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The Role of Lysophosphatidylcholine Acyltransferase (LPCAT) 3 in Macrophage Inflammatory Responses

Okorie, Confidence Obianuju

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Plymouth University

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The Role of Lysophosphatidylcholine Acyltransferase (LPCAT) 3 in Macrophage Inflammatory Responses

Confidence Obianuju Okorie

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(ResM)

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School of Biomedical and Healthcare Sciences

Plymouth University Peninsula Schools of Medicine and Dentistry

The John Bull Building

Plymouth Science Park

Research Way

Plymouth, PL6 8BU

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ABSTRACT

Sepsis is the systemic response to infection and is characterised by the presence of Severe Inflammatory Response Syndrome (SIRS) in addition to a documented or presumed infection. It is an important reason for admission into the Intensive Care Unit (ICU), the leading cause of death in non-coronary ICU's and one of the most common causes of death among hospitalized patients. It affects 18 million people worldwide each year with over 7.3 million fatalities. Molecules such as endotoxin, the Lipopolysaccharide (LPS) present in the outer membrane of Gram-negative bacteria, are key factors in the pathogenesis of sepsis. The activation of macrophages with LPS results in rapid changes in the expression of genes encoding cytokines and other inflammatory mediators. The excessive, prolonged and unregulated activation of macrophages in sepsis leads to a vicious cascade of the release of these inflammatory mediators that augments the inflammation to the detriment of the host. The Lysophosphatidylcholine acyltransferase (LPCAT) enzymes, expressed in monocytes and macrophages among other cells and tissues have been found to be very important in the regulation of membrane phospholipids' fatty acid composition and together with phospholipase A2 enzyme, control the phospholipids' re-modelling process in the plasma membrane and are required for the inflammatory function of macrophages. There have been indications that the induction of LPCAT 3 results in the priming of macrophages for subsequent eicosanoid secretion. Investigating the activity of this enzyme, particularly, its contribution to inflammatory processes in these cells informed this research. A murine macrophage cell line, RAW 264.7, was used in this study. To investigate the effect of LPCAT 3 on LPS induced inflammatory responses, LPCAT3 was silenced using the RNA interference (RNAi) technology and this was confirmed using reverse transcription coupled real time PCR. Macrophage stimulation was achieved using LPS. The optimization of reference genes was considered important for the standardisation of results as the conventional practice of utilising one reference gene for result optimisation has been found unreliable. This was done using real time PCR, as was the comparative quantitation of two cytokines: Tumour Necrosis Factor alpha (TNF-alpha) and Interleukin-10 (IL-10). The protein expression of TNF-alpha was analysed using ELISA. Results from the experiments showed the reference genes GAPDH, RPL13A and CASC3 to be the most stably expressed in the PCR analysis of the expression of the genes of interest. Normalization of PCR results was done using the normalization factor generated by the geometric mean of the cycle threshold values of these reference genes. A strong relationship between LPCAT 3 silencing and a statistically significant reduction in the LPS-induced expression of the pro-inflammatory cytokine TNF-alpha was also observed while this relationship with regards to IL-10 was inconclusive. In conclusion, LPCAT 3 is involved in the inflammatory responses of macrophages and its silencing results in a significant reduction in the LPS-induced expression of TNF-alpha. Further investigations into the mechanisms of action of this enzyme would further strengthen the possibility of the emergence of novel and specific anti-sepsis therapies in the near future.

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List of Abbreviations

AA: Arachidonic acid

ACT-B: β -Actin

AcylCoA: Acyl Coenzyme A

AKT: Protein Kinase B

API: Baculoviral IAP repeat-containing protein3

ATP: Adenosine Triphosphate

ATP5B: Adenosine Triphosphate Synthase subunit Beta

B2M: β 2- Microglobulin

BI: BestKeeper Index

C5AR: C5a Receptor

CASC3: Cancer Susceptibility Candidate 3

CD14: Cluster of Differentiation

CD36: Cluster of Differentiation 36

cDNA: Complementary Deoxyribonucleic acid

CoAIT: CoA-Independent Transacylase

COX: Cyclo-oxygenase

CP: Crossing Point

CT: Cycle Threshold

CYPA: Cyclophilin A

DMEM: Dulbecco's Modified Eagle Medium

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic acid

DOK1: Docking protein 1

DOK2: Docking protein 2

ECACC: European Collection of Cell Cultures

EIF4A2: Eukaryotic initiation factor 4A-II

EJC: Exon Junction Complex

ELISA: Enzyme Linked Immunosorbent Assay

EPA: Eicosapentaenoic acid

FADD: Fas (TNFRSF6)-associated via death domain

FBS: Foetal Bovine Serum

FDM: First Derivative Maximum

GAIT: Gamma Interferon-Activated Inhibitor of Translation

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

GPC: GlyceroPhosphatidylcholine

GUSB: Beta-glucuronidase

HMBS: Hydroxymethylbilane synthase

HMGB1: High-mobility group protein B1

HPRT1: Hypoxanthine-guanine phosphoribosyltransferase 1

IFN-beta: Interferon beta

IFN-gamma: Interferon gamma

IKBk-alpha: Nuclear Factor-Kappa-B inhibitor Kinase alpha

IKK-alpha: Inhibitor of nuclear factor kappa-B kinase subunit alpha

IKK-beta: Inhibitor of nuclear factor kappa-B kinase subunit beta

IKK-gamma: Inhibitor of nuclear factor kappa-B kinase subunit gamma

IL-1: Interleukin 10

IL-10: Interleukin 10

IL-6: Interleukin 6

IL-8: Interleukin 8

INK: A tumour suppressor protein that acts to inhibit CDk4 and CDk6 upon binding, causing the arrest of the cell cycle in G1

IRAK1: Interleukin-1 receptor-associated kinase 1

IRAK4: Interleukin-1 receptor-associated kinase 4

IRAKM: Interleukin-1 receptor-associated kinase M

IRF2: Interferon regulatory factor 2

IRF3: Interferon regulatory factor 3

LPA: Lysophosphatidic acid

LPC: LysoPhosphatidylcholine

LPCAT: Lysophosphatidylcholine acyltransferase

LPEAT: Lysophosphatidylethanolamineacyltransferase

LPS: Lipopolysaccharide

LPSAT: Lysophosphatidylserine Acyltransferase

LXR: Liver X Receptor

MAPK: Mitogen-activated protein kinases

MBOAT: Membrane bound O-acyl transferase

MD2: Lymphocyte antigen 96

MHC: Major Histocompatibility Complex

MIF: Migratory Inhibitory Factor

MIQE: Minimum Information for publication of Quantitative real-time PCR Experiments

mRNA: Messenger Ribonucleic acid

MVP: Mid-Value Point

MyD88: Myeloid differentiation primary response gene 88

NF-KB: Nuclear factor kappa-light-chain-enhancer of activated B cells

NMD: Nonsense-Mediated mRNA Decay

OPTI-MEM: Reduced-Serum Medium

PAF: Platelet Activating Factor

PAFAT: Platelet Activating Factor Acetyl Transferase

PC: Phosphatidylcholine

PCR: Polymerase Chain Reaction

PE: Phosphatidylethanolamine

PI3K: Phosphoinositide 3-kinase

PL: Phospholipid

PLA2: Phospholipase A2

PPIB: cyclophilin B

PS: Phosphatidylserine

r: Pearson Correlation Coefficient

REST: Relative Expression Software Tool

RIP-1: Receptor interacting Protein 1

RNA: Ribonucleic acid

RPL13A: 60S Ribosomal Protein L13A

rRNA: Ribosomal Ribonucleic Acid

RT: Reverse Transcription

RT-qPCR: Reverse Transcription quantitative/real time Polymerase Chain Reaction

SD: Standard deviation

SDHA: Succinate dehydrogenase complex, subunit A

SDM: Second Derivative Maximum

SE: Standard Error

Sf9: *Spodoptera frugiperda* 9

SHIP: SH2 domain-containing Inositol Phosphatase

SIGIRR: Single immunoglobulin IL-1R-related molecule

siRNA: Small Interfering Ribonucleic Acid

ST2: A member of the interleukin 1 receptor family encoded by the IL1RL1 gene

TAB1: Mitogen-activated protein Kinase 7 Interacting Protein 1

TAB2: Mitogen-activated protein Kinase 7 Interacting Protein 2

TAK1: Transforming growth factor -Beta Activated Kinase 1

TAZ: Tafazzin

TBK1: TRAF family member-associated NF-kappa-B activator Binding Kinase 1

TBP: TATA Box Binding Protein

TBP: TATA-box binding protein

Th1: T helper cells 1

Th17: T helper cells 17

Th2: T helper cells 2

THP-1: A human monocytic cell line commonly used in research

TICAM-1: TIR domain-containing adapter molecule 1

TICAM-2: TIR domain-containing adapter molecule 2

TIRAP-1: TIR domain-containing adapter protein 1

TIRAP-2: TIR domain-containing adapter protein 2

TLR2: Toll-Like Receptor 2

TLR4: Toll-Like Receptor 4

TLR5: Toll-Like Receptor 5

TLR9: Toll-Like Receptor 9

TNF-alpha: Tumour Necrosis Factor – alpha

TOLLIP: Toll Interacting Protein

TRAF6: TNF receptor associated factor 6

TRIAD3: Triad domain-containing protein 3

TRIF: TIR-domain-containing adapter-inducing interferon- β

TRIS-base: Tris(hydroxymethyl)aminomethane-Base

UBC: Ubiquitin C

UBC13: Ubiquitin 13

Uev1A: Ubiquitin-Conjugating Enzyme E2 Variant 1

VLDL: Very Low Density Lipoprotein

YWHAZ: Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide

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Dedication

This research is dedicated to the blessed memory of Obianuju Gift Ohiri (18/11/2014 - 2/11/2015), my niece and namesake who lost the battle to Severe Sepsis before her first birthday; and to countless others whose lives inspire us to continue to research.

Author's declaration

At no time during the registration for the Research Masters degree has the author been registered for any other University award without prior agreement of the Graduate Committee.

The laboratory experiments and data analysis presented in this project were carried out by the author.

Relevant scientific seminars and conferences were regularly attended and research work has been presented.

Presentation and Conferences Attended:

- Plymouth University Biomedical Research Celebration and Systems Biology Centre Launch (2nd September 2014): Poster presentation: The Role of Lysophosphatidylcholine Acyltransferase (LPCAT) 3 in Macrophage Inflammatory Responses.
- Plymouth University Postgraduate Society Conference (22nd October, 2014): Oral Presentation: The Role of Lysophosphatidylcholine Acyltransferase (LPCAT) 3 in Macrophage Inflammatory Responses.

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Date

CHAPTER ONE: INTRODUCTION

1.1 SEPSIS

Sepsis is a spectrum of non-specific inflammatory responses with evidence or suspicion of a microbial origin. The continuum that is characteristic of this disease process informed the necessity of the birth of a definition that would incorporate the various aspects typically defining its presence. This need led to a consensus conference focusing on a way to clinically define sepsis by the American College of Chest Physicians/Society of Critical Care Medicine (ACCP/SCCM), in 1992 (Bone et al, 1992).

The description published by the consensus committee, of the defining criteria for systemic inflammatory response syndrome and specific definitions for sepsis, severe sepsis, septic shock and multiple organ dysfunction syndrome is presented in Table 1.

ACCP/SCCM named condition	Defining criteria
SIRS	Core body temperature $>38^{\circ}\text{C}$ or $<36^{\circ}\text{C}$ HR ≥ 90 bpm Respirations $\geq 20/\text{min}$ (or PaCO ₂ <32 mmHg) WBC $\geq 12,000/\mu\text{l}$ or $\leq 4000/\mu\text{l}$ or $>10\%$ immature forms
Sepsis	At least two SIRS criteria caused by known or suspected infection
Severe sepsis	Sepsis with acute organ dysfunction (including hypo-perfusion and hypotension) caused by sepsis
Septic shock	Sepsis with persistent or refractory hypotension or tissue hypo-perfusion despite adequate fluid resuscitation
MODS	The presence of organ dysfunction in an acutely ill patient such that homeostasis cannot be maintained without intervention

ACCP: American College of Chest Physicians; HR: Heart rate; MODS: Multiple organ dysfunction syndrome; PaCO₂: Partial pressure of carbon dioxide in the blood; SCCM: Society of Critical Care Medicine; SIRS: Systemic inflammatory response syndrome; WBC: White blood cell.

Table 1: *Defining criteria of ACCP/SCCM named conditions (Martin, 2012)*

The incidence of sepsis is increasing dramatically despite the advantages of modern medicine including vaccines, antibiotics and intensive care. Worldwide, 20 to 30 million patients are estimated to be afflicted every year, with over 6 million cases of neonatal and early childhood sepsis and over 100,000 cases of maternal sepsis (The Global Sepsis Alliance, 2015). Annual mortality is over 7.3 million (Angus, 2011). Sepsis is one of the leading causes of death in the developed world, equalling myocardial infarction in its annual prevalence and resulting in considerable costs to the health economy (Richards, 2013). International estimates of incidence differ, but consensus suggests about 300 cases per 100,000 each year. As comparators, myocardial infarction affects 208 patients per 100,000 per year and stroke 223 (Yeh et al. 2010 and Feigin et al, 2009). In the USA, sepsis incidence has been dramatically increasing by an annual rate of between 8-13 % (Vincent et al. 2006, Hall et al. 2011) over the last decade and now claims more lives than bowel cancer and breast cancer combined (Richards, 2013). The mortality from sepsis in a recent major European study was 36%. In 2010, 5.1% of deaths in England were without doubt associated with sepsis. Bringing cases which are probably associated with sepsis into the picture increases this figure to 7.7% of all deaths (McPherson et al. 2013). In the United Kingdom, it is conservatively estimated from Intensive Care National Audit and Research Centre (ICNARC) data that the yearly incidence of sepsis stands at 102,000 cases, resulting in 36,800 deaths and costing the NHS approximately £2.5 billion (Richards, 2013).

1.1.1 Pathophysiology of Sepsis: The role of LPS and Toll-like Receptor 4 (TLR4)

LPS or endotoxin is a predominant, integral structural component of the outer membrane of Gram-negative bacteria and is one of the most potent microbial initiators of inflammation and sepsis (Dobrovolskaia and Vogel, 2002). LPS activates monocytes and macrophages to produce cytokines such as TNF- α , IL-1, and IL-6, which, in turn, serve as endogenous mediators of inflammation through receptor-mediated interactions with various target cells. Although cytokine production is important for the efficient control of growth and dissemination of invading pathogens, overproduction of cytokines is harmful for the host, since it may lead to multiple organ failure and death. Such multiple organ dysfunction caused by excessive production of inflammatory cytokines is a primary characteristic of the septic shock

syndrome (Dobrovolskaia and Vogel, 2002, Cohen 2002, Heumann and Roger, 2002 and Van Amersfoort et al. 2003).

LPS typically consists of a hydrophobic domain known as lipid A (or endotoxin) which consists of two glucosamine units with attached acyl chains and usually containing one phosphate group on each carbohydrate. It makes up the outer monolayer of the outer membranes of most Gram-negative bacteria. A non-repeating “core” oligosaccharide and a distal polysaccharide (or O-antigen) are two other components which are generally not required for growth in the laboratory, but enable bacteria resist antibiotics, the complement system, and other external factors (Raetz and Whitfield, 2002).

A substantial amount of proof backs a model where LPS or LPS-containing bodies (including intact bacteria) form complexes with a serum protein known as LPS-binding protein the role of which is to bring LPS to the cell surface by binding to LPS and forming a ternary complex with the LPS receptor molecule CD14 (Dauphinee and Karsan, 2006); the LPS in this complex is subsequently transferred to another protein which binds LPS, CD14 and Toll-like receptor 4 (TLR4)-MD-2 complex (Figure 1) (Ulevitch and Tobias 1999 and Heumann and Roger 2002).

Since CD14 lacks a transmembrane domain, it lacks the ability to initiate intracellular signalling events. Intensive efforts over several years have led to the discovery of the TLR4/MD2 receptor complex as the signalling entity for LPS (Lamitaire et al. 1996; Medzhitov et al. and 1997 Shimazu et al. 1999). MD2 is a secreted glycoprotein that functions as an indispensable extracellular adaptor molecule for LPS-initiated signalling events, perhaps by aiding in ligand recognition (Shimazu et al. 1999).

Toll-like receptor 4 (TLR4) is a member of a family of innate immune receptor proteins and has been found to detect LPS from Gram-negative bacteria and is thus important in the activation of the innate immune system (Medzhitov et al. 1997). A fusion protein comprising a CD4 extracellular domain and the intracellular region of TLR4 was engineered, and its expression in the human macrophage cell line THP-1, led to the induction of IL-1, IL-8 and B7.1 gene expression, and in the presence of IFN-gamma, to an elevation of IL-6 expression. It has also been discovered that when the intracellular signalling pathway by constitutively active TLR4 in Jurkat cells is activated, a nuclear factor kappa B (NF-KB)-driven pathway is also activated

(Medzhitov et al. 1997). These findings lead to the conclusion that human TLR4 played a fundamental role in this early stage of the induction of adaptive immunity.

Members of this family of (currently) thirteen Toll-like receptors possess a wide range of ligand specificity including bacterial, fungal and yeast proteins (Shi et al. 2011). Thus, TLR4 is the LPS receptor (Medzhitov et al. 1997), TLR2 is predominantly responsible for recognizing Gram-positive cell-wall structures (Yoshimura et al. 1999), TLR5 is the receptor for flagellin (Hayashi et al. 2001) and TLR9 recognizes CpG elements in bacterial DNA (Takeshita et al. 2001).

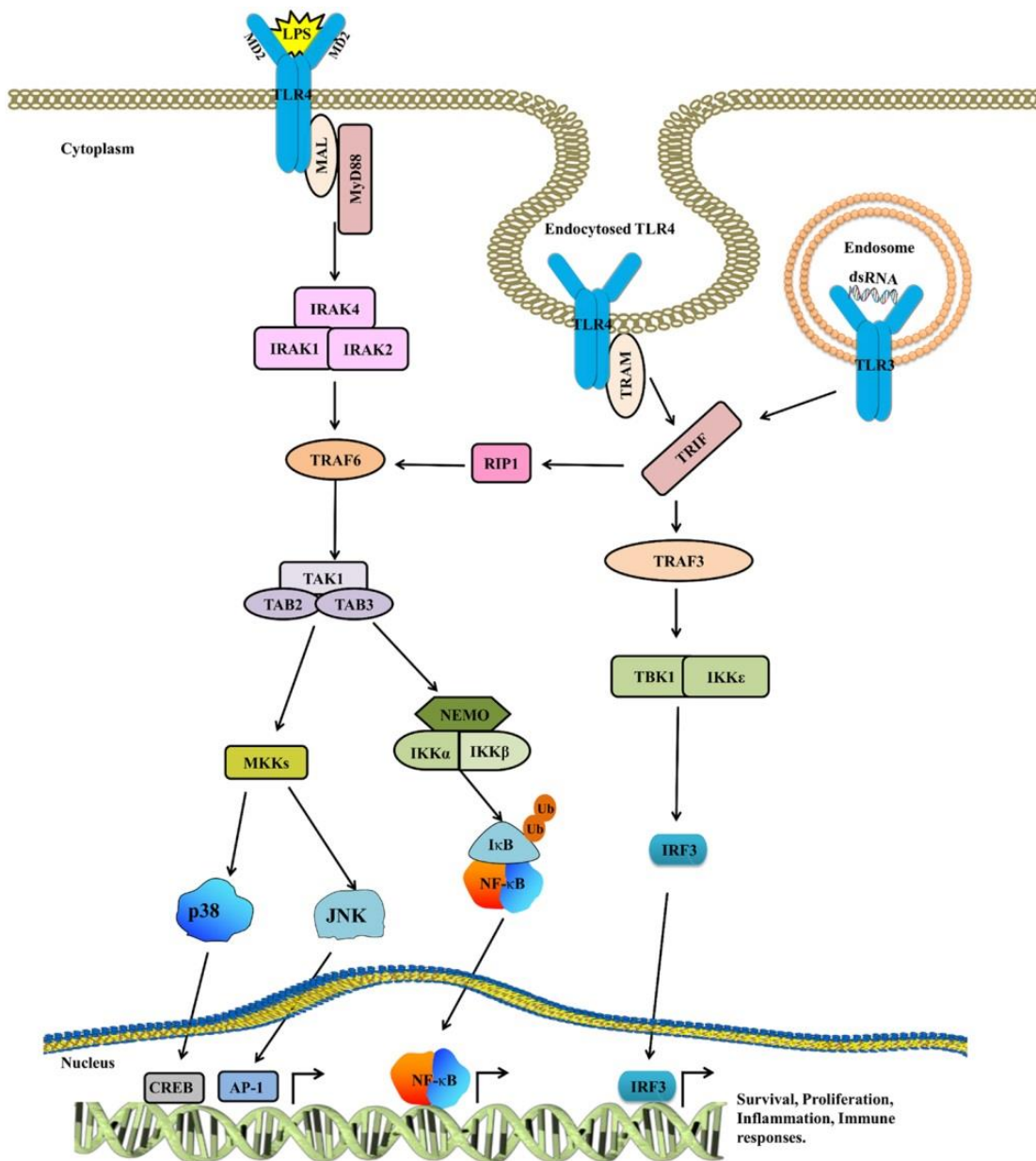


Figure 1: **The TLR4 signaling pathway.** TLR4 is activated by LPS, whereas CD14 and MD2 act as accessory proteins for LPS/TLR4 binding. Upon ligand binding, TLR4 dimerizes, and recruits downstream adaptor molecules such as MyD88/MAL and TRIF/TRAM to mount an inflammatory response. The activated MyD88/MAL then activates IRAK4, TRAF6, TAK1, and IKK complexes, while TRIF/TRAM signals through RIP1 to TRAF6/TAK1 and IKK. After this, both these pathways converge at NF- κ B. The cytoplasmic NF- κ B complex is maintained in the inactive state by I κ B, which is in turn degraded by proteasomes, resulting in the translocation of NF- κ B into the nucleus. Besides activating NF- κ B, TAK1 also phosphorylates MAPKs to further reinforce the inflammatory response. The TRIF/TRAM pathway not only activates NF- κ B but also triggers IRF3 to mount an antiviral response. Cumulatively, all these signaling pathways assist in eradicating infection as well as play an important role in sustaining the normal physiological functions in intestinal epithelial cells (Figure and legend taken from Yesudhas et al, 2014).

The TLR4 signalling cascade initiated following LPS binding is enhanced by homodimerization of the receptor (Zhang et al. 2002) and the signal propagation involves recruitment of cellular adaptor molecules for TLRs such as myeloid differentiation factor 88 (MyD88), MyD88 adaptor-like protein (Mal), also called TIRAP, TIR-containing adaptor inducing interferon (IFN) β (TRIF), also known as TIRAP-1 (TICAM-1), and TRIF-related adaptor molecule (TRAM), also called TIRAP-2 (TICAM-2); as well as kinases including IRAKs and MAPKs (Lee et al. 2006).

Furthermore, activation of TLR4 leads to stimulation of both a MyD88-dependent and a MyD88-independent pathway. The major players involved in eliciting the functional effects of LPS are activated through the NF- κ B, mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)/Akt pathways. These pathways control the equilibrium between cell viability and inflammation (Figure 2) (Dauphinee and Karsan, 2006).

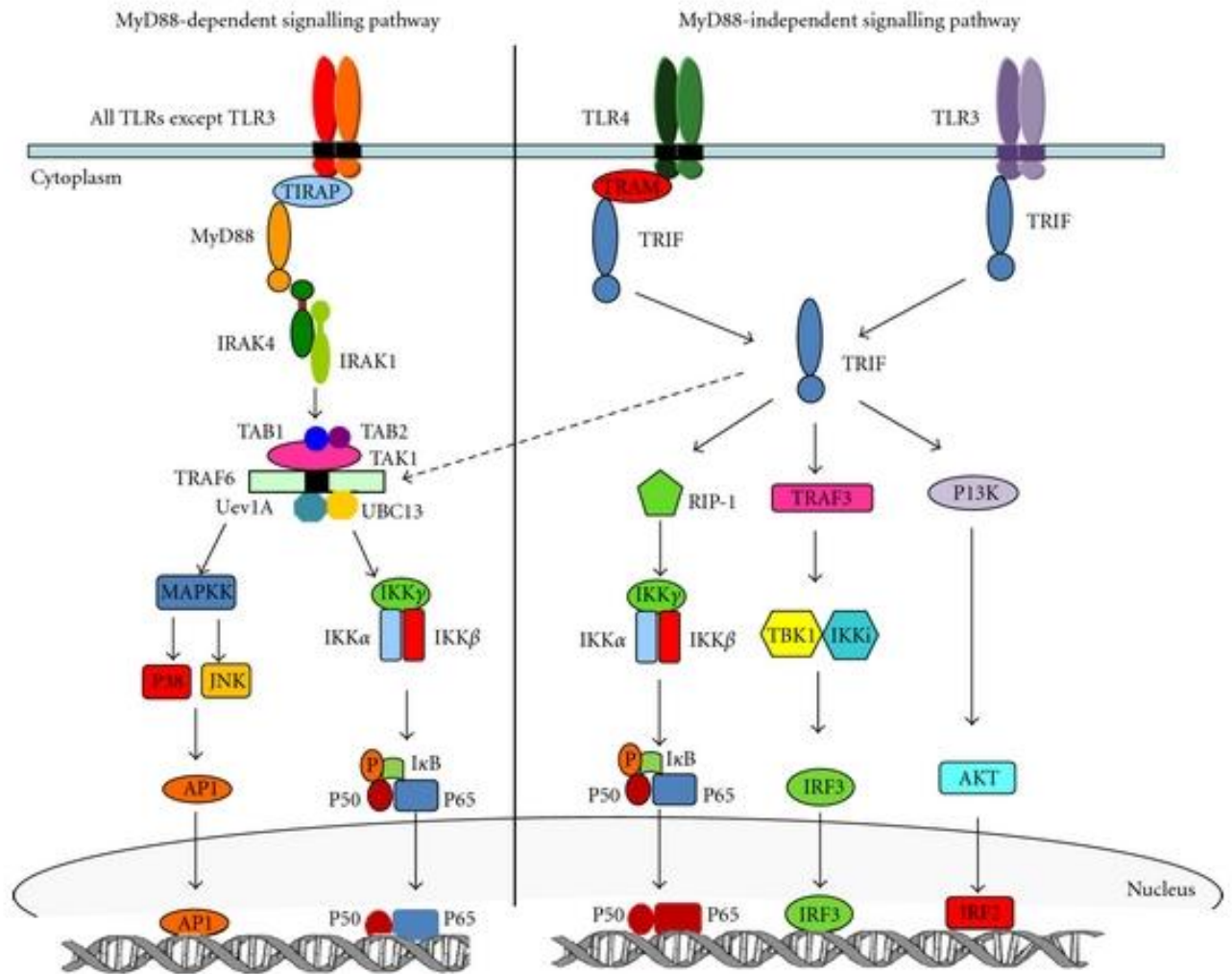


Figure 2: TLR signalling pathway. MyD88-dependent signalling pathway is used by all TLRs except TLR 3. Signalling through the MyD88-dependent pathway leads to the activation of MAPKK and IKK complex resulting in activation and nuclear translocation of AP-1 and NF-κB, respectively. TLR 4 is capable of signalling through the MyD88-independent pathway as well; however this is the sole signalling mechanism for TLR 3. TRIF is the main adaptor protein in the MyD88-independent pathway and can associate with TRAF6 to activate AP-1 and NF-κB. Alternatively it also activates NF-κB by interacting with RIP-1. TRIF can further interact with TRAF3 and the phosphatidylinositol 3-kinase (PI3K)-AKT pathway resulting in the nuclear translocation of IRF3 and IRF2, respectively (Figure and Legend taken from Patel et al, 2012).

The activation of macrophages with LPS results in rapid changes in expression of genes encoding cytokines and other inflammatory mediators (Flad et al.1993; Rietschel et al. 1994 Cowdery et al. 1996 and Brunialti et al. 2006). The various cytokines released from macrophages exert diverse cellular effects and they are central to the inflammatory process in sepsis. The excessive, sustained and

uncontrolled activation of macrophages in sepsis leads to a vicious cascade of inflammatory mediators' release that amplifies the inflammation to the detriment of the host (Rangel-Frausto et al. 1995).

1.1.2 Pathophysiology of Sepsis: The role of Macrophages

The discovery of macrophages as cardinal actors in the immune system can be traced to observations and experiments conducted by Ukrainian scientist Elie Metchnikoff, for which he won a Nobel Prize in 1908. Apart from their crucial role of antigen presentation, it has been long recognised that the release of cytokines from primed macrophages is an integral event in the pathogenesis of sepsis. Ayala et al. (1992) discovered that the cytokines IL-1, IL-6 and TNF-alpha were released from macrophages in response to a polymicrobial infection.

In another research, TNF-positive mononuclear phagocytes were found in sheep lungs alongside a progressive rise in the plasma levels of the cytokine with continuous LPS infusion. It was concluded that these cells probably contribute to both the rise in the circulating levels of TNF-alpha and the development of acute lung injury (Cirelli et al. 1995). Also, following injection of LPS in mice, peritoneal and alveolar macrophages were found to release TNF in *in vitro* cultures (Fitting et al. 2004). Ge and colleagues have also shown that after injection of LPS or bacteria, *in situ* analysis shows that spleen macrophages produce abundant amounts of IL-1 (Ge et al. 1997). Furthermore, bone marrow monocytes contain TNF, and Kupffer cells are also an important source of IL-1 and TNF (Cavallion and Adib-Conquy, 2005).

It has also been discovered that haemorrhage-induced suppression of macrophage and splenocyte functions leads to a decline in cytokine production and therefore decreases susceptibility to sepsis (Ertel et al. 1992).

1.2 LYSOPHOSPHATIDYLCHOLINE ACYLTRANSFERASES (LPCAT)

The biomembrane lipid bilayer serves as an important structure that compartmentalizes living cells and also forms organized intracellular membrane organelles for various physiological functions. The foremost and important structural components of membrane lipid bilayers are phospholipids (PLs) including

phosphatidylcholine (PC), phosphatidylserine (PS), and phosphatidylethanolamine (PE) with phosphatidylcholine being the most abundant in mammals (Zhao et al. 2008). As major components of the external plasmalemma, phospholipids form a membrane system which provides the cell with a protective barrier that has selective permeability for different metabolites. Within the cell, phospholipids are the major constituents of an intrinsic membrane network that delineates individual organelles and provides a uniquely ordered system for intracellular metabolic processes (Choy et al. 1997).

Phospholipid metabolism is made possible by two major pathways: The de novo pathway and the remodelling pathway. By the use of acyl-Coenzyme A (CoA's) and Sn-3-Glycerophosphate, phospholipids are formed from diacylglycerol by the de novo pathway, originally described in 1956 (Kennedy and Weiss, 1956). But, the acyl groups in glycerophospholipids show great diversity and are distributed in an asymmetrical way. Saturated and monounsaturated fatty acids are usually esterified at the sn-1 position, whereas polyunsaturated acyl groups, including those contained in arachidonic acid are located at the sn-2 position. The Kennedy pathway does not fully explain this diversity and asymmetry. The rapid and continuous reproduction of the sn-2 acyl moiety of glycerophospholipids was described by Lands as a remodelling pathway (Lands' cycle) (Lands, 1958) and is ascribed to the combined activation of phospholipase A2s (PLA2s) and lysophospholipid acyltransferases (LPLATs) including lysophosphatidylcholine acyltransferase (LPCAT) enzymes acting on phosphatidylcholine (Hishikawa et al. 2008).

LPCAT activity has been found to reside primarily in microsomal fractions of tissues and characterization of the activity of this group of enzymes using microsomes from various tissues has led to the hypothesis that multiple forms of enzymes may exist in different tissues with different substrate specificity of fatty acyl-CoAs (Choy et al. 1997). Until recently, attempts to purify the enzyme(s) have not been successful largely due to their membrane protein nature and the fact that LPCAT activity was mostly lost during solubilisation and/or further fractionation (Zhao et al. 2008). However, by genomic methods, LPCAT1 (first in this family of enzymes) was successfully cloned and characterised by two independent groups (Chen et al. 2006 and Nikanishi et al. 2006) which subsequently paved the way for cloning and

characterisation of three more enzymes with LPCAT activity: LPCAT2, LPCAT3 and LPCAT4.

1.2.1 Brief description, activities and specificities of the LPCAT enzymes

The LPCAT enzymes, so far characterized, are found in specific locations, show preference for definite types of fatty acids and demonstrate particular enzyme activities (Shindou et al. 2009).

LPCAT1 possesses both acyltransferase and acetyltransferase activities. Its activity is calcium-independent. It mediates the conversion of 1-acyl-sn-glycero-3-phosphocholine (LPC) into phosphatidylcholine (PC) and displays a clear preference for saturated fatty acyl-CoAs, and 1-myristoyl or 1-palmitoyl LPC as acyl donors and acceptors, respectively. It has been found to synthesize dipalmitoylphosphatidylcholine in pulmonary surfactant, thereby playing a fundamental role in respiratory physiology (Chen et al. 2006; Nikanishi et al. 2006 and Bridges et al. 2001).

LPCAT2 demonstrates both acyltransferase and acetyltransferase activities, but unlike LPCAT1, its activity is calcium-dependent. It has been discovered to be involved in platelet-activating factor (PAF) biosynthesis by catalysing the conversion of the PAF precursor, 1-O-alkyl-sn-glycero-3-phosphocholine (lyso-PAF) into 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine (PAF). It also converts lyso-PAF to 1-O-alkyl-2-acyl-sn-glycero-3-phosphocholine (PC), a major component of cell membranes and a PAF precursor. Acyltransferase activity is preferred in resting conditions. However, upon acute inflammatory stimulus, acetyltransferase activity is heightened and PAF production rises (Shindou et al. 2007). It also catalyses the conversion of 1-acyl-sn-glycero-3-phosphocholine to 1,2-diacyl-sn-glycero-3-phosphocholine (Argawal and Garg, 2010 and Moessinger et al. 2011).

LPCAT3 is active in mediating 1-O-alkyl-sn-glycero-3-phosphocholine acylation with long chain fatty acyl-CoAs to generate 1-O-alkyl-phosphatidylcholine, a very essential constituent of the cell membrane systems of mammals (Kazachkov et al. 2008). The enzyme has been found to be localised to the endoplasmic reticulum and most abundantly expressed in the liver, pancreas and adipose. LPCAT3

overexpression in tissue culture has been found to increase the amount of phospholipids with relatively more saturated acyl chains, while its inhibition has also been found to result in an increase in the amount of phospholipids with more unsaturated acyl chains (Jain et al. 2009). Importantly, LPCAT3 prefers unsaturated fatty acyl-CoAs as substrates and appears to be the only enzyme in the liver responsible for this function (Zhao et al. 2008).

LPCAT4 is richly expressed in the brain, corresponding to the abundance of phosphatidylethanolamine in this tissue. Mouse LPCAT4 mRNA is highly expressed in the epididymis, brain, testis, and ovary (Shindou et al. 2009). LPCAT4 also exhibits significant acyl-CoA-dependent acyltransferase activity toward 1-O-alkenyl-lysophosphatidylethanolamine, lysophosphatidylglycerol, 1-O-alkyl-lysophosphatidylcholine, lysophosphatidylserine, and lysophosphatidylcholine but lacked significant acylating activity toward glycerol 3-phosphate, lysophosphatidic acid, lysophosphatidylinositol, and diacylglycerol, showing several but selective functions of LPCAT4 as an enzyme involved in phospholipid remodelling (Cao et al. 2008). Its activity is not affected by Calcium. When overexpressed in mammalian cells, LPCAT4 is seen to be localized to the endoplasmic reticulum (Cao et al. 2008).

1.2.2 Enzyme activity: the acyl transferase function

The differences in the turnover rates of the fatty acyl and glycerol moieties of phospholipids led Lands to the finding of the presence of acyl-CoA: lysophospholipid acyltransferase in rat lungs (Lands, 1958b). The enzyme system acylating the sn-1 position of 2-acyl-glycerophosphocholine (GPC) was shown to be more active with saturated acyl-CoAs, whereas the one acylating the sn-2 position of 1-acyl-GPC was more active with unsaturated acyl-CoAs (The LPCAT enzymes have since been found to be in this group). These observations emphasized the importance of these enzyme systems in the selective placement of different fatty acids between the two positions of glycerophospholipids. Acyl- CoA: lysophospholipid acyltransferase (and by extension LPCAT) activities are widely distributed in various cells and tissues, and are tightly bound to microsomal and plasma membranes (Yamashita et al. 1997).

The basic enzyme activity of the LPCAT family is the transfer of an acyl group (RCOO-) to Lysophosphatidylcholine to form Phosphatidylcholine in the deacylation-reacylation reactions of the Lands cycle as shown in Fig. 3 below.

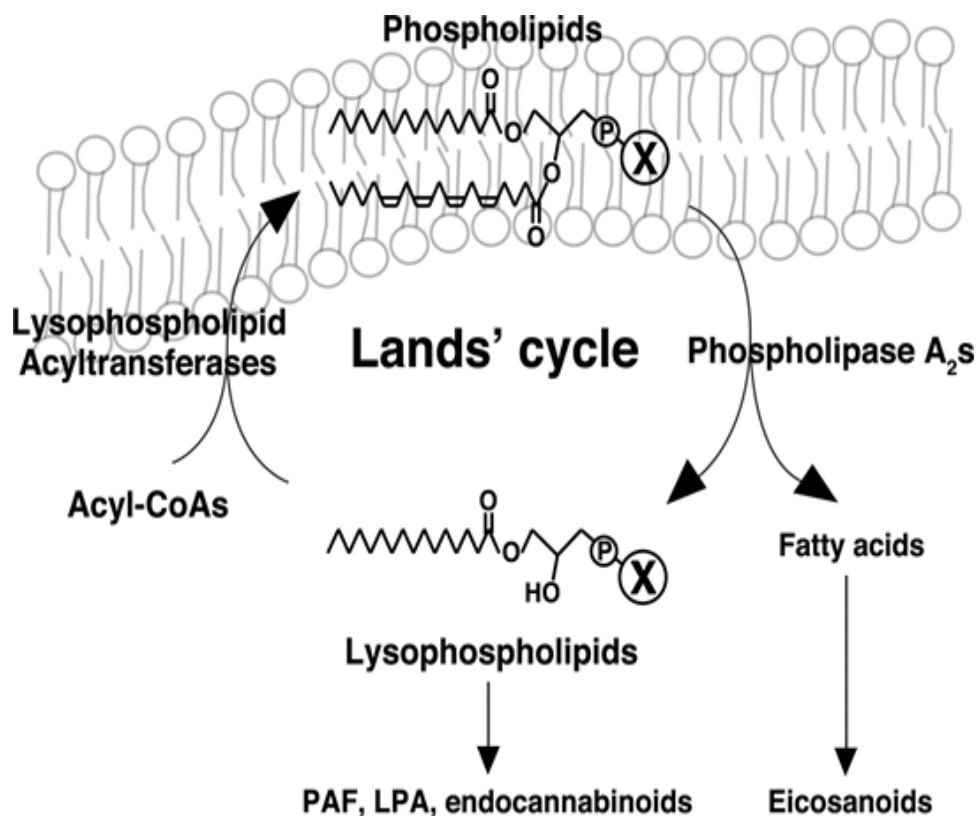


Figure 3: The rapid deacylation/reacylation of cellular phospholipids is known as the Lands cycle. In this cycle, cellular phospholipases produce lysophospholipids (LPLs) and free fatty acids, which are then recycled to generate novel or different arrangements of phospholipids and fatty acids. The Lands cycle also constitutes the foremost pathway for incorporation and release of free arachidonic acid into cellular phospholipid. (Figure from Hishikawa et al. 2008).

First, Phosphatidylcholine is deacylated to a Lysophosphatidylcholine by PLA₂, and then subsequently reacylated back to Phosphatidylcholine with a different acyl chain composition. The reacylation of Lysophosphatidylcholine to Phosphatidylcholine is catalysed by the action of Lysophosphatidylcholine:acyl-CoA acyltransferase (EC 2.3.1.23, 1-acylglycerophosphocholine acyltransferase, LPCAT) in the following reaction:

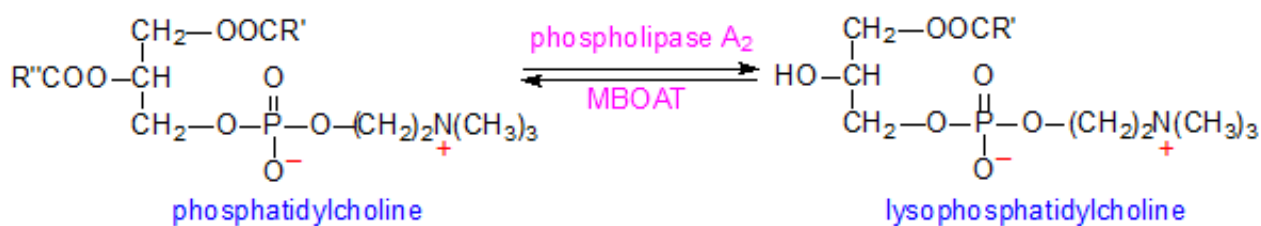


Figure 4: The MBOAT (membrane bound O-acyl transferase) family of membrane proteins is a family of various acyltransferase enzymes including enzymes involved in neutral lipid biosynthesis, protein/peptide acylation and phospholipid remodelling (Figure taken from Lipidlibrary.aocs.org (2015)).

This acylation-deacylation of membrane phospholipids is a mechanism for incorporating unsaturated fatty acids (mainly arachidonic acid) into different phospholipids to provide a range of lipid mediators (Jackson et al. 2008(b)) which are essential in various cellular functions. The Lands cycle may be critical for proper membrane fluidity, vesicle trafficking, signal transduction and other relevant biological functions (Hishikawa et al. 2014). In pancreatic cells for instance, phospholipid remodelling is believed to play an essential role in amplifying the signal for insulin secretion; and in hepatocytes, proper phospholipid composition appears vital for very low-density lipoprotein assembly and secretion (Tran et al. 2006 and Nolan et al. 2006).

1.2.3 Enzyme activity: the acetyltransferase function

Platelet-activating factor (1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine or PAF) and alkyl-lysophosphatidylcholine (Lyso-PAF) are phosphatidylcholine-derived molecules with important roles in inflammatory processes. PAF can be synthesized through two different pathways: the de novo and a recovery pathway. The recovery pathway involves the hydrolysis of alkyl-PC at sn-2 position by PLA2 with the production of lyso-PAF which can be acetylated, in the presence of acetyl-CoA, in a reaction catalysed by the major enzyme lyso-PAF:acetyl-CoA acetyltransferase (PAF-AT, EC 2.3.1.67) (Stanca et al. 2013).

It has been reported that LPCAT1 also possesses a lyso-PAF acetyltransferase activity. The amino acid residues of LPCAT1 that are essential for each activity (LPC-Acyltransferase or Lyso-PAF acetyltransferase activity) were identified by site-

directed mutagenesis. The LPCAT1 remodelling pathway for PAF synthesis has been found to be non-inflammatory/constitutive (Harayama et al. 2008).

LPCAT2 (also called LysoPAFAT), has also been found to catalyse both PAF and PC synthesis mainly in inflammatory cells. Thus, a single enzyme catalyses membrane biogenesis (LPC-acyltransferase activity) of inflammatory cells, while producing PAF (lyso-PAF acetyltransferase activity) in response to external stimuli. This activity of LPCAT2 is inducible and activated by inflammatory stimulation in contrast to that of LPCAT1 which is constitutive (Shindou et al. 2007 and Harayama et al. 2008). The lyso-PAF acetyltransferase activity is shown in the reaction below:

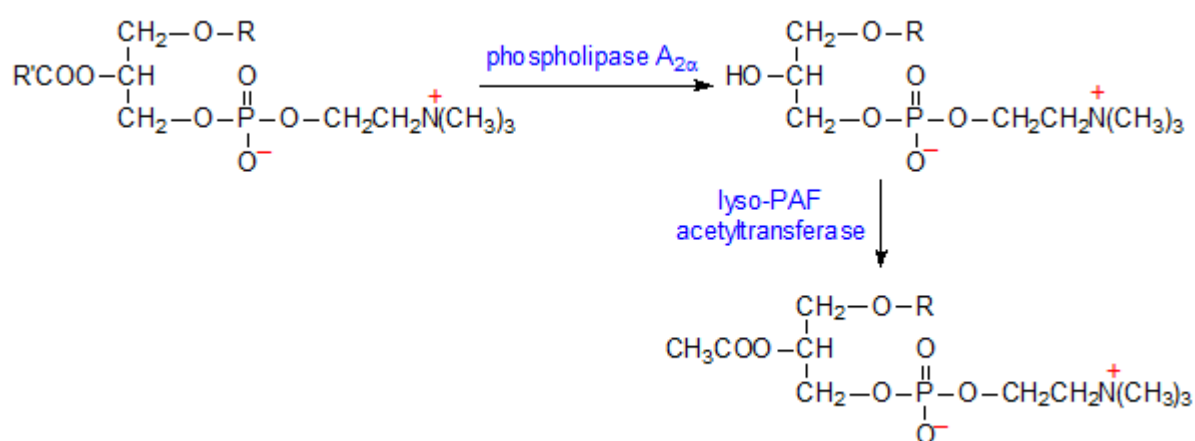


Figure 5: The acetyltransferase activity of the LPCAT enzymes. Both LPCAT1 and LPCAT2 demonstrate lyso-PAF acetyltransferase activity (Figure taken from Lipidlibrary.aocs.org (2015)).

1.3 RESEARCH PROGRESS IN THE DISCOVERY OF THE LPCAT ENZYMES

The LPCAT enzyme activity was first described in rat liver microsomes, in a paper which described the properties of the microsomal system which catalyses the acylation of lysolecithin to form lecithin (Lands, 1958a). The enzymes were further identified in bacteria in 1966 when a group of researchers gave evidence that *Escherichia coli* contains enzymes catalysing the conversion of Lyso-PC and Lyso-PE to their corresponding diacyl analogs by an acylation reaction (Proulx and Van Deenen, 1966). Further discoveries were made of the enzymes in plants (1971), insects (1977), fish (1976) and in mammals (1989).

Despite the crucial importance of the phospholipid profiles of the plasma membrane for proper membrane fluidity, vesicle trafficking and special domain formation for signalling, the understanding of the mechanistic specifics of the remodelling process was hindered by the lack of suitable molecular methods. However, in 1996, Fyrst and colleagues found that the enzyme LPCAT can be isolated in newly formed PC vesicles by solubilisation of rat liver microsomes with the two substrates lysoPC and acyl-CoA. They sought to enhance the conditions for the formation of PC vesicles and investigated the lipid composition and enzyme activity of the newly formed vesicles. This method was further used to obtain initial characterisation and sequence data on human monocyte LPCAT (Fyrst et al. 1996).

Through DNA sequencing methods which have become available, the characterization of the enzymes has been made possible. Full-length rat and mouse cDNAs coding for LPCAT have been cloned (Nikanishi et al, 2006) and the characterization of three important enzymes with different substrate specificities and tissue distributions (LPCAT 3, LPCAT 4 and LPEAT1) through gene-based techniques were carried out (Hishikawa et al. 2008 and Zhao et al. 2008). Both LPCAT 1 and LPCAT 2 were also characterized (Shindou et al. 2007 and Shindou et al. 2009). The enzymes have since been characterized in mouse and human lungs, platelets, lymphocytes and phagocytic cells (Jackson et al. 2008b).

1.4 EXPRESSION AND BIOLOGICAL ROLES OF THE LPCAT ENZYMES IN MAMMALIAN CELLS AND TISSUES

Lysophosphatidylcholine and Lysophosphatidic acid have been associated with a number of biological activities from blood vessel development to myelination, and substantial work is in progress to understand the metabolism of these lipids. The use of Lysophospholipids by acyltransferases is expected to be essential in the regulation of their availability and hence activity (Jackson et al. 2008b).

1.4.1 Inflammatory cells

The enzymes have been found to participate in the control of arachidonate levels in inflammatory cells. Studies have suggested that the initial incorporation of arachidonic acid into 1-acyl-2-lyso-GPC of these cells involves a CoA-dependent acyltransferase activity which is selective for 1-acyl-linked phospholipids (Chilton et al. 1996).

It has also been shown that both CoA-dependent and CoA-independent acyltransferases are important in macrophage activation (Jackson, 1997). Phosphorylation of LPCAT 2 at Ser34 has been discovered to enhance platelet-activating factor production in endotoxin-stimulated macrophages (Morimoto et al. 2010).

There has also been evidence that the enzymes may mediate both the priming reactions and the inflammatory response of monocytes to the cytokine interferon-gamma (Jackson and Parton, 2004 and Schmid et al. 2003). IFN-gamma has been discovered to cause a significant increase in the activity of the LPCAT and CoAIT enzymes in the microsomal fraction of human monocyte cell line MonoMac 6 (MM6), at concentrations and over a time-course consistent with a significant role for these enzymes in the sensitization of monocytes, thereby providing evidence to highlight the role of acyltransferases as part of the molecular mechanism underlying inflammation (Neville et al. 2005).

LPCAT 2 has been identified as the long-sought lyso-PAF acetyltransferase involved in PAF biosynthesis in the remodelling pathway. Its expression is mainly observed in inflammatory cells such as peritoneal macrophages and induction and activation of LPCAT 2 is regulated by LPS (Shindou et al. 2013).

The results of the investigation of the details of biosynthesis of PAF by Lyso-PAF acetyltransferase (Lyso-PAFAT) have suggested that one (LysoPAFAT/LPCAT2) is inducible and activated by inflammatory stimulation, and the other (LPCAT1) is constitutively expressed; and also, that each Lyso-PAFAT biosynthesises inflammatory and physiological amounts of PAF, depending on the cell type (Harayama et al. 2009).

A signalling role for LPCAT3 has been found in receptor-regulated arachidonic acid reacylation reactions in human monocytes. In a research considering this activity of the enzyme, in vitro activity measurements indicated that the receptor-sensitive step of the AA re-acylation pathway was the acyltransferase using lysophosphatidylcholine (lysoPC) as acceptor, and inhibition of LPCAT3 by specific siRNA resulted in inhibition of the stimulated incorporation of AA into phospholipids (Perez-Chacon et al. 2010).

1.4.2 Platelets

LPCAT activities in human platelet lysates have been studied. Linoleic acid as fatty acid substrate has the highest affinity to LPCAT with LysoPC as variable substrate, followed by eicosapentaenoic acid (EPA) and arachidonic acid (AA) (Bakken and Farstad, 1992).

1.4.3 Metabolic tissues

LPCAT3 is localized within the endoplasmic reticulum and is primarily expressed in metabolic tissues including the liver, adipose, and pancreas. In human hepatoma Huh7 cells, RNA interference-mediated silencing of LPCAT3 resulted in almost complete loss of membrane LPCAT activity, suggesting that LPCAT3 is predominantly responsible for hepatic LPCAT activity (Zhao et al. 2008).

There have been indications that the four LPCAT enzymes are expressed in the kidney and that they seem to play a physiologically critical role in reducing the pool of free arachidonic acid in the kidney (Yamazaki et al. 2009).

Liver X receptors (LXRs) are oxysterol-activated nuclear receptors that are highly expressed in macrophages and regulate lipid homeostasis and inflammation. LXR alpha was found to be involved in the regulation of cholesterol homeostasis in mammals and in the control of fat synthesis. In 2011, a group of researchers aimed

at identifying new LXR genes and found LPCAT 3 as a likely LXR target (Demeure et al. 2011) suggesting an important role for the enzyme in phospholipid homeostasis.

LPCAT 3 knockdown has also been found to significantly reduce hepatic triglycerides. Results from laboratory experiments have also indicated that hepatic LPCAT3 modulates VLDL production by regulating LysoPC levels and microsomal triglyceride transfer protein expression (Li et al. 2012).

Using C3H10T1/2, a cell line capable of differentiating to adipocyte-like cells *in vitro*, changes of LPCAT activities were recently investigated. LPCAT, LPEAT and LPSAT activities were enhanced, especially with 18:2-CoA, 20:4-CoA as donors. Similarly, mRNA expression of LPCAT3, which possesses LPCAT, LPEAT and LPSAT activities with high specificity for 18:2- and 20:4-CoA, was up-regulated during adipogenesis suggesting that increased phospholipid remodelling by LPCAT3 may be connected with adipocyte differentiation (Eto et al. 2012).

1.4.4 Lungs and surfactant production

Pulmonary surfactant is a complex of lipids and proteins most of which are produced and secreted by alveolar type II cells, which provide the low surface tension at the air–liquid interface. There have been significant indications that LPCAT plays a key role in regulating surfactant phospholipid biosynthesis in alveolar type II cells and that understanding the regulation of the enzyme will provide important insights into surfactant phospholipid biosynthesis (Chen et al. 2006 and Harayama et al. 2009). The cloning and characterization of a cDNA for mouse lung-type LPCAT (LPCAT1) have further strengthened these indications (Nikanishi et al. 2006).

To investigate the role of LPCAT 1 in surfactant production, Bridges and colleagues generated mice bearing a hypomorphic allele of LPCAT1 (here referred to as LPCAT1GT/GT mice). New-born LPCAT1GT/GT mice developed serious respiratory difficulties of different degrees resulting in the death of some of the animals. Also, there was a significant decline in LPCAT 1 activity and saturated PC content in the same group. Surfactant isolated from dead LPCAT1GT/GT mice was unable to reduce minimum surface tension to wild-type levels. Taken together, their data showed that full LPCAT1 activity is essential to the achievement of the levels of saturated PC required for the transition to air breathing (Bridges et al. 2010).

1.4.5 Brain

The deacylation–reacylation cycle is an important mechanism responsible for the introduction of polyunsaturated fatty acids into neural membrane glycerophospholipids (Kitson et al. 2012). It involves four enzymes, namely acyl-CoA synthetase, acyl-CoA hydrolase, acyl-CoA: lysophospholipid acyltransferase, and phospholipase A2 (Kitson et al. 2012). All of these enzymes have been purified and characterised from brain tissue (Farooqui et al. 2000).

All four isoforms of LPCAT have been found to be expressed in brain tissue where they are involved in infant neurodevelopment and neurological diseases associated with aging such as Alzheimer's disease and cognitive decline (Kitson et al. 2012).

1.5 LPCAT ENZYMES AND DISEASE PROCESSES

1.5.1 Brain injury

The accumulation of arachidonic acid and lysophospholipids alongside lipid peroxides and eicosanoids have been discovered to result in neural inflammation, oxidative stress and neurodegeneration. The deacylation-reacylation (Land's cycle) in which the LPCAT enzymes play a prominent role maintains an equilibrium between free and esterified fatty acids leading to low levels of these compounds. Thus, the Land's cycle is essential for not only normal membrane integrity and function, but also for the optimal functioning of the membrane-bound enzymes, receptors, and ion channels involved in normal signal-transduction processes (Farooqui et al. 2000). Hence, a disturbance in the equilibrium maintained by the Land's cycle could play a key role in the pathophysiological processes in brain injury.

1.5.2 Barth Syndrome

First described in 1983, Barth syndrome is broadly regarded as a rare X-linked genetic disease characterised by skeletal myopathy, cardiomyopathy, neutropenia, growth delay, and increased urinary excretion of 3-methylglutaconic acid (Barth et al. 1983 and Clarke et al. 2013). Tafazzin (TAZ) has been identified as the gene responsible for the condition and is located on region q28 of the X chromosome. It has significant protein homology to other acyltransferase family members and is believed to affect lipid metabolism (Jackson et al. 2008(b)). This suggests a possible

role of the acyltransferases in the pathogenesis of this disease or a potential therapeutic target.

1.5.3 Endometriosis

Current non-invasive diagnostic methods for endometriosis lack sensitivity and specificity (Vouk et al. 2012). Results from laboratory experiments conducted by Vouk and colleagues showed that endometriosis is associated with elevated levels of sphingomyelins and phosphatidylcholines, which may contribute to the suppression of apoptosis and affect lipid-associated signalling pathways; suggesting novel potential routes for therapy by specifically blocking highly up-regulated isoforms of Phospholipase A2 and LPCAT 4 (Vouk et al. 2012).

1.5.4 Cancer

Lysophospholipids stimulate the growth, survival and migration of tumour cells from various origins (ovary, prostate, glioblastoma), they are amply expressed in malignant effusions and the Lysophospholipid-producing enzymes have been found to be tumorigenic (Raynal et al. 2005).

The identification of cancer biomarkers is crucial for target-linked cancer therapy. The overall level of phosphatidylcholine is elevated in colorectal cancer (Merchant et al. 1991 and Mansilla et al. 2009). Currently existing data show the potential usefulness of PC (16:0 / 16:1) for the clinical diagnosis of colorectal cancer and associate LPCAT4 with the elevated expression of PC (16:0 / 16:1) in the disease (Kurabe et al. 2013).

The mRNA and protein of LPCAT1 have been found to be more abundantly expressed in hepatocellular carcinoma cells than in the surrounding parenchyma. In cell line experiments, LPCAT1 overexpression promoted cell proliferation, migration, and invasion while its silencing did the opposite (Morita et al. 2013).

LPCAT1 expression shows a relationship with the development of prostate cancer and is believed to be a new biomarker in diagnosis, prognosis and studying the pathogenesis of the malignancy (Zhou et al. 2012). The prognostic impact of LPCAT1 expression has been found to be independent of histological and clinical parameters. Therefore, LPCAT1 measurement, either alone or in combination with other prognostic markers of prostate cancer, may be employed for better clinical

decision-making in the management of the malignancy (Grupp et al. 2013). It has also been shown that PAF might play pivotal roles in the progression of prostate cancer (Xu et al. 2013). It is noteworthy that this lipid signalling molecule can be generated by the remodelling of phosphatidylcholine: an activity of LPCAT.

Bussolati and fellow researchers conducted experiments whose results suggested that PAF, produced and released by breast cancer cells, can contribute to tumour progression by enhancing cell motility and proliferation and by stimulating the angiogenic response (Bussolati et al. 2000), again highlighting the function of LPCAT in the disease process.

1.5.5 Chronic inflammatory disorders

The activities of the LPCAT enzymes have been associated with the pathogenesis of rheumatoid arthritis, multiple sclerosis, pulmonary fibrosis and hepatitis (Sevastou et al. 2012). In particular, the ratio of PC/LysoPC in plasma have been found to be a reliable measure of inflammation in rheumatoid arthritis and possibly other inflammatory conditions (Fuchs et al. 2005).

1.5.6 Diabetic retinopathy

Elevated levels of PAF and LPC are associated with the onset of diabetic retinopathy and neurodegeneration (Cheng et al. 2009). LPCAT 1 is abundantly expressed in the retina and has been found to be involved in the anti-inflammatory responses to diabetic retinopathy (Cheng et al. 2009). In a research conducted by Cheng and colleagues, LPCAT1 mRNA levels and acyltransferase activity toward lyso-PAF and LPC were markedly down-regulated in retina and brain tissues in response to the onset of diabetes in mouse models of Type 1 and Type 2 Diabetes. On the other hand, treatment of one of the mouse models with rosiglitazone, a hypoglycaemic agent resulted in a significant up-regulation of LPCAT1 mRNA levels simultaneously with increased acyltransferase activity in the retina and brain. Taken together, these findings revealed a novel regulatory role of LPCAT1 in catalysing the inactivation of inflammatory lipids in the retina of diabetic mice (Cheng et al. 2009).

1.5.7 Sepsis

The LPCAT enzymes have been associated with the overwhelming response of macrophages to LPS and therefore, in sepsis. Jackson and colleagues showed that LPCAT regulates inflammatory responses to LPS and other microbial stimuli (Jackson et al. 2008a). In the experiments, specific inhibition of LPCAT down-regulated inflammatory cytokine production in monocytes and epithelial cells by preventing translocation of Toll-like receptor 4 into membrane lipid raft domains. Their observations reveal a new regulatory mechanism that facilitates the innate immune responses to microbial molecular patterns and suggested a possible pathway for the anti-inflammatory activity seen in many phospholipid metabolites. This provided the possibility of the development of new classes of anti-inflammatory and antisepsis agents (Jackson et al. 2008(a)).

Drobink and colleagues (2003) analysed the plasma levels of Ceramide and LPC by quantitative tandem mass spectrometry in 102 sepsis patients starting with the day at which the sepsis criteria were fulfilled for the first time, as well as on day 4 and day 11. The values were compared with 56 healthy controls and showed a positive relationship with sepsis-related mortality within 30 days of study entry. In the study, Ceramide species were increased in sepsis patients, while all LPC species were significantly decreased. In addition, the molar ratios with their precursor molecules sphingomyelin (SPM) and phosphatidylcholine (PC) were determined, which reflect the enzymatic reactions responsible for their formation. Species-specific as well as total Ceramide-SPM ratios were increased, whereas LPC-PC ratios were decreased in sepsis patients. The increased Ceramide-SPM ratios as well as the decreased LPC-PC ratios showed a strong predictive power for sepsis-related mortality.

1.6 THE LPCAT ENZYMES AS NOVEL TARGETS FOR ANTI-SEPSIS THERAPIES

A number of challenges have been encountered in the search for specific therapies against inflammatory diseases, despite recent successes. The major impediments have been lack of responsiveness to these medications, drug resistance, problems with drug delivery and the high cost of manufacture. Signal transduction has been identified as a strategy for the development of effective therapies against these diseases. (O'Neill, 2006). The modulation of inflammatory gene expression by lipids,

particularly through the Toll-like receptors, has encouraged the development of new classes of anti-inflammatory molecules based on lipid modifications (Lee and Hwang, 2006).

A previous study investigated the ability of two structurally diverse inhibitors (SK&F 98625 and SK&F 45905) of CoA-Independent Transacylase, (an enzyme which has been proposed to mediate the movement of arachidonate between specific phospholipid subclasses) to inhibit the enzyme using two assay systems: the transacylation of 1-alkyl-2-lyso-sn-glycero-3-phosphocholine (GPC) and the transfer of [14C]arachidonate from 1-acyl-2-[14C]arachidonoyl-GPC to lyso-PE. Both compounds inhibited CoA-IT activity in both assays, but had little or no effect on other lipid-modifying activities, including CoA-dependent acyltransferase or acetyltransferase. In intact human neutrophils, both compounds completely blocked the movement of [3H] arachidonate from 1-acyl-linked phospholipids into 1-alkyl-2-arachidonoyl-GPC and 1-alk-1'-enyl-2-arachidonoyl-GPE. Both compounds also inhibited PAF production and arachidonic acid release (Winkler et al. 1995). These results showed that the enzyme activities of CoA-Independent transacylase which are important in arachidonate remodelling between phospholipids, PAF production, arachidonic acid release and the formation of eicosanoid products can be successfully inhibited and this can be used as an effective pathway for the production anti-sepsis therapies as these activities of the enzyme are known to be fundamental to the pathophysiology of sepsis.

Experiments demonstrating the effectiveness *in vitro* and studies showing the therapeutic effects of lysoPA and lysoPC in experimental models of sepsis and organ failure have demonstrated the possible role of LPCAT inhibition in the treatment of sepsis and potentially other inflammatory conditions (Yan et al. 2004 and Murch et al. 2006). In one of such experiments, treatment with LPC markedly enhanced clearance of intraperitoneal bacteria and blocked Caecal Ligation and Puncture (CLP) - induced deactivation of neutrophils, *in vivo*. *In vitro*, LPC increased bactericidal activity of neutrophils by enhancing Hydrogen peroxide production in neutrophils that ingested *E. coli* (Yan et al. 2004).

The use of acyltransferase inhibitors as probes for studying the potential role of lysophospholipid acyltransferases in intracellular membrane trafficking in the

secretory and endolytic pathways has been put forth. CI-976 has been referred to as a useful addition to the pool of small molecule inhibitors that can be used to study secretory and endocytic membrane trafficking pathways (Brown and Schmidt, 2005).

Also along the line of the LPCAT enzymes being potential targets for anti-sepsis therapies, Jackson and colleagues have used selective inhibitors for LPCAT identified by high-throughput screening. 5 hydroxyethyl 5,3' thiophenyl pyridine, a non-competitive specific inhibitor of CoA-dependent LPCAT was identified as a promising candidate. A cDNA sequence for LPCAT from human monocytes has also been identified and this sequence is being used to develop inhibitory RNA sequences as potential LPCAT inhibitors (Jackson et al. 2008a).

It is to be noted that inhibitory RNA sequences have the advantage of specificity over chemical inhibitors, and for that reason, they were utilized for gene silencing in this research.

1.7 STATEMENT OF THE PROBLEM

Conventional treatments have focused on source control, antimicrobials, vasopressors, and fluid resuscitation with minimal success at salvaging the lives of sepsis patients.

Research has shown that severely septic patients continue to experience undue morbidity and mortality even with recent progress in critical care. Although substantial resources have been invested in new treatments, almost all have failed to improve outcomes (Rice, 2006). Also, the injurious effects of sepsis continue beyond the acute process. Patients who survive the initial episode experience higher rates of death for the first year after hospital discharge compared with age-matched controls (Weycker et al. 2003).

The history of therapeutic interventions in clinical trials for sepsis has been referred to as the "graveyard for pharmaceutical companies." (Riedemann et al. 2003). Corticosteroids, Anticoagulant therapy, Activated protein C, High Mortality Group B1 protein (HMGB1), Macrophage Migration Inhibitory Factor (MIF), Complement C5a and its receptor C5aR and apoptosis inhibitors have all been through clinical trials

and so far, most clinical trials have generally not improved the overall outcome (enhanced survival) of sepsis patients (Riedemann et al. 2003).

The success of anti-sepsis therapies clearly depends on adequately modulating the immune response (with the use of specific treatments) with the least possible adverse effect to the host. It is therefore important to understand in more detail the various dynamics of pathophysiological responses that lead to hyperactive or suppressed immune and inflammatory responses (Riedemann et al. 2003).

The LPCAT enzymes offer novel possibilities of specific therapies for the overwhelming inflammatory responses of sepsis; namely, the control of the PC/LysoPC balance. There have been indications that the induction of the Isoform LPCAT 3 which has been characterised in Sf9 insect cells (Jain et al. 2009) results in the priming of macrophages to release inflammatory mediators (Ishibashi et al. 2013). This research was conducted based on the hypotheses that the LPCAT 3 enzyme is involved in the inflammatory response of macrophages in the regard that it plays an important role in the release of inflammatory mediators from macrophages, in response to LPS. It therefore provides answers to the primary research questions which bordered on the involvement of the LPCAT3 enzyme in the inflammatory response of macrophages, the effect of LPCAT3 knockdown on the expression of cytokines and the most suitable reference gene for the analysis of these results. The outcome of this research would broaden the knowledge base available as regards possible candidates for specific anti-sepsis therapies in the near future.

1.8 PURPOSE OF THE STUDY

1.8.1 Aim

This research was aimed at investigating the role of Lysophosphatidylcholine acyltransferase 3 (LPCAT 3) in macrophage inflammatory responses following suitable reference gene identification. This aim was achieved with the following objectives:

1.8.2 Objectives

1. To identify the most suitable reference gene in the analysis of the response of the LPCAT3 gene to LPS-induced inflammation.
2. To establish the involvement of the LPCAT 3 enzyme in the inflammatory response of macrophages.
3. To investigate the effect of LPCAT 3 gene knockdown on Lipopolysaccharide-induced inflammatory responses in macrophages.

CHAPTER 2: MATERIALS AND METHODS

2.1 MATERIALS

MATERIAL	SOURCE AND COUNTRY
CELL CULTURE	
Dimethyl Sulfoxide (DMSO)	Fisher Scientific UK Ltd., UK
DMEM culture media	Lonza Group Ltd., UK
<i>E. coli</i> Lipopolysaccharide (LPS) O111:B4	Sigma-Aldrich Co. LLC., UK
Fetal bovine serum (FBS)	Biosera (Labtech) International Ltd, UK
L-glutamine	Lonza Group Ltd., UK
Optimem culture media	Life Technologies Ltd., UK
RAW264.7 cell line	European Collection of Cell Cultures (ECACC), Health Protection Agency (HPA),UK
Transfection reagent (Interferin)	Polyplus-transfection Inc., UK
ELISA	
Bovine Serum Albumin	Sigma-Aldrich Co. LLC., UK
ELISA TMB Substrate	Insight Biotechnology Ltd., UK
Maxisorp 96-well ELISA plates	Fisher Scientific UK Ltd., UK
Mouse TNF-alpha ELISA duo set-kit	R&D Systems, UK
Plate sealers	Fisher Scientific UK Ltd., UK
Tween-20	Fisher Scientific UK Ltd., UK
Versa Max Microplate Reader with Softmax Pro Software	Molecular devices, USA

PLASTIC AND GLASS WARE	
6 well plates	Greiner (Scientific Supplies) Ltd., UK Laboratory
25cm ² tissue culture flasks	Greiner (Scientific Supplies) Ltd., UK Laboratory
75cm ² tissue culture flasks	Greiner (Scientific Supplies) Ltd., UK Laboratory
Scrapers	Greiner (Scientific Supplies) Ltd., UK Laboratory
Universal containers	Greiner (Scientific Supplies) Ltd., UK Laboratory
REVERSE TRANSCRIPTION AND PCR	
High Capacity RNA – to - cDNA Kit	Life Technologies Ltd., UK
Mini plate spinner centrifuge	Labnet International. Inc, USA
Nanodrop systems	GE Healthcare Life Sciences, USA
PCR plates	Life Technologies Ltd., UK
Primers	Eurofins MWG Operon, Germany
Real time PCR kit	Life Technologies Ltd., UK
StepOnePlus Real- Time PCR System	Life Technologies Ltd., UK
Veriti 96 well Thermal Cycler	Life Technologies Ltd., UK

RNA EXTRACTION

Bromochloropropane

Sigma-Aldrich Co. LLC., UK

Ethanol

Fisher Scientific UK Ltd., UK

Glacial acetic acid

Fisher Scientific UK Ltd., UK

Guanidinium thiocyanate

Fisher Scientific UK Ltd., UK

Mecarptoethanol

Fisher Scientific UK Ltd., UK

Sarkosyl

Fisher Scientific UK Ltd., UK

Sodium acetate

Fisher Scientific UK Ltd., UK

TRIS-base

Fisher Scientific UK Ltd., UK

Water saturated Phenol

Fisher Scientific UK Ltd., UK

2.2 METHODS

2.2.1 CELL CULTURE

RAW 264.7 cells (Adherent macrophage cell line derived from an Abelson murine leukaemia virus-induced tumour and isolated in 1978) were maintained in DMEM media supplemented with 10% Foetal Bovine Serum and 1% L-glutamine as suggested by the supplier. The cells were cultured at 37⁰C in a humidified CO₂ incubator. The cells were sub-cultured in a class 2 cabinet twice a week under sterile conditions with a media change session in between.

2.2.1.1 Determination of Cell Viability

Cell viability was determined by the Trypan blue dye exclusion method. This involved a 1:8 serial dilution of the cells and then, cell staining with an equal volume of the Trypan blue dye. The percentage of unstained cells (viewed and counted under the microscope at X10 magnification) correlated with the percentage of viable cells.

Cell viability of 90% and above was considered acceptable.

2.2.1.2 FREEZING AND STORAGE OF CELLS.

Upon delivery, the cells were stored in liquid Nitrogen at -180⁰C. The cryovials of the cells were taken out from the liquid Nitrogen tank following the SOP for Liquid Nitrogen handling /sample storage and retrieval from liquid Nitrogen freezers. The liquid Nitrogen tanks were also used for the long term storage of the cells till they were ready to be used.

2.2.1.3 THAWING AND GROWING FROZEN CELLS.

Thawing of the cells (for ~3 minutes in a water bath at 37⁰C) was done in preparation for cell culture. The cell suspension was then transferred from the cryovial to a universal container containing 10ml of cell culture medium and centrifuged for 5 minutes at a speed of 900g. The pellets were then re-suspended in 10ml of fresh medium and transferred into tissue culture flasks and cultured as stated in section 2.2.1 above.

2.2.3 LPCAT3 GENE SILENCING

RAW 264.7 cells at a concentration of 8×10^4 cells/mL/well were seeded onto 6-wells (RT-PCR) and 7×10^4 cells/mL/well onto 12-wells plates (ELISA) in DMEM culture medium supplemented with foetal bovine serum and L-glutamine (complete medium) and the cells were incubated for 24 hours at 37°C. The cells were then transfected in reduced serum OPTI-MEM medium with a control negative siRNA (Ambion) or siRNA directed against LPCAT-3 (Ambion siRNA ID s67014) at final concentration of 5nM using INTERFERin transfection reagent (A non-liposomal cationic amphiphile transfection reagent developed for the delivery of siRNA into the cells in culture). After 24 hours, the medium was changed and a complete DMEM medium added and the cells were stimulated with LPS. The expression of LPCAT-3, the inflammatory cytokines and reference genes mRNA were measured by quantitative real time PCR after reverse transcription (as described in section 2.2.5.3) while the released TNF- α was assayed by ELISA using a DuoSet kit (as described in section 2.2.6).

2.2.4 STIMULATION OF CELLS WITH LPS

RAW 264.7 cells were stimulated with *E. coli* Lipopolysaccharide (LPS) O111:B4 at a concentration of 1 μ g/ml for 4 hours (RT-PCR) and for 24 hours (ELISA, for which the supernatants were collected and stored at -80°C until assayed). This was done 24 hours after LPCAT3 gene silencing.

2.2.5 REAL TIME QUANTITATIVE POLYMERASE CHAIN REACTION (RT-qPCR)

2.2.5.1 TOTAL RNA EXTRACTION.

The total RNA was extracted using the Acid Guanidinium Thiocyanate-Phenol-Chloroform extraction method as described by Chomczynski and Sacchi (1987) with minor modifications which included the use of Bromochloropropane instead of Chloroform to introduce the liquid interphase and the re-suspension of the RNA pellet with 75% Ethanol to dissolve residual Guanidinium Thiocyanate, twice, as against its re-suspension only once, described in the original extraction method. Denaturation was done with Guanidinium Thiocyanate denaturing solution (containing Mearpto-ethanol) and Sodium acetate pH4 was used for acidification.

RNA precipitation was done with Isopropanol. The pellets were air-dried and subsequently dissolved in RNase and DNase-free water and stored at -80°C (Chomczynski and Sacchi, 1987).

2.2.5.2 TOTAL RNA QUATITATION.

The total RNA in each sample was quantified using the Nanodrop machine and software. RNA purity was assessed by observing the ratio of absorbance at 260nm and 280nm. A ratio of 1.8-2.1 was accepted as pure.

The RNA quantitation results are presented in table 2 below.

Experiment	RNA concentration (µg/ml)	Ratio of absorbances (A260/A280)
Experiment 1		
Sample 1	255	2.13
Sample 2	116	2.24
Sample 3	172	2.23
Experiment 2		
Sample 1	376	1.97
Sample 2	472	2.03
Sample 3	400	1.87
Experiment 3		
Sample 1	436	1.94
Sample 2	482	1.96
Sample 3	430	1.89

Table 2: RNA quantitation results, including data for the assessment of RNA purity. The ratio of absorbances at the wavelengths of 260nm and 280nm indicated the purity of the RNA samples. A ratio of 1.8-2.1 was accepted as pure.

2.2.5.3 REVERSE TRANSCRIPTION

Total RNA (0.5µg) were reverse transcribed to cDNA using High capacity RNA to cDNA reverse transcriptase Kit in thin walled PCR tubes following the manufacturer's instruction. The Veriti 96 well Thermal Cycler machine was used to reverse transcribe the RNA to the cDNA. The samples were stored at - 20° C until used.

2.2.5.5 PRIMER DESIGN

The primers for LPCAT-3, TNF, IL-10 and all the reference genes (Table 4) were designed using Primer Express software (Life Technologies Ltd, UK) to amplify 100bp product for all the targets. The specificity of each sequence was checked by NCBI nucleotide Basic Local Alignment Search Tool (BLAST) (Altschul et al. 1990). The sequences were also checked for hairpin, self-dimer and heterodimer using OligoAnalyser 3.1 (Integrated DNA Technologies).

2.2.5.4 REAL TIME PCR.

cDNA from the reverse transcription reaction (please see section 2.2.5.3) was amplified using Power SYBR Green kit according to the supplier's instructions. 0.6µl of cDNA from RT reaction was used for each PCR reaction. Negative controls consisting of reactions lacking RT were run in some of the experiments to check the purity of the RNA samples (for any DNA contamination). Reaction conditions were 95°C for 10 minutes at the holding stage, followed by 40 cycles at 95°C for 15 seconds and 60°C for 60 seconds at the cycling stage. For the melting curve stage, reaction conditions were 95°C for 15 seconds, 60°C for 60 seconds and 95°C for a further 15 seconds.

The melting curve analysis was used to assess whether the PCR assays have produced single, specific products. Results from this analysis showed that the melting temperatures of the PCR products were optimal and the graphs showed satisfactory evidence that the products were specific and that there were no primer dimers present in the reaction plates.

2.2.6 ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA).

ELISA experiments were performed to assay the protein expression of TNF-alpha, following the manufacturer's instructions. Briefly, 0.8µg/ml of capture antibody was made to bind to the wells of a Maxisorp 96-well ELISA plate (The wells were coated with diluted capture antibody, sealed and incubated overnight at room temperature). Any nonspecific binding sites on the surface were blocked. The antigen-containing sample was then applied to the plate which was washed to remove unbound antigen.

50ng/mL of the detection antibody was then added with a subsequent washing to remove the unbound antibody-enzyme conjugates. This was followed by the addition of Streptavidin-HRP, the substrate solution and the stop solution. The absorbance of the plate wells was measured using the Versa Max Microplate Reader with Softmax Pro Software to determine the presence and quantity of antigen.

2.2.6 DATA ANALYSIS.

At least, three independent experiments (for PCR and for ELISA) were set and done in duplicate.

PCR results were analysed using a number of algorithms. The PC Miner software (Zhao et al. 2005) was used to calculate the Cycle Threshold and Efficiency values of the genes of interest. Best Keeper (Pfaffl et al. 2004), geNorm (Vandesompele et al. 2002) and Normfinder (Andersen et al. 2004) algorithms were used to determine the most stably expressed reference genes. The Relative Expression Software Tool (REST) (Pfaffl et al. 2002) was used to normalise the expression of the genes of interest. The T test provided by the GraphPad software was used to determine statistically significant results for both PCR and ELISA experiments. A P-value <0.05 was considered significant.

Details of the reference genes used in this research are shown in Table 3.

Reference gene	Basic function	Functional Class	Accession Number
RNA Polymerase 13A (RPL13A)	Associated with ribosomes but is not required for canonical ribosome function and has extra-ribosomal functions. Component of the GAIT (gamma interferon-activated inhibitor of translation) complex which mediates interferon-gamma-induced transcript-selective translation inhibition in inflammation processes	Ribosomal proteins	NM_009438
Adenosine Triphosphate Synthase subunit Beta (ATP5B)	Encodes a subunit of mitochondrial ATP synthase which catalyzes ATP synthesis, utilizing an electrochemical gradient of protons across the inner membrane during oxidative phosphorylation	Mitochondrial	NM_016774
β -Actin (ACTB)	Encodes a protein that is involved in cell motility, structure and integrity	Cytoskeletal	NM_007393
β 2- Microglobulin (B2M)	Encodes a protein that associates with the alpha chain of MHC class I molecules and class I-like molecules such as CD1 and Qa	HLA/Immunoglobulin/Cell recognition	NM_009735
Cancer Susceptibility Candidate 3 (CASC-3)	Encodes a protein which is a core component of the exon junction complex (EJC), a protein complex that is deposited on spliced mRNAs at exon-exon junctions and functions in nonsense-mediated mRNA decay(NMD).	Cancer related genes/Transporters	NM_138660.2
Eukaryotic initiation factor 4A-II (EIF4A2)	Encodes the ATP-dependent RNA helicase which is a subunit of the eIF4F complex involved in cap recognition and is required for mRNA binding to ribosome. In the current model of translation initiation, eIF4A unwinds RNA secondary structures in the 5'-UTR of mRNAs which is necessary to allow efficient binding of the small ribosomal	Translation factors	NM_001123038

	subunit, and subsequent scanning for the initiator codon		
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	Encodes an enzyme of ~37kDa that catalyzes the sixth step of glycolysis and thus serves to break down glucose for energy and carbon molecules	Carbohydrate metabolism	NM_008084
Succinate dehydrogenase complex, subunit A (SDHA)	Encodes the protein complex that catalyzes the oxidation of succinate (succinate + ubiquinone => fumarate + ubiquinol)	Citric acid cycle	NM_023281.1
Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (YWHAZ),	Encodes a protein that belongs to the 14-3-3 family of proteins which mediate signal transduction by binding to phosphoserine-containing proteins	Kinases/Signalling	NM_011740
Ubiquitin C (UBC)	Encodes a protein which is required for ATP-dependent, non-lysosomal intracellular protein degradation of abnormal proteins and normal proteins with a rapid turnover	Proteosome	NM_019639

Table 3: Details of all 10 reference genes analysed in this research, including their basic functions, functional classes and accession numbers. It was important that all reference genes be selected from different functional classes to avoid the problem of co-regulation.

CHAPTER 3: RESULTS

3.1 IDENTIFYING THE MOST SUITABLE REFERENCE GENE IN THE ANALYSIS OF THE RESPONSE OF THE LPCAT3 GENE TO LPS-INDUCED INFLAMMATION.

It has been established that in the internal standardization of target gene expression data, the stability of the expression of a reference gene is of utmost importance. This reference gene, often referred to as an endogenous control is instrumental to accurate calibration in many biotechnological applications and genomic studies, qRT-PCR being one of the most important. In fact, the standardization with an endogenous control whose expression is believed to be constant has been considered the method of choice in relative quantitation experiments (Suzuki et al, 2000).

Because these endogenous controls are required for the maintenance of basal cellular functions essential to the existence of a cell, they are expected to be stably expressed in all cells of an organism regardless of tissue type, cell cycle state, developmental stage or external signal (Schmittgen and Zakrajsek, 2000, Radonić et al. 2004 and Jain et al. 2006).

In this research, it was deemed important that the results from the qRT-PCR be appropriately calibrated with endogenous controls. While the conventional practice has been the use of one control gene in the standardization of the results from experiments, this practice has been considered unreliable as many so called endogenous controls with assumed stable expression have been found to be capable of exhibiting either up- or down-regulation under some experimental conditions (de Jonge et al. 2007, Goidin et al. 2001, Ross et al. 2000 and Vandesompele et al. 2002). Consequently, it was considered essential to analyse the stability of the expression of ten endogenous controls and to use the most stably expressed genes in the standardization of the experiments.

Reference Gene	Sequence (5' -> 3') Foward	Reverse	MeltingTemp erature (°C)
mSDHA	TTACCTGCGTTTCCCCTCAT (20)	CAGCAAGTACTACCAAGCTGTTGATC (26)	60
mUBC	CAAGAAGGTCAAACAGGAAGACAGA (25)	AAGACACCTCCCCCATCACA (20)	60
mYWHAZ	GTTGCTGCTGGTGATGACAAGA (22)	GGTGTGTCGGCTGCATCTC (19)	60
mGAPDH	CCTCGTCCCGTAGACAAAATG (21)	TCTCCACTTTGCCACTGCAA (20)	60
mIL-10			
mTNF	AGGACCCAGTGTGGGAAGCT (20)	AAAGAGGAGGCAACAAGGTAGAGA (24)	60
mATP5B	AGGGTGGGAAAATCGGACTC (20)	AAATCATTGCCCTCACGGGT (20)	60
mB2M	GCTATCCAGAAAACCCCTCAAA (22)	GCGGGTGGAAGTGTGTTACG (20)	60
mEIF4A2	TGTTGAGCGAGAGGAGTGGAA (21)	CACCTTGCCTTGTATTGA (20)	60
mRPL13A	CCTGCTGCTCTCAAGTTGTT (21)	CTGTCACTGCCTGGTACTTCCA (22)	60
mβ-Actin	GGCTCTTTTCCAGCCTTCCT(20)	GTCTTTACGGATGTCAACGTCACA(24)	60
mCASC-3	GCTCCGGGTGTCATGAATTT(20)	GAGCCTGCGTGTTAGGATACAGA(23)	60
LPCAT-3	TTCTGGTCCGCTGCATGTA(20)	CCGACAGAATGCACACTCCTT(21)	60

Table 4: Sequences of primers for real time quantitative PCR

3.1.1 Determination of the CT and Efficiency values of candidate genes by PC Miner

The PC Miner software developed by Zhao and Fernald (2005) was used for the objective determination of the CT and Efficiency values of all the endogenous controls and genes of interest analysed in this study. The software algorithm prevented researcher biases, offered noise resistance and determined the CT and Efficiency values without the need for the preparation of standard curves.

3.1.1.1 Results: CT and Efficiency Values

Raw PCR data from all independent experiments were submitted to the PC Miner software. Utilizing the above-described algorithms, PC Miner objectively calculated the Average efficiency and CT values of all reference genes.

The PC Miner results of all 10 endogenous controls are shown in the appendix.

3.1.1.2 Expression profiles of candidate reference genes

The expression profiles of these 10 reference genes varied widely with CT values ranging from 14 to 26 cycles (Figure 6), and most of the CT values were between 16 and 20 cycles. β -Actin was the most abundantly transcribed with CT values of less than 15 cycles; ATP5B, UBC, RPL13A, B2M and GAPDH were relatively moderately expressed mRNAs with most of the CT values between 15 and 20 cycles; YWHAZ and SDHA showed CT values between 21 and 24 or slightly higher. However, EIF4A2 and CASC3 showed the lowest level of expression in all samples with CT values as high as 25 cycles.

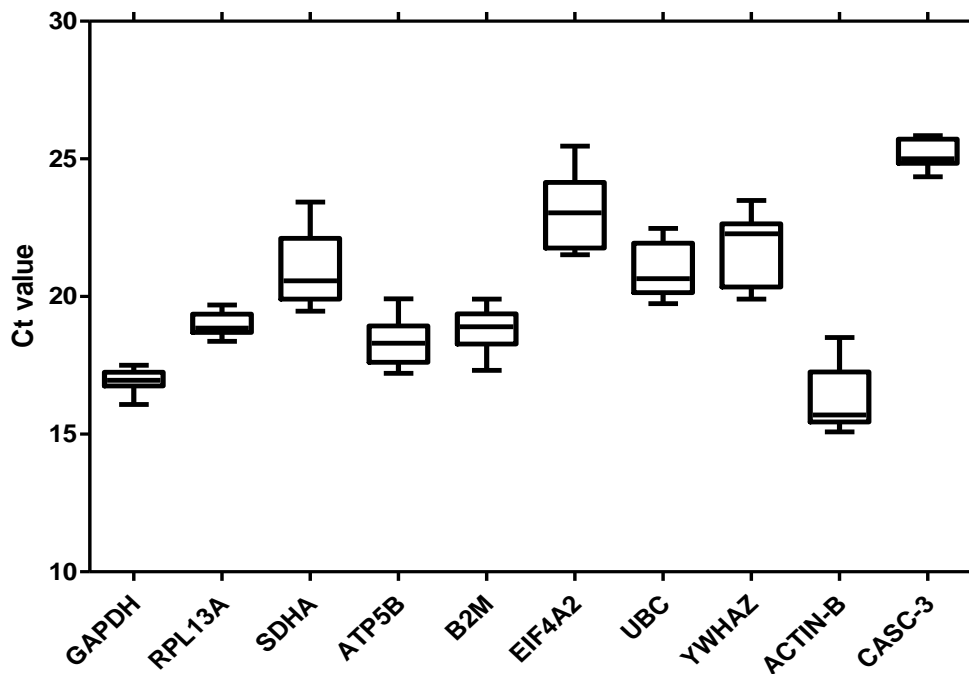


Figure 6: Expression values of candidate reference genes. Boxes indicate the 25 and 75 percentile, medians are indicated by lines across boxes, and whiskers represent maximum and minimum data points. CT values of candidate genes ranged from 14 to 26 cycles, with most of the CT values being between 16 and 20 cycles. ACT-B was the most abundantly transcribed with CT values of less than 15 cycles. EIF4A2 and CASC3 showed the lowest level of expression in all samples with CT values as high as 25 cycles.

3.1.2 Determination of the most stably expressed endogenous control by BestKeeper

Bestkeeper is an Excel-based tool that uses pair-wise correlations to determine the reference genes with the least variability. All data processing using this tool are based on crossing points (CP) or threshold cycles (CT) generated by any real time PCR platform (Pfaffl et al. 2002).

The determination of genes with the most stable expressions was based on the coefficient of correlation (r) to the BestKeeper Index (BI) and those showing a strong correlation with the BestKeeper index were regarded as stable reference genes. The descriptive statistics for the individual candidate genes are first generated. Then, their expression stabilities are determined, based on the inspection of calculated variations (Standard Deviation and Coefficient of Variance values). According to the variability observed, the reference genes are arranged from the most stably expressed, exhibiting the lowest variation, to the least stable one, exhibiting the highest variation (Pfaffl et al. 2002).

Bestkeeper generates an index using the geometric mean of the CT values of the best candidate genes under study. To assess inter-gene relationships of all possible reference gene pairs, numerous pair-wise correlation analyses are performed. Within each such correlation, the Pearson correlation coefficient (r) and the probability (P-value) are calculated. All those highly correlated reference genes are combined into an index. Then, correlation between each candidate reference gene and the index is calculated, giving a description of the relationship between the index and the contributing candidate reference gene (Pfaffl et al. 2002). Reference genes with P-values ≤ 0.05 (highlighted in green; Table 5b) are considered having a significant correlation with the calculated index.

Insight into the stability of the reference genes can also be gained by considering the information presented in Figure 7. The degree of tortuosity of the lines running parallel to the X-axis serves as an indication of how stably expressed the genes are.

The BestKeeper analysis revealed that when ten reference gene samples were introduced, the best correlations were obtained for CASC3 ($r = 0.768$), RPL13A ($r = 0.689$), B-Actin ($r = 0.647$) and GAPDH ($r = 0.633$) with p-values highlighted in green (Table 5b). The high Pearson's coefficients of correlation indicate that the expression profiles of the above gene pairs were similar. UBC and YWHAZ were determined as the genes with the least stability and therefore not proper to be used as normalization standards (Table 5b).

The ranking of gene expression stability based on the standard deviation of the candidate genes showed GAPDH, RPL13A and CASC-3 to be the most stably expressed genes (Figure 8).

<i>CT data of housekeeping Genes:</i>										
	GAPDH	RPL13A	SDHA	ATP5B	B2M	EIF4A2	UBC	YWHAZ	ACTIN-B	CASC-3
geo Mean [CT]	16.96	18.97	20.93	18.34	18.80	23.03	20.99	21.64	16.29	25.14
ar Mean [CT]	16.97	18.97	20.97	18.36	18.81	23.07	21.01	21.68	16.32	25.14
min [CT]	16.08	18.37	19.47	17.21	17.32	21.52	19.74	19.91	15.08	24.35
max [CT]	17.51	19.69	23.43	19.91	19.91	25.46	22.47	23.49	18.51	25.84
std dev [± CT]	0.26	0.33	1.01	0.64	0.66	1.11	0.85	1.19	0.98	0.43
CV [% CT]	1.54	1.74	4.82	3.49	3.52	4.80	4.03	5.50	6.02	1.72
min [x-fold]	-1.85	-1.51	-2.76	-2.20	-2.78	-2.86	-2.38	-3.32	-2.31	-1.73
max [x-fold]	1.46	1.65	5.64	2.97	2.16	5.38	2.79	3.60	4.67	1.63
std dev [± x-fold]	1.20	1.26	2.02	1.56	1.58	2.15	1.80	2.28	1.98	1.35

Table 5(a): Results from the BestKeeper analysis. The standard deviations, the geometric and the arithmetic mean of the CT-values, as well as minimum and maximum CT-values are listed for the ten reference genes. The genes whose standard deviations are higher than 1 are considered inconsistent and are highlighted in red, above; i.e. SDHA, EIF4A2 and YWHAZ.

Pearson correlation coefficient (r)										
vs.	GAP DH	RPL 13A	SDHA	ATP5 B	B2M	EIF4 A2	UBC	YWH AZ	ACTI N-B	CASC3
RPL13A	0.465	-	-	-	-	-	-	-	-	-
p-value	0.070	-	-	-	-	-	-	-	-	-
SDHA	0.632	0.069	-	-	-	-	-	-	-	-
p-value	0.009	0.799	-	-	-	-	-	-	-	-
ATP5B	0.010	0.650	-0.506	-	-	-	-	-	-	-
p-value	0.969	0.006	0.046	-	-	-	-	-	-	-
B2M	0.358	0.171	0.517	0.107	-	-	-	-	-	-
p-value	0.175	0.526	0.040	0.695	-	-	-	-	-	-
EIF4A2	0.693	0.101	0.940	0.385	0.713	-	-	-	-	-
p-value	0.003	0.710	0.001	0.141	0.002	-	-	-	-	-
UBC	0.064	0.088	-0.027	0.204	-0.172	0.122	-	-	-	-
p-value	0.854	0.793	0.938	0.544	0.616	0.720	-	-	-	-
YWHAZ	0.080	0.143	-0.118	0.134	0.379	0.234	-0.788	-	-	-
p-value	0.816	0.677	0.727	0.691	0.250	0.490	0.004	-	-	-
ACTIN-B	0.184	0.568	-0.464	0.851	-0.045	0.334	0.444	-0.296	-	-
p-value	0.495	0.022	0.070	0.001	0.867	0.205	0.170	0.377	-	-
CASC3	0.736	0.367	0.515	0.176	0.465	0.525	0.363	-0.337	0.355	-
p-value	0.001	0.161	0.041	0.514	0.070	0.037	0.272	0.312	0.177	-
BestKeeper vs.	GAP DH	RPL 13A	SDHA	ATP5 B	B2M	EIF4 A2	UBC	YWH AZ	ACTI N-B	CASC3
coeff. of corr. [r]	0.633	0.689	0.214	0.629	0.549	0.389	0.145	0.128	0.647	0.768
p-value	0.008	0.003	0.426	0.009	0.028	0.136	0.670	0.706	0.007	0.001

Table 5(b): Pearson Correlation and the determination of the P-value of reference genes by BestKeeper. Genes whose P-values are highlighted in green have the best correlations and are thus considered most stably expressed.

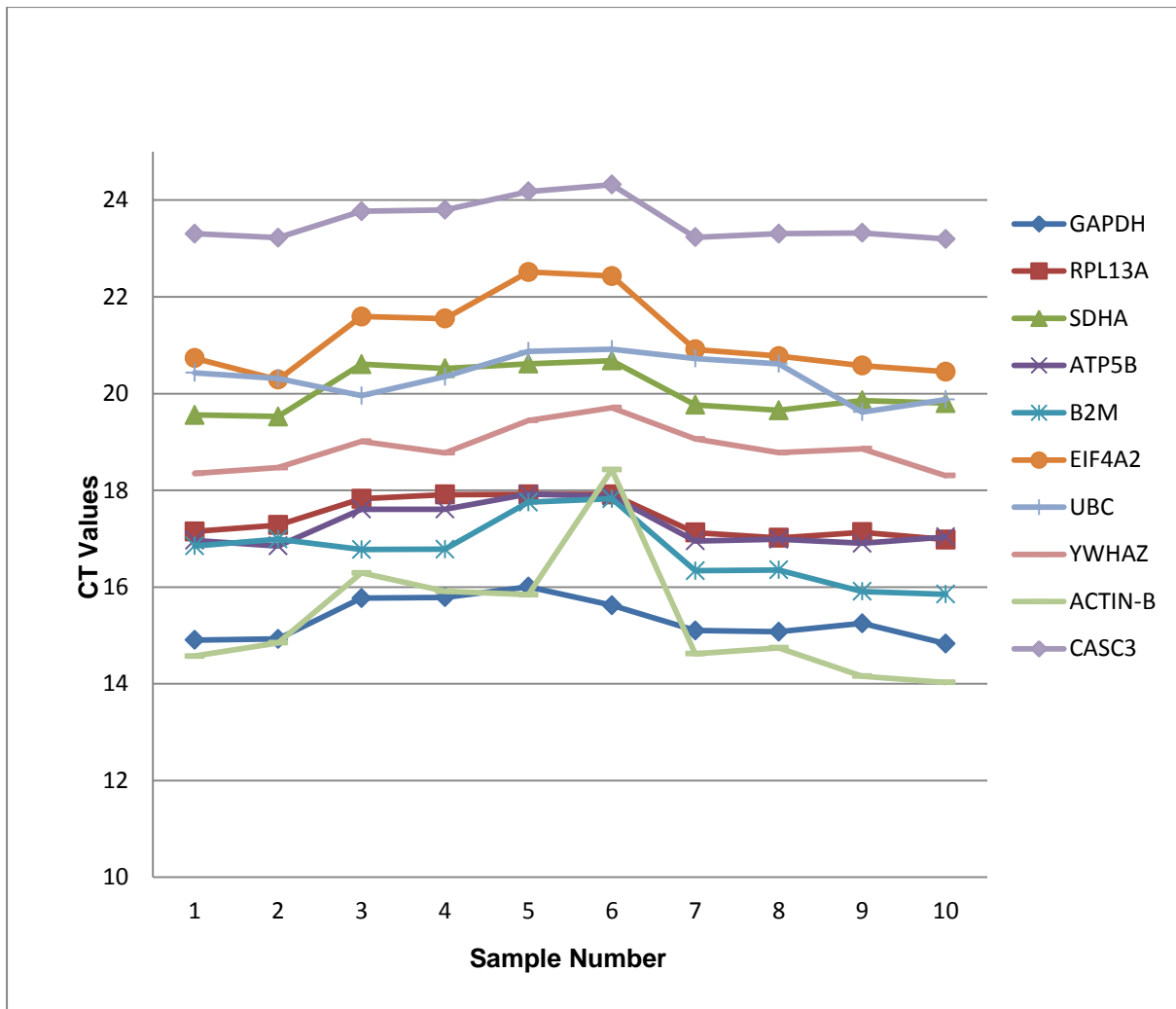


Figure 7: Line graph of the CP of all reference genes plotted against the sample number as generated by the BestKeeper software. The variabilities of the expression of each reference gene is depicted by the degree of tortuosity of the lines running parallel to the X axis.

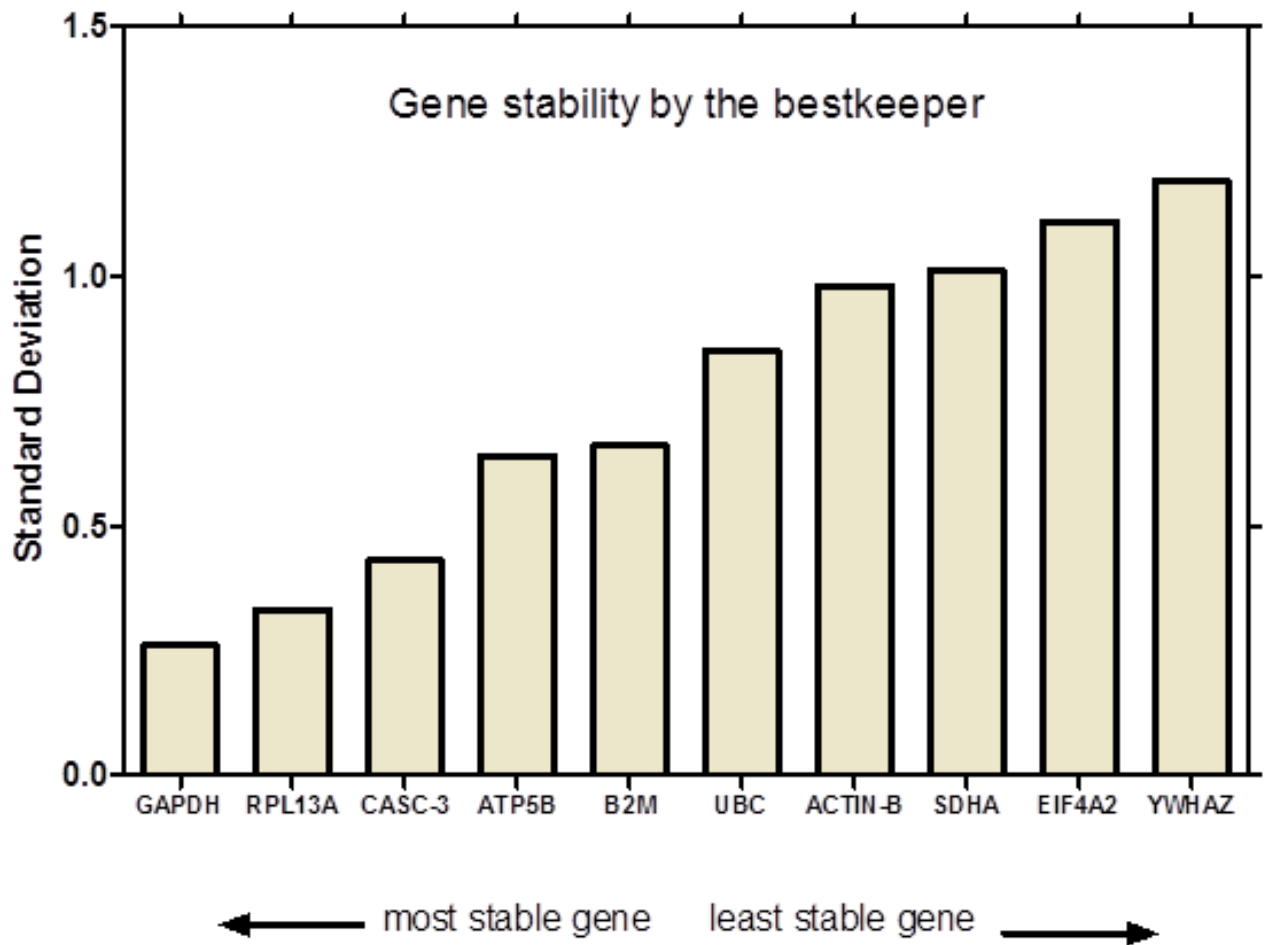


Figure 8: Ranking of gene expression stability (based on standard deviation) of the ten candidate genes as predicted by Bestkeeper. GAPDH, RPL13A and CASC3 were selected as the most stably expressed whilst YWHAZ was selected to be the least stably expressed gene.

3.1.3 Determination of the most stable reference gene by the Normfinder software

NormFinder uses an ANOVA-based model to estimate stability variation, and combines these estimates to provide a direct measure of the variation in expression for each gene (Andersen et al. 2004). More stable gene expression is indicated by lower average expression stability values. The stability values of all 10 reference genes were calculated by Normfinder using the Cycle Threshold (CT) values generated from the PC Miner software.

The stability values as calculated by Normfinder are presented in the appendix, while the ranking of the genes is shown in figure 9 below.

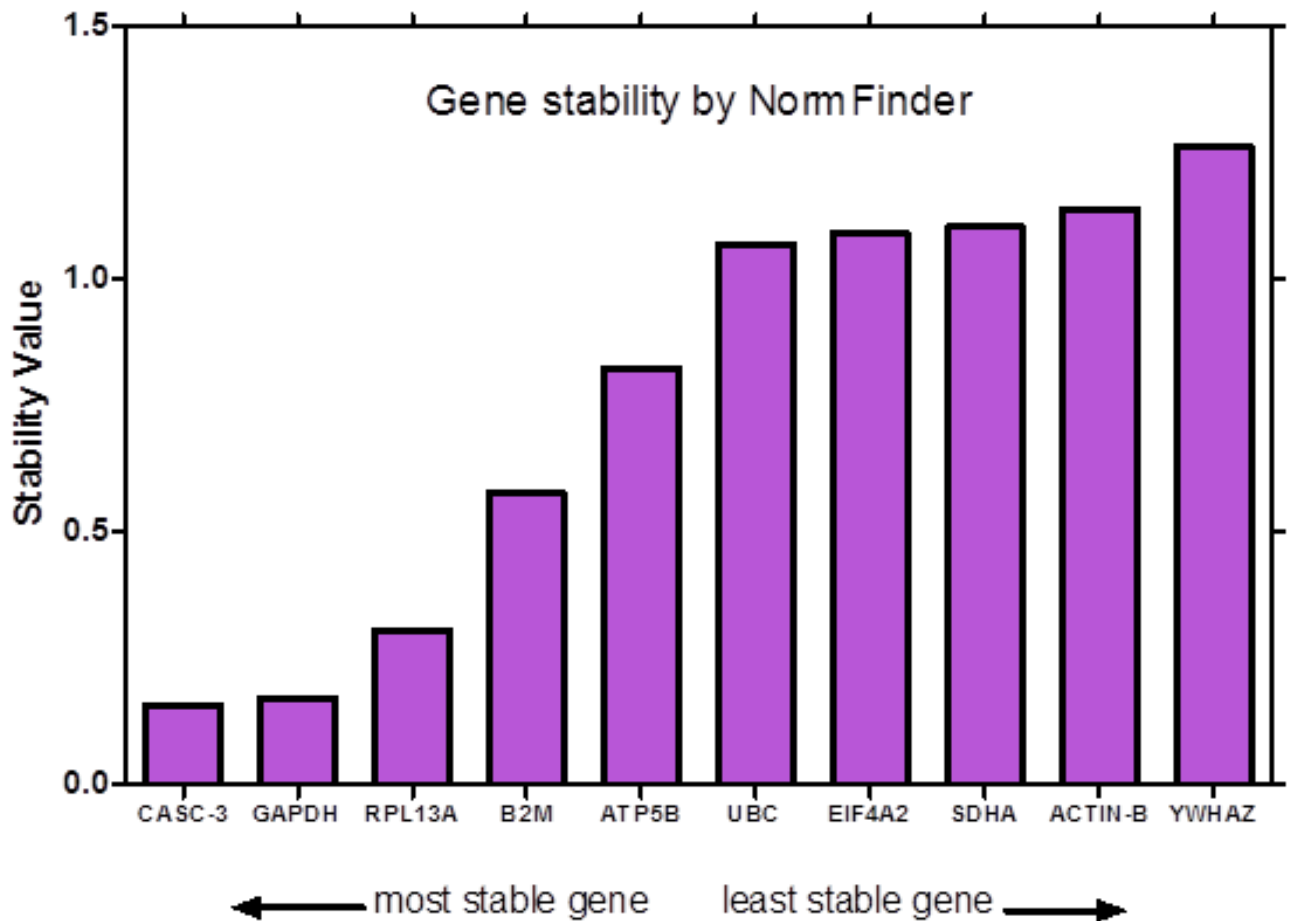


Figure 9: Gene expression stability of the ten candidate genes as predicted by Normfinder. CASC3 was considered the most stably expressed gene while YWHAZ was considered the least stably expressed.

3.1.4 Determination of the most stable reference gene by the geNorm software

geNorm is an application integrated in the qbase+ software for real-time PCR data analysis in Windows, Mac and Linux (available from Biogazelle). It automatically calculates the gene stability measure (M) for all control genes in a given set of samples. For every control gene, the pairwise variation (V) with all other control genes is determined as the standard deviation of the logarithmically transformed expression ratios, and defines the internal control gene-stability measure (M) as the average pairwise variation of a particular gene with all other control genes (Vandesompele et al. 2002). An M value of 1.5 is recommended as the screening criteria and those genes with M value below this threshold are recognised as ideal reference genes with stable expression. Genes with the lowest M values are believed to have the most stable expression (Vandesompele et al. 2002).

The pair-wise variation (V_n/V_{n+1}) was determined as an indicator, which represents the effect of adding further reference genes on the normalization factor (that is calculated as the geometric mean of the expression values of the selected reference genes). It is feasible to add additional reference genes to the normalization factor until the pair-wise variation (V_n/V_{n+1}) drops below a given threshold (0.15), recommended by Vandesompele et al. (2002) which means the added gene has no significant effect and the inclusion of an additional reference gene is not required (Vandesompele et al. 2002).

The analysis in this study showed that M values of all ten selected genes were less than 1.5, which indicates that all of them conformed to the basic requirement for reference genes. As shown in Table 7 below, GAPDH and CASC3 jointly showed the lowest M values and were thus adjudged the most stable reference genes while YWHAZ was found to have the highest M value being the least stable gene.

The stability values (M) of all 10 reference genes were calculated by geNorm and are shown in the appendix while the ranking of the genes is shown in figure 10 below.

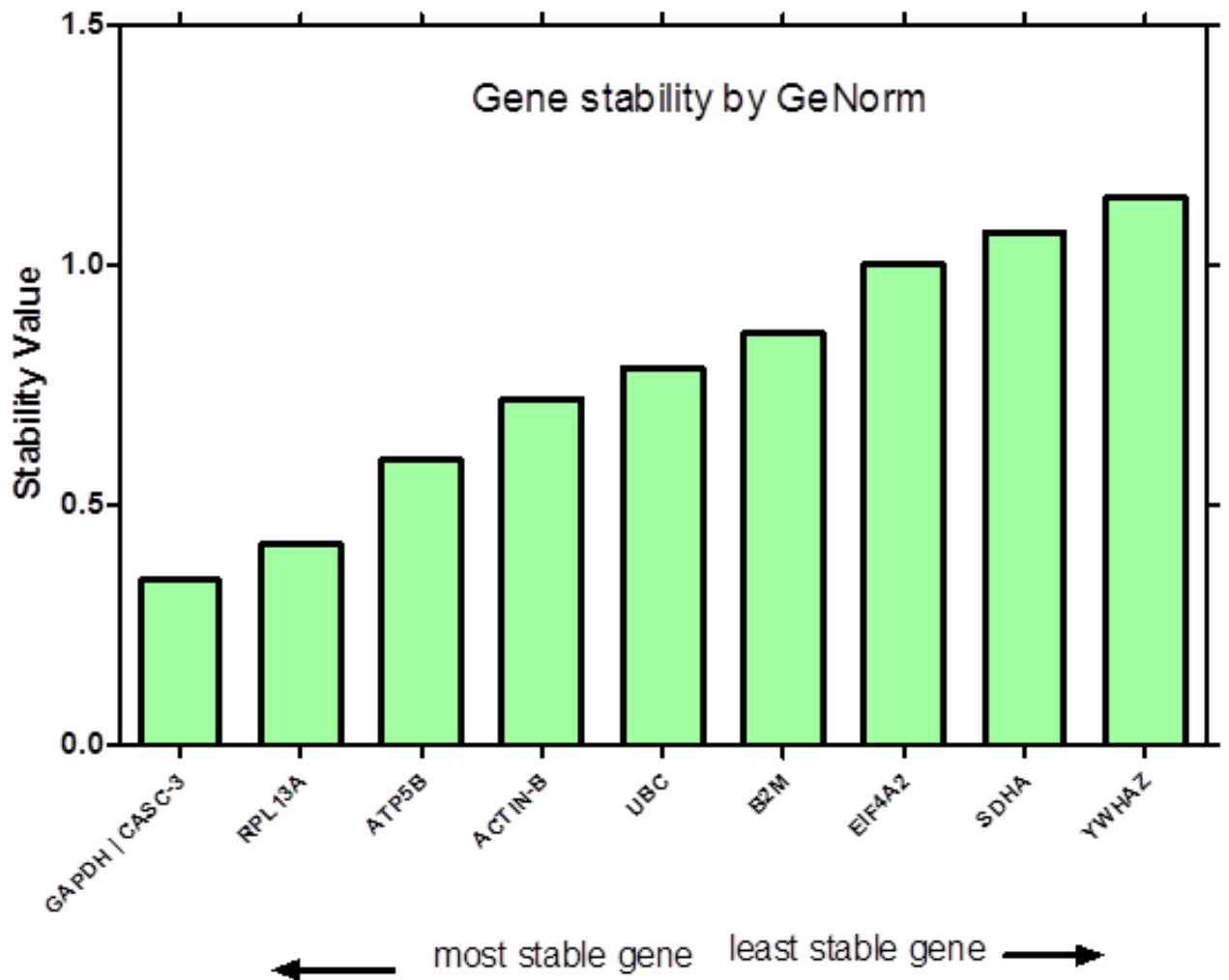


Figure 10: Gene expression stability of the ten candidate genes as determined by geNorm. GAPDH and CASC3 were jointly adjudged the most stably expressed genes while YWHAZ was adjudged the least stably expressed gene.

3.1.5 Normalization strategy

There is no unanimously accepted approach to normalization as there is no error-free procedure (Huggett et al. 2005). However, it is important that the best available normalization strategy be employed for optimal results to be obtained. In this research, all three software algorithms showed a great deal of similarity in ranking the first three (most stably expressed) genes: GAPDH, RPL13A and CASC3. YWHAZ was unanimously ranked least stably expressed while all other reference genes showed varying positions among the algorithms except Ubiquitin which was ranked sixth by all three. Therefore, normalization was done by obtaining the geometric mean of the CT values of the three genes unanimously selected by all the

reference gene standardization software algorithms (GAPDH, RPL13A and CASC3) and then determining the expression of the target genes relative to this normalisation factor. Thus, the Pfaffl equation (Pfaffl, 2001), shown below was utilized.

$$\text{Fold increase} = \frac{(1+E_{\text{target}})^{\Delta\text{CT}_{\text{target}} \text{ (control - sample)}}}{(1+E_{\text{ref}})^{\Delta\text{CT}_{\text{ref}} \text{ (control - sample)}}$$

The Relative Expression Software Tool (REST) (Pfaffl, 2001) was utilized in the calculation of the fold increase/decrease of the genes of interest for each experimental condition. The unpaired student t-test provided by the GraphPad prism software was used to determine the statistical significance of each data set and from its results, conclusions were drawn.

3.1.6 Melting Curve Analysis for the PCR experiments

To evaluate whether the PCR assays have produced single, specific products, Melting curve analyses were conducted. These demonstrated that there were satisfactory amplifications of specific products, thereby ruling out the possibility of the presence of primer dimers in the reaction plates (Figure 11).

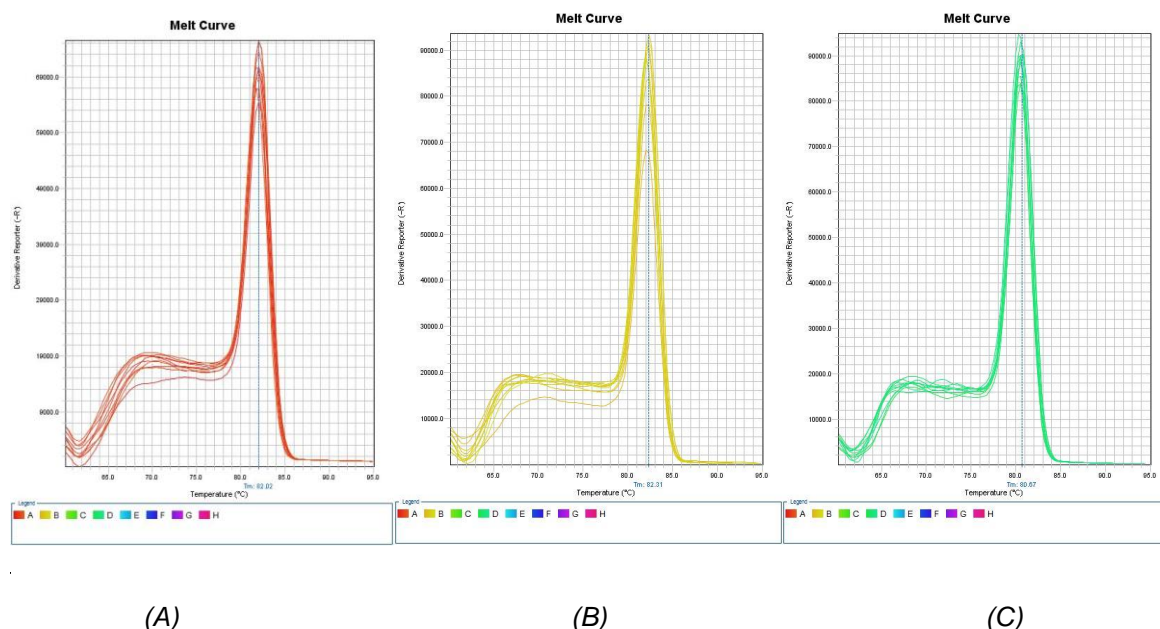


Figure 11: The melting curve analyses which were used to assess whether the PCR assays have produced single, specific products (Figure 9(A) for GAPDH, 9(B) for RPL13A and 9(C) for TNF-alpha), showed optimal PCR product melting temperatures as well as satisfactory amplifications of single, specific products (Figures are representative).

3.2 INVESTIGATING THE EFFECT OF LPCAT 3 GENE KNOCKDOWN ON LIPOPOLYSACCHARIDE-INDUCED INFLAMMATORY RESPONSES IN MACROPHAGES

3.2.1 LPCAT3 gene silencing

To establish the involvement of the LPCAT3 enzyme in the inflammatory responses of macrophages, LPCAT3 gene silencing and subsequent stimulation with Lipopolysaccharide were first carried out.

The achievement of a satisfactory gene silencing efficiency was important as this would determine the reliability of subsequent results. In this research, approximately 80% LPCAT3 gene silencing was achieved. This is shown in Figure 12 below.

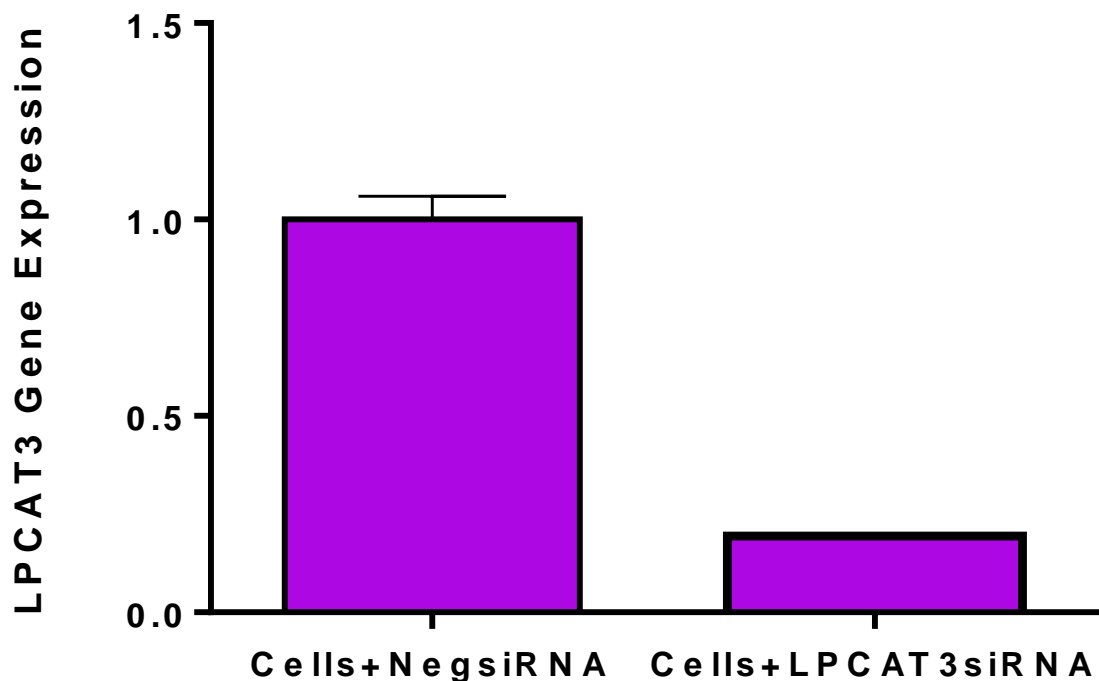


Figure 12: Efficiency of LPCAT3 gene silencing. Normalised against the geometric mean of the CT Values of GAPDH, RPL13A and CASC3, LPCAT3 was down-regulated by a mean factor of 0.806 (~80% silencing) (SE \pm 0.0065).

3.2.2 TNF-alpha expression

Following the successful silencing of the LPCAT3 gene, further experiments were conducted to compare the gene expression of the pro-inflammatory cytokine TNF-alpha, between samples containing the Negative siRNA and LPS, LPCAT3 siRNA and LPS (Silenced LPCAT3 genes) and the controls (Cells only). The results of the comparison are illustrated in the graph below:

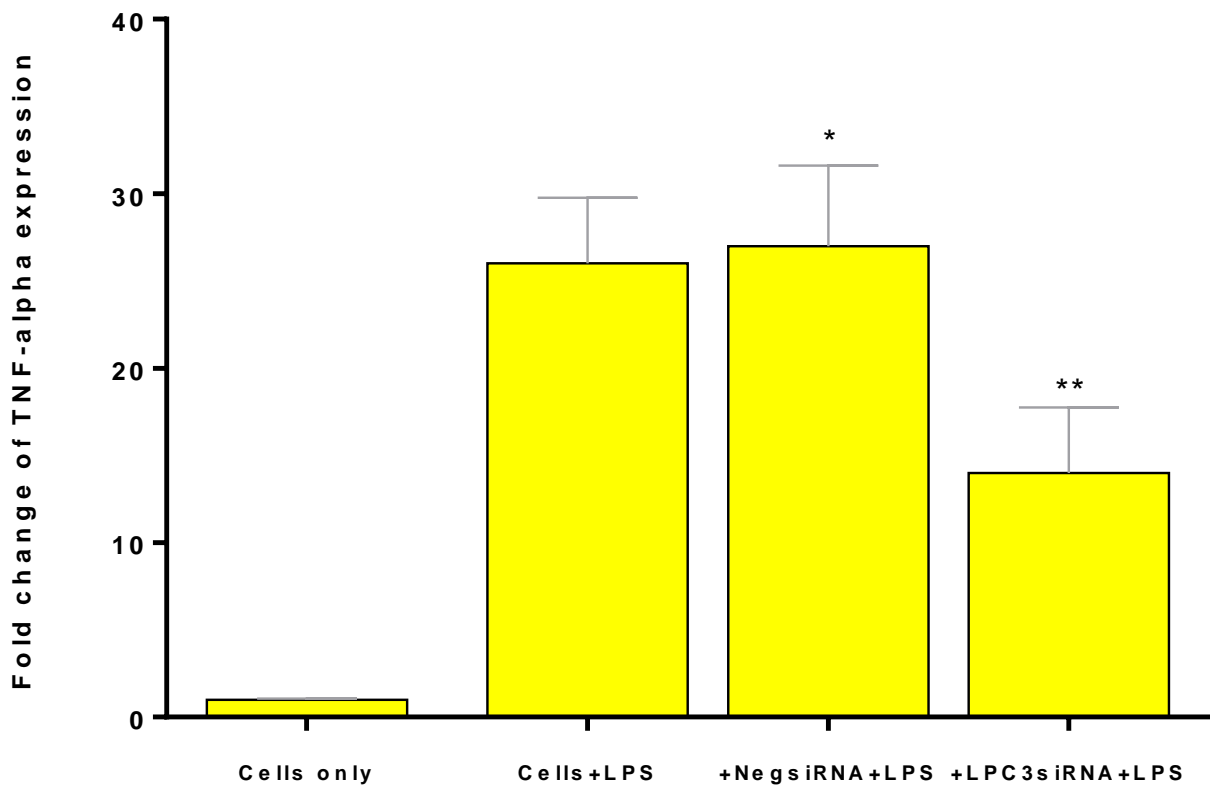


Figure 13: LPCAT3 silencing down-regulates inflammatory responses to Lipopolysaccharide. Normalised against the geometric mean of the CT values of GAPDH, RPL13A and CASC3, LPCAT3 silencing using the siRNA technology was effective in significantly reducing the gene expression of TNF-alpha in response to LPS in RAW 264.7 cells. Cells+LPS are representative. Results are the Mean±SE from three independent experiments; * = P Value <0.05; ** = P value <0.01.

Comparing against the control (Cells only), there was a 27 (SE: ± 4.62 ; n=3) fold increase in the expression of TNF-alpha on addition of LPS (P-value: 0.02). Samples containing LPCAT3 silenced genes and LPS (shown as Cells+LPCAT3siRNA+LPS) showed a much reduced fold increase (14; SE ± 3.75 ; n=3) in the expression of TNF-alpha (P-value: 0.0238) when compared with samples without LPCAT3 silencing (shown as Cells+NegsiRNA+LPS).

Comparing the expression of TNF-alpha between samples without LPCAT3 silencing (shown as Cells+NegsiRNA+LPS) and samples with LPCAT3 silencing (shown as Cells+LPCAT3siRNA+LPS), using the former as the control, there was an approximately 2-fold decrease (SE ± 0.06 , P Value=0.002; n=3) in the expression of the cytokine.

3.2.3 IL-10 expression

Experiments were also conducted to compare the expression of the anti-inflammatory cytokine IL-10, between samples containing the negative siRNA and LPS, LPCAT3 siRNA and LPS (silenced LPCAT3 genes) and the control (Cells only). The results of the comparison are illustrated in Figure 14.

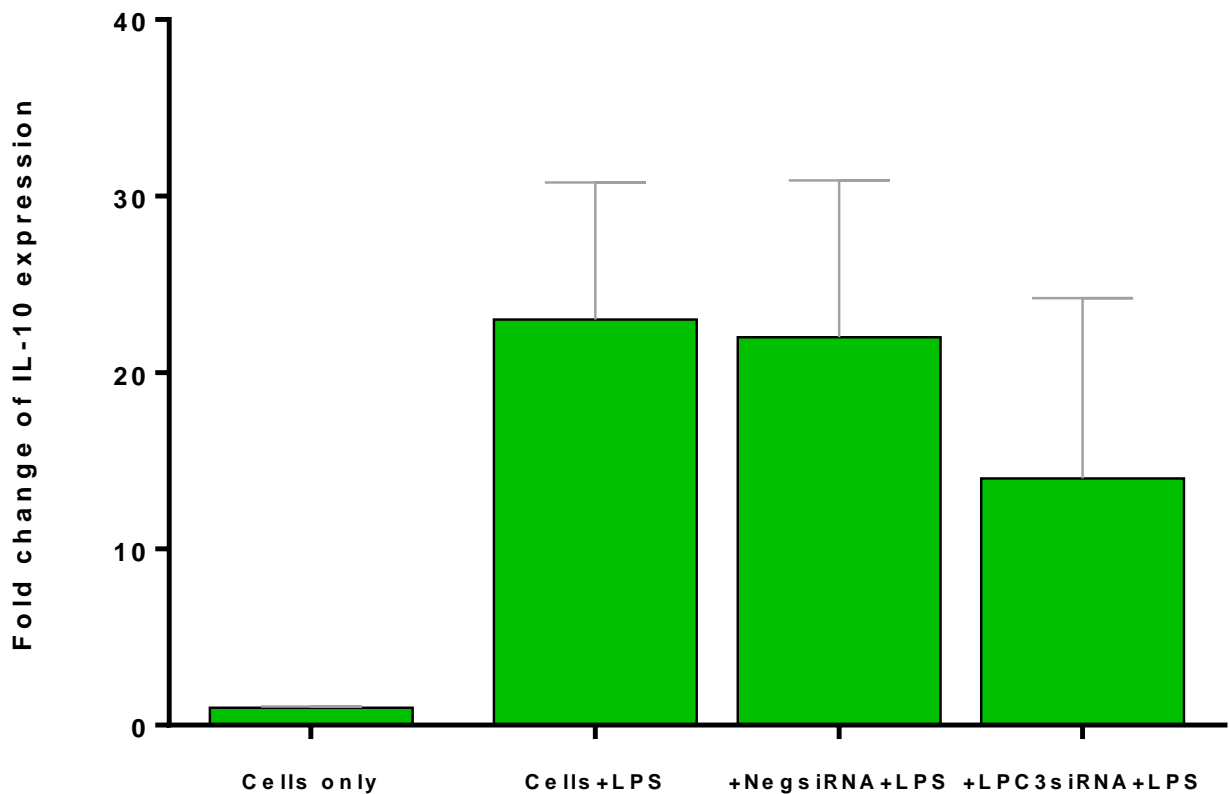


Figure 14: LPCAT3 silencing down-regulates inflammatory responses to Lipopolysaccharide. Normalised against the geometric mean of the CT values of GAPDH, RPL13A and CASC3, LPCAT3 silencing using the siRNA technology resulted in a statistically insignificant reduction in the gene expression of IL-10 in response to LPS in RAW 264.7 cells. Cells+LPS are representative. Results are the Mean±SE from three independent experiments; P Value 0.09.

Comparing against the control (cells only), there was a 22 (SE: ± 8.89; n=3) fold increase in the expression of IL-10 on addition of LPS (P-value: 0.0710). Samples containing LPCAT3 silenced genes and LPS (Cells+LPCAT3siRNA+LPS) showed a much reduced fold increase (14; SE ±10.22; n=3) in the expression of IL-10 (P-value: 0.2607) when compared with samples without LPCAT3 silencing (Shown as Cells+NegsiRNA+LPS).

Comparing the expression of IL-10 between samples without LPCAT3 silencing (Shown as Cells+NegsiRNA+LPS) and samples with LPCAT3 silencing (Shown as Cells+LPCAT3siRNA+LPS), using the former as the control, there was an approximately 2 fold decrease (SE ±0.23, P Value=0.09; n=3) in the expression of the cytokine.

3.2.4 Enzyme-Linked Immunosorbent Assay (ELISA) for TNF-alpha expression

A sandwich ELISA was performed with the aim of comparing the protein expression of the cytokine TNF-alpha between samples in which the LPCAT3 gene had been silenced and the controls. Three independent experiments were performed. The supernatants were collected post stimulation with LPS. The TNF-alpha expression was analysed and the results are presented below:

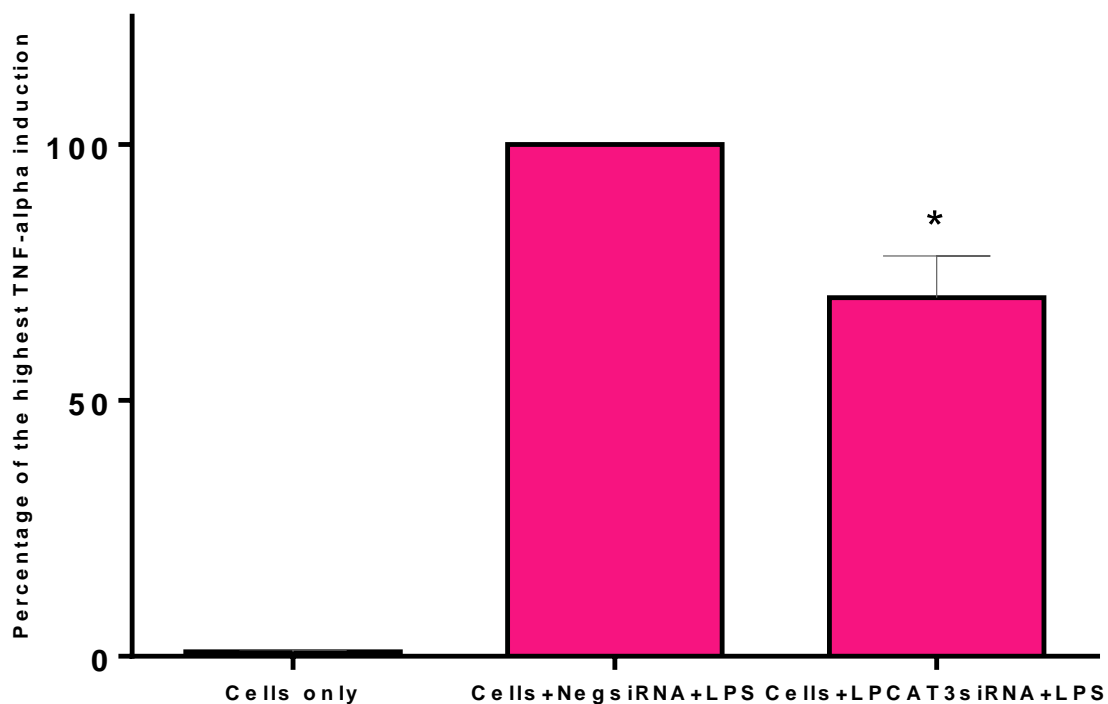


Figure 15: LPCAT3 silencing down-regulates inflammatory responses to Lipopolysaccharide. LPCAT3 silencing using the siRNA technology was effective in significantly reducing the protein expression of TNF-alpha in response to LPS in RAW 264.7 cells. Results are the Mean±SE from three independent experiments; * = P Value <0.05

Considering the protein expression of TNF-alpha in samples without LPCAT3 silencing (Shown as Cells +NegsiRNA+LPS) to be 100% as these samples showed the highest TNF-alpha concentrations, samples with LPCAT3 silencing showed a 30% decrease in the expression of TNF-alpha (SE ± 4.72; P-Value=0.021; n=3).

Taken together, LPS stimulation was found to result in a significant increase in the expression of both TNF-alpha and IL-10. LPCAT3 silencing was found to cause a significant decrease in the gene expression as well as the protein expression of

TNF-alpha (P-Values 0.002 and 0.021 respectively); while its effect on the gene expression of IL-10 was statistically insignificant (P-Value 0.09).

CHAPTER 4: DISCUSSION

4.1 IDENTIFYING THE MOST SUITABLE REFERENCE GENE IN THE ANALYSIS OF THE RESPONSE OF THE LPCAT3 GENE TO LIPOPOLYSACCHARIDE-INDUCED INFLAMMATORY RESPONSES IN MACROPHAGES

4.1.1 Endogenous controls and their relevance

In recent years, the profiling of mRNA transcription has become a popular research field following the establishment of the fact that it is the link between DNA and proteins. Common methods for RNA detection include: in situ hybridisation, Northern blotting, qualitative RT-PCR, RNase protection assay, competitive RT-PCR, microarray analysis, and quantitative real-time PCR (qPCR) (Radonić et al. 2004).

Among these methods, real-time PCR has become an established technique for quantifying mRNA in biological samples due to its benefits over other conventional methods. These benefits include its sensitivity, large dynamic range, and the potential for high throughput as well as accurate quantification. Its enhanced specificity is particularly useful for immunological research, which frequently involves analysis of proteins derived from different splice variants of the original transcript (Atamas, 1997). Furthermore, many of the key proteins (e.g. cytokines and transcription factors) are found in such low abundance that real-time RT-PCR quantification of their mRNAs represents the only technique sensitive enough to measure reliably their expression *in vivo* (Huggett et al. 2005).

Despite its advantages over other RNA quantification techniques, real-time PCR does have its draw-backs, among which are the inherent variability of RNA, RNA recovery, RNA integrity, variability of extraction protocols that may co-purify inhibitors, different reverse transcription and PCR efficiencies and differences in the overall transcriptional activity of the tissues or cells analysed (Bustin and Nolan, 2004 and Andersen et al. 2004). Consequently, the choice of an accurate calibration parameter aimed at the normalisation of the above-mentioned inconsistencies is of utmost importance. Among the several normalisation strategies that have been proposed (discussed in detail in section 4.1.2), the most frequently used is the endogenous control.

Endogenous controls are typically constitutive genes that are required for the maintenance of basic cellular function. They are expected to be expressed in all cells of an organism under normal and patho-physiological conditions, irrespective of tissue type, developmental stage, cell cycle state, or external signal. It is expected that the level of expression of endogenous controls should not vary during cell growth, differentiation and transformation or in response to experimental treatment (Zampieri et al. 2010). Reliable reference genes have been referred to as a vital prerequisite for any functional study employing RT-qPCR for analysing gene expression (Maeß et al. 2010). Gene expression results in real time PCR experiments have also been found to be significantly dependent on the choice of reference genes (Piehler et al. 2010).

In recent years, it has become clear that no single gene is constitutively expressed in all cell types and under all experimental conditions, implying that the expression stability of the intended control gene has to be verified before each experiment (Andersen et al. 2004). Also, the mRNA levels of commonly-used reference genes such as ACT-B and GAPDH have been shown to vary significantly under particular developmental contexts and experimental conditions (Blanquicett et al. 2002, Thellin et al. 1999, Tricarico et al. and de Jonge et al. 2007), and the use of only a single reference gene as internal control can lead to erroneous normalisation with transcript levels differing up to 20-fold (Vandesompele et al. 2002). Consequently, it was deemed essential that a robust verification process aimed at determining the most stably expressed reference genes suitable for normalisation in the PCR experiments for this research be undertaken.

There are a number of commonly used endogenous controls. Ten of them were chosen for analysis and the selection criteria took account of the importance of choosing reference genes from different classes in order to avoid the problem of co-regulation. The details of all 10 reference genes analysed in this research, including their basic functions, functional classes and accession numbers were presented in Table 3 of Chapter 2.

4.1.1.1 Expression profiles of all candidate genes: Relevance in normalization

The expression profiles of all reference genes were measured by the CT value, which is the number of PCR cycles needed to reach a specific threshold level of

detection and is inversely correlated with the quantity of initial RNA template present in the sample (Walker, 2002).

For qRT-PCR normalization, a moderately expressed reference gene is preferred because extremely high or low expression of a reference gene could introduce variability to data analysis (Tan et al. 2012). This point was taken into consideration in the selection of reference genes for normalisation. The CT values of all candidate genes were in the range of 15 and 25 cycles which was optimal for normalisation.

4.1.1.2 Determination of the CT and Efficiency values of candidate genes by PC Miner

The Steponeplus software (used for the PCR experiments in this research) does allow researcher biases in the determination of the Cycle Threshold (CT) values and the Efficiency (E) of the genes analysed. This necessitated the search for an objective and more accurate means of calculating these values. The PC Miner software provided an objective and noise-resistant technique of calculating the CT values and the Efficiencies of all the genes tested without the need of preparing a standard curve (Zhao and Fernald, 2005 and Speiss et al. 2008).

It has been established that for accuracy of PCR results, the threshold used to compute the CT must be within the exponential phase such that it reflects initial template differences rather than just a change in reaction kinetics. To meet this important requirement, the developers of the software used a four-parameter logistic model to fit the raw fluorescence data as a function of PCR cycles to identify the exponential phase of the reaction and then fit the exponential phase with a three parameter simple exponent model using an iterative non-linear regression algorithm. This is a more accurate determining measure of the exponential phase as the Steponeplus software permits the researcher to subjectively identify the exponential phase which action can be riddled with bias and consequently lead to experimental errors.

For the objective determination of CT values, the developers of the PC Miner software chose the first positive Second Derivative Maximum (SDM) from the logistic model.

This choice was made by the developers of the software after putting up a number of methods to a robust comparison. The Taqman threshold method (MyIQ software),

the First Derivative Maximum (FDM) method and the Mid-Value Point (MVP) method were compared using known serial dilutions. The second derivative maximum (SDM) method was found to be the most accurate method as it produced the lowest mean square error. The SDM method also provided a very good estimate among replicates with a lower mean coefficient of variation for CT than did the three other methods (Figure 16).

The efficiency of genes is computed through the calculation of a weighted average from an iterative non-linear regression. It has been the conventional practice to calculate the efficiency of genes using a linear (Peirson et al. 2003; Ramakers et al. 2003) or a non-linear regression (Tichopad et al. 2003) for the points found in the exponential phase. The developers of the PC Miner software found that there was a slight miscalculation of the efficiency when the linear regression is used. The non-linear regression showed more corresponding efficiency values with those derived from the standard curve and was thus adopted. The efficiency calculated from the CTs based on the SDM was found to be more stable (having a smaller standard deviation) than that computed by the CT's based on the Taqman threshold.

As discovered by the developers of the PC Miner software, the SDM method also effectively solves the problem of the noise due to the randomness of PCR amplification and fluorescence detection, while still retaining the essential CT set-point of being within the exponential phase. While the Mid-value point method targets the beginning of the exponential phase and thus may be more accurate at calculating the CT, it is hugely influenced by noise which renders its calculation less reliable than other methods. The first derivative maximum values are found outside the exponential phase where all the kinetic curves tend to be saturated and then merge. While the utilization of this algorithm tends to completely avoid the PCR noise, this method does not meet the requirement for the calculation of an accurate CT, which is that the amplification threshold for quantification must be set within the exponential phase of the PCR. The SDM method sets the threshold for the CT determination within the exponential phase, overcomes the influence of PCR noise and when compared with other commonly used methods, was proven to provide the closest CT values to those generated by known serial dilutions (Zhao and Fernald, 2005). Thus, it was adjudged the best available method to be used for the accurate analysis of the PCR results in this research.

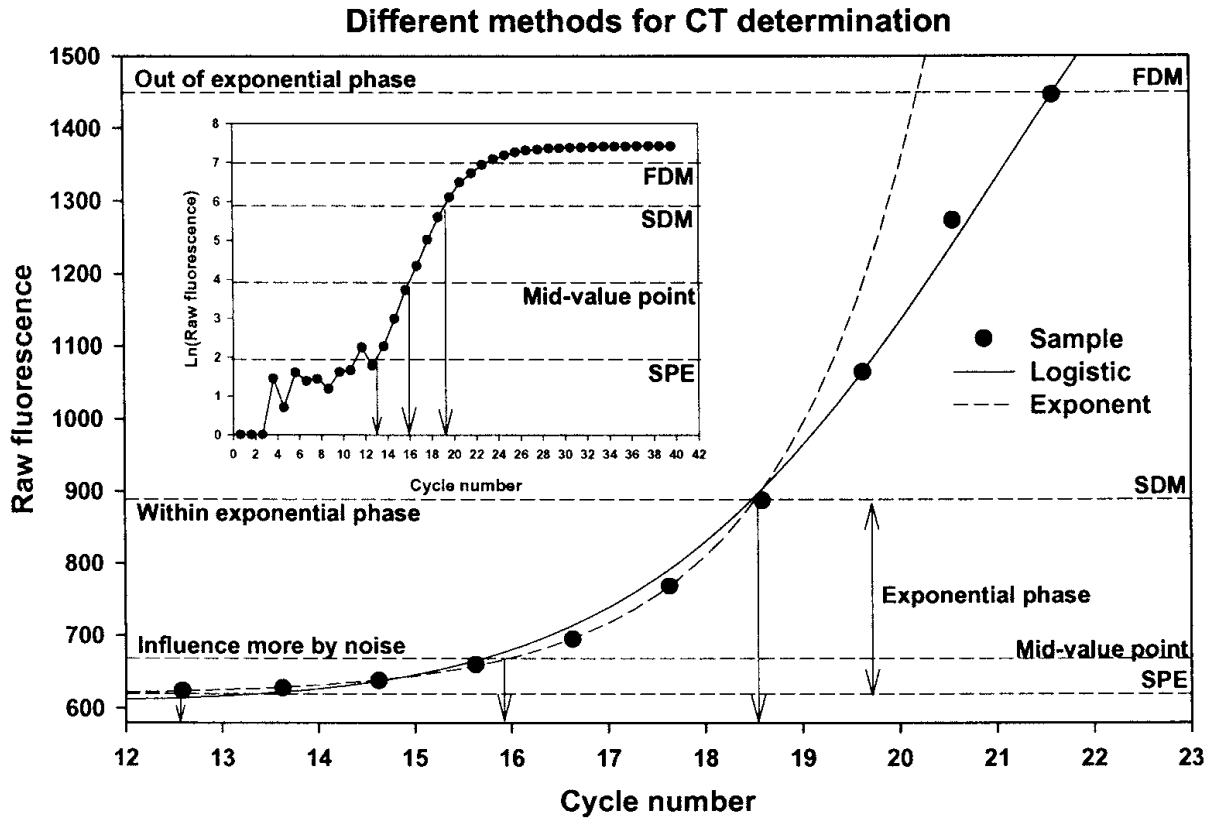


Figure 16: Comparison of methods for CT determination: CT determination using FDM, SDM, and mid-value point. The SDM method sets the threshold for the CT determination within the exponential phase, overcomes the influence of PCR noise and when compared with other commonly used methods, was proven to provide the closest CT values to those generated by known serial dilutions (Figure and legend reproduced with permission from Zhao and Fernald, 2005; Please, see E-mail correspondence in Appendix).

Taken together, the flowchart for the algorithm used by the PC Miner software for the analysis of raw PCR data is presented in Figure 17.

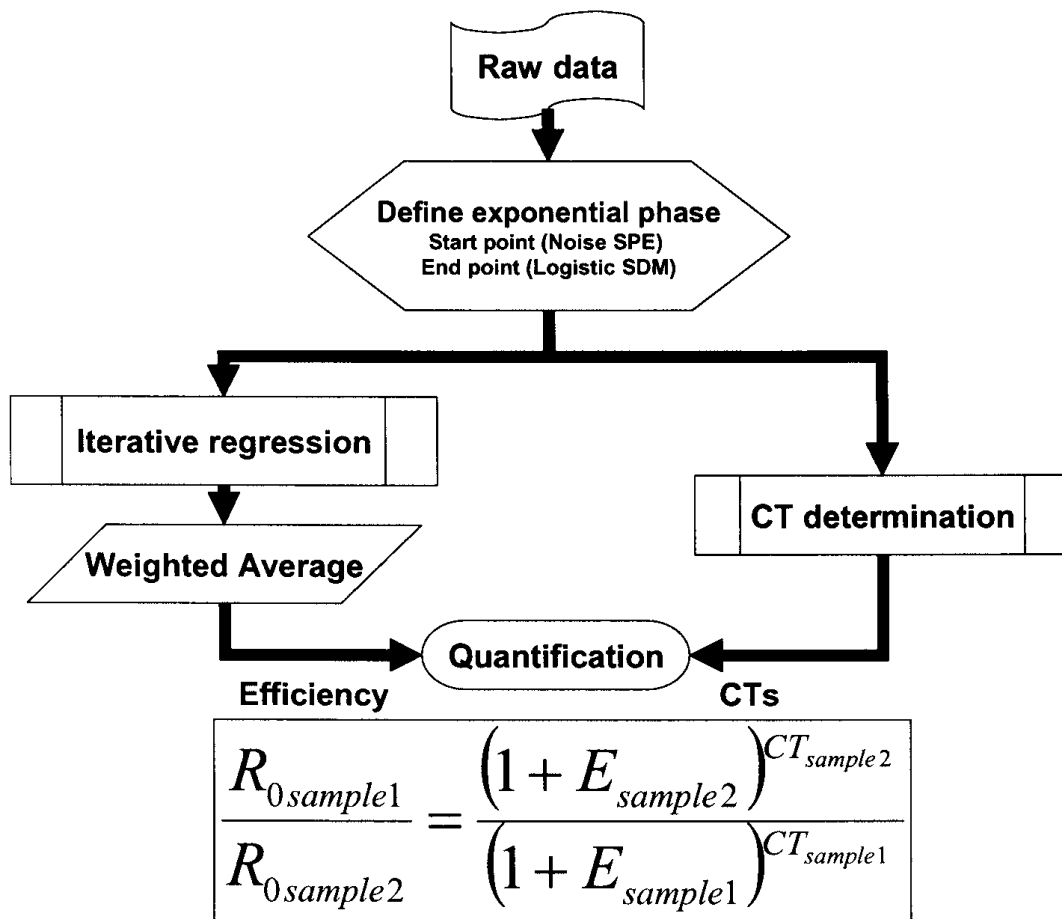


Figure 17: Flow chart showing steps in implementing the PC Miner algorithm described. The process of quantification includes exponential phase determination, efficiency estimation, CT calculation, and comparison among samples. R_0 is the start template concentration; E represents the efficiency of the PCR reaction (Figure and legend reproduced with permission from Zhao and Fernald, 2005; Please, see E-mail correspondence in Appendix).

4.1.2 Comparison of results generated by the three software applications used: Bestkeeper, geNorm and Normfinder.

Normalisation with multiple reference genes is becoming more prevalent and it has been suggested by a good number of studies that this strategy is currently the most effective in ensuring that accurate results are obtained in real-time PCR experiments. The selection of the software applications for the analysis of the most stable reference genes was based on convincing evidence that there was a good measure of accuracy in the methodology utilized by these algorithms in the standardization of the genes. The statistical models used are known standards for the calculation of the values for correlation, and robust testing was carried out by the developers of the

models using biological samples with satisfactory results obtained. The details of their various methodologies have been discussed in the previous chapter.

Analysis with geNorm has been described as an easy method to determine the optimal number of stable housekeeping genes for accurate normalization (Vasompele et al. 2002 and Jian et al. 2008), whereas NormFinder and Bestkeeper can be and has been used to assess and further validate the quality of the ranking obtained by geNorm (Maeß et al. 2010).

All three software applications showed close similarity in ranking the first three (most stably expressed) genes: GAPDH, RPL13A and CASC3. YWHAZ was unanimously ranked least stably expressed while all other reference genes showed varying positions among the three algorithms except Ubiquitin which was ranked sixth by all three. This is the exact observation put forward by Maeß and fellow researchers while investigating the selection of reliable reference genes during THP-1 monocyte differentiation into macrophages (Maeß et al. 2010). The reproducibility of the top rankings gives high assurance regarding the actual stability of the selected reference genes. The lack of confidence within the middle positions might be due to only very slight differences in expression stability within that range (Maeß et al. 2010). It is therefore safe to infer that minimal differences within the calculation algorithms of each software application may have caused the observed differences in ranking positions.

A comparison with recently published studies involving inflammatory cells further highlights the importance of individual validation of reference genes even if comparable cell models are used. In a study conducted by Piehler and co-workers, twelve candidate reference genes were quantified by RT-qPCR in LPS-stimulated human monocytes and evaluated using the programs geNorm, Normfinder and BestKeeper. geNorm ranked PPIB (cyclophilin B), B2M (beta-2-microglobulin) and PPIA (cyclophilin A) as the best combination for gene expression normalization in LPS-stimulated monocytes. Normfinder suggested TBP (TATA-box binding protein) and B2M as the best combination (Piehler et al. 2010). It is to be noted that in this research, B2M was ranked 7th by geNorm, 4th by Normfinder and 5th by Bestkeeper, from a total of 10. This underscores the need for appropriate and careful validation of

reference genes for cell culture models, as the ranking for one cell model may not be applicable to others.

Zampieri et al. (2010) evaluated the expression stability of 5 frequently used reference genes (18s rRNA, ACTB, GAPDH, HPRT1 and GUSB) in human peripheral blood mononuclear cells with respect to aging, using the geNorm and Normfinder software applications. Both algorithms unanimously ranked GAPDH and ACT-B 2nd and 4th respectively. This is similar to the ranking of GAPDH in this research which is 2nd, by both applications. geNorm also produced similar results with ACT-B ranking it 4th while Normfinder showed a completely opposite result, ranking ACT-B 9th out of 10. This further shows the importance of ascertaining the gene expression stabilities of reference genes to be used for normalization using varying methods, before normalization is done.

Another study aimed at the evaluation of stable housekeeping genes for gene expression normalization in carbon nanoparticle-induced acute pulmonary inflammation in mice (Yin et al. 2010). 11 frequently used housekeeping genes, namely 18S rRNA, ACTB, B2M, CYPA, GADPH, GUSB, HMBS, HPRT1, RPL13A, SDHA and TBP were selected for the study. geNorm, NormFinder and BestKeeper programs were used for the analyses. All three algorithms ranked RPL13A and ACT-B among the most stable reference genes while GAPDH and B2M were considered among the least stable. This is partly contrary to the results from this research in the sense that although RPL13A has also been considered a stable reference gene from my results, GAPDH which Yin and fellow researchers found not to be stably expressed in lung tissues, was found stable.

4.1.2.1 A brief critique of all three normalization algorithms

It is note-worthy that a number of studies have critiqued the accuracy of the widely-used software algorithms BestKeeper, geNorm and Normfinder. It has been observed that these algorithms are based on the main supposition that none of the investigated candidate reference genes show systematic variation in their expression profile across the samples being considered. However, Khanlou and Bockstaele, 2012 have argued that this conjecture does not always reflect reality.

Although geNorm has been commended for its two main advantages, namely: that the raw data are used as an input file and do not need to be normally distributed, and that the algorithm does not need a large sample size to provide reliable results (Serrano et al. 2011); the analysis relies on the principle that the expression ratio of two 'ideal' reference genes should be identical in all samples, regardless of the experimental condition or cell type (Vandesompele et al. 2002). geNorm top ranks those genes with the highest degree of similarity in their expression profile, rather than with minimal variation (Andersen et al. 2004), because geNorm investigates the expression ratio of candidate genes and does not take the variation across sample sets into account. It is therefore crucial that the two 'ideal' reference genes top ranked by geNorm should independently be regulated and not co-regulated, because co-regulated genes have a high similarity in their expression profile (Khanlou and Bockstaele, 2012). This problem was anticipated in this research and since BestKeeper and Normfinder do not suffer from this problem, they were used to confirm the results generated by geNorm.

The major advantage of BestKeeper has been considered to be the determination of standard deviation for each reference gene. BestKeeper then eliminates the reference genes which show high standard deviation ($SD C > 1$) from further calculation. However, a major draw-back of BestKeeper is that the Pearson correlation coefficient used by this algorithm is only valid for normally distributed data with a homogeneous variance. If the data do not meet these assumptions, using this parametric test can lead to false results (Khanlou and Bockstaele, 2012). As the method used by geNorm handles this problem effectively, there was a degree of confidence derived from the fact that both algorithms produced similar results.

The NormFinder algorithm uses a model-based approach which takes variations across subgroups into account and avoids artificial selection of co-regulated genes (Andersen et al. 2004). This is its major advantage. However, the accuracy of the result achieved by NormFinder is still based on the assumption that the average of the tested candidate genes should show no systematic variation. As this is not always the case, NormFinder has been found to be deficient in this area.

The use of all three algorithms strengthened the accuracy of the results obtained as the concerns which might result from the pitfalls of one application were seen to be

effectively addressed by the strengths of the others. Although their methods cannot be seen to be flawless, a combination of all three methods does possess some measure of accuracy and can therefore, be considered appropriate for normalization.

4.1.3 Normalization strategy

One of the most important problems affecting the accuracy of quantitative expression data is the choice of an appropriate normalization strategy. Worryingly, this is still a not widely appreciated or acknowledged step in gene quantification analysis and therefore requires targeted experimental investigation (Zampieri et al. 2010).

Taking into consideration the work of researchers on this subject, there were substantial indications that normalisation using the geometric mean of the most stably expressed genes selected by robust algorithms was the most satisfactory strategy currently available.

After identification of the most stably expressed reference genes in primary human monocytes stimulated with LPS, Piehler and colleagues were interested in validating their data and ascertaining whether different normalization strategies significantly change gene expression results. For this, they quantified the mRNA expression of two target genes (TNF- α and IL10) known to be significantly upregulated upon LPS stimulation of monocytes and, based on their data, used different approaches to normalize the expression levels of these target genes. The following normalization approaches were applied in their research:

1. A normalization factor calculated by geNorm based on the three most stably expressed genes (PPIB, B2M and PPIA),
2. The geometric mean of the best combination of two genes as recommended by Normfinder (B2M and TBP),
3. The single best gene as suggested by Normfinder (ACTB), and
4. Another gene frequently used for gene expression normalization, GAPDH.

Their results indicated that normalization of RT-qPCR data to GAPDH or ACTB alone leads to imprecise gene expression results in LPS-stimulated monocytes and

that the employment of several, stably expressed reference genes is crucial (Piehler et al. 2010).

Another study focused on the selection of reference genes required for reliable performance of RT-qPCR in human normal and cancer cell lines. Two software applications were used for the validation of selected reference genes. The researchers found the results from their analysis unsatisfactory and therefore came to a conclusion that for the establishment of a set of reference genes for target gene normalization in an experimental setup, the use of ideally 3 reference genes selected by at least 3 stability algorithms should be undertaken. Another important recommendation was that it should be considered that ideal reference genes can vary with the set of cell lines under investigation and therefore these genes should be carefully selected for individual studies with the best possible compliance with the MIQE (Minimum Information for publication of Quantitative real-time PCR Experiments) guidelines (Jacob et al. 2013).

In another study, suitable control genes for quantitative PCR studies in plasma DNA for non-invasive prenatal diagnosis were evaluated and validated. BestKeeper, geNorm and Normfinder were used for the analysis. The validation of the results was satisfactory and the researchers were confident to draw conclusions on their study, based on the analysis of the software applications used (Yang et al. 2014).

A study which focused on the selection of reliable reference genes during THP-1 monocyte differentiation into macrophages also used the software applications employed in this study. The validation of the results obtained using CD36 known to be regulated during THP-1 monocyte differentiation into macrophages was satisfactory and the researchers therefore recommended the genes considered most stably expressed by the applications for further studies involving THP-1 monocyte differentiation (Maeß et al. 2010).

From the fore-going, it is of great importance that the results of gene-expression experiments be normalised using multiple genes. Normalisation improves the accuracy and reliability of results, and was therefore utilised in this study.

4.2 INVESTIGATING THE EFFECT OF LPCAT 3 GENE SILENCING ON LIPOPOLYSACCHARIDE-INDUCED INFLAMMATORY RESPONSES IN MACROPHAGES

4.2.1 LPCAT3 gene silencing

Silencing the LPCAT3 gene was the first step towards the investigation of the role of the enzyme in the inflammatory response of macrophages. This is based on the premise that if LPCAT3 were relevant in the inflammatory response of macrophages, successfully silencing the gene encoding the enzyme would result in a significant reduction in the expression of selected inflammatory cytokines. The small interfering RNA (siRNA) technique (Hamilton and Baulcombe, 1999) was used. This technique which involves the introduction of double stranded RNA (dsRNA) into a cell which results in the destruction of mRNAs whose sequences share homology to the dsRNA; was initially reported in plants, but later on, RNAi-related events were described in almost all eukaryotic organisms, including protozoa, flies, nematodes, insects, parasites, and mouse and human cell lines (Agarwal et al. 2003). The excellent sequence-specificity (Deng et al. 2014) which places a good degree of confidence in the preciseness of LPCAT3 silencing (as against the co-silencing of other LPCAT enzymes) and the high throughput property (Gao et al. 2014) of the siRNA technology, make it stand-out among other gene-silencing techniques; and led to its use in the silencing of the LPCAT3 gene in this study.

Satisfactory evidence of LPCAT3 gene silencing was obtained in the experiments. Normalised against the geometric mean of the CT values of GAPDH, RPL13A and CASC3, approximately 80% (SE \pm 0.0065) LPCAT3 gene silencing was achieved.

Assessing the protein expression of this enzyme would have provided a further confirmation of the existence of its silencing, but for time constraints, this experiment could not be carried out. The assessment of this confirmation is hereby suggested for further studies that would be involving this enzyme.

4.2.2 TNF-alpha expression

Tumour necrosis factor (known to be produced chiefly by activated macrophages) is a cell signalling protein involved in systemic inflammation and is one of the pro-inflammatory cytokines that make up the acute phase reaction (a group of systemic and metabolic changes that occur within hours of an inflammatory stimulus). The primary role of TNF is in the regulation of immune cells. It is known to be able to induce fever, apoptotic cell death, cachexia, inflammation and to inhibit tumourigenesis and viral replication.

Having been considerably observed to play a significant role in inflammation and sepsis (Spooner et al, 1992, Kumar et al. 1996, Rigato et al. 1996, Lv et al. 2014 and Schulte et al. 2013), it would not be inappropriate to say that changes in the expression of the gene encoding this cytokine, in response to LPCAT3 down-regulation, should be considered relevant; and insinuations derived from this, on the role of LPCAT3 in the inflammatory response of macrophages would be appropriate. Thus, the cytokine was selected as a suitable candidate for observation in this research.

Normalised against the geometric mean of the CT values of GAPDH, RPL13A and CASC3, there was a 27 fold increase (SE: ± 4.62 , P value: 0.0170) in the expression of TNF-alpha on the addition of LPS in three independent experiments; This suggests that macrophage stimulation with LPS results in a significant increase in the expression of the pro-inflammatory cytokine, TNF-alpha. This has been the observation of numerous studies (Nathan, 1987, Shapira et al, 1994, Matsukawa et al. 1997 and Kuwata et al. 2003).

Also normalised against the geometric mean of the CT values of GAPDH, RPL13A and CASC3, using the samples with negative siRNA + LPS as the control, there was an approximately 2 fold decrease (SE ± 0.06 , P Value=0.002) in the LPS-induced expression of TNF-alpha in the samples in which LPCAT3 was silenced (Cells+LPCAT3siRNA+LPS), in three independent experiments. This implies that LPCAT3 silencing results in a significant decrease in the LPS-induced expression of the pro-inflammatory cytokine, TNF-alpha.

4.2.2.1 Enzyme-Linked Immunosorbent Assay (ELISA) analysis for TNF-alpha protein expression

The central principle of ELISA is the use of an enzyme to deliver a signal that a particular antigen-antibody reaction has occurred and to what extent (Butler, 2000). Enzymes are highly specific, and their catalytic properties can enhance a non-enzymatic reaction a billion-fold (Fersht, 1977). This highly sensitive immunoassay, employing enzyme-conjugated antibodies measures changes in enzyme activities proportional to the antigen or antibody concentrations involved in the underlying immune reactions.

It was considered appropriate that a confirmation of the protein expression of at least one of the cytokines assayed, be conducted. This would further buttress the results obtained from real time PCR and enable conclusions to be reached more confidently.

TNF-alpha was selected for this assay. Normalization of the results of the ELISA experiments was done by calculating the percentage concentration of the cytokine in the three different treatments used, considering the treatment with the highest concentration (Cells+Negative siRNA+LPS) as 100%. In the three independent experiments conducted, there was a significant (30%) reduction ($SE \pm 4.72$; P value: 0.0214) in the protein expression of TNF-alpha in the samples in which LPCAT3 was silenced.

The foregoing strongly suggests that LPCAT3 gene silencing results in a significant reduction in the protein expression of the pro-inflammatory cytokine TNF-alpha.

4.2.3 IL-10 expression

Interleukin-10 (IL-10) is a key immune-regulatory cytokine that can be produced by almost all leukocytes, including innate immune cells such as monocytes, macrophages, mast cells, natural killer cells, eosinophils, and neutrophils, as well as adaptive immune cells.

The main targets of IL-10 on immune cells are antigen-presenting cells and lymphocytes. It has been observed that IL-10 inhibits the antigen-presenting capacity of monocytes and macrophages by down-regulating cell surface levels of MHC class II, co-stimulatory molecules such as CD86 and adhesion molecules such as CD58

(Ding et al. 1993) and also inhibits the function of dendritic cells by down-regulating the production of IL-12 and expressions of MHC class II and co-stimulatory molecules (McBride et al. 2002). IL-10 also promotes the development of a type 2 cytokine pattern by inhibiting the IFN- γ production of T lymphocytes (Romagnani, 1995), directly inhibits the proliferation of CD4⁺ T cells and production of cytokines such as IL-2, IFN- γ , IL-4, IL-5, and TNF-alpha (Joss et al. 2000), consequently impairing cellular immune responses, and regulates Th1/Th2 imbalance. IL-10 has also been found to reduce the secretion of IL-23 by macrophages, which is essential for the existence of Th17 cells (Schuetze et al. 2005).

Thus, there was sufficient evidence that this anti-inflammatory cytokine was deeply involved in the dynamics associated with inflammation and the development of sepsis. Therefore, it was selected as an appropriate candidate in this research.

Normalised against the geometric mean of the CT values of GAPDH, RPL13A and CASC3, there was a 22 (SE: \pm 8.89, P-value: 0.0710) fold increase in the expression of IL-10 on the addition of LPS in three independent experiments. This suggests that macrophage stimulation with LPS results in an increase in the expression of the anti-inflammatory cytokine, IL-10, although this increase did not prove to be statistically significant in this research. A number of studies have observed an LPS-induced increase in the expression of IL-10 (Barsig et al. 1995, Liu et al. 2006, Chanteux et al. 2007 and Iyer et al. 2010).

Also normalised against the geometric mean of the CT values of GAPDH, RPL13A and CASC3, there was an approximately 2 fold decrease (SE \pm 0.23, P Value=0.09) in the LPS-induced expression of IL-10 in the samples in which LPCAT3 was silenced (Cells+LPCAT3siRNA+LPS), in three independent experiments. Because the PCR results from the experiments for IL-10 were not statistically significant, there is therefore unconvincing evidence, from this research, that LPCAT-3 gene silencing results in a significant change in the expression of the anti-inflammatory cytokine, IL-10.

4.3 CONCLUSION AND RECOMMENDATIONS

Normalisation with multiple reference genes is becoming more prevalent, but studies that apply this normalisation approach are limited. This research undertook this important aspect of real time PCR experiments to ensure that valid results are obtained and accurate conclusions are arrived at. A combination of the methodology employed by the three software applications used, provided some measure of accuracy in the results produced, because the pitfalls of each algorithm were effectively addressed by the strengths of the others.

To the best of the researcher's knowledge, there have been no published studies on the most stably expressed reference genes suitable for RT-qPCR normalization involving RAW cells. The contribution to knowledge afforded by this research can therefore be considered novel and relevant. It would be of relevance to other researchers that the geometric mean of the objectively determined CT values of the reference genes GAPDH, RPL13A and CASC 3 would serve as an appropriate normalization factor in real-time PCR studies involving RAW cells.

Secondly, as has been observed in numerous studies, LPS stimulation of macrophages results in a significant increase in the expression of the pro-inflammatory cytokine TNF-alpha. Its role in the gene expression of the anti-inflammatory cytokine IL-10 is inconclusive, from the results of this study.

Thirdly, The LPCAT3 enzyme does have an important role in the inflammatory response of macrophages. Silencing the gene encoding this enzyme results in a significant reduction in the gene and protein expression of TNF-alpha. Its role in the expression of IL-10 is yet inconclusive from the results of this study.

Conclusions from a study conducted by Jackson and colleagues have suggested that specific inhibition of LPCAT leads to the down-regulation of inflammatory cytokine production in monocytes and epithelial cells by preventing translocation of TLR4 into membrane lipid raft domains (Jackson et al. 2008a). In the experiments conducted by Jackson and colleagues, after stimulation with LPS, a significant fraction of TLR4 was seen to be located in the lipid raft fractions of the cell membrane. When the cells were treated with the LPCAT inhibitor, 5 hydroxyethyl 5.3' thiophenyl pyridine, and stimulated with LPS, TLR4 was prevented from

translocating into the raft fractions (Jackson et al. 2008a). This strongly suggests that LPCAT plays a key role in the important signal transduction processes that are central to the LPS-induced inflammatory responses by regulating the translocation of TLR4. The possible co-localisation of this class of enzymes with the TLR4-CD14 signalling complex, also points to this mechanism of action (Abate et al. 2015). This is a possible mechanism by which the silencing of LPCAT3 results in a significant reduction in the gene and protein expression of the cytokine TNF- α .

LPCAT could be playing this role by regulating membrane fluidity (Hishikawa et al. 2014 and Rong et al. 2015) thus controlling movements of molecules within the membrane and in and out of the lipid raft domains, or perhaps by acting as an intermediate molecule in possible biochemical reactions that may be working to regulate the inflammatory responses to LPS. This could be a potential research question to be investigated in future.

4.3.1 Implications for Sepsis

Numerous studies have been conducted aimed at the discovery of therapies whose mechanisms of action border on the modulation of the inflammatory process. In one study, the effect of TNF-alpha blockade on the inflammatory response in cirrhotic rats with induced bacterial peritonitis was evaluated. Serum TNF-alpha decreased significantly in rats treated with ceftriaxone plus anti-TNF-alpha monoclonal antibody but not in rats treated with antibiotics alone (Sanchez et al. 2013). This further supports the observation that effective modulation of cytokine release is an important anti-inflammatory mechanism which should be given more research attention.

Another study investigated the mechanisms by which inflammatory responses contribute to apoptosis in human neuroblastoma cells treated with fipronil. Results from this study suggested that Meloxicam (a COX-2 inhibitor) may exert anti-apoptotic effects against fipronil-induced cytotoxicity by both attenuating oxidative stress and inhibiting the inflammatory cascade via inactivation of MAPK and p53 signalling, both important elements in the TLR4 signalling cascade. The results from this study put forward the point that further study into the activities of COX-2

inhibitors might reveal other pathways in their mechanisms of action that could be explored in the search for specific anti-inflammatory therapies.

Pavlov and fellow researchers have described a role for central muscarinic acetylcholine receptors in the activation of the cholinergic anti-inflammatory pathway. Activation of this pathway inhibits the production of TNF and other cytokines and protects animals from the inflammatory damage caused by endotoxemia and severe sepsis (Pavlov et al. 2006). This discovery paved the way for a new research area in drug discovery for sepsis: The exploration of the activities of central muscarinic receptors. Research into the treatment of sepsis, thus suggests very diverse pathways to the attainment of that goal, which should all be continuously explored until much better advances are made towards the emergence of specific therapies for the disease condition.

Lithium, a therapeutic agent used for bipolar disorder and neurodegenerative disease has also been explored for anti-inflammatory properties. In a research that investigated the effect of lithium on ceecal ligation and puncture (CLP)-induced tissue injury in the lungs, Lithium decreased both the pro-inflammatory cytokine response (IL-1, IL-6 and TNF-alpha) and the generation of Reactive Oxygen Species (ROS), increased by polymicrobial sepsis (Albayrak et al. 2013).

In this research, the role of phospholipids in the inflammatory processes in sepsis was investigated and results suggest an important role for them in this disease process, especially in response to a powerful inducer of the inflammatory response: LPS. While other studies on sepsis, some of which have been highlighted above are as important, research into LPS-induced inflammatory responses explores a specificity in the therapy against bacterial sepsis.

Recent studies have investigated agents involved in LPS-induced inflammatory responses. Granzymes which are serine proteases released by cytotoxic lymphocytes have been found to bind to Gram-negative bacteria and LPS, lowering the threshold for monocyte activation by LPS, by synergistically increasing LPS-induced release of proinflammatory cytokines *in vitro* and *in vivo* (Wensink et al. 2014).

Specific Inhibition of Histone Deacetylase 8 has also recently been found to result in significant decreases in the LPS-induced gene expression of the pro-inflammatory

cytokines IL-1 β , IL-1 α , TNF α , and IL-6 in peripheral blood mononuclear cells (Li et al. 2015).

Results from another study showed that Lactic acid attenuates LPS-induced mast cell activation. In this study that utilised murine bone marrow mast cells, pre-incubation with lactic acid significantly reduced cytokine (TNF, IL-6) and chemokine secretion following LPS stimulation (Caslin et al. 2015).

It is note-worthy that it is this specificity in activity, explored by the studies investigating LPS-induced responses, including this research, that is crucial in the search for novel anti-sepsis agents.

The involvement of LPCAT3 in macrophage inflammatory responses has been strongly suggested in this study; specifically that LPCAT3 gene silencing results in a significant decrease in the expression of the pro-inflammatory cytokine TNF-alpha. This discovery does have important implications in the search for novel targets for anti-sepsis therapies. As previously observed, Jackson and colleagues identified a promising non-specific inhibitor of CoA-dependent LPCAT, 5 hydroxyethyl 5.3' thiophenyl pyridine, and inhibitory RNA sequences as potential LPCAT inhibitors are also being developed (Jackson et al. 2008a). The inhibition of LPCAT3 would result in a significant inhibition of the release of a key pro-inflammatory cytokine TNF-alpha. This development would be an important step towards the modulation of a crucial inflammatory response in macrophages, namely TNF-alpha release.

Inflammatory roles have been ascribed to LPCAT1 and LPCAT2 (Morimoto et al. 2010, Jackson and Parton, 2004, Schmid et al. 2003, Shindou et al. 2013 and Harayama et al. 2009). There have also been indications that the induction of LPCAT 3 results in the priming of macrophages to release inflammatory mediators (Ishibashi et al. 2013). This study strengthens that observation and further broadens the knowledge base available in this research area.

This study utilised well-reputed techniques in its investigation, a robust PCR calibration system, the RAW 264.7 cell line proven to be a suitable transfection host, *E.coli* LPS for cell stimulation very well reputed for inducing strong inflammatory responses and the use of the ELISA technique to further buttress the results of the PCR experiments.

While the quality and accuracy of results from this research were vigorously tested, the sample size (number of experiments conducted) was the accepted minimum. This would have been increased to further strengthen the reproducibility of the outcomes, but for time constraints. Performing the experiments using a human cell line would have also provided more reliable outcomes, as the anti-sepsis therapies being targeted by the investigation into the phospholipid remodelling enzymes are for the benefit of humans. However, obtaining human macrophages in sufficient quantities for repeat experiments is challenging. Furthermore, such cells are more difficult to keep in culture and there are genetic differences between cells from different patients which add to the variability in the experimental results. In addition, they are more difficult to transfect and would require ethical approval (Wilding and Bodmer, 2014). Apart from human monocytes or macrophages, human cell lines such as MonoMac6 could be used, as these retain many characteristics of human monocytes but are continuously growing (Moesby et al., 1999).

So, while the RAW 264.7 cell line provided a good measure of consistency and transfection stability from experiment to experiment, and was therefore considered appropriate for this initial investigation, reproducing the results of these experiments in human cells would provide more relevant results and further strengthen the prospects of specific anti-sepsis therapies as suggested by this study.

Further studies on this subject are highly recommended. A study of the effect of LPCAT3 enzyme over-expression on cytokine release would further underscore the role of the enzyme in inflammation and sepsis while an investigation into the mechanism of action of this enzyme would provide insight into further potential drug targets. Further research aimed at the discovery of specific inhibitors of this enzyme and their effects on LPS-induced cytokine expression would offer further understanding of the nature of the drug formulations that would possibly be developed into specific anti-sepsis treatments. If the results of such studies support and improve upon the conclusions that have been arrived at in this research, there is a great possibility that specific anti-sepsis therapies have good prospects of being developed in the near future.

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Appendix

Experiments/ Endogenous controls	ATPB5	B2M	EIF4A2	SDHA	GAPDH	RPL13A	ACTB	CASC3	UBC	YWHAZ
Experiment 1	83.8	83.5	88.1	89.3	79.5	83.3	84.5	85.4	86.8	87.2
Experiment 2	86.9	86.6	85.9	91.7	82.3	83.1	91.7	84.9	87.7	88.4
Experiment 3	87.6	86.2	87.5	92.0	82.5	83.8	91.8	86.4	88.2	86.6

Table 6(a): Average Efficiency of all reference genes (expressed in percentages) as calculated by the PC Miner software

Samples/Endogenous controls	ATPB5	B2M	EIF4A2	SDHA	GAPDH	RPL13A	ACTB	CASC3	UBC	YWHAZ
Cells only	16.907	16.921	20.511	19.541	14.915	17.215	14.709	23.263	20.372	18.410
Cells+Negative siRNA+LPS	17.609	16.778	21.572	20.564	15.778	17.870	16.103	23.783	20.155	18.894
Cells+LPCAT3 siRNA+LPS	16.970	15.881	20.516	19.829	15.041	17.056	14.093	23.258	19.747	18.582

Table 6(b): Average CT Values of all reference genes as calculated by the PC Miner software (1st independent experiment)

Samples/Endogenous controls	ATPB5	B2M	EIF4A2	SDHA	GAPDH	RPL13A	ACTB	CASC3	UBC	YWHAZ
Cells only	17.5247	19.8556	23.581	21.530	16.979	15.408	15.725	25.651	20.567	22.638
Cells+Negative siRNA+LPS	17.890	19.168	24.702	22.842	17.434	15.585	15.551	25.792	20.396	23.292
Cells+LPCAT3 siRNA+LPS	17.490	18.711	23.984	22.313	16.926	15.296	15.159	24.838	19.912	22.430

Table 6(c): Average CT Values of all reference genes as calculated by the PC Miner software (2nd independent experiment)

Samples/Endogenous controls	ATPB5	B2M	EIF4A2	SDHA	GAPDH	RPL13A	ACTB	CASC3	UBC	YWHAZ
Cells only	17.208	19.909	24.197	21.235	16.943	15.236	15.473	24.863	20.142	22.279
Cells+Negative siRNA+LPS	17.973	19.384	25.460	23.430	17.508	15.540	15.434	25.804	20.645	23.489
Cells+LPCAT3 siRNA+LPS	17.505	19.290	24.727	22.536	17.071	15.300	15.079	24.848	19.740	22.492

Table 6(d): Average CT Values of all reference genes as calculated by the PC Miner software (3rd independent experiment)

Gene name	Stability value
GAPDH	0.245
RPL13A	0.336
SDHA	0.609
ATP5B	0.382
B2M	0.501
EIF4A2	0.568
UBC	0.668
YWHAZ	0.864
Beta Actin	0.626
CASC-3	0.118

Table 7: Normfinder analysis using the CT values from the PC Miner. NormFinder ranked CASC3, GAPDH and RPL13A as the three most stably expressed genes among ten tissue samples, having stability values of 0.118, 0.245 and 0.336 respectively. YWHAZ was found to be the least stable gene, having the highest expression stability value of 0.864.

Gene name	Stability value
GAPDH/CASC3	0.344
RPL13A	0.419
ATP5B	0.596
ACT-B	0.721
UBC	0.787
B2M	0.860
EIF4A2	1.001
SDHA	1.068
YWHAZ	1.140

Table 8: geNorm analysis of the most stably expressed genes. GAPDH and CASC3 were adjudged the most stable reference genes having the least expression stability value of 0.344. YWHAZ was found to be the least stable gene, having the highest expression stability value of 1.140.

From: Confidence Okorie
Sent: 10 February 2015 13:32
To: 'windupzs@gmail.com'
Subject: RE: Request for Permission

Thank you, Professor Zhao.
Kind regards,
Confidence.

From: Sheng Zhao [<mailto:windupzs@gmail.com>]
Sent: 10 February 2015 01:00
To: Confidence Okorie
Subject: RE: Request for Permission

Hi, Okorie,
Thanks for the request. Yes, you can use them for your research.

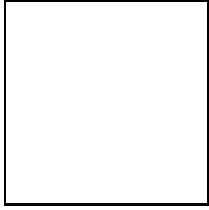
赵 晟 (Sheng Zhao)

Associate Professor
Dept. of Biochemistry and molecular biology,
Medical School, Southeast University,
#87, Ding Jia Qiao, Gu Lou Qu,
Nanjing, Jiangsu, China, 210009

Cell phone in China: +86 18551669724 and +86 13675130010 (Feixin)
Cell phone in USA: +1 (510) 854-6689 (voice and text only)
Email: shengzhao@seu.edu.cn, windupzs@gmail.com
QQ/gchat/MSN/Skype: windupzs@gmail.com (or QQ number: 1217953800)

From: Confidence Okorie [<mailto:confidence.okorie@plymouth.ac.uk>]
Sent: Thursday, February 5, 2015 5:40 PM
To: windupzs@gmail.com
Subject: Request for Permission

Dear Professor Zhao,
I am a postgraduate student conducting research in Cellular and Molecular Immunology at the University of Plymouth, United Kingdom.
I did find your research paper: Sheng Zhao, Russell D. Fernald. Comprehensive algorithm for quantitative real-time polymerase chain reaction. J. Comput. Biol. 2005 Oct;12(8):1045-62.; as well as the PC Miner software particularly useful in my research.
I wish to obtain permission to use Figures 1 and 5(a) in my research project. I guarantee they would be properly referenced.
Thank you in anticipation.
Yours faithfully,
Confidence.



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