AYTL1: AGPAT11)

Chromosome 16

Gene ID: 54947

mRNA sequence: NM\_017839.4

Primers used in this study:

**Primer 1 sequence:** LD1F and LD1R. Product length = 184 bp**Primer 2 sequence:** LE1F and LE1R. Product length = 182 bp**Primer 3 sequence:** LF1F and LF1R. Product length = 150 bp

Primers used in literature:

hAGPAT11 primer sequence; product length= 64 bp. Agarwal and Garg, 2010.

lysoPAF-AT primer sequence; product length= 178 bp. Shindou *et al.*, 2007.

GCCTGGGGCGTGTACAGCCGAAGGGGCAGGGCGGCAGCAGCAGGCGTC  
TAAGTAACTTCAGCGCCTGCGCAGAGGCTCCCCAGCGTCGCCCTAGGCTG  
GGACTCTAGTAGGTCTTCGGCTCAGTTTTGGCTGCAGCGCCCGCGTAGAT  
CGCTTCGGCCGGTCTACGCCCGGCTCAACTATGAGCCGGTGCGCCAG  
GCGGCGGAAGTGGCGGCCACAGTGCCAGGTGCCGGCGTCGGGAACGTGG  
GGCTGCGGCCGCCATGGTGCCCCGTCAGGCGTCCTTCTTCCCGCCGCCG  
GTGCCGAACCCCTTCGTGCAGCAGACGCAGATCGGCTCCGCGAGGCGGGT  
CCAGATTGTCCTTCTTGGGATTATCTTGCTTCCAATTCGTGTCTTATTGG  
TTGCGTTAATTTTACTTGCATGGCCATTTGCTGCAATTTCAACAGTAT  
GCTGTCCTGAAAAGCTGACCCACCCAATAACTGGTTGGAGGAGGAAAATT  
ACTCAAACAGCTTTGAAATTTCTGGGTCGTGCTATGTTCTTTTCAATGG  
GATTTATAGTTGCTGTAAAAGGAAAGATTGCAAGTCCTTTGGAAGCACCA  
GTTTTTGTTGCTGCCCTCATTCAACATTCTTTGATGGAATTGCCTGTGTTG  
TAGCTGGGTTACCTTCTATGGTATCTCGAAATGAGAATGCACAAGTCCCT  
CTGATTGGCAGACTGTTACGGGCTGTGCAACCAGTTTTGGTGTCCCGTG  
TAGATCCGGATTCCCGAAAAACACAATAAATGAAATAATAAAGCGAAC  
AACATCAGGAGGAGAATGGCCCCAGATACTAGTTTTCCAGAAGGTA  
GTTACTAATCGTTCCTGTTTGAATTTTAAACCAGGAGCCATTCATCCA  
GGAGTTCCAGTGCAGCCAGTCCTCCTCAGATACCCAAACAAGCTGGATA  
CTGTGACCTGGACATGGCAAGGATATACATTCATTCAGCTTTGTATGCTTA

CTTTCTGCCAGCTCTTCACAAAGGTAGAAGTTGAGTTTATGCCAGTTCAAG  
TACCAAATGATGAAGAAAAAATGATCCTGTCCTTTTTGCCAATAAAGTC  
CGGAATTTAATGGCAGAAGCTCTGGGAATACCAGTAACAGATCATACCTA  
TGAAGACTGCAGATTGATGATTCAGCAGGACAGCTAACATTGCCTATG  
GAAGCTGGGCTGGTGGAAATTTACTAAAATTAGCCGAAAATTGAAATTAG  
ATTGGGATGGTGTTCGTAAGCATTGGATGAATATGCATCTATTGCGAGTT  
CCTCAAAGGGAGGAAGAATTGGAATTGAAGAATTCGCCAAGTATTTAA  
AGTTGCCTGTTTCAGATGTCTTGAGACAACCTTTTTGCACTCTTTGACAGG  
AACCATGATGGCAGCATTGACTTCCGAGAGTATGTGATTGGCCTGGCT  
GTCTTGTGCAACCCTTCCAACACAGAGGAGATCATCCAGGTGGCATTAA  
GCTGTTTGACGTTGATGAGGATGGCTACATAACGGAGGAAGAGTTCTCCA  
CCATTCTACAGGCTTCCCTTGGAGTGCCTGACCTTGATGTTTCTGGTCTCT  
TCAAGGAAATAGCCCAAGGGGACTCAATTTCTATGAGGAATTTAAAAG  
TTTTGCCTTAAAGCATCCAGAATATGCTAAGATATTTACAACATACCTAG  
ACCTCCAGACGTGCCATGTGTTTTTATTACCAAAGAAGTCCAGACAACC  
CCCTCCACCGCCAGTAATAAAGTCAGCCCTGAAAAGCATGAAGAGAGTA  
CCTCAGACAAAAAAGATGACTGAAAGCAGTATTTCCAATAAGGAAAACA  
CAGTAGCTTTTGCTTGAAATTGTAAAGGCACTTATTGATAATACTTTAAT  
GTGTTGGTAATGATGTTTAAAATTGAAAGATTTTTAAAACAAAAATGATA  
GATTTTCTTACTAAAAATGTTTTTATTAACCTTGCTTTTATTGGAAAAAT  
CAAGCAATATTTTCGTTTTCTTTGTGTTATATTGTACTTTACTGATTCATTT  
ACTGGTGATACATATGTTTTTATGGATTTTCCAGTTTAATTTGCATATACA  
AATGAATGCAATGGTCTATTGGTGAGCATTGAGCAACACTGTATAAAGTT  
TAAAAATGTAAACACTTTTTAATCTACTTTCCTCTAAAAATCAATAATAT  
TCTATTATTTCTAATCCTTTTCCACTTGGGAAATAACAATGAAGAATCTGA  
GAATTTGACATCTATAACTTTACAGATTCATTTTTCCATTTAAATTTTCAGTT  
TCTTGGATCACTGAATATGGGAAGGGAGAGCTTCACTAATTAGACGCAGC  
TTCTTAAGAACTTATATTCTTTTGACATACATCTCTATTGTAGTTTTTTGT  
TTTGTTTTGTTTTTTGGAGATGGAGTCTTGCTCTGTCACCCAGGCTGGAGTG  
CAGTGGTGCAATCTCAGCTCACTGCAACCTCTGCCTCCTGGGTTC AAGTG  
ATTCTCGTACCTCAGACTCCCGAGTAGTTGGGATTACAGGTGCCACCAC  
CACACCCGACTAATTTTTGTATTTTTAGTAGCCATGTGGTTTTTGCCATGTT  
GGCCAGGCTGGTTTCGAACTCCTGACCTCAGGTGATCCACCCACCTCAGC  
CTCCCAAAGTGCTGGGATTACAGGTGTGAGCCACCGTTCCCGGCCTATTG

TAGATTTTCTTAACTACTTGCTAAGGAAATCATATCCTTTTACATGAACTA  
CAGGTTTAGAACTTGGTTTTAAGACAACCTGCTATGGCCAGAAGGTAAATG  
GGAATTGCCTTATTGAAGGTAACATTGATTGCCTAATAAGAAAATGAATT  
GTTTGCCACAGAGTTGAATTTAATTTGAGTTAGATAGTTCAGAATGTAGC  
ACTTGCCCTATAAATGAATCAGATTTGTTCTATTTATATAATATTAGAATT  
AATATATTATCATGTAAGTGGGAATTTTATTTTGTTAAGTGGACTCTCAA  
TTTTAGAACTTGTGTGAAATATCTCCAGAAAACACAAAAGGTTTCAAGG  
TATCACCCAAAGCTAGGGAATCAACAAACGTACTTTATTAATGCAAGACC  
ACAATTTAAGCCCCAGGCAGGAATCCAACAGTAACATTTTTCTTTAGGT  
TAAGCAGTAATTTTTTTGAATTGTCATTTTTAATGGGTCTCACACATGCAT  
ATTGCTAATATCATTTCATGTTTGAGGTTTTCAACAAAGAACCAATTTATTA  
ATATAATGTACAAAAATAGTTGGTTCATTTTTCTGAATTCAGTCTTTGAAAT  
AAAACCTCTTGTTTTGGCCGTGTTTCTGAATGTATTGGTGATTCACCCCCA  
AGCTAATTTTTTAAAGTCATTTTTGAAGTTGGGAAGTCTCATGAGAGATGT  
TGAAGTATGTATTTAATCAAGAGTCATGATTTCAAACCTAGTTTTACATATT  
AAGCAGTTAGTGTCTAATTTAATGGGTAAATGTGTGTTTGGATAAATATC  
TGAAAATTTTATCCTTAAGTATATATAATTATTTGCCTCTTATATGTCTTAA  
AGCTATTTAAACAAGGTGTTAAATGAGCCAAAACAATTAAGTAATTAGAA  
CAGTACATTTTTATCAGAGTGTCTGTCATATGCAATGAATGTATGTAATAC  
TAAAAAATCATGACTACTTTTATCAAAGAAAAACCACACATTAATCCTAT  
TAATCATGAAAGCGTAGCATTGTAAATTAAGGTTTTCTTTGAGGCTCTTG  
AAAGTGATCCCATTTGCTTTCTGTTTTAAAAATATTTTATGCTCTTTATTT  
CACTTCTGTGAATGTGATATTTCTATTTTGTGATTATGTTACTGAATAAAC  
AACTTGCTACATAAAATTCTTAGCAATTAAAAAAATTCTGATTCTGCCAT  
TTTGTCTCAAATGTAATATACCCTTTAATATCATATGTTTACATATTTATG  
AATGATTAATCATTTTTGTTTGCATTAAACTTTATGAAATGTCAGAAATGA  
TTTTACTCTAATGAACTCAAATTTTGCCCAAAGTAGCAATATTCTTAAAG  
ATGTTGAATTTTACTGGTTGAATTTTATATAGTAACACACAAGTCGTGAT  
CATCAATATTATTGCAGACCAGGACTCTGCTTATATGCCTTGCTAACAAA  
GATTTCTTTCACAGAGGCTTTTCTCCTCTCAAGACTTAAAGTAAGAATTAC  
ATTTATTAAGTCAGTTAAATGCACCATGGCTTCATATAGTAATATAAAA  
AACTCTTTGAAGTGAGAAATATTATATCCTAAAACCTCTAAACCACAAA  
CATTCAATTGAAAGAGTTCTGTTGAAGGATAATGACATACTGACTGCTTA  
CAGACCAAGTTGCTTGCATTTTGTATGTTTAGCCCTCCTTTGCCACTGCTTT



TAGAGCCTTGGAAGGCTAAGTGTGATAGTAATGCTAGCTCTAATGCATAT  
TTAAAGGAGACTGCCTCGCTTTTAGAAGACATCTGGTCTGCTCTCTGCATG  
AGGCACAGCAGTAAAGCTCTTTGATTCCCAGAATCAAGA ACTCTCCCCTT  
CAGACTATTACCGAATGCAAGGTGGTTAATTGAAGGCCACTAATTGATGC  
TCAAATAGAAGGATATTGACTATATTGGAACAGATGGAGTCTCTACTACA  
AAAGTCTTTGGGTATTTGTTTCTTACATAGAAAATGCTAACATGAATAGA  
AAGATACTGGTGCAAGACCATTCCCGGGAAAGTAGACATACTTACATTTT  
TTTCCTTTTCTGCTCATTGGAATGAACTCAATTTTATGTTAAATTGTTATAT  
CAGAGTGACAAATAAGTGCTATGGCTTGATAGAAGTGAAGCTCTTCACAT  
ATATTCAAATACATATCACAACTTTGGTAAATAGGATAGTAATCTGAA  
GAACTTTTGCCCTTTTTACCCCATTTACTGTA ACTCTTGTTTCTAGGTAATC  
GTTCTCTCTCAACAACTTCTCAAGCGTCTGTGTAACAAGCCACATGTTCT  
AACAAATTGTCTCCATCGCACTTCAACAGCCAGGTCCCTATTTTTTATAAC  
GTATTAACCTTATTATTTTCTTATTATTTTAAAAGAATCTATGCACATTAG  
CAAATTTAAAAGATAGAGAAAATATAAACAGAAAAAATTATGTTTAC  
TTCTACCACCCTAAATCAACTATTATCAATTTTATACATATTTTACTCCATC  
TTTTTCAAAGTTTCTTACATTTTCCAATGTCATTAAAATTCTCTGTGAATG  
TAAATTTTAAA ACTGTACCTACTGTTTTTTGGAAATCTGTAACAAGCTAT  
GTGTAATCATTCTTCTAATATTAACATATTGTGTCCAGAATTTAAAAAAA  
A

**LPCAT3** (MBOAT5)

Chromosome 12

Gene ID: 10162

mRNA sequence: NM\_005768.5

Primers used in this study:

**Primer 1 sequence:** LG1F and LG1R. Product length = 154 bp**Primer 2 sequence:** LH1F and LH1R. Product length = 198 bp

Primers used in literature:

hLPCAT3 primer sequence; location 434. Product length = 91 bp. Matsuda *et al.*, 2008hLPCAT3 primer sequence; location 795. Product length = 125 bp. Zhao *et al.*, 2008.hLPCAT3 primer sequence; Product length = 495bp. Gijón *et al.*, 2008.

AATGCGCACGCCGTGGGCGGGCCCCGAGCCGGAATTGGGGGTGAAGCGA  
TAGCGTTTTGCCCGCATTTCGGGGCGCGCGGAGCTGGGGGGTCCCTGTGGG  
GCTCCCGGAGTTAAGATGGCGTCCTCAGCGGAGGGGGACGAGGGGACTG  
TGGTGGCGCTGGCGGGGGTTCTGCAGTCGGGTTTCCAGGAGCTGAGCCTT  
AACAAGTTGGCGACGTCCCTGGGCGCGTCAGAACAGGCGCTGCGGCTGAT  
CATCTCCATCTTCCTGGGTTACCCCTTTGCTTTTATCGGCATTACCTT  
TTCTACAAGGAGACCTACCTCATCCACCTCTTCCATACCTTTACAGGCCTC  
TCAATTGCTTATTTAACTTTGGAAACCAGCTCTACCACTCCCTGCTGTGT  
ATTGTGCTTCAGTTCCTCATCCTTCGACTAATGGGCCGCACCATCACTGC  
CGTCCTCACTACCTTTTGCTTCCAGATGGCCTACCTTCTGGCTGGATACTA  
TTACTGCCACCGGCAACTACGATATCAAGTGGACAATGCCACATTGT  
GTTCTGACTTTGAAGCTGATTGGTTTGGCTGTTGACTACTTTGACGGAGGG  
AAAGATCAGAATTCCTTGTCCTCTGAGCAACAGAAATATGCCATACGTGG  
TGTTCCCTCCCTGCTGGAAGTTGCTGGTTTCTCCTACTTCTATGGGGCCTTC  
TTGGTAGGGCCCCAGTTCTCAATGAATCACTACATGAAGCTGGTGCAGGG  
AGAGCTGATTGACATACCAGGAAAGATAACAAACAGCATCATTCCTGCTC  
TCAAGCGCCTGAGTCTGGGCCTTTTCTACCTAGTGGGCTACACACTGCTCA  
GCCCCACATCACAGAAGACTATCTCCTCACTGAAGACTATGACAACCAC  
CCCTTCTGGTTCCGCTGCATGTACATGCTGATCTGGGGCAAGTTTGTGCTG

TACAAATATGTCACCTGTTGGCTGGTCACAGAAGGAGTATGCATTTTGAC  
GGCCTGGGCTTCAATGGCTTTGAAGAAAAGGGCAAGGCAAAGTGGGAT  
GCCTGTGCCAACATGAAGGTGTGGCTCTTTGAAACAAACCCCCGCTTCAC  
TGGCACCATTCCTCATTCAACATCAACACCAACGCCTGGGTGGCCCGCT  
ACATCTTCAAACGACTCAAGTTCCTTGGAATAAAGAAGTCTCTCAGGGT  
CTCTCGTTGCTATTCCCTGGCCCTCTGGCACGGCCTGCACTCAGGATAACC  
TGGTCTGCTTCCAGATGGAATTCCTCATTGTTATTGTGGAAAGACAGGCTG  
CCAGGCTCATTCAAGAGAGCCCCACCCTGAGCAAGCTGGCCGCCATTACT  
GTCCTCCAGCCCTTCTACTATTTGGTGCAACAGACCATCCACTGGCTCTT  
CATGGGTACTCCATGACTGCCTTCTGCCTCTTCACGTGGGACAAATGGCT  
TAAGGTGTATAAATCCATCTATTTCCCTTGGCCACATCTTCTTCCTGAGCC  
TACTATTCATATTGCCTTATATTCACAAAGCAATGGTGCCAAGGAAAGAG  
AAGTTAAAGAAGATGGAATAATCCATTTCCCTGGTGGCCTGTGCGGGACT  
GGTGCAGAAACTACTCGTCTCCCTTTTCACAGCACTCCTTTGCCCCAGA  
GCAGAGAATGGAAAAGCCAGGGAGGTGGAAGATCGATGCTTCCAGCTGT  
GCCTCTGCTGCCAGCCAAGTCTTCATTTGGGGCCAAAGGGGAAACTTTTTT  
TTGGAGAAGGCGTCTTGCTTTGTCACCCACGCTGGAATGCAGTGGCGGGA  
TCTCAGCTCACCGCAACCTCCACCTCCTGGGTTCAAGTGATTTTCCTGCCT  
CAGCCTCCCAAGTAGCTGGGAATACAGGCACGCCACCATGCCAGCTAAT  
TTTTGTATTTTCAGTAGAAACGGGATTTACCACGTTGGCCAGGCTGGTCT  
CGAACTCCTGACCGCAAGTGATCCACCCGCCTCCGCCTCCCAAAGTGCTG  
GGATTACAGGCGTGAGCCACCGTGCCCGGCCAAAGGGGAAACTCTTG TG  
GGAGGAGCAGAGGGGCTCACATCTCCCCTCTGATTCCCCCATGCACATTG  
CCTTATCTCTCCCATCTAGCCAGGAATCTATTGTGTTTTTCTTCTGCCAAT  
TACTATGATTGTGTATGTGCCGCTACCACCACCCCCCATGGGGGGGT  
GGAGAGGGGTGCAAGGCCCTGCCTGCTCCACTTTTTCTACCTTGGAAGT  
TATTAGATAAAATCACTTCTGTTTGTTTCAGTTTTTCA

**LPCAT4** (MBOAT2)

Chromosome 2

Gene ID: 129642

mRNA sequence: NM\_138799.2

Primers used in this study:

**Primer 1 sequence:** LL1F and LL1R. Product length = 153 bp**Primer 2 sequence:** LM1F and LM1R. Product length = 169 bp**Primer 3 sequence:** LN1F and LN1R. Product length = 171 bp

Primers used in literature:

**hLPCAT4 Reverse primer sequence; Product length = bp. Gijón *et al.*, 2008**

GGCCTTCCCCGCGCAGAGCTCCGACCGCGGGCGGCCAGGGGCGGGCGC  
GCCGCTGCATCCCCATCCTCGTCGTCGCCCGGCACAGCGCGAGCGGGCGA  
GCGGCGCGGGCGGCCGGAGCGCCGAGGCCCGGCCATGGCCACCACCAGC  
ACCACGGGCTCCACCCTGCTGCAGCCCCTCAGCAACGCCGTGCAGCTGCC  
CATCGACCAGGTCAACTTTGTAGTGTGCCAACTCTTTGCCTTGCTAGCAGC  
CATTTGGTTTCGAACTTATCTACATTCAAGCAAACTAGCTCTTTTATAAG  
ACATGTAGTTGCTACCCTTTGGGCCTTTATCTTGCACTTTTTTGCTTTGGA  
TGGTATGCCTTACACTTTCTTGTACAAAGTGGAATTTCTACTGTATCATG  
ATCATCATAGGAGTGGAGAACATGCACAATTACTGCTTTGTGTTTGCTCTG  
GGATACCTCACAGTGTGCCAAGTTACTCGAGTCTATATCTTTGACTATGGA  
CAATATTCTGCTGATTTTTTCAGGCCCAATGATGATCATTACTCAGAAGATC  
ACTAGTTTGGCTTGCGAAATTCATGATGGGATGTTTCGGAAGGATGAAGA  
ACTGACTTCCTCACAGAGGGATTTAGCTGTAAGGCGCATGCCAAGCTTAC  
TGGAGTATTTGAGTTACAACCTGTAACCTTCATGGGGATCCTGGCAGGCCCA  
CTTTGCTCTTACAAAGACTACATTACTTTCATTGAAGGCAGATCATACCA  
TATCACACAATCTGGTGAAAATGGAAAAGAAGAGACACAGTATGAAAGA  
ACAGAGCCATCTCCAATACTGCGGTTGTTTCAGAAGCTCTTAGTTTGTGG  
GCTGTCCTTGTATTTCACTTGACCATCTGTACAACATTACCTGTGGAGT  
ACAACATTGATGAGCATTTTCAAGCTACAGCTTCGTGGCCAACAAAGATT  
ATCTATCTGTATATCTCTCTTTTGGCTGCCAGACCCAAATACTATTTTGCA  
TGGACGCTAGCTGATGCCATTAATAATGCTGCAGGCTTTGGTTTCAGAG

GGTATGACGAAAATGGAGCAGCTCGCTGGGACTTAATTTCCAATTTGAGA  
ATTCAACAAATAGAGATGTCAACAAGTTTCAAGATGTTTCTTGATAATTG  
GAATATTCAGACAGCTCTTTGGCTCAAAAGGGTGTGTTATGAACGAACCT  
CCTTCAGTCCAACCTATCCAGACGTTTCATTCTCTCTGCCATTTGGCACGGGG  
TATACCCAGGATATTATCTAACGTTTCTAACAGGGGTGTTAATGACATTA  
GCAGCAAGAGCTATGAGAAATAACTTTAGACATTATTTTCATTGAACCTTC  
CCAACCTGAAATTATTTTATGATGTTATAACATGGATAGTAACTCAAGTAG  
CAATAAGTTACACAGTTGTGCCATTTGTGCTTCTTTCTATAAAACCATCAC  
TCACGTTTTACAGCTCCTGGTATTATTGCCTGCACATTCTTGGTATCTTAGT  
ATTATTGTTGTTGCCAGTGAAAAAACTCAAAGAAGAAAGAATACACATG  
AAAACATTCAGCTCTACAATCCAAAAAGTTTGATGAAGGAGAAAATTCT  
TTGGGACAGAACAGTTTTTCTACAACAAACAATGTTTGCAATCAGAATCA  
AGAAATAGCCTCGAGACATTCATCACTAAAGCAGTGATCGGGAAGGCTCT  
GAGGGCTGTTTTTTTTTTTTGATGTTAACAGAAACCAATCTTAGCACCTTT  
TCAAGGGGTTTGAGTTTGTGGAAAAGCAGTTAACTGGGGGGAAATGGAC  
AGTTATAGATAAGGAATTTCTGTACACCAGATTGGAAATGGAGTGAAAC  
AAGCCCTCCCATGCCATGTCCCCGTGGGCCACGCCTTATGTAAGAATATTT  
CCATATTTTCAGTGGGCACTCCCAACCTCAGCACTTGTCCGTAGGGTCACA  
CGCGTGCCCTGTTGCTGAATGTATGTTGCGTATCCCAAGGCACTGAAGAG  
GTGGAAAATAATCGTGTCAATCTGGATGATAGAGAGAAATTAACCTTTTC  
CAAATGAATGTCTTGCCTTAAACCCTCTATTTCTAAAATATTGTTCCCTAA  
ATGGTATTTTCAAGTGTAATATTGTGAGAACGCTACTGCAGTAGTTGATGT  
TGTGTGCTGTAAAGGATTTTAGGAGGAATTTGAAACAGGATATTTAAGAG  
TGTGGATATTTTAAAATGCAATAAACATCTCAGTATTTGAAGGGTTTTCT  
TAAAGTATGTCAAATGACTACAATCCATAGTGAAACTGTAAACAGTAATG  
GACGCCAAATTATAGGTAGCTGATTTTGCTGGAGAGTTTAATTACCTTGTG  
CAGTCAAAGAGCGCTTCCAGAAGGAATCTCTTAAAACATAATGAGAGGTT  
TGGTAATGTGATATTTTAAAGCTTATCTTTTTCTTAAAAGAGAGAGGTGAC  
GAAGGAAGGCAGGAATGAAGAAGCACTGCGTGGCCTCCGGTGGAAATGCA  
CGGGGCACAGCCGCGACTCTGCAGGCAGCTTCCCCCATGCCAGGGCTC  
TGCGCCGTCATGTGAGACTTAAAAAAAAGTTGAATGACTTCGTGATACT  
TTGGACTTCTAAATTAATTTATCAGGCATAAATTATGTAGAATTAGAGG  
CTTTGAAAATAATACTGGTAGGTTGCTCAAAGGTTTTGAAAGAGAAATCG  
CTAGGTAGGTTACTATCTGGCTAATCCATTTCTTATCCTTGACAATTTAAT

TCATATTTGGGAAACTTTTAGGGAAATGAAAAATAAAAGTCACTGAGTCT  
GGGTGACATTTTTTAAGAATAATATAAATTCAGTTTCAAACCTCTTCTCACA  
TTAAAATTTTGCTGTGAACTCTTACTAAAATGAGTTTTAGGTTCTGTAAGT  
GGAAAAATGTGCTTTTATTTTATGGGCCATTTTACCACAACTAATCTTG  
CCTTGGATTACTAAGCATCTCCTGCGATCCCACAGAGGACTGTGGTGGCC  
ACAGGAGCTGAAAGCAGAAGAGTGGGATTTGATGCCAGGCAGTGGAGTG  
GCCTAGCCCCAGATTGTACCTCCTGCCCTGTAGGAGGGGAGGGGGCA  
AAGCCTTCTGACTTCACCTTTGTTTGACCTATGTATGGAACCTACTTTTACT  
TTTTGCCTTAAATTTTTAATGAAATGCAAATTTTCTGTGATGGGGTTCTCT  
CTCTCTTTTTTTTCGGGGGGTGGAGTCACTAATAAATTTGCAAATGAAGTTA  
AAGACAAGGCAACCATCTGGCTTATGCTATATAATACTTCATTTAAAGAA  
GAAAGGAAAAGCAAATGCACTTGCAGCTTTTGAGGTCTCAGCAAAAATG  
GGCATGTGTCTTTTTTTGAAGTTTAGAAATATCCTAATCTATTTTTATTTATC  
TAAAAGTAAGTGTTTTCCGGCTGATAAGGCTAACCCCTACCCAGGAAAGGA  
TTGATAACTAAATAAATTTCTCTGTTTTCCCATGCATTGAAATTATGTTG  
GCTGAGCATGGTGGCTCACACCTGTAATCCTAGCACTTTGGGAGGCCGAG  
GTGGGCGGATCACTTGAGGTCAGGAGTTGGAGACCAGCCTGGCCAACGT  
GGTGAATCCCCGTCTCTACTGAAAACACAAAAATTAGACGGGCATGGTGG  
CGCACACCTGTAATCCCAGCTACTTGGGAGGCTGAGGCAGGAGAATTGCT  
TGAACCTGGGAGGTGGAGGTTGCAGTGAGCTAAAATTGTGCCACTGCACT  
CCAGCCTGGGTGACAGAGGAAGACTCCGTCTCAC

**LPEAT2** (AGPAT7: AYTL3)

Chromosome 15

Gene ID: 254531

mRNA sequence: NM\_153613.2

Primers used in this study:

**Primer 1 sequence:** LI1F and LI1R. Product length = 167 bp**Primer 2 sequence:** LJ1F and LJ1R. Product length = 198 bp**Primer 3 sequence:** LK1F and LK1R. Product length = 167 bp

Primers used in literature:

hLPEAT 2 primer sequence. Product length = 370 bp. Ye, Guang-Ming *et al.*, 2005hLPEAT 2 primer sequence; location 212. Product length = 101 bp. Cao *et al.*, 2008.

AGCGCCGCCGCCGCCGCTGCTGCAGCAGCAGCTGCTCTGCAGAGTGGTGG  
 CCGGGGCCAGGGCCGGGGTGCCCTCCCTCCCACCTTCTCCCGCCATGAGC  
 CAGGGAAGTCCGGGGGACTGGGCCCCCTAGATCCCACCCCCGGACCCCC  
 AGCATCCCCCAACCCCTTCGTGCATGAGTTACATCTCTCTCGCTCCAGAG  
 GGTAAAGTTC**TGCCTCCTGGGGGCATTGCTGGCCCCATCCGAGTGCTTCT**  
**GGCCTTTATCGTCCTCTTTCTCCTCTGGCCCTTGCCTGGCTTCAAGTGGC**  
**CGGTCTTAGTGAGGAGCAGCTTCAGGAGCCAATTACAGGATGGAGGAA**  
**GACTGTGTGCCACAACGGGGTGCTAGGCCTGAGCCGCCTGCTGTTTTCTCT**  
**GCTGGGCTTCTCCGATTTCGCGTTCGTGGCCAGCGAGCCTCTCGCCTTCA**  
**AGCCCCTGTCCTTGTTGCTGCCCCACA****CTCCACTTTCTTTGACCCCA**ATTG  
 TTCTGCTGCCCTGTGACCTGCCCAAAGTTGTGTCCCGAGCTGAGAACCTTT  
 CCGTTCCTGTC**ATTGGAGCCCTTCTTCGATTCAACCAAGCCATCCTGGTA**  
**TCCCGGCATGACCCGGCTTCTCGACGCAGAGTGGTGGAGGAGGTCCGAAG**  
**GCGGGCCACCTCAGGAGGCAAGTGGCCGCAGGTGCTATTCTTTCCTGAGG**  
**GCACCTGTTCCAACAAGAAGGCTTTGCT**TAAGTTCAAACCAGGAGCCTT  
 CATCGCAGGGGTGCCTGTGCAGCCTGTCCCTCATCCGCTACCCCAACAGTC  
 TGGACACCACCAGCTGGGCATGGAGGGGTCTGGAGTACTCAAAGTCCTC  
 TGGCTCACAGCCTCTCAGCCCTGCAGCATTGTGGATGTGGAGTTCCTTCTC  
 GTGTATCACCCAGCCCTGAGGAGAGCAGGGACCCACCCCTCTATGCCAA  
 CAATGTTCAAGAGGGTCATGGCACAGGCTCTGGGCATTCCAGCCACCGAAT

GTGAGTTTGTAGGGAGCTTACCTGTGATTGTGGTGGGCCGGCTGAAGGTG  
GCGTTGGAACCACAGCTCTGGGAACTGGGAAAAGTGCTTCGGAAGGCT  
GGGCTGTCCGCTGGCTATGTGGACGCTGGGGCAGAGCCAGGCCGGAGTC  
GAATGATCAGCCAGGAAGAGTTTGCCAGGCAGCTACAGCTCTCTGATCCT  
CAGACGGTGGCTGGTGCCTTTGGCTACTTCCAGCAGGATACCAAGGGTT  
TGGTGGACTTCCGAGATGTGGCCCTTGCACTAGCAGCTCTGGATGGGGGC  
AGGAGCCTGGAAGAGCTAACTCGTCTGGCCTTTGAGCTCTTTGCTGAA  
GAGCAAGCAGAGGGTCCCAACCGCCTGCTGTACAAAGACGGCTTCAGCA  
CCATCCTGCACCTGCTGCTGGGTTACCCCCACCCTGCTGCCACAGCTTTGC  
ATGCTGAGCTGTGCCAGGCAGGATCCAGCCAAGGCCTCTCCCTCTGTCAG  
TTCCAGAACTTCTCCCTCCATGACCCACTCTATGGGAAACTCTTCAGCACC  
TACCTGCGCCCCCACACACCTCTCGAGGCACCTCCCAGACACCAAATGC  
CTCATCCCCAGGCAACCCCACTGCTCTGGCCAATGGGACTGTGCAAGCAC  
CCAAGCAGAAGGGAGACTGAGTGCCTCAGCCTCTCACCCCCTCCTCCTC  
AGGGCAGCGCTAGGGGCCTCCCCTATGCCTCAGCCCCATCTCTGCTCCTGT  
TTGAATTTTGTATTGTTGTTTGGTTGTTGTTTTTTAAGTTGATTTTAATTT  
TTTGTTTGGTTGATTTTTTTGTAAAAAACTATTTTATATATAAATATAAATC  
TATATCTATATCTATTAAAAAAATGAAGTCCAGTCATATTGATGTTACCA  
TTA



## RuBisCO

*Senecio Vulgaris* Ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCO) small subunit mRNA sequence and primers.

dbEST Id: 37236834

EST name: SV\_CP\_06\_F12

GenBank Acc: DY664462

GenBank gi: 89508666

Primers used in this study:

**Primer sequence:** RuBisCOF and RuBisCOR. Product length = 83 bp.

TGGATCCCCCGGGCTGCAGGTAGAAGTCCATTCCTAAGTAACAACACTACTA  
AAATGGCTTCAATCTCTTCTTCCGCGGTCCGCACTGTTAGCAGGACCCCTG  
CTCAAGCTAGCATGGTCGCTCCATTTACCGGTCTTAAGTCTAACGTCGCCT  
TCCCAGTTACCAAGAAGGCTAACAATGACTTCTCCACCCTTCCCTCTAACG  
GCCGAAGAGTCCAATGCATGAAGGTGTGGCCACCTTTGGGTTTGAAGAAG  
TACGAGACCCTTTCATACCTTCCACCATTGACCGAAATCCAATTGGCTAA  
GGAAGTCGACTACCTTCTCCGCAACAAGTGGGTTCCCTTGTTTGGGAATTCG  
AAGTCGAGCACGGTTTCGTTTACCGTGAGCACGGCAACACCCCCGGATAC  
TATGACGGAAGATACTGGACAATGTGGAAGTTGCCTATGTTTCGGGTGCAC  
CGACTCCGCTCAAGTGTTGAAGGAGCTAGCTGAGTGCAAGAAGGAGTA  
CCCTGAGGCCTTCATCCGTATCATCGGATTCGACAACGTTCGTCAAGTG  
CAATGTGTCAGTTTCATCGCCTCCAAGCCAGCTGGCTACTAAGCAACAAA  
ATTTTCTATTTCTACCGGAACCTTTCGATTAACCCACTTATAACGAGTAGGG  
CTTACTTGAATGTTTAGGGTTTTGTTATATTTTTTTTTTCGTTTTAAATTTAT  
CATTTTCCTAATTGTCTTGTAATTCATTTTCGTTGTTTGTTCGGATTTCCG  
AT

APPENDIX 2 – Composition of buffers

TRIS Borate–EDTA buffer (TBE) (pH 8.0) 2 mM EDTA, 90 mM Boric acid 90 mM Tris-base	2L Transfer buffer (pH 7.2) 6.06 g Tris-base 28.8 g Glycine 200 ml methanol
Triton X-100 Extraction buffer (pH 7.6) 10 mM Tris-base 150 mM NaCl, 1 mM EDTA 1 mM EGTA 1 % Triton X-100 Protease inhibitor cocktail tablet	Tris-buffered Saline (TBS) (pH 7.2-7.4) 20 mM Tris-base 150 mM NaCl For TBS-T add 0.1 % Tween 20
MES buffered Saline (MBS) (pH 6.5) 150 mM NaCl, 20 mM MES 1 % Triton X-100 Protease inhibitor cocktail tablet	Red Blood Cell Lysis buffer (x10) (pH 7.2-7.4) 1.5 M NH <sub>4</sub> Cl 100 nM KHCO <sub>3</sub> 10 nM Na <sub>4</sub> EDTA
XT-MOPS running buffer (pH 7.7) 1 M MOPS 1 M Tris-base 69.3 mM SDS 20.5 mM EDTA	50 ml Denaturing Solution 25 g guanidinium thiocyanate 1.76 ml 0.75 M sodium citrate (pH 7.0), 2.64 ml 10 % sarkosyl
	The working Denaturing solution can be prepared by adding 72 µL of 98 % 2-mercaptoethanol to 10ml of stock solution