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The role of Lysophosphatidylcholine acyltransferase-2 (LPCAT-2) in inflammatory responses

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**The role of Lysophosphatidylcholine
acyltransferase-2 (LPCAT-2) in inflammatory
responses**

By

HANAA SALIH ABD ALI ALRAMMAH

A thesis submitted to Plymouth University in partial
fulfilment for the degree of

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The role of Lysophosphatidylcholine acyltransferase-2 (LPCAT-2) in inflammatory responses

Hanaa Salih Abd Ali Alrammah

Abstract

Sepsis is the overwhelming inflammatory response to infection, especially bacterial infection and associated bacterial products. It has major healthcare impacts, affecting an estimated 19-30 million persons/year worldwide with a mortality of 30-70%. Despite intense research, no specific therapy has been established for sepsis and in addition to the high mortality, the associated economic costs are very high. For example, recent data shows that the annual cost of patients with sepsis is more than \$20 billion in the USA, and £2.5 billion in the UK. Therefore, novel targets and new therapies for sepsis are required which will have an important impact on both mortality and economic benefits. Recent work has demonstrated that the phospholipid modifying enzyme, LPCAT, has a role in the regulation of inflammatory responses to bacterial infections. However, the mechanism of action in this regard is not well understood. This project aimed to identify the role of LPCAT-2 in inflammatory response to infections. This project has utilized the RAW264.7 murine macrophage cell line as an experimental model and LPS or Pam3CSK4 as infectious stimuli to investigate the role of overexpressing LPCAT-2 as well as silencing the over-expressed LPCAT-2 using siRNA technique. RAW264.7 cells transiently or stably transfected with the LPCAT-2 gene were used to study the role of LPCAT-2 in the inflammatory responses of macrophages. LPCAT-2 was successfully over-expressed in RAW264.7 cells and the overexpression was successfully confirmed with real time polymerase chain reaction (RT-PCR) and western blotting. The overexpression of LPCAT-2 significantly upregulated the pro-inflammatory cytokines TNF- α and IL-6, at both gene expression, and protein level, while the anti-inflammatory cytokine, IL-10, was down regulated in these cells. Moreover, overexpression of LPCAT-2 significantly decreased the expression of TLR4, peroxisome proliferator-activated receptors γ (PPAR γ) and CD206 (a marker of M2 macrophages) while it significantly increased CD14, TLR2, COX-2 and iNOS (M1 markers). LPCAT-2 gene expression was also increased when PPAR γ was blocked with the selective (PPAR- γ) antagonist T0070907. Importantly, silencing the transiently over-expressed murine LPCAT-2 resulted in a significant reduction in TNF-alpha and a significant increase in IL-10 gene expression. Both the transient and stably transfected RAW264.7 cells have been used to study the role of LPCAT-2 in regulating inflammatory responses in macrophages. The results have significantly added to knowledge of the role of LPCAT-2 in the inflammatory response and will aid in the development of novel therapies for inflammatory conditions such as sepsis.

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Gentle, Hermanus Koopman, Connor Wood, Dr Shagun Khera, Rafie Pajouheshnia, Dr Hanaa Thigeel, Afak Alzaidi, Shaymaa Aljoubory, Sanna Hadi, Ali Hadi, Mashael Alkhanbashi, Ibrahim Alhallawani, Khalid Hazim Al-Muhannah, Nicolaus Kairyasis, Khalid Alshaghдали, Dr Hadil Hadi, Dr Sahib Mohammadbakir, Luma Abdulqadir, Dr Wasan Gharby, Dr Tamara Natiq, Nuha Khalaf, Dr Samah Jassam, Dr Inam lafta, Aman Niyazi, Jekaterina Kockina, Soulaf Jabbar Kakel, Dr Jwan Hussain, Ali Hussain, Dr Ali Al-Omari, Rose and Terry, Ahmed Alaazawi, Oluwamuyiwa Soewu, Deepak Dharan Padmini, Dr. Basil Saati and Ranj Salaie, Akhil Alsadwi, Musaab Al Ameer, Monkith Gailan, Entisar Hussien, Lubna Tahir Khalaf, Wijdan Ali Omran, Taha Ibrahim, Dr Aseel Hussein, Wameidh Potrus, and Dr Isaac Shawa for their supports and continued encouragement and motivation.

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I would like to thank the examiner committee, Prof Ken Jones, Dr Simon Fox and Dr Craig Donaldson for their valuable amended corrections to make my thesis more valuable.

Dedication.

I dedicate this thesis

To my sponsor, the Higher Committee for Education Development in Iraq who was unconditionally supportive.

To my mother, the most precious person for me ever who spent her life so far to support me, my sisters and brothers with smiley face even though, she was suffering in silent, her smile which I really missed every single day was the only thing that could make the darkness fade away and shine my life, I am praying to Almighty Allah to keep her safe and strong and all my family members until I will be able to see them all again.

To my aunt (Kadhimia) who passed away and I was not being able to say Good-Bye.

To my friend Suad Alsoleh (2nd Feb 1975- 24th March 2016) who passed away lonely in DerriFord Hospital. Till now, I am regretting the moment that I was not been able to be there beside her for the last time.

To all my friends who have killed as well as all the innocence victims due to the massive explosion in Alkaradda shopping centre on the 3rd July 2016, which turned their Eid event, the most happily moments into such sorrow days.

To all innocence people who have been killed in my beloved city, Baghdad and all Iraqi cities due to continued explosions without any mercy nor a sense of humanity toward innocence people who supposed to live happily and peacefully in their home country since 2003 and so far.

To my best friend Nasr Al-Dulaimi who was always inspiring and motivating me whenever I was down, unfortunately, he is fighting the malignant cancer since October 2016. I hope he will survive, if not, it will be very hard to accept it. Although, his name will last here and in my memory too.

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To the one who make me reach the other edge of the river.

Author's Declaration

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award without prior agreement of the Doctoral College Quality Sub-Committee. Work submitted for this research degree at the University of Plymouth has not formed part of any other degree either at the University of Plymouth or at another establishment.

This study was financed with the aid of Prime Minister Office/ Higher Committee for Education Development in Iraq and carried out in collaboration with collaborating institution, University of Plymouth.

During the course of the doctoral study programme of advanced study was undertaken, which included taught modules taken; full attendance of research methods and skills in biological sciences, BIO5124, included the literature review and research project proposal that credited as a requirements for PhD programme, informal attendance of immunology module with no credit. Also a number of relevant postgraduate courses were attended to gain transferable skills such as; introduction to EndNote, introduction to SPSS, Nvivo worksop, introduction to R.

Project management, what is LaTeX? an introduction, careers in academia, making progress in your research degree - avoiding defeatism and self sabotage, overview to searching and accessing information resources, careers: simulated assessment centre, LaTeX? getting started, citadel hill seminar: James Lea, Plymouth growing futures gardening project launch. An introduction to applying for research funding, an invitation for Charles Clarke 150 Prestige Lecture, An introduction to applying for research funding, scientific writing skills for environmental scientists workshop (twice), Relevant scientific seminars and conferences were attended at University of Plymouth such as; Ecotoxicology and chemistry research conference- Plymouth University 13th July 2013

(attendance).

International symposium on environmental radioactivity 3rd - 4th September 2012: invitation for the social event and inaugural session, Being a focus group member in adopts a staff member with Plymouth University, human resources from February 2012 until June 2012. VC's Teaching and Learning Conference including TEL showcase 6th July 2012 (attendance), Vice-Chancellor's enterprise award finalist, 2013. Attending the international intreprenuer conference 2014, 22nd March 2014.

Centre for research in translational biomedicine (CRTB) research day 4th July 2013, and 2nd September 2014, Postgraduate society committee conferences 11th March 2013 at which work was presented.

The following external institutions were visited for consultation purposes: Bugs Busters Immunity activity 30th Nov-1st December 2013 British society of immunology, Liverpool as a volunteer to present immunity activity for public, Research employability event 18th April 2012 (Bristol Trip). Fisher trip to Science World (21st Science World celebrate with us) 19th April 2012. British society for immunology (BSI) congress meeting in Brighton (1- 4 December 2014) at which work was presented.

Workshop

Practical Techniques in molecular biology workshop, 16-19 July, 2012, Plymouth University. Gas cylinder and gases safety awareness workshop 26th June 2012, cryogenic safety workshop 25th June 2012, Practical Lab Induction 3rd May 2012. Alpha Labs Pipetting Academy workshop 3rd of May 2012.

Publications

Alammah H, Woldie WA, Avent N & Jackson SK 2014 'Overexpression of lysophosphatidylcholine acyltransferase 2 (LPCAT-2) up-regulated LPS-induced

responses in a murine macrophage' *Immunology*, 143, pp104-105. Poster presentation delivered at December, 2014 (Published, January 2015) BSI congress meeting in Brighton (1- 4 December 2014).

Presentations and Conferences attended.

Alrammah, H. Abate, W. Avent, N. Jackson, SK. (2016) Overexpression of Lysophosphatidylcholine acyltransferase 2 (LPCAT-2) up-regulated LPS-induced responses in a murine macrophage April, 2016. Unpublished poster presentation. Centre for Research in Translational Biomedicine Annual Research Day, Plymouth University.

Alrammah, H. Abate, W. Avent, N. Jackson, SK. (2015) Overexpression of Lysophosphatidylcholine acyltransferase-2 (LPCAT-2) induced M1 Phenotype in LPS induced response in murine macrophages. Unpublished poster presentation delivered at the Centre for Research in Translational Biomedicine research day, Plymouth, UK. July 2015.

Alrammah, H. Abate, W. Avent, N. Jackson, SK. (2014) Over-expression of Lysophosphatidylcholine acyltransferase-2 (LPCAT-2) in a stably transfected RAW264.7 cells. Oral presentation, 22nd October and (Poster presentation Centre for Research in Translational Biomedicine research day, Plymouth, UK. September 2014). (Published, January 2015) BSI congress meeting in Brighton (1- 4 December 2014).

Alrammah, H. Abate, W. Avent, N. Jackson, SK. (2014) Lysophosphatidyl-choline acyltransferase (LPCAT) - 2 overexpression up-regulates LPS-induced inflammatory response in macrophages. Unpublished poster presentation delivered at centre for research in translational biomedicine (CRTB), research day 2nd September 2014. Awarded the first best poster (£50 amazon Voucher).

Alrammah, H. Abate, W. Avent, N. Jackson, SK. (2013) The cloning and over-expression of Lysosphatidylcholine acyltransferase (LPCAT) in RAW264.7 cells. Unpublished poster presentation delivered at Centre for Research in Translational Biomedicine (CRTB), Research day 4th July 2013. Awarded the second best poster (£20 amazon Voucher).

Alrammah, H. Abate, W. Avent, N. Jackson, SK. (2012) The role of Lysophosphatidylcholine acyltransferase (LPCAT) in the inflammatory responses. Unpublished poster presentation delivered to Postgraduate Society with Plymouth University conference, March 2012.

Other activitie.

Apart from my PhD study, I have gained valuable and useful skills, with practical leadership experiences that developed my personality so far to be such a useful student not only a calssical ordinary PhD student within the University of Plymouth community to contribute more in building up its wellbeing to make a different by adding my fingerprint after leaving back home. It will be mentioned here:

- Work as a laboratory demonstrator, volunteer work to supervise the biomedical and healthcare sciences undergraduate and master students wthin Simon Jackson research group along with my PhD laboratory work. I also work as demonstrator (paid as teaching support assistants) in genetics subject that including two parts in his workshop; population genetics and pedigree analysis for 8 hours to assist with two sets of first year workshops in May 2016 with Dr David Price.
- Work as postgraduate society with Plymouth University represented, volunteer work in graduate review meetings since September 2014 and so far.

- Work as a postgraduate society with Plymouth University represented, volunteer work in Doctoral College meetings (sub-committee meetings) since September 2014 and so far.
- Work as a biomedical sciences school represented, volunteer work in Doctor Training Centre (DTC) meetings since September 2014.
- Work as resident assistant in Francis Drake Halls since 9th September 2013 until 6th September 2014, and resident assistant in Mary Newman Halls since 6th September 2014 until 6th July 2015.
- Work as a chairman of the postgraduate society committee, volunteer work since September 2014 and so far, and as a secretary since April 2012 until September 2014, organising and managing the leisure activities for postgraduate students, their families and friends as well as annually three scientific conferences that I have attended all to encourage them to represent their researches for public with rewards prizes for best posters and presentations.
- Work as a chairman of association of Iraqi students at University of Plymouth since 1st July 2014 until 30th August 2015 , and as a deputy chair since 8th July 2013 until 1st July 2014.
- Work as a chairman of Plymouth cultural café committee since January 2014 and so far, and as a secretary since January 2013 until January 2014.
- Member of the of the British society for immunology (BSI) since 24th August 2013 and so far.
- Member of the Iraqi Veterinary Medical Syndicate since August 6th August 2001 and so far.
- A member of the veterinarian Association for the period from 2002 and so far.

Awards.

- Centre for Research in Translational Biomedicine (CRTB) research day (award second best poster, £ 20 amazon voucher).
- Centre for Research in Translational Biomedicine (CRTB) research day (award First best poster, £ 50 amazon voucher).
- Vice-Chancellor's Enterprise Award finalist, 2013.

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A handwritten signature in blue ink, appearing to be 'H. H. H.', is written over a faint, circular stamp or watermark.

Signed.....

Date **26/02/2018**

List of abbreviations.

Abbreviation	Glossary
Ac Wt	Actual weight
AA	Arachidonic acid
ACT-B	β -actin
AcylCoA	Acyl co-enzyme A
AGPAT	1-acylglycerol-3-phosphate O-acyltransferase
AKT	Protein kinase B
ANOVA	Analysis of variance
APC	Antigen presenting cell
ATP	Adenosine triphosphate
ATP5B	Adenosine triphosphate synthase sub-unit beta
BSA	Bovine serum albumin
BMDMs	Bone marrow –derived macrophages
C5AR	C5a receptor
CD	Cluster of differentiation
CD14	Cluster of differentiation 14
cDNA	Complementary deoxyribonucleic acid
CoAIT	CoA-independent transacylase
COX	Cyclo-oxygenase
cPLA2	cytosolic phospholipase A2
CT	Cycle threshold
CYPA	Cyclophilin A
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulfoxide

DNA	Deoxyribonucleic acid
DOK1	Docking protein 1
DOK2	Docking protein 2
DPBS	Dulbecco's phosphate buffered saline
<i>E. coli</i>	<i>Escherichia coli</i>
ECACC	European collection of cell cCultures
EDTA	Ethylene- Di-amine tetra-acetic acid
EIF4A2	Eukaryotic initiation factor 4A-II
EJC	Exon junction complex
ELISA	Enzyme linked immunosorbent assay
EPA	Eicosapentaenoic acid
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
FADD	Fas (TNFRSF6)-associated via death domain
FA	Fatty acid
FBS	Foetal bovine serum
FW	Molecular weight
G418	Geneticin antibiotic
GAIT	Gamma interferon-activated inhibitor of translation
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GPC	Glycerophosphatidylcholine
GUSB	Beta-glucuronidase
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HETP	5 hydroxyethyl 5,3' thiophenyl pyridine
HMBS	Hydroxymethylbilane synthase
HMGB1	High-mobility group protein B1

HPRT1	Hypoxanthine-guanine phosphoribosyltransferase 1
HTS	High-throughput screening
HRP	Horse radish peroxidase
ICNARC	Intensive care national audit and research centre
ICU	Intensive care unit
IFN- β	Interferon beta
IFN- γ	Interferon gamma
IgG	Immunoglobulin G
I κ B- α	Kappa-B Inhibitor subunit alpha
IKK- α	Inhibitor kappa-B kinase subunit alpha
IKK- β	Inhibitor of kappa-B kinase subunit beta
IKK- γ	Inhibitor of kappa-B kinase subunit gamma
IL	Interleukin
IL-1 α	Interleukin 1 alpha
IL-1 β	Interleukin 1 beta
IL-10	Interleukin 10
IL-6	Interleukin 6
IL-8	Interleukin 8
IL-18	Interleukin 18
iNOS	Inducible nitric oxide synthase
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
Jak	Janus kinase

JNK	Jun N-terminal kinase
KAN	Kanamycin
KD	Kilo Dalton
Kdo	3-deoxy-D-manno-oct-2-ulosonic acid
L	Litter
LB-Agar	Luria Britani agar
LB-broth	Luria Britani broth
LBP	LPS-binding protein
LDS	Lithium dodecyl sulphate
LPA	Lysophosphatidic acid
LPAAT	Lysophosphatidic acid acyltransferase
LPAT	Lysophospholipid acyltransferase
LPC	Lysophosphatidylcholine
LPCAT	Lysophosphatidylcholine acyltransferase
LPEAT	Lysophosphatidylethanolamineacyltransferase
LPS	Lipopolysaccharide
LPSAT	Lysophosphatidylserine acyltransferase
Lyso-PAF	Lyso-platelet activating factor
lyso-PAFAT	lyso-PAF acetyltransferase(Lyso-platelet activating factor acetyltransferase)
M1	Classically activated macrophage or pro-inflammatory macrophage

M2	Alternatively activated macrophage or anti-inflammatory macrophage
M	Molar
Mal	MyD88 adaptor-like protein
MAPK	Mitogen-activated protein kinase
MK2	MAPK-activated protein kinase 2 (MAPKAP kinase 2)
MBOAT	Membrane bound O-acyl transferase
mcPAF	methylcarbanyl-PAF
MD2	Myeloid differentiation factor 2
MyD88	Myeloid differentiation primary response 88
MHC	Major histocompatibility complex
MIF	Migratory inhibitory factor
NCBI	National centre for biotechnology information
<i>NRAMP1</i>	Natural resistance- associated macrophage protein gene 1
µg	Microgram
µL	Microlitter
mM	Milli molar
mRNA	Messenger ribonucleic acid
NEM	N-ethylmaleimide
NEMO	NF-Kappa-B essential modulator protein
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
Ng	Nanogram
NMD	Nonsense-mediated mRNA decay
NO	Nitric oxide
NODs	Nucleotide oligomerisation domains
NR	Nuclear receptor

OPTI-MEM	Reduced-serum medium
OxPAPC	Oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine
PA	Phosphatidic acid
PAM3CSK4	N-Palmitoyl-S-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-[R]-cysteinyl-[S]-seryl-[S]-lysyl-[S]-lysyl-[S]-lysyl-[S]-lysine
PAF	Platelet activating factor
PAF-AH	PAF acetylhydrolase
PAFAT	Platelet activating factor acetyl transferase
PAFR	PAF receptor
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffer saline
PC	Phosphatidylcholine
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
PE	Phosphatidylethanolamine
PG	Prostaglandin
PI	Phosphatidylinositol
PI3K	Phosphoinositide 3-kinase
PKC	Protein kinase C
PL	Phospholipid
PLA2	Phospholipase A2
PPAR α	Peroxisome proliferator-activated receptor alpha

PPAR γ	Peroxisome proliferator-activated receptors gamma
PRR	Pattern recognition receptor
PS	Phosphatidylserine
PVDF	Polyvinylidene fluoride
qPCR	Quantitative polymerase chain reaction
RAW 264.7	Macrophage; Abelson murine leukaemia virus transformed cell line
sCD 14	Soluble cluster of differentiation 14
REST	Relative expression software tool
RIP-1	Receptor interacting protein 1
RIPA buffer	Radioimmunoprecipitation assay buffer
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
RPL13A	60S ribosomal protein L13A
rRNA	Ribosomal ribonucleic acid
RT	Reverse transcription
RT-PCR	Real-time– polymerase chain reaction
SD	Standard deviation
SDHA	Succinate dehydrogenase complex, subunit A
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SE	Standard error
Sf9	Spodoptera frugiperda 9
SHIP	Src homology 2-containing inositol-5'-phosphate
SI	Supporting information

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
STAT	Signal transducers and activators of transcription
T0070907	2-Chloro-5-nitro-N-4-pyridinylbenzamide
TAB1	TAK1-binding protein 1
TAB3	TAK1-binding protein 3
TAE	Tris-acetic acid EDTA
TAG	Triacylglycerol
TAK1	Transforming growth factor -Beta Activated Kinase 1
TAZ	Tafazzin
TBK1	TRAF family member-associated NF-Kappa-B activator Binding Kinase 1
TB	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
TIR	Toll-interleukin-1 receptor
TICAM-2	TIR domain-containing adapter molecule 2
TRAF6	TNF Receptor Associated Factor 6
TIRAP-1	TIR domain-containing adapter protein 1

TIRAP-2	TIR domain-containing adapter protein 2
TLR 4	Toll-like receptor four
TLR2	Toll-Like Receptor two
TLR3	Toll-Like Receptor three
TLR5	Toll-Like Receptor five
TLR9	Toll-Like Receptor nine
TMB	3,3',5,5'-Tetramethylbenzidine
TNF-alpha	Tumour Necrosis Factor – alpha
TNF α	Tumor Necrosis Factor Alpha
TOLLIP	Toll Interacting Protein
TRAF6	TNF receptor associated factor 6
TRAM	TRIF related adapter molecule
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
UV	Ultra violet
V/V	Volume by volume
VLDL	Very Low Density Lipoprotein
W/V	Weight by volume
WB	Western Blotting
	Tyrosine 3-monoxygenase/tryptophan 5-mono-oxygenase
YWHAZ	activation protein, zeta polypeptide

Chapter 1:
General introduction
&
literature review

1. General introduction and literature review.

1.1. Sepsis.

1.1.1. Definitions and Incidence.

Sepsis is defined as ‘life-threatening organ dysfunction caused by a dysregulated host response to infection’ (Singer *et al.*, 2016). The continuum that is characteristic of the development of this disease informed the necessity of a definition which would combine the various aspects typically defining its presence.

Sepsis has been frequently referred to as a systemic response to infection due to the presence of bacteria and bacterial products, such as lipopolysaccharide (LPS), in the blood (Adib-Conquy *et al.*, 2000; Kawai *et al.*, 2010; Adib-Conquy *et al.*, 2012; Guzzo *et al.*, 2012; Hagar *et al.*, 2013; Kayagaki *et al.*, 2013; Boomer *et al.*, 2014). It is a major healthcare problem that annually affects 19 - 30 million patient worldwide with a mortality of 25 – 70 %, and the incidence is increasing compared to 20-40 years ago because the medications was not well developed, the patient with this illness would have been died or even died due to any accident before being able to discover the exact reason of the death, compared to the high medical technology that have prolonged the patient life in intensive care units (ICU) (Bloos *et al.*, 2014; Boomer *et al.*, 2014; Ramachandran, 2014; Page *et al.*, 2015; Global Sepsis Alliance, 2016; Papali *et al.*, 2017; Patel *et al.*, 2017).

Despite the fact that sepsis is a major cause of morbidity and mortality worldwide due to the impact of multiple bacterial infection, there remains no specific therapy (Brun-Buisson *et al.*, 1995; Angus *et al.*, 1997; Brightbill, 1999; Schorr *et al.*, 2007; Logters *et al.*, 2009; Adhikari *et al.*, 2010; Paul *et al.*, 2010; Daniels, 2011; Pavlaki *et al.*, 2013; Ramachandran, 2014; Papali *et al.*, 2015; Powell *et al.*, 2015; Global Sepsis Alliance, 2016; Papali *et al.*, 2017; Patel *et al.*, 2017; Rannikko *et al.*, 2017). It is significantly common than heart attack, and leading cause of death than any cancer (Changsirivathanathamrong *et al.*, 2011; Darcy *et al.*, 2011; Dellinger *et al.*, 2013;

Wilding & Bodmer, 2014; Cohen *et al.*, 2015; Papali *et al.*, 2017; Patel *et al.*, 2017; Rannikko *et al.*, 2017). Nevertheless, even in the most developed countries less than half of the adult population have heard of it. It also remains the leading cause of death in the least developed countries (Chalupka *et al.*, 2012; Perner *et al.*, 2012; Wiens *et al.*, 2012; Langley *et al.*, 2013; Boomer *et al.*, 2014; Wiersinga *et al.*, 2014; Mickiewicz *et al.*, 2014; Global Sepsis Alliance, 2016; Papali *et al.*, 2017; Patel *et al.*, 2017; Rannikko *et al.*, 2017).

In the United States alone, sepsis affects more than 500,000 patients a year, with a mortality of 30 -70 % (Angus *et al.*, 2006; Cheng & Shi, 2009; Guzzo *et al.*, 2011). In addition, sepsis is considered the leading cause of death in intensive care units (ICUs) (Hoesel & Ward, 2004, Zahorec *et al.*, 2005; Daniels, 2011; Gaieski *et al.*, 2013; Reinhart *et al.*, 2013; Fujishima *et al.*, 2014; Rhee *et al.*, 2015; Falcone *et al.*, 2016; Civitarese *et al.*, 2017; Rannikko *et al.*, 2017).

The economic impact is also significant with estimated costs in the USA of \$ 22,000 per case and an annual cost of \$ 16.7 Billion (Angus *et al.*, 2001; Schorr *et al.*, 2007; Lozano *et al.*, 2014). The need to better clinically define sepsis led to a consensus conference by the American College of Chest Physicians/Society of Critical Care Medicine (ACCP/SCCM), in 1992 (Bone *et al.*, 1992).

The definitions published by the consensus committee, were of the essential criteria for the systemic inflammatory response syndrome (SIRS) and specific characterizations (definitions) for sepsis, severe sepsis, septic shock and multiple organ dysfunction syndromes (MODS).

The criteria for SIRS is defined by two or more of the following; core body temperature is $> 38\text{ }^{\circ}\text{C}$ or $< 36\text{ }^{\circ}\text{C}$, Heart rate (HR) is ≥ 90 beat per minute (bpm), Respiration is ≥ 20 per minute (or Partial pressure of carbon dioxide (CO₂) in the blood (PaCO₂) is < 32 mm Hg) White blood cell (WBC) count is $\geq 12,000 / \mu\text{l}$ (mm^3) or $\leq 4000 / \mu\text{l}$ or $> 10\%$ immature

forms (Shen *et al.*, 2010; Seymour *et al.*, 2012; Wiens *et al.*, 2012; Schulte *et al.*, 2013; Schorr *et al.*, 2014; Rhee *et al.*, 2014; Yealy *et al.*, 2014; Rhee *et al.*, 2015; Seymour *et al.*, 2016; Shankar-Hari *et al.*, 2016; Rhodes *et al.*, 2017).

The definition of sepsis was set as a clinical definition of SIRS criteria caused by known or suspected infection. However, these definitions of sepsis and ‘severe sepsis’ were found to be misleading and SIRS criteria could be achieved in non-life-threatening situations.

Therefore, following the 2016 consensus meeting it was suggested that sepsis should be defined as life-threatening organ dysfunction caused by a dysregulated host response to infection and septic shock should be defined as a subset of sepsis in which particularly profound circulatory, cellular, and metabolic abnormalities are associated with a greater risk of mortality than with sepsis alone (Singer *et al.*, 2016).

Patients vary in their responses; the severity of their sepsis and the speed in which its development is affected by their inherited characteristics and the presence of coexisting illnesses, and the number and virulence of infecting microbes (Martin *et al.*, 2003; Kisson *et al.*, 2011; Martin *et al.*, 2012; Angus & van der Poll, 2013; Jovanovich *et al.*, 2014; Kumar *et al.*, 2014; Nygard *et al.*, 2014; Mayr *et al.*, 2014; Assuncao *et al.*, 2014; Moore *et al.*, 2015; Jiménez-Sousa *et al.*, 2015; Jolley *et al.*, 2015; Moskowitz *et al.*, 2017; Minasyan *et al.*, 2017).

Most types of micro-organisms can cause sepsis, including bacteria, fungi, viruses and parasites. However, viral infections accompanied by seasonal influenza viruses, the Dengue and Ebola viruses could progress to an acute organ damage resulting in death from multiple organ dysfunction and septic shock (Clark *et al.*, 2004; Maier *et al.*, 2004; Harrison *et al.*, 2006; Kim *et al.*, 2008; Feldman & Geisbert, 2011; Hall *et al.*, 2011; Jawad *et al.*, 2012; Lagu *et al.*, 2012; Paessler & Walker, 2013; Iwashyna *et al.*, 2014; Liu *et al.*,

2014; Hashmi, 2015; Ojard *et al.*, 2015; Guirgis *et al.*, 2016; Kempker & Martin, 2016; Jeganathan *et al.*, 2017).

Gram-positive organisms causing sepsis to have increased in frequency over time and are now almost as common as gram-negative infections (Martin *et al.*, 2003; Finfer *et al.*, 2004; Vincent *et al.*, 2006; Anzaldi *et al.*, 2011; Dellinger *et al.*, 2013; Packiriswamy *et al.*, 2013; Hoeniglet *et al.*, 2014; Henriksen *et al.*, 2015; Kempker & Martin, 2016). This almost equal cause between Gram-positive and Gram-negative bacteria contrasts with the situation 40 years ago, when the majority were Gram-negative. Currently, most patients with sepsis seen in ICU and have indwelling catheters that could introduce skin flora internally and the majority of such organisms are Gram-positive (Karlsson *et al.*, 2007; Iwashyna *et al.*, 2010; Husak *et al.*, 2010; Kissoon *et al.*, 2011; Iskander *et al.*, 2013; Mayr *et al.*, 2014; Kumar, 2014; Lozano *et al.*, 2014; Ogura *et al.*, 2014; Singer *et al.*, 2014; Henriksen *et al.*, 2015; Singer *et al.*, 2016; Jeganathan *et al.*, 2017).

The more frequent use of broad-spectrum antibiotics in the treatment of increasingly ill patients who remain in the ICU for longer periods of time has probably contributed to an increased bacterial resistance over time (Reacher *et al.*, 2000; Carlet *et al.*, 2004; Ranieri *et al.*, 2012; Schmerler *et al.*, 2012; Standage *et al.*, 2012; Rogers *et al.*, 2014; Caironi *et al.*, 2014; Lozano *et al.*, 2014; Asfar *et al.*, 2014; Cohen *et al.*, 2015; Jolley *et al.*, 2015; Gutierrez *et al.*, 2015; Singer *et al.*, 2016).

Sepsis occurs due to the presence of bacteria and bacterial products, such as lipopolysaccharide (LPS), in the circulation. Despite the advantages of modern medicine including vaccines, antibiotics and intensive care, the incidence of sepsis is increasing. Worldwide yearly estimated morbidity rate is approximately 20 to 30 million patients to be affected, with over 6 million cases of neonatal and early childhood sepsis (Clark *et al.*, 2004; Iwashyna *et al.*, 2010; Lagu *et al.*, 2012; Guzzo *et al.*, 2012; Iskander *et al.*, 2013; Hagar *et al.*, 2013; Iwashyna *et al.*, 2014; Kaukonen *et al.*, 2014). In addition, the maternal

incidence of sepsis is over 100,000 cases (Angus *et al.*, 2013; The Global Sepsis Alliance, 2015; The Global Sepsis Alliance, 2016).

Sepsis, is higher in infants and elderly people than in other age groups. It is also higher in males than in females, and blacks than whites (Adrie *et al.*, 2005; Mayr *et al.*, 2010; Hall *et al.*, 2011; Gutierrez *et al.*, 2015; The Global Sepsis Alliance, 2016). Patients with chronic illnesses, for example, diabetes, cancer, AIDS, renal or hepatic diseases, are also at higher risk, as are pregnant women and those with a severe burn or physical injuries (The Global Sepsis Alliance, 2015, 2016; Jeganathan *et al.*, 2017).

Furthermore, the annual mortality is over 7.3 million (Angus, 2011). Sepsis is one of the leading causes of death in the advanced world, corresponding to myocardial infarction in its yearly incidence combined with a high impact on the economy (Seymour *et al.*, 2010; Wiens *et al.*, 2012; Richards, 2013; Schorr *et al.*, 2014; The Global Sepsis Alliance, 2016; Adrie *et al.*, 2017).

Recent estimates suggest an annual incidence of sepsis of about 300 cases per 100,000 people; in comparison, myocardial infarction has been estimated to affect 208 per 100,000 per year while stroke is 223 per 100,000 (Feigin *et al.*, 2009; Yeh *et al.*, 2010; Hashmi, 2015; The Global Sepsis Alliance, 2016). The annual rate of sepsis incidence, in the USA has been increasing between 8-13 % (Vincent *et al.*, 2006, Hall *et al.*, 2011; Wiens *et al.*, 2012).

In addition, Sepsis is considered as the leading cause of death in intensive care units (ICUs) (Hoesel & Ward, 2004; Karlsson *et al.*, 2007; Daniels, 2011; Myr *et al.*, 2010; McPherson *et al.*, 2013; Richards, 2013; Assuncao *et al.*, 2014; Arefian *et al.*, 2017; Adrie *et al.*, 2017; Jeganathan *et al.*, 2017). Many individuals who survive severe sepsis are completely recovered with no long-term effects but others who survived to the hospital discharge system after sepsis remained at increased risk for death during the following

months and years (Rice, 2006; Winters *et al.*, 2010; Phua *et al.*, 2011; Bloos & Reinhart, 2014; Schorr *et al.*, 2014; Adrie *et al.*, 2017; Jeganathan *et al.*, 2017).

Those who survived also could have physical or neuro-cognitive functioning impairments, mood disorders, and a low quality of life Iwashyna *et al.*, (2010). Wang *et al.*, (2012) stated: “We do not know for certain how many people are affected by such problems, but it is likely to be at least 20% of survivors”. Moreover, there is some evidence that an episode of severe sepsis can disorder a person’s immune system, making them more susceptible to future infections (Wang *et al.*, 2012; Henriksen *et al.*, 2015).

The agency for healthcare research and quality has listed sepsis as the most expensive status treated in U.S. hospitals, which has been costed more than \$20 billion in 2011 increasing annually average of 11.9 % (Torio *et al.*, 2011).

In the UK, the intensive care national audit and research centre (ICNARC) data estimates that over 140,000 patients each year develop sepsis and the mortality rate is increasing at a percentage rate of 1.5 % per annum. In addition, in 2010 about 78,000 of these patients admitted to intensive care units (ICU), with a mortality of 30 – 80 % (Daniels, 2011) with an approximate cost to the NHS of £ 2.5 billion (Dombrovskiy *et al.*, 2007; Danial, 2009; Richards, 2013; Vincent *et al.*, 2014; Ferrario *et al.*, 2016; Esposito *et al.*, 2017).

Sepsis should be treated as an emergency (Daniels, 2011; McPherson *et al.*, 2013; Asfar, *et al.*, 2014; Vincent *et al.*, 2014; Adrie *et al.*, 2017; Jeganathan *et al.*, 2017). Recent studies and quality enhancement initiatives have been demonstrated that the early recognition of the condition was following by the intravenous fluids administration and antibiotics are key to survival (Barochia *et al.*, 2010; Dellinger *et al.*, 2013; The ProCESS Investigators, 2014; Investigators *et al.*, 2014; De Backer *et al.*, 2014; Corrêa *et al.*, 2015; De Pascale *et al.*, 2016; Adrie *et al.*, 2017; Jeganathan *et al.*, 2017).

1. 2. Pathophysiology of Sepsis.

1.2.1. Lipopolysaccharide.

Sepsis is triggered by pathogens or their products in blood. Pathogen-associated molecular patterns (PAMP) is a term that refers to pathogen molecules that induce a host response including production of inflammatory mediators (Cohen, 2002).

Examples of PAMPs include molecules from Gram-negative and Gram-positive bacterial membranes. Of these, the lipopolysaccharide (LPS) (also called endotoxin) present in the outer membrane of Gram – negative bacteria have been strongly associated with the induction of sepsis (Rietschel *et al.*, 1994; Van Amersfoort *et al.*, 2003; Jackson & Parton, 2004; Harrison *et al.*, 2006; Rosenfeld & Shai, 2006; Guzzo *et al.*, 2011).

Understanding and modulating the production of LPS-induced mediators such as TNF α has therefore been the focus of research aimed for developing specific therapies for septic shock (Seymour *et al.*, 2012; Schorr *et al.*, 2014, Wensink *et al.*, 2014).

LPS can be released into the circulation from invading bacteria, either spontaneously during growth or due to immune-mediated lysis of bacteria (Wright *et al.*, 1990; Miyake, 2004; Wiersinga *et al.*, 2014).

It has three regions (Figure 1), a lipid A molecule that anchors the LPS in the outer membrane, a core sugar consisting of 3-deoxy-D-manno-oct-2-ulosonic acid (Kdo) moieties, and the O antigen, which consists of repeating oligosaccharide units. O antigen is an extremely variable region since variety bacteria possess diversity repeating numbers of polysaccharide (Raetz & Whitfield, 2002; Holst, 2011).

The lipid A element of LPS is recognized by Toll-like receptor 4 (TLR4) with its co-receptor MD-2 on host cells (Kawai and Akira, 2007; Kawai & Akira, 2010; Wiersinga *et al.*, 2014; Wensink *et al.*, 2014; Kim *et al.*, 2017). Lipid A, the hydrophobic domain of LPS, is composed of two glucosamine units with attached acyl chains and generally containing one phosphate group on each carbohydrate (Figure 1). It is the main

component of the outer monolayer of the outer membranes of Gram-negative bacteria. i.e. lipid A; a membrane anchored phospholipid contains 6 acyl groups and 2 phosphate groups. Numbers of acyl and phosphate groups could vary from one bacterial species to another, for examples the difference between the chemical structures of the major species of lipid A produced by *E. coli* (Figure 2) (Zähringer *et al.*, 1999; Hirschfeld *et al.*, 2001; Holst, 2011).

Attached to the lipid A are non-repeating “core” oligosaccharide and a distal polysaccharide (or O-antigen) that are not essential for bacterial growth in the laboratory but, in vivo are required for bacteria to resist some antibiotics, the complement system, and other external factors (Raetz & Whitfield, 2002).

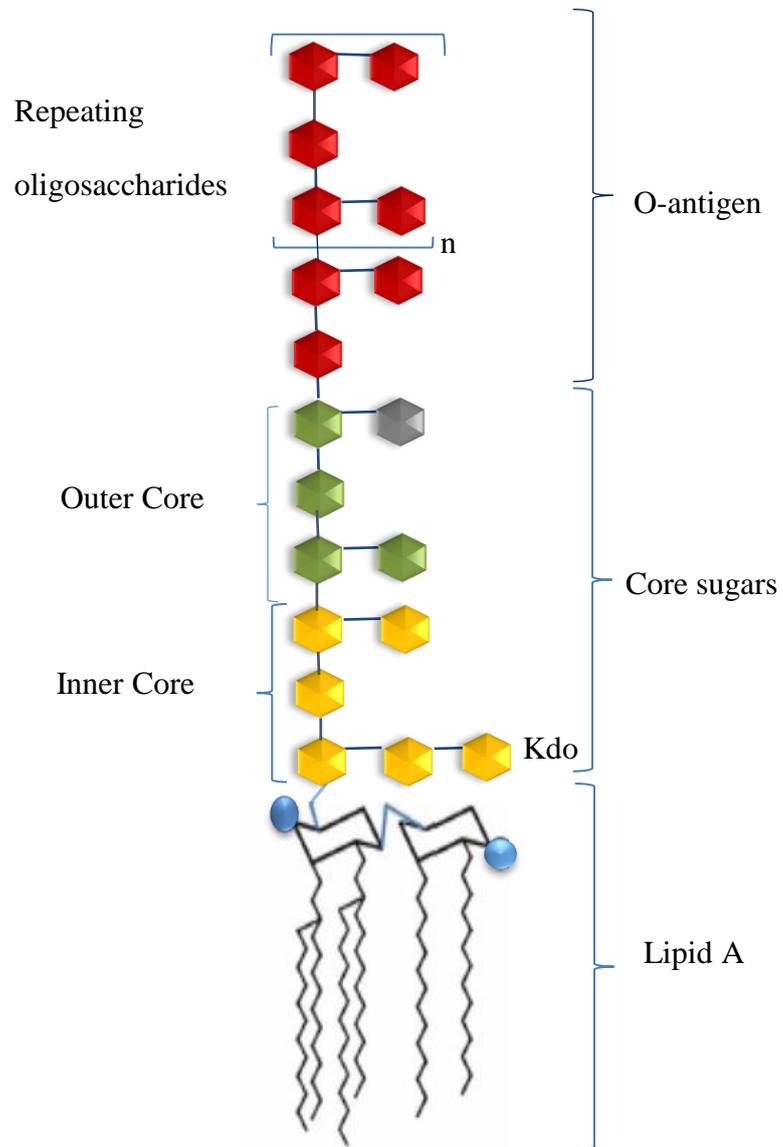


Figure 1. Schematic of the basic structure of lipopolysaccharide.

LPS consists of three regions: lipid A (chair structure indicates di-glucosamine head group, blue circles indicate phosphate groups, wavy lines indicate acyl chains), core sugars, and O-antigen, that consists of repeating units (denoted in brackets, with an “n”) of oligosaccharides (Adopted from Raetz & Whitfield, 2002).

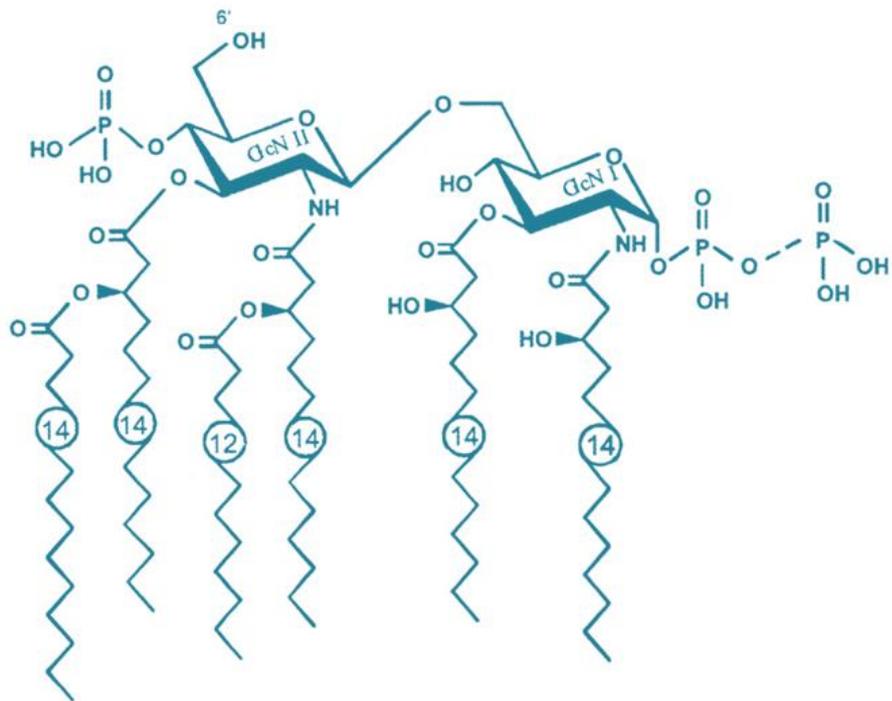


Figure 2. Chemical structure of *E. coli* lipid A.

The main structural differences between types of LPS in different bacteria is depended on the nature and number of fatty acids, presence or absence of the second phosphate in position 4', and substitution of the position 1 phosphate. For more detailed comparison is presented in reference (Zähringer *et al.*, 1999).

1. 3. The innate immune system and the response to LPS.

The innate immune system represents the first line of host defense during infection and thus plays an essential role in the priming recognition followed by triggering of immunological mediators for instance cytokines and chemokines (Medzhitov *et al.*, 1997, 1998; Yang *et al.*, 1997; Dinarello, 2000; Miyake, 2004; Guzzo *et al.*, 2012; Hagar *et al.*, 2013). The adaptive immune system is responsible for eradication of pathogenic microbes in the late stage of infection (Hashimoto-Tane *et al.*, 2010; Owen *et al.*, 2013).

As part of the innate immune system, the (PRR) are a family of receptors present on the cell wall or in the cytosol of most cell types such as monocytes, macrophages and dendritic cells that recognise pathogen-associated molecular patterns (PAMPs) (Hirschfeld *et al.*, 2002; Heumann *et al.*, 2002; Hořejší *et al.*, 2005; Kim *et al.*, 2007; Akira *et al.*, 2009; Aliche-Djoudi *et al.*, 2011; Płóciennikowska *et al.*, 2015). Toll-like receptors (TLRs) are type of Pattern recognition receptors (PRR) trans-membrane protein which were first discovered in *Drosophila* flies (Nüsslein-Volhard & Wieschaus, 1980). It is known that LPS signals via TLR 4, is one of the most potent molecules triggers an overwhelming immune response that might lead to severe health conditions and even death (Akira *et al.*, 2001; Akira, 2003; Akira & Takeda, 2004 & 2005; Akira *et al.*, 2006; Akira *et al.*, 2009; Yamamoto *et al.*, 2011; Schorr *et al.*, 2014; Płóciennikowska *et al.*, 2015).

1.3.1. The role of Macrophages.

The early information about macrophages as a fundamental actor in the immune system could be outlined to the observations and experiments conducted by the Ukrainian scientist Ilya Metchnikoff, for which he won a Nobel Prize in 1908. Apart from their central role of antigen presentation, it has been long recognised that the release of cytokines from

primed macrophages is a vital event in the pathogenesis of sepsis (Nathan, 1987; Ayala *et al.*, 1992).

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. Furthermore, functions of macrophages are essential to generation and resolution of inflammation and tissue repair (Mosser & Edwards, 2008; Guzzo *et al.*, 2012; Ha *et al.*, 2012; Hamazaki *et al.*, 2013; Hashimoto *et al.*, 2013).

Cirelli and colleagues, (1995) showed that TNF-positive mononuclear phagocytes were found in sheep lungs alongside a progressive increase within the plasma levels of the cytokine with continuous LPS infusion (Cirelli *et al.*, 1995). It was also determined that these cells possibly contribute to both the increasing circulating levels of TNF-alpha and the development of acute lung injury. In addition, following LPS injection 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 & Adib-Conquy, 2005). Ertel and his colleagues (1992) discovered that haemorrhage-induced suppression of macrophage as well as the splenocyte functions which leads to a decreasing in cytokine production. Therefore, it reduced susceptibility to sepsis (Ertel *et al.*, 1992).

Macrophages show much plasticity in their functions and their phenotype can be described between to polar extremes from 'classical activation' or M1 polarization in which they express anti-bacterial and inflammatory mediators, to 'alternative activation' or M2 polarization when they function in resolution of inflammation and tissue repair

(Benoit *et al.*, 2008; Shaykhiev *et al.*, 2009; Mantovani & Sica, 2013; Liu *et al.*, 2014; Martinez & Gordon, 20014; Chang *et al.*, 2015).

In vitro experiments have shown that stimulation of macrophages with lipopolysaccharide and interferon- γ (IFN γ) promotes the differentiation of “classically activated” M1 macrophages that release destructive proinflammatory mediators (Ding *et al.*, 1988; Mitchell *et al.*, 2011; Mills, 2012).

In contrast, interleukin (IL)-4 and IL-10 induce an “alternatively activated” M2 phenotype that possesses neuroprotective properties (Bouhlef *et al.*, 2007; Kigerl *et al.*, 2009; Goerdts *et al.*, 1999; Mandal *et al.*, 2011; Durafourt *et al.*, 2012; Ming *et al.*, 2012; Mingeot-Leclercq *et al.*, 2012; Mills, 2012; Mills *et al.*, 2014).

1.3. 2. Toll-like receptors.

Innate immunity is triggered by the recognition of pathogen-associated molecular patterns (PAMPs), by pattern recognition receptors expressed by macrophages and other host cells (Lien, 1999; Lien, 2000; Kumar *et al.*, 2009a; Kumar *et al.*, 2011). This primary recognition is crucially important for the rapid innate immune response, and for directing the later pathogen-specific adaptive immune responses. Since the identification of TLR4 as the lipopolysaccharide (LPS) receptor in 1998, it has long been expected to trigger all the responses to LPS (Poltorak *et al.*, 1998; Aderem & Ulevitch, 2000; Beutler *et al.*, 2001; Beutler & Poltorak, 2001; Lee & Hwang, 2006; Liu *et al.*, 2008; Kumar *et al.*, 2009b).

LPS triggers monocytes and macrophages to produce cytokines such as TNF- α , IL-1, and IL-6 (Akira *et al.*, 1990; Shapira, *et al.*, 1994; Kielian *et al.*, 2004; Płóciennikowska *et al.*, 2015). These cytokines serve as endogenous mediators of inflammation via receptor-mediated interactions with different target cells. Cytokine production is important for the efficient control of growth and diffusion of invading pathogens (Rothe *et al.*, 1993; Tracy & Cerami, 1994; Armstrong *et al.*, 1996; Chen *et al.*, 2011).

However, cytokine overproduction is harmful for the host, and may lead to multiple organ failure and death. The primary characteristic feature of severe sepsis is multiple organ dysfunction caused by excessive production of inflammatory cytokines (Dobrovolskaia & Vogel, 2002; Cohen, 2002; Heumann & Roger, 2002; Van Amersfoort *et al.*, 2003; Liu *et al.*, 2006; Iwamura, & Nakayama, 2008; Hashimoto-Tane *et al.*, 2010; Iskander *et al.*, 2013; Hussell *et al.*, 2014).

Upon recognition of the PAMPs, TLRs trigger production of pro-inflammatory mediators helping to eradicate infection. Currently, thirteen TLRs have been identified and described in mammals, twelve of which are expressed in mice and ten in humans (Chen *et al.*, 2011; Płóciennikowska *et al.*, 2015).

A considerable amount of evidence backs a model where LPS or LPS-containing bodies (including intact bacteria) form complexes with a serum protein known as LPS-binding protein (LBP) 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 & Karsan, 2006; Kumar *et al.*, 2009b; Płóciennikowska *et al.*, 2015; Kim *et al.*, 2017).

Then, the LPS-LBP complex is transferred and binds to soluble CD14 (sCD14) or the membrane-bound CD14 that binds to the extracellular domain of TLR4-MD2 complexes (Ulevitch & Tobias, 1999; Heumann & Roger, 2002; Płóciennikowska *et al.*, 2015), followed by TLR4 oligomerisation and activation of the signaling cascade (Jin *et al.*, 2008; John *et al.*, 2010; Płóciennikowska *et al.*, 2015; Kim *et al.*, 2017).

The dimerization of the receptor complex induces the assembly of TIRAP, MyD88, and IRAK kinases in a myddosome at the TIR domain of TLR4 prompting a signaling pathway leading to production of pro-inflammatory cytokines (Kawai & Akira, 2010; Kainu, 2012; Płóciennikowska *et al.*, 2015; Kim *et al.*, 2017).

After endocytosis, TRAM and TRIF associate with TLR4 triggering a signaling pathway which controls production of type I interferons and some other cytokines as shown in

figure 2. CD14 is a cell surface GPI-anchored protein that lacks a transmembrane domain and thus lacks the capability to directly initiate intracellular signaling events (Kim *et al.*, 2007; Kawai *et al.*, 2010; Kennedy *et al.*, 2011; Płóciennikowska *et al.*, 2015; Kim *et al.*, 2017).

In addition, MD2 is a secreted glycoprotein which is a crucial extracellular adaptor molecule for LPS-initiated signaling events, possibly by aiding in ligand recognition (Shimazu *et al.*, 1999; Kim *et al.*, 2007; Kim *et al.*, 2017). The discovery of the role of TLRs has significantly advanced the field of innate immunology and was honored with the Nobel Prize to Jules Hoffmann and Bruce Beutler in 2011.

Beutler's group first revealed that TLR4 is activated by lipopolysaccharide (LPS, endotoxin) (Figure 3) (Płóciennikowska *et al.*, 2015). It was revealed that when the intracellular signaling pathway of constitutively active TLR4 in Jurkat cells is activated, a transcription factor, nuclear factor kappa B (NF- κ B) is activated (Medzhitov *et al.*, 1997; Lee *et al.*, 2006; Jin *et al.*, 2007; Kim *et al.*, 2017). These findings have led to the assumption that human TLR4 played a vital role in this early stage of the induction of innate immunity.

While TLR4 is the LPS receptor, ligands for the other TLRs have been discovered. TLR2 is mainly responsible for Gram-positive cell-wall structures recognition (Takeuchi, 1999; Takeuchi *et al.*, 1999; Yoshimura *et al.*, 1999; Lucero *et al.*, 2004; Lu *et al.*, 2008; Takeuchi & Akira, 2010), while TLR5 is the receptor for flagellin recognition (Hayashi *et al.*, 2001; Takeda *et al.*, 2003) and TLR9 is responsible for recognition of coupled protein G (CpG) elements in bacterial DNA (Takeshita *et al.*, 2001; Takeda *et al.*, 2003; Kumar *et al.*, 2011; Packiriswamy *et al.*, 2013).

Then, the activated MyD88/MAL activates IRAK4, TRAF6, TAK1, and IKK complexes, while TRIF/TRAM signals by RIP1 to TRAF6/TAK1 and IKK. Next, both these

pathways unite at NF- κ B (Luo *et al.*, 2003; Lu *et al.*, 2008; Takeuchi & Akira, 2010; Płóciennikowska *et al.*, 2015).

The cytoplasmic NF- κ B complex is retained in the inactive state through I κ B that is in turn degraded via proteasomes, resulting in the translocation of NF- κ B into the nucleus. Moreover, activating NF- κ B, TAK1 also phosphorylates MAPKs to further reinforce the inflammatory response (Figure 3) (Ninomiya-Tsuji, 1999; O'Neill, 2006; Carmody *et al.*, 2007; Boomer *et al.*, 2014; Płóciennikowska *et al.*, 2015)

However, the TRIF/TRAM pathway triggers IRF3 to mount an antiviral response. As a conclusion, all these signaling pathways contribute to eradicating infection and play an important role in sustaining the normal physiological functions in intestinal epithelial cells as well (Yesudhas *et al.*, 2014; Płóciennikowska *et al.*, 2015).

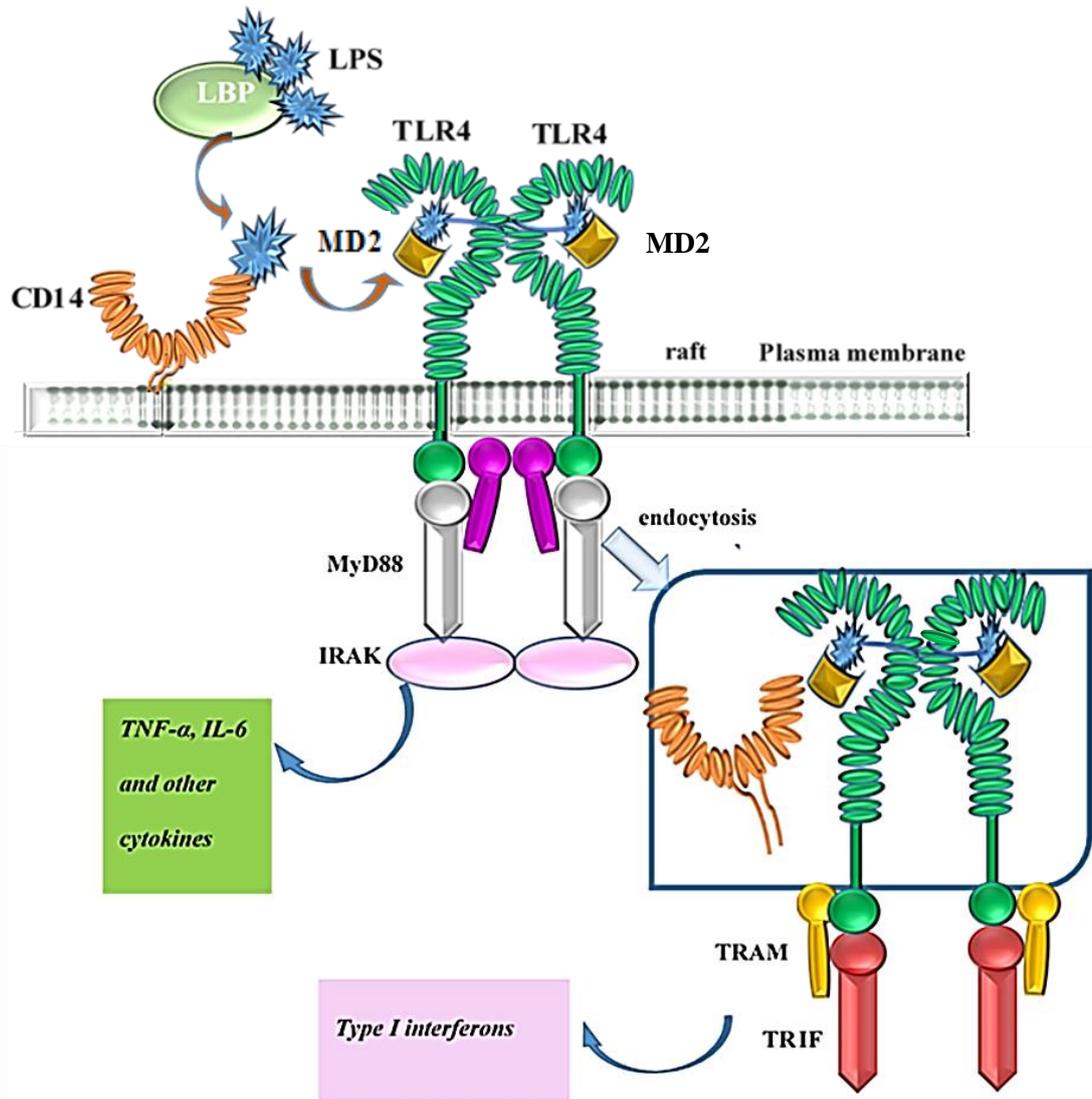


Figure 3. Initiation of TLR4 signaling pathway mediated by LPS.

LPS is bonded to the LBP, to bring the LPS to the cell membrane surface via forming LBP-LPS complex, this complex then, jointed the membrane-bound CD14, CD14 which is binding to the extracellular domain of TLR4-MD2 complexes, then followed by TLR4 oligomerisation and activation of the signaling cascade pathway through either the endocytosis or MyD88-dependent pathway to control the synthesis of which controls production of type I interferons and proinflammatory cytokines. (Figure adapted from (Płóciennikowska *et al.*, 2015)).

Furthermore, the TLR4 signaling cascade originated following LPS binding is boosted by homodimerization of the receptor (Zhang *et al.*, 2002; Lu *et al.*, 2008; Płóciennikowska *et al.*, 2015). Then, the signal propagation involves recruitment of cellular adaptor molecules for TLRs such as myeloid differentiation factor 88 (MyD88), MyD88 adaptor-like protein (Mal), which is termed TIRAP, TIR-containing adaptor inducing interferon (IFN) β (TRIF), that is known as TIRAP-1 (TICAM-1), and TRIF-related adaptor molecule (TRAM), also termed TIRAP-2 (TICAM-2). Kinases including IRAKs and MAPKs as well (Lee *et al.*, 2006; Kim *et al.*, 2017).

Moreover, the TLR4 activation leads to induction of both a MyD88-dependent and a MyD88-independent pathway. The main players involved in eliciting the functional effects of LPS are activated via the NF- κ B, mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)/Akt pathways. These pathways controlled the equilibrium between cell viability and inflammation (Dauphinee & Karsan, 2006; Lee *et al.*, 2006; Jin *et al.*, 2007; Kim *et al.*, 2017).

Furthermore, the TLR signaling pathway is initiated by MyD88-dependent signaling pathway that is used by all TLRs except TLR3. Then, the signaling through the MyD88-dependent pathway leads to the MAPK and IKK complex activation resulting in activation and nuclear translocation of AP-1 and NF- κ B, respectively. In this step, the TLR4 is capable of signaling via the MyD88-independent pathway as well. The MyD-88 pathway this is the single signaling mechanism for TLR 3 (Jin *et al.*, 2007; Płóciennikowska *et al.*, 2015).

In addition, TRIF is the main adaptor protein in the MyD88-independent pathway and can be associated with TRAF6 to activate AP-1 and NF- κ B. On the other hand, it also activates NF- κ B by interacting with RIP-1. TRIF could further interact with TRAF3 and the phosphatidylinositol 3-kinase (PI3K)- KT pathway resulting in the nuclear translocation of IRF3 and IRF2, respectively (Figure 4) (Jin *et al.*, 2007).

The stimulation of macrophages with LPS results in rapid changes in the expression of genes encoding cytokines and other inflammatory mediators. The numerous cytokines released from macrophages exert diverse cellular effects and they are essential to the inflammatory process in sepsis (Flad *et al.*, 1993; Rietschel *et al.*, 1994; Cowdery *et al.*, 1996; Brunialti *et al.*, 2006; Płóciennikowska *et al.*, 2015; Kim *et al.*, 2015).

The excessive, sustained and uncontrolled activation of macrophages in sepsis leads to a vicious cascade of inflammatory mediator release that amplifies the inflammation to the detriment of the host (Rangel-Frausto *et al.*, 1995; Kim *et al.*, 2007; Płóciennikowska *et al.*, 2015).

Furthermore, the pro-inflammatory activity of TLR4 is related to pathological responses to endogenous ligands in autoimmune disorders and chronic inflammatory conditions associated with the development of atherosclerosis, neurodegenerative diseases, and others, which fuels interest in TLR4 signaling (O'Neill *et al.*, 2009; den Dekker *et al.*, 2010; Trotta *et al.*, 2014).

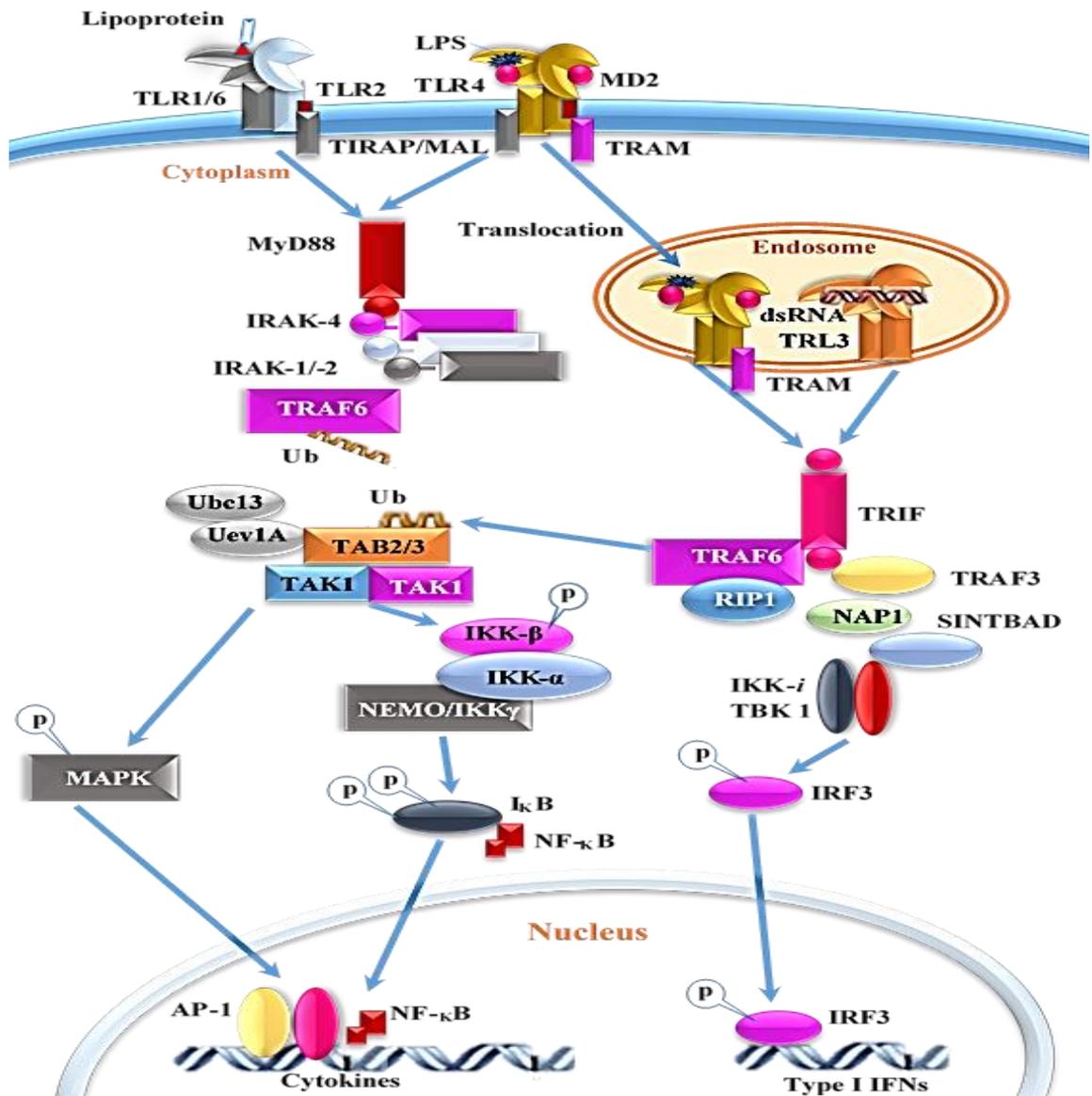


Figure 4. TLR2, TLR3, and TLR4 Signaling Pathways.

Lipoproteins and LPS were recognized on the cell surface by a heterodimer of TLR1/6 and TLR2, and via 2 sets of TLR4/MD2 complexes, respectively. TLR signaling pathway is initiated by MyD88-dependent signaling pathway that is used by all TLRs except TLR 3. The signaling through the MyD88-dependent pathway leads to the MAPK and IKK complex activation resulting in activation and nuclear translocation of AP-1 and NF- κ B, respectively. TRIF is the main adaptor protein in the MyD88-independent pathway and can be linked with TRAF6 to activate AP-1 and NF- κ B. However, it also activates NF- κ B by interacting with RIP1. While TRAF3 is responsible for phosphorylation of IRF3 by TBK1/IKK-i. NAP1 and SINTBAD were required for the activation of TBK1/IKK-i. Phosphorylated IRF3 trans-located into the nucleus to induce expression of type I IFN genes (Figure adapted from *Jin et al., 2007*).

1.3.3. NOD- Like receptors (NLRs).

Nucleotide-binding oligomerisation domain (NOD) -Like receptors (NLRs) are important elements of innate immunity which have been detected in species as simple as sea urchins. Additionally, NLR- like proteins were observed in plants and they were functionally and structurally similar to the mammalian one (Lespinet *et al.*, 2002; Ogura, *et al.*, 2003; Martinon & Tschopp, 2005; Hibino *et al.*, 2006; Proell *et al.*, 2008; Laing *et al.*, 2011; Lange *et al.*, 2011). NLRs are cytosolic receptors which are expressed predominately by macrophages and dendritic cells. To date, in humans, 23 NLRs are described and 33 once in mice.

Generally, NLRs are a cytoplasmic PRR family containing three domains: C- terminal region a leucine –rich repeats (LRR) domain that utilised for the ligand sensing, the central nucleotide domain NATCH (well-known as NOD) which is required for the self-oligomerisation as well as activation, and the effector domain at the N-terminus that contained either the caspase recruitment domain (CARD) or the pyrin domain (PYD) which is mediated interactions with other signaling proteins (Kanneganti *et al.*, 2007) (Figure 5).

The TLRs, LRR structures in NLRs are essential to the recognition of PAMPs, but direct evidence of the interaction with identified NLR-agonists has not yet been demonstrated. The fundamental central nucleotide domain, NATCH is sharing structural similarities with the dominant motif of apoptotic protease-activating factor 1, that promotes formation of oligomeric structures. Consequently, a parallel mechanism has been suggested for the formation of the NLR inflammasomes platform (Martinon & Tschopp, 2004; Faustin *et al.*, 2007; Church *et al.*, 2008; Moher *et al.*, 2009; Conforti-Andreoni *et al.*, 2011). Martinon *et al.*, (2002) have described the multi-protein cytoplasmic complexes which are facilitating the proinflammatory caspases activation, termed inflammasomes.

NALP subfamily of NOD family which has 14 members, each processed a PYD domain (Tschop *et al.*, 2003; Murray, 2009; Conforti-Andreoni *et al.*, 2011). Numerous NALPs are shown to form inflammasomes after activation led to processing IL-1 β (Figure 5). Three inflammasomes have been identified according to the NLR protein they engage: the NLRP1, NLRP3 and IPAF inflammasomes. Inflammasomes complex formation led to the recruitment of ASC, ASC contained both the CARD and PYD domains, which is mediating docking of other CARD-containing proteins into inflammasomes, for example, caspase-1.

Therefore, the recruitment and activation of caspase-1 lead into the processing of the IL-1 β pro-form (Figure 5) (Martinon *et al.*, 2004; Mariatthasan *et al.*, 2004; Mariatthasan *et al.*, 2006; Masters *et al.*, 2011; Brown *et al.*, 2011). Inflammasome complex formation is leading to ASC recruitment. Then ASC is containing both the CARD and the PYD domains as well, would mediate docking of other CARD-containing proteins into inflammasome, like caspase-1. Then, caspase-1 recruitment and activation will lead to the performing of the IL-1 β pro-form which is revealed to another possible mechanism of action of immune response toward sepsis through the formation of inflammasomes complexes and the processing and releasing of pro-form of IL-1 β and IL-18 by NOD1 and NOD2 which is recognising the bacterial molecules that involving in peptidoglycan metabolism (Agostini *et al.*, 2004; Mariatthasan *et al.*, 2006; Ogura *et al.*, 2006; Brown *et al.*, 2011). While the stimulation of TLR will induce the activation of NF- κ B which will lead to pro-IL-1 β production (Brown *et al.*, 2011).

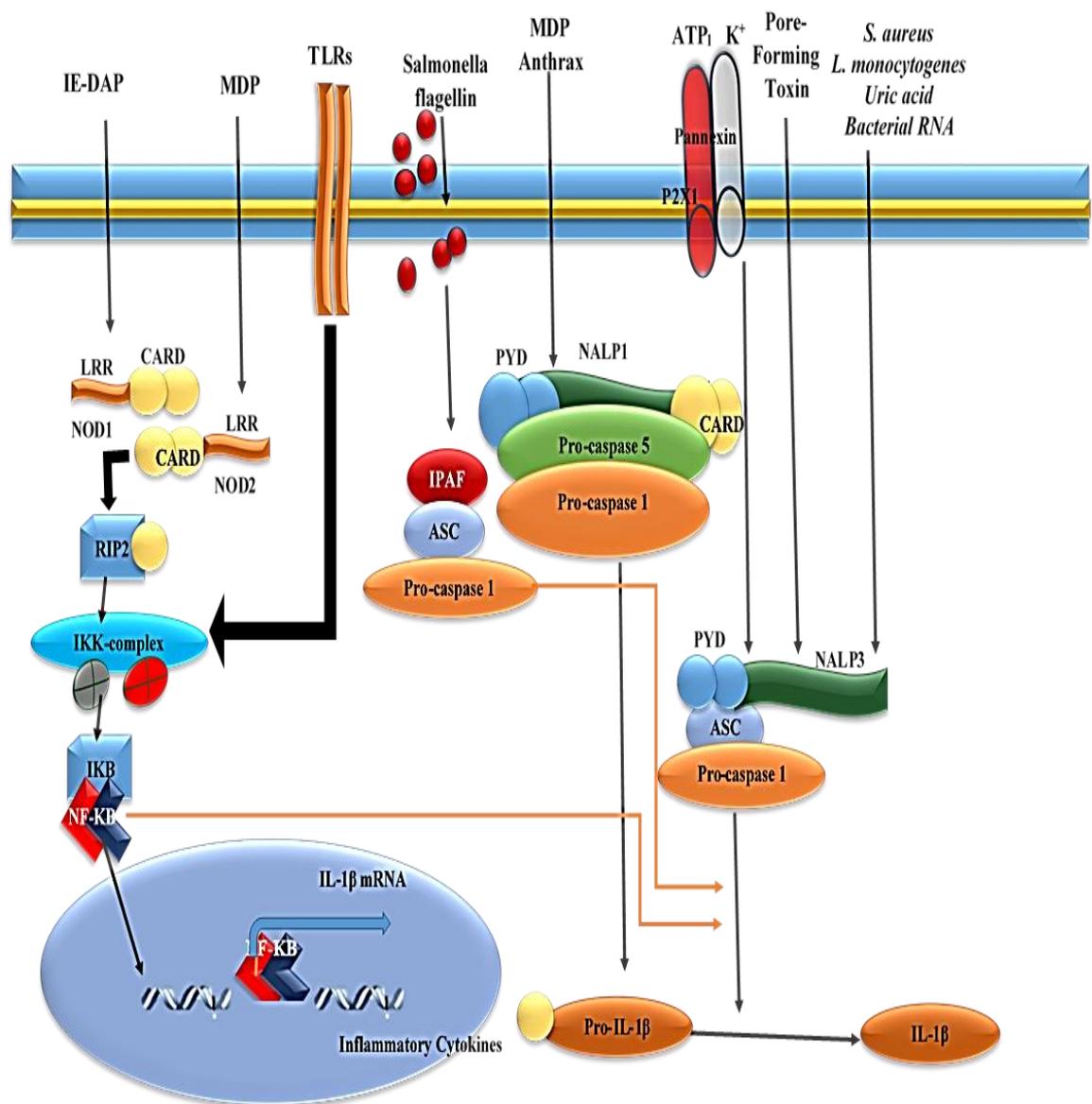


Figure 5. NOD signaling pathways. The NLR family is possessing various effector domains which is altering protein interactions. Moreover, the NOD and IPAF subfamily has a CARD effector domain, but the NALP subfamily is possessing a PYD domain. It showed different mechanism of action of the immune responses depending on the causative agents which lead to inflammasomes complexes formation and release of cytokines (pro-IL-1 β) in presence of NOD1 and NOD2 that recognise the bacterial molecules involving in peptidoglycan metabolism, for example, IE-DAP as well as MAD. While numerous NALPs could form the inflammasomes on activation leading to IL-1 β processing. The IPAF-dependent activation of caspase-1 could be induced by the flagellin from Salmonella and Legionella. While Anthrax toxin and MAP could activate caspase-1 in NALP- dependent pathway. (Figure adapted from Brown *et al.*, 2011).

1.4. Proinflammatory cytokines.

Cytokines are host response regulators toward normal and pathological environments for instance, infection, immune responses, inflammation, and trauma. Some cytokines act as proinflammatory, whereas others act anti-inflammatory to reduce inflammation and promoting healing. In the human body, cytokines have been intended to prompt biological effects in their local microenvironment where they were produced after exposing to pathological infections. In normal tissues, the incidence of cytokine-mediated tissue damages is mainly due to highly regulated cytokines release. In addition, excessive cytokine secretion from the activated cells through LPS for instance resulting in a local tissue damage (Beutler *et al.*, 1986; Dinarello & Wolff, 1993, Shimazu *et al.*, 1999; Płóciennikowska *et al.*, 2015; Kim *et al.*, 2017).

1.4.2. Tumour Necrosis Factor Alpha (TNF- α).

TNF- α is a proinflammatory cytokine released via a number of cells including activated macrophages or T-cells as a response to invading microbes or other agents. TNF- α plays an essential role in an inflammation initiation in lungs and other tissues (Driscoll *et al.*, 1997). TNF- α is a key mediator in sepsis development (Tracy & Cerami, 1994; Lee *et al.*, 2006; Płóciennikowska *et al.*, 2015; Kim *et al.*, 2017). It is one of the most essential mediators of the inflammatory diseases, in which it is increasing in some pathogenic conditions and potential toxic effects, that resulting in hypersensitivity reactions with chronic inflammation (Morrison *et al.*, 1994; Levine *et al.*, 1990; Lee *et al.*, 2006; Kim *et al.*, 2017).

The encoding gene for TNF- α is located on chromosome 6. TNF- α is a proinflammatory cytokine released by a number of cells including activated monocytes and macrophages. TNF- α is one of the TNF family of ligands that signals by two receptors, TNFR1 and TNFR2. TNF- α is cytotoxic to numerous cancer cells and considered a vital factor in

mediating the immune response against the bacterial infections (Curtis *et al.*, 2007; Lee *et al.*, 2006; Kim *et al.*, 2017).

Although, TNF- α plays a crucial role in the activation of innate response. The excessive production of TNF- α could initiate pathological changes subsequently from chronic inflammation and tissue damage. The TNF- α increasing levels could also play a role in the inflammation stimulation as well as septic shock, moreover, in the pathogenesis of several chronic diseases such as auto-immune diseases, rheumatoid arthritis (Kodama *et al.*, 2005), and diabetes (Shiau *et al.*, 2003).

1.4.3. Interleukin-1.

IL-1 is an effective intercellular mediator which is involved in inflammatory and immunological responses.

It is induced by monocytes and macrophages during antigen presentation and in response to a variety of other stimuli including endotoxin (Dinarello & Thompson, 1991; Płóciennikowska *et al.*, 2015). The gene for IL -1 α and β are located on chromosome 2. IL-1 α is one of the interleukin 1 family that is synthesised by induced macrophages, neutrophils, epithelial cells, endothelial cells and monocytes.

IL-1 β can promote pathogenesis of disorders associated with tissue damage, for instance, inflammasome mediated IL-1 β over-production which is involved in the pathogenesis of type 2 diabetes, liver damage and muscular dystrophy (Rawat *et al.*, 2010; Masters *et al.*, 2010; Tsutsui *et al.*, 2010). IL-1 β is involved in the early pathogenesis and the continued severity of a broad pattern of diseases, many arthritic diseases and septic shock (Dinarello, 2009).

While IL-1 α is pyrogenic during an inflammatory response (Dube *et al.*, 2001) and it has multi-inflammatory response controlling functions. Ingeborg *et al.*, (1996) suggested that fibroblasts could be involved in turning off the inflammatory response (IL-1 α releasing

through the activated keratinocytes could act as stimulator for the expressing and releasing of IL-8 and IL-6 yield) by reducing IL-1 levels, most, likely through IL-1 receptor-mediated uptake.

1.4.4. Interleukin-8.

Interleukin-8 (IL-8) was discovered in 1987 as a novel type of neutrophil activating proinflammatory cytokine which is acting as a chemotactic component and an activator to neutrophils, basophil and T-cells (Mukaida *et al.*, 1997) at the inflammation site when its released by monocytes and macrophages due to the inflammatory stimulants (Baggiolini *et al.*, 1989, Baggiolini and Clark-Lewis, 1992).

IL-8 is generated in response to inflammatory stimuli containing the proinflammatory cytokines IL-1 (Eckmann *et al.*, 1993b) and TNF (Li *et al.*, 2002), and cellular stress (Shapiro and Dinarello, 1995), bacterial (Eckmann *et al.*, 1993a) and viral products (Mastrorade, 1998). Moreover, it has been proposed that the polymorphism of these genes is accompanied with rheumatoid arthritis (Troughton *et al.*, 1996) and Alzheimer's disease (Vendramini *et al.*, 2007).

1.4.5. Peroxisome proliferator-activated receptors –gamma (PPAR- γ), Cyclo-oxygenase enzymes (COX-2), iNOS and CD206.

Peroxisome proliferator-activated receptors –gamma (PPAR- γ) is one of the three members of the subfamily of ligand-dependent nuclear receptor (NR) transcription factors which regulated lipid metabolism, glucose homeostasis, tumor progress and inflammation (Evans, 1988; Blumberg and Evans, 1998; Mukherjee *et al.*, 1997; Elbrecht *et al.*, 1996; Crosby *et al.*, 2005; Bouhrel *et al.*, 2007; Chawla *et al.*, 2010).

Karp *et al.*, (1991) documented that during the development of sepsis, the monocytes/macrophages shown a hyper-inflammatory status that last from hours to days, after which their status is shifted to lower-inflammatory conditions considered by stimulation with

LPS *in vitro*. Necela *et al.*, (2008) revealed that TLR4 mediate crosstalk between PPAR γ and NF-KB in macrophage.

Mueller *et al.*, (1998) revealed that PPAR γ are crucial in a diversity of biological processes, consists of; adipogenesis, glucose metabolism, and inflammation. Additional difficulties in interpretation of studies of PPAR ligands is that some act via both PPAR-dependent and PPAR-independent pathways (Crosby *et al.*, 2005; Croasdell *et al.*, 2015; Assunção *et al.*, 2017).

The PPAR γ antagonist, the synthetic compound, 2-chloro-5-nitro-N-4-pyridinylbenzamide (T0070907) that is bonded with PPAR γ , but has no capability to stimulate the transcriptional action of PPAR γ , rather antagonised PPAR γ agonised to trigger transcriptional and adipogenic actions of this receptor (Wright *et al.*, 2000; Yoshikai, 2001; Funk, 2001; Croasdell *et al.*, 2015).

Prostaglandins (PGs) are produced from arachidonic acid which is released from cell membrane by phospholipase A2 and then altered by the cyclooxygenase enzymes (COX-1 and COX-2) to enter the PG pathway (Yoshikai, 2001; Funk, 2001; Choi *et al.*, 2005; Chien *et al.*, 2013).

Funk, (2001) revealed that COX-1 is constitutively active, while COX-2 is induced under inflammatory situations. COX-2-derived PGs are involved in a diversity of pro- and anti-inflammatory progressing (Funk, 2001; Matsuoka & Narumiya, 2008).

The involvement of COX-1 and COX-2 in controlling inflammation is proved via the increased cardiovascular risk connected with the inhibition of COX-2 (Choi *et al.*, 2009; Cannon & Cannon, 2012).

Fredenburgh *et al.*, (2011) revealed that COX-2 associated with increased intestinal epithelial permeability and leads to exaggerated bacterial translocation and raised mortality during peritonitis-induced sepsis. Their results have also suggested that

epithelial expression of COX-2 in the ileum is a vital modulator of tight junction protein expression and intestinal barrier function during sepsis.

NF- κ B pathway is also well known as the major transcription factor for most inflammatory mediators, for instance, inducible isoform of nitric oxide synthase (iNOS), COX-2, ICAM-1 (Barnes and Karin, 1997; Chu *et al.*, 1998; Yamamoto and Gaynor, 2001, Ye, 2001; Corrêa *et al.*, 2007; Reuter *et al.*, 2010).

Kilburn & Griffith, (1992) stated that the bacterial infection or immunological stimuli such as LPS, IFN- γ or IL-1 is causing the expression of iNOS that, when it expressed, producing excessive amount of nitric oxide (NO). Rees *et al.*, (1990) reported that the excess NO production via macrophages and other cells which were exposing to endotoxin might contribute to septic shock, cerebral injury (Dawson *et al.*, 1993), myocardial ischemia (Matheis *et al.*, 1992), local inflammatory disorders such as polyarthritis, osteoarthritis or systemically, for instance, diabetes, arteriosclerosis (AS), and other diseases (Stefanovic-Racic *et al.*, 1993; Connor *et al.*, 1995; Wu & Thiemermann, 1996; Corrêa *et al.*, 2007). The network of M1 and M2 phenotyping shifting in macrophages due to different microbial stimuli (Green *et al.*, 2013; Martinez & Gordon, 2014) is shown in the schematic figure 6. i.e. this project hypothesized that the over-expressed LPCAT-2 might promote murine macrophages into M1 by upregulating iNOS (M1-macrophage phenotype marker), COX-2 and down regulating CD206 (M2-macrophage phenotype marker) and the opposite picture when the LPCAT-2 expressed gene is knocked down.

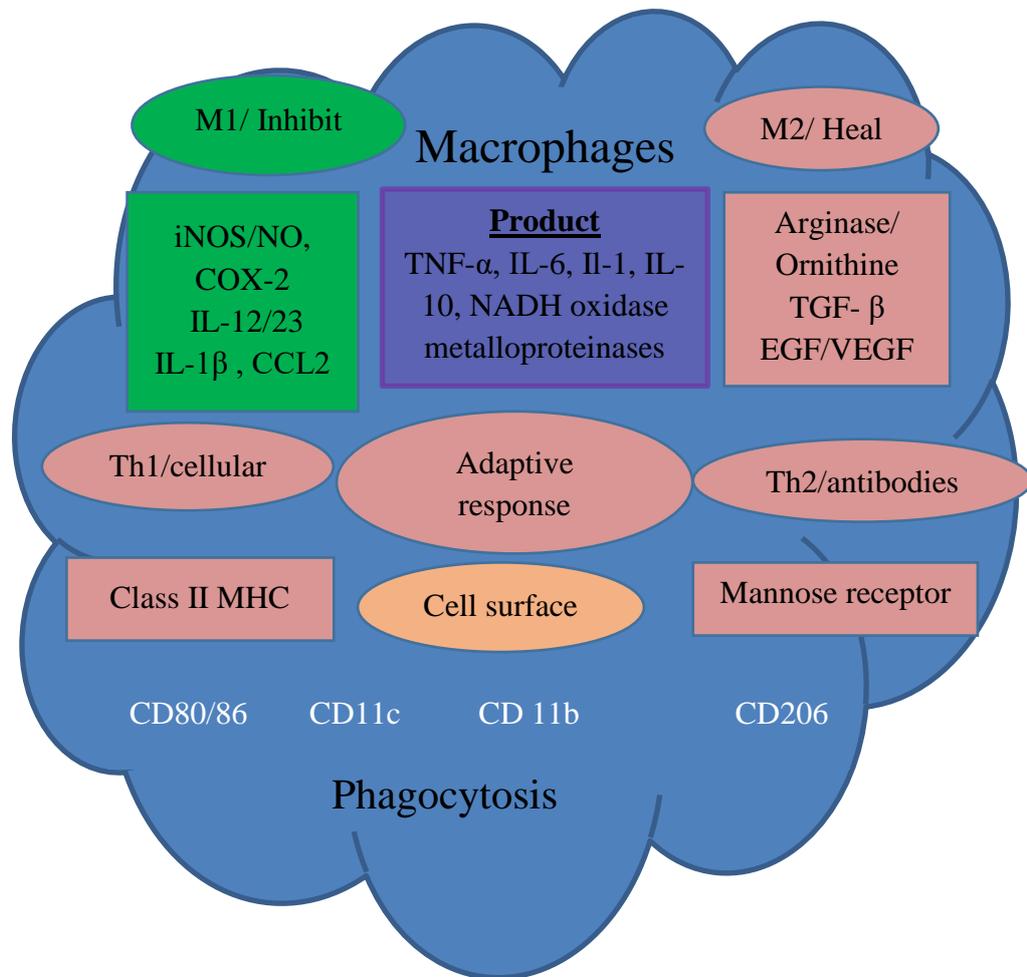


Figure 6. Schematic picture of the network of M1/M2 – macrophage polarization.

Shows the M1 and M2 characteristic products and functions. Adapted from Martinez & Gordon, 2014; Green *et al.*, 2013. *Immunol.*; 190: 270 -277.

1.5. Phospholipid metabolism and Cell membranes.

1.5.1. Phospholipid turnover.

The bio-membrane lipid bilayer serves as a vital structure that classifies living cells and forms organized intracellular membrane organelles for numerous physiological functions. The primary and key 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). Phospholipids form a membrane system that provides the cell with a protective barrier which has selective permeability for different metabolites.

Within the cell, phospholipids are the major constituents of an essential membrane system which delineates individual organelles and provides an individually ordered system for intracellular metabolic processes (Choy *et al.*, 1997). In addition, the fatty acid composition of phospholipids is key to the many different roles that these molecules impart to the cell membrane as membrane curvature, fluidity and dynamics are essential for the normal functioning of cells.

Phospholipid composition is controlled by two major pathways: The *de novo* pathway and the remodeling pathway. Through the use of Acyl-Coenzyme A (CoA's) and Sn-3-Glycerophosphate, phospholipids may be formed from diacylglycerol by the *de novo* pathway, originally described in 1956 (Kennedy and Weiss, 1956).

The acyl groups in glycerophospholipids show a great diversity and are distributed in an asymmetrical way. The saturated and the monounsaturated fatty acids are usually esterified at the sn-1 position, while the polyunsaturated acyl groups, including those contained in arachidonic acid, are located at the sn-2 position. Furthermore, the 'Kennedy' pathway does not fully explain this diversity and asymmetry.

Therefore, the rapid and continuous replacement of the sn-2 acyl moiety of glycerophospholipids has been described by Lands as a remodeling pathway (Lands'

cycle) (Lands, 1958). In the Land's cycle, it is the concerted activation of phospholipase A2s (PLA2s) that remove fatty acyl groups from the sn-2 position and lysophospholipid acyltransferases (LPLATs) that replace fatty acyl moieties at these positions that control the phospholipid composition. For the most abundant phosphatidylcholine, it is lysophosphatidylcholine acyl-transferase (LPCAT) enzymes that re-acylate the lysophosphatidylcholine and are key to the PC membrane composition. (Hishikawa *et al.*, 2008) (Figure 7& 8).

The activity of LPCAT is found to reside primarily in microsomal fractions of tissues and recognition of the activity of this group of enzymes using microsomes from different tissues which has been led to the hypothesis that multiple forms of enzymes may exist in diversity of tissues with different substrate specificity of fatty acyl-CoAs (Choy *et al.*, 1997).

There were attempts to purify the enzyme(s) which have not been successful mainly due to their membrane protein nature and the fact that LPCAT activity was typically lost during solubilisation and/or further fractionation (Zhao *et al.*, 2008). However, by applying genomic methods, LPCAT1 (the first in this family of enzymes) has been successfully cloned and characterised by two independent groups (Chen *et al.*, 2006; Nakanishi *et al.*, 2006) which then paved the way for cloning and characterisation of three more enzyme isoforms with LPCAT activity: LPCAT-2, LPCAT3 and LPCAT4. Nakanishi *et al.*, (2006) identified and characterised a mouse lung-type LPCAT expressed in alveolar type II cells. While Chen *et al.*, (2006) and Shindou *et al.*, (2007) have identified and characterised a LPCAT in alveolar type II cells termed LPCAT1. LPCAT enzyme played a critical role in the regulation of surfactant phospholipid biosynthesis.

1.5.2. The LPCAT family of enzymes.

The LPCAT enzymes, so far characterized, are found in specific locations, demonstrate preference for certain types of fatty acids and validating enzyme activities (Shindou *et*

al., 2009). LPCAT1 has both acyltransferase and acetyltransferase activities and its activity is calcium-independent. Moreover, it has been mediated the conversion of 1-acyl-sn-glycero-3-phosphocholine (LPC) into phosphatidylcholine (PC). It is shown a clear preference for saturated fatty acyl-CoAs as well as 1-myristoyl or 1-palmitoyl LPC as acyl for both donors and acceptors, respectively.

Furthermore, the dipalmitoylphosphatidylcholine synthesis has been found in pulmonary surfactant, thus playing a vital role in respiratory physiology (Bridges *et al.*, 2001; Chen *et al.*, 2006; Nakanishi *et al.*, 2006). LPCAT-2 also displays acyltransferase and acetyltransferase activities. However, unlike LPCAT1, its activity is calcium-dependent. It has been revealed to be involved in platelet-activating factor (PAF) biosynthesis through 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 changes lyso-PAF to 1-O-alkyl-2-acyl-sn-glycero-3-phosphocholine (PC), which is a key component of cell membranes and a PAF precursor.

In addition, acyltransferase activity is preferred in inactive conditions (Figure 4). On the other hand, upon the acute inflammatory stimulus, the acetyltransferase activity has been heightened and PAF production is increased (Shindou *et al.*, 2007). Additionally, it catalyses the transformation of 1-acyl-sn-glycero-3-phosphocholine to 1,2-diacyl-sn-glycero-3-phospho-choline (Agarwal & Garg, 2010; Moessinger *et al.*, 2011).

LPCAT3 and LPCAT4 from the membrane bound O-acyltransferase (MBOAT) family LPCAT3 (called MBOAT5) has been discovered by two independent groups (Hishikawa *et al.*, 2008; Zhao *et al.*, 2008).

LPCAT3 is active in mediating 1-O-alkyl-sn-glycero-3-phosphocholine acylation within a long chain fatty acyl-CoAs to produce 1-O-alkyl-phosphatidylcholine, a very crucial constituent of the cell membrane systems of mammals (Kazachkov *et al.*, 2008). The enzyme is normally localised to the endoplasmic reticulum and most richly expressed in

the liver, pancreas and adipose tissues. The overexpression of LPCAT3 in tissue culture has been established to increase the amount of phospholipids within a relatively more saturated acyl chains. However, its inhibition has also been established to result in a rise in the amount of phospholipids with more unsaturated acyl chains (Jain *et al.*, 2009). Vitrally, the preferred substrate of LPCAT3 is unsaturated fatty acyl-CoAs which appears to be the only enzyme in the liver responsible for this function (Zhao *et al.*, 2008). LPCAT4 is abundantly expressed in the brain, equivalent to the richness of phosphatidylethanolamine in this tissue. Mouse LPCAT4 mRNA has been highly expressed in the epididymis, brain, testis, and ovary (Shindou *et al.*, 2009).

In addition, LPCAT4 displays a significant Acyl-CoA-dependent acyltransferase activity toward 1-O-alkenyl-lysophosphatidylethanolamine, Lysophosphatidylglycerol, 1-O-alkyl-lysophosphatidylcholine, Lysophosphatidylserine, as well as Lysophosphatidylcholine.

Although, it lacked vital acylation activity toward glycerol 3-phosphate, lysophosphatidic acid, glycerophosphatidylinositol, and diacylglycerol as well, that has been shown several but selective functions of LPCAT4 as an enzyme involved in phospholipid remodeling (Cao *et al.*, 2008).

Its activity was not affected by Calcium. While the over-expressed in mammalian cells, LPCAT4 is seen to be localized to the endoplasmic reticulum (Cao *et al.*, 2008) (Figure 10 Appendix 1).

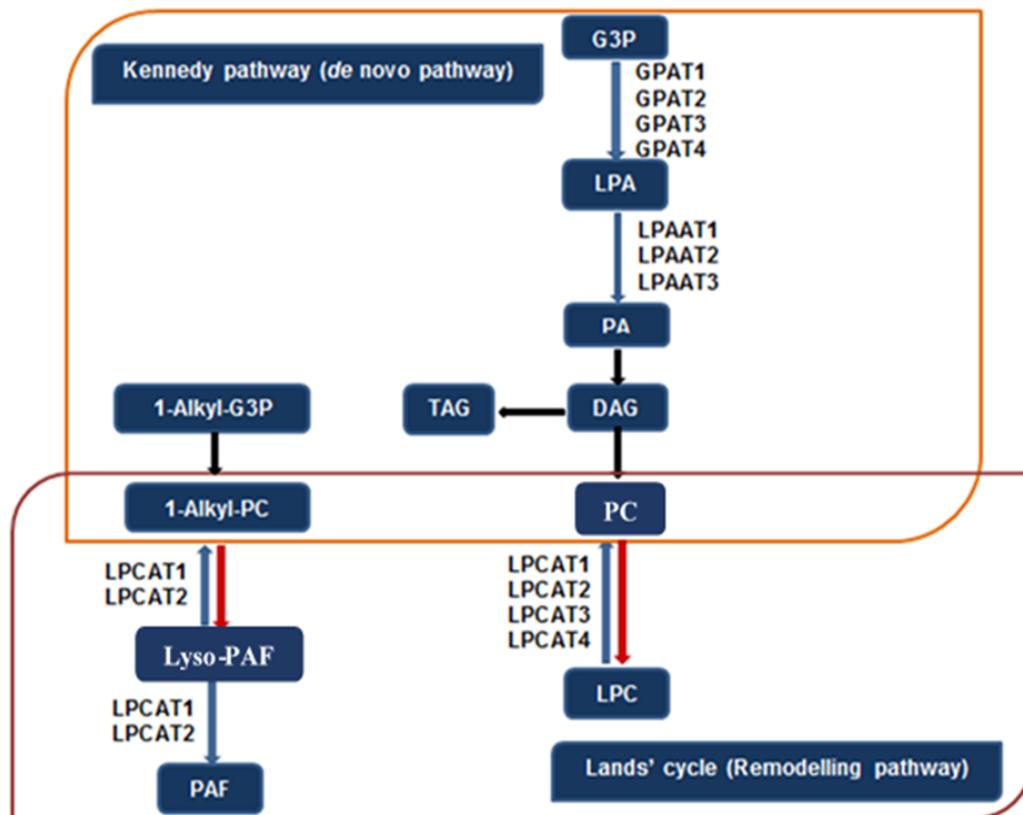


Figure 7. Glycerophospholipids biosynthesis Pathways. The Kennedy pathway (de novo pathway).

Is the initial synthesis of glycerophospholipids while they are then modified through the remodeling pathway (Lands' cycle). Red and blue arrows indicate acyltransferases and PLA2s, respectively. G3P, glycerol 3-phosphate; TAG, Triacylglycerol; DAG, Diacylglycerol; LPA, Lyso-phosphosphatidic acid; PC, Phosphatidylcholine; LPC, lyso-PC; PAF, Platelet activating factor, Adapted from Shindou and Shimizu, 2009. *Biol. Chem.*; 284:1-5.

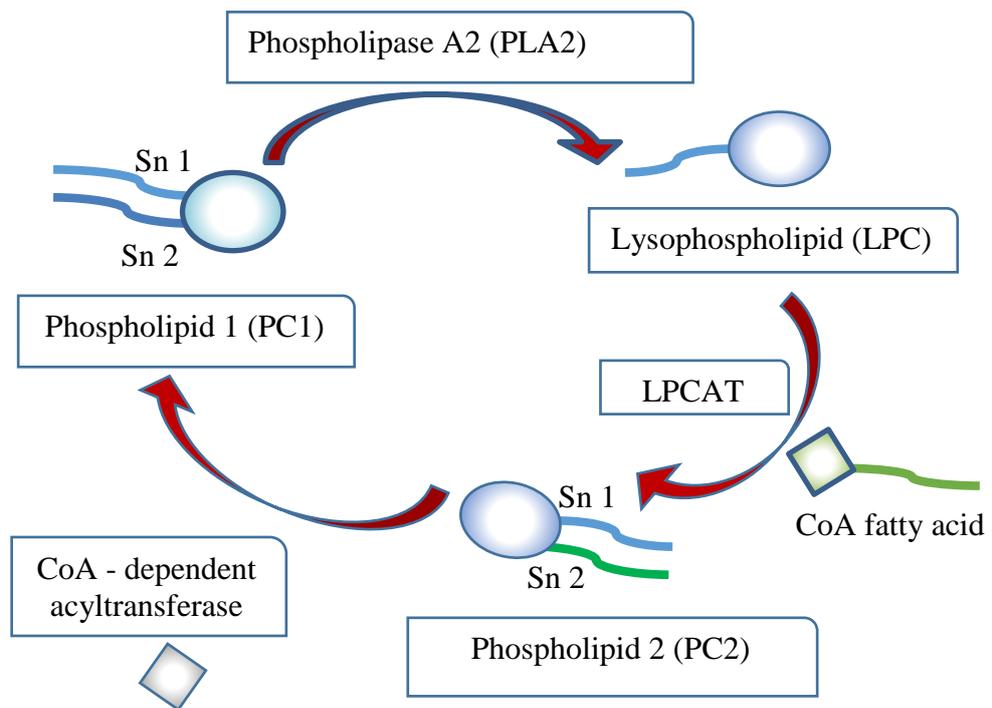


Figure 8, Land Cycle (the remodeling pathway) Membrane phospholipids

Are remodelled through the concerted actions of phospholipase A2 (PLA₂) and lysophospholipid acyltransferases. In the case of phosphatidylcholine (PC), PLA₂ removes a fatty acyl group from the *sn*-2 position to produce lysoPC and this is replaced by a different fatty acyl group (shown in green) by LysoPC acyltransferase (LPCAT) utilising fatty acyl-CoA as donor. This continuing cycle allows the composition of phospholipids to be remodelled for different cellular functions. Adapted from Jackson & Parton (2004). *Immunobiol.*; 209:31-38.

1.5.4. LysoPAF Acetyltransferase activity.

The amino acid residues of LPCAT1 which are essential for each activity (LPC-acyltransferase or lyso-PAF acetyltransferase activity) were recognized by site-directed mutagenesis. Furthermore, the LPCAT1 remodeling pathway for PAF synthesis has been found to be non-inflammatory/constitutive (Harayama *et al.*, 2008; Morimoto *et al.*, 2010; Morimoto *et al.*, 2014).

Furthermore, LPCAT-2 (also termed LysoPAFAT), has been found to catalyse both PAF and PC synthesis which occurs mainly in inflammatory cells. Thus, a single enzyme catalyses membrane LPC-acyltransferase activity of inflammatory cells, whilst, producing PAF (lyso-PAF acetyltransferase activity) in response to external stimuli (Morimoto *et al.*, 2010; Morimoto *et al.*, 2014).

Therefore, this activity of LPCAT-2 enzyme is inducible and activated by inflammatory stimulation in contrast to that of LPCAT1 which is constitutive (Shindou *et al.*, 2007; Harayama *et al.*, 2008; Morimoto *et al.*, 2010, Morimoto *et al.*, 2014). The lyso-PAF acetyltransferase activity is shown in the reaction below (Figure 9).

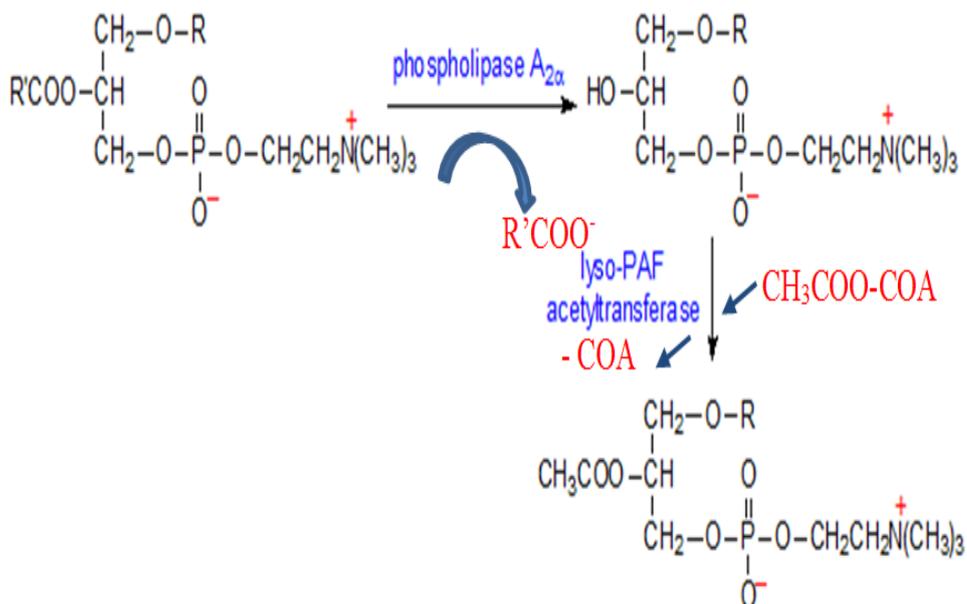


Figure 9. The acetyltransferase activity of the LPCAT-2/LPAFAT enzyme.

Both LPCAT1 and LPCAT-2 have lyso-PAF acetyltransferase activity by which PAF is biosynthesized via the *de novo* pathway (under physiological conditions i.e. non-inflammatory) or remodeling pathway (inflammatory conditions). However, this figure shows the remodeling pathway (inflammatory conditions), alkyl-PC is cleaved at *sn*-2 position via cytosolic Phospholipase 2 α (cPLA₂ α) (under inflammatory conditions), generating the lyso-PAF(alkyl-LPC), then, PAF is biosynthesized from lyso-PAF through lyso-PAF acetyltransferase (LPAFAT/LPCAT-2). (Figure taken from Lipidlibrary.aocs.org, 2015).

1.6. The biological roles and expression of LPCAT in pro and anti-inflammatory processes.

Lysophosphatidic acid and lysophosphatidylcholine have been associated with a number of a different biological activities from blood vessel development to myelination. The use of lysophospholipid by acyltransferase is likely to be essential in the regulation of their availability and hence activity (Jackson *et al.*, 2008b).

1.6.1. Inflammatory cells.

The enzymes have been found to play a part in the control of arachidonate levels in inflammatory cells. Studies have been 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 have an important in activation of macrophage (Jackson, 1997).

Additionally, the phosphorylation of LPCAT-2 at Ser34 enhance platelet-activating factor production in endotoxin-stimulated macrophages (Morimoto *et al.*, 2010; Morimoto *et al.*, 2014). Several studies have reveal that the enzymes might mediate both the initiating reactions and the inflammatory response of monocytes to cytokine interferon-gamma (Jackson and Parton, 2004 and Schmid *et al.*, 2003).

IFN-gamma cause a significant rise 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 revealing the evidence to highlight the role of acyltransferases as part of the molecular mechanism underlying inflammation (Neville *et al.*, 2005).

Morimoto *et al.*,(2010) have attempted to find out which kinase is related to LPS-induced phosphorylation of LPCAT-2 by using inhibitors and siRNA against mitogen-activated

protein kinases (MAPK) cascades which verified that LPCAT-2 phosphorylation through LPS-TLR4 signaling that may directly depend on MAPK-activated protein kinase 2 (MAPKAP kinase 2 or MK2).

Their findings indicated that under LPS stimulation, LPCAT-2 phosphorylation depended on the MyD88 (myeloid differentiation primary response gene 88), TAK1, p38 α , and MK2 signaling pathway.

Furthermore, the only phosphorylated site of LPCAT-2 that enhanced its catalytic activities was Ser34 (Morimoto *et al.*, 2010). Lipopolysaccharide (LPS) (Jackson & Parton, 2004).

LPCAT-2 has been recognized as the long-sought lyso-PAF acetyltransferase which is involved in PAF biosynthesis within the remodeling pathway. Therefore, its expression is mainly detected in inflammatory cells such as peritoneal macrophages and stimulation and activation of LPCAT-2 is regulated by LPS (Shindou *et al.*, 2013).

Furthermore, investigation of PAF by Lyso-PAF acetyltransferase (Lyso-PAFAT) which have been proposed that one (LysoPAFAT/LPCAT-2) is inducible and activated by inflammatory stimulation, and the other (LPCAT1) is constitutively expressed. In addition, each Lyso-PAFAT biosynthesises inflammatory and physiological amounts of PAF were depending on the cell type (Harayama *et al.*, 2009).

1.6.4. Lungs and surfactant production.

The pulmonary surfactants are complexes of lipids and proteins most of which are formed and secreted by alveolar type II cells that provided the low surface tension at the air-liquid interface. There have been significant evidences that the LPCAT enzyme is playing a key role in regulating the surfactant phospholipid biosynthesis in alveolar type II cells. Thus, this understanding of the regulation of the enzyme might provide key insights into surfactant phospholipid biosynthesis (Chen *et al.*, 2006; Harayama *et al.*, 2009). In

addition, 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 LPCAT1 in surfactant production, Bridges and colleagues generated mice bearing a hypomorphic allele of LPCAT1 (here referred to as LPCAT1GT/GT mice).

The newly-born LPCAT1GT/GT mice have been developed serious respiratory complications of different degrees resulting in the death of some of the animals. In addition, there was a substantial decline in LPCAT1 activity and saturated PC content in the same group. The surfactant that isolated from the dead LPCAT1GT/GT mice was unable to reduce minimum surface tension to the wild-type levels.

Therefore, their taken together data has been showed that the full LPCAT1 activity is essential to the success of the levels of saturated PC which is required for the transition to air breathing (Bridges *et al.*, 2010).

1.6.5. Brain.

The deacylation and reacylation cycle is a key mechanism responsible for the composition of polyunsaturated fatty acids into neural membrane glycerophospholipids (Kitson *et al.*, 2012).

It four enzymes implication, specifically Acyl-CoA synthetase, Acyl-CoA hydrolase, Acyl-CoA: lysophospholipid acyltransferase, and phospholipase A2 (Kitson *et al.*, 2012).

These enzymes have been purified and categorized from brain tissue (Farooqui *et al.*, 2000). Furthermore, all the four LPCAT isoforms have been found to be expressed in brain tissue where they were involved in infant neurodevelopment and neurological diseases accompanying with aging such as Alzheimer's disease and cognitive decline (Kitson *et al.*, 2012).

1.7. The LPCAT enzyme as a novel target for anti-sepsis therapy.

Despite intensive research and advances in medical care and technology, there is currently no specific treatment for sepsis and mortality and morbidity continues to increase. (Paul *et al.*, 2010; Winters *et al.*, 2010; Wiens *et al.*, 2012; Wiens *et al.*, 2013; Wang *et al.*, 2013(a,b,c); Vincent *et al.*, 2014; Wang *et al.*, 2014(a); Nygard *et al.*, 2014; Wang *et al.*, 2015; Shankar-Hari *et al.*, 2016 Tsertsvadze *et al.*, 2016)

In addition, a lack of responsiveness to antibiotics, new and emerging infections, drug resistance, as well as the high costs of drug discovery and manufacture have impacted on the lack of advancement for sepsis therapies.. Therefore new approaches to sepsis therapy including signal transduction pathways have been identified as an approach for new therapies against sepsis (Yan *et al.*, 2006; Schorr *et al.*, 2007; O'Neill, 2006; Vincent *et al.*, 2010; Phua *et al.*, 2011; Wu *et al.*, 2013; Vincent *et al.*, 2014; Wong *et al.*, 2015; Tsertsvadze *et al.*, 2016; Rodriguez *et al.*, 2016).

Several studies have indicated the therapeutic potential of lysoPAF and lysoPC (LPC) in experimental models of sepsis and organ failure that suggest the potential role of LPCAT inhibition in the treatment of sepsis and possibly other inflammatory conditions (Yan *et al.*, 2004; Murch *et al.*, 2006; Nakanishi *et al.*, 2006; Morimoto *et al.*, 2010; Morimoto *et al.*, 2014). While *in vitro*, LPC has been shown to increase the bactericidal activity of neutrophils by enhancing hydrogen peroxide production (Yan *et al.*, 2004).

LPCAT-2 also converts lyso-PAF to PC, which are a main component of cell membranes and a precursor of PAF. In addition, PAF production in the body is increased in response to extracellular inflammatory stimuli such as bacterial endotoxin (Servillo *et al.*, 2006; Morimoto *et al.*, 2010; Shindou *et al.*, 2013; Stanca *et al.*, 2013; Morimoto *et al.*, 2014).

In addition, Brown & Schmidt (2005) attempted to use acyltransferase inhibitors as probes to study the possible role of lysophospholipid acyltransferases in intracellular membrane transferring in the secretory and endolytic pathways has been put into view.

CI-976 has also been stated as a useful addition to the pool of small molecule inhibitors that could be used for studying secretory and endocytic membrane trafficking pathways. Jackson and colleagues showed that LPCAT regulates inflammatory responses to LPS and other microbial stimuli (Jackson *et al.*, 2008a). They showed that inhibition of LPCAT resulted in inhibition of TLR4 translocation into membrane lipid raft domains. Their observations suggest a new regulatory mechanism that facilitates the innate immune response to microbial molecular patterns and has been suggested a possible pathway for the anti-inflammatory activity seen in many phospholipid metabolites.

This delivered the possibility of the development of new classes of anti-inflammatory and antiseptis agents (Jackson *et al.*, 2008a). Furthermore, LPCAT enzymes are potential targets for anti-sepsis therapies, Jackson and colleagues have used selective inhibitors for LPCAT recognised through high-throughput screening. 5 hydroxyethyl 5,3' thiophenyl pyridine, a non-competitive specific inhibitor of CoA-dependent LPCAT has been identified as a promising candidate.

Also a cDNA sequence for LPCAT from human monocytes has been identified and this sequence is being used to develop inhibitory RNA sequences as potential LPCAT inhibitors (Jackson *et al.*, 2008(a)). Inhibitory RNA sequences have the advantage of specificity over the chemical inhibitors, and for that reason, they were utilized for gene silencing in this research. LPCAT-2 unlike LPCAT1 is vital for stimulation of macrophage cytokine gene expression and release in response to stimulation of TLR2 and TLR4 through bacterial ligands; but not for TLR-independent stimuli. These findings were revealed by performing small interfering RNA (siRNA) knockdown (Abate & Jackson, 2015).

1.8. Experimental models.

1.8.1. LPS and Pam3CSK4.

This project has chosen LPS and Pam3CSK4 (C81H156N10O13S) (TLR4 and TLR2 ligands respectively) as surrogate models of Gram negative or Gram-positive bacterial infections.

As both Gram-negative and Gram-positive bacterial infections contribute almost equally overall to the incidence of sepsis, it was important to include molecular stimuli from both these bacterial classes in experimental models of inflammation and sepsis. LPS recognition and signaling has been detailed in sections 1.2 and 1.3. Pam3CysSerLys4 (PAM3CSK4) is a synthetic tripalmitoylated lipopeptide that mimicks the acylated amino terminus of bacterial lipoproteins. Pam3CSK4 is recognized by TLR2 and signals via TLR2/TLR1 heterodimers to activate NFkB pathways Which led to cell activation and apoptosis through bacterial lipoproteins by TLR2 (Aliprantisao *et al.*, 1999; Ozinsky a. *et al.*, 2000).

Sepsis is induced by bacteria and bacterial products such as Lipopolysaccharides (LPS), Lipoproteins, peptidoglycan, which are recognised via pattern recognition receptors (PPR) such as CD14 and Toll like receptors (TLRs) (Aderen & Ulevich, 2000; Takeda & Akira, 2005). Bacterial products such as LPS (Rietschel *et al.*, 1994, Van Amersfoot *et al.*, 2000), and Pam3CSK4, which is a synthetic analogue of Gram-positive bacterial cell wall structures, induce over production of cytokines from key immune cells such as macrophages (Ozinsky *et al.*, 2000; Soong *et al.*, 2004) and this dysregulated over production of cytokines is at the centre of the pathophysiology of sepsis (Jackson *et al.*, 2008a).

1.8.2. Macrophages, the murine RAW264.7 cell line.

Macrophages are prominent innate immune cells that recognize and respond to bacteria and their products. The activation of macrophages with LPS results in rapid changes in expression of genes encoding cytokines and other inflammatory mediators (Brunialti *et al.*, 2006). This project has used the murine macrophage, RAW264.7 cell line as a model cell line to study the role of LPCAT-2 in inflammatory response to bacterial ligands.

Moreover, several studies have used RAW264.7 cell line as a model for investigation of inflammatory responses, Chiou *et al.*, (2000) have utilised RAW264.7 cell line to investigate the possible suppression mechanism of the inducible nitric oxide synthase (iNOS) expression in macrophages via Andrographolide.

Huttunen *et al.*, (2000) have also used RAW264.7 cells to study the inflammatory responses in macrophages that caused by Mycobacterial strains, which is isolated from mold-buildings. While Barthel *et al.*, (2001) studied the effect of bovine natural resistance- associated macrophage protein gene 1 (bovine NRAMP1) gene on *Brucella abortus* survival by establishing a stable transfection of bovine NRAMP1 gene into murine RAW264.7 cells.

Furthermore, Li *et al.*, (2000) have investigated the possible mechanism of peroxisome proliferator-activated receptor gamma (PPAR- γ) –dependent suppression of the iNOS gene in murine macrophages by using 1 μ g/ml of LPS.

In addition, in 2002, Shiojiri and co-workers used RAW264.7 cells to explore the possible effects of PPAR- γ specific ligands, rosiglitazone or pioglitazone as suppressors for both nitro-tyrosine formation and the expression of inflammatory mediators in adjuvant-induced murine rheumatoid arthritis (Shiojiri *et al.*, 2002).

Not only are RAW264 cells well validated as models of leukocyte responses to LPS and other bacterial ligands, they also providing ease of use for transfection experiments critical for the current investigation. Indeed, Shindou *et al.*, (2007) used RAW264.7 cells

to overexpress LPCAT-2 for the first time and study its role as a catalyser of platelet-activating factor (PAF) production.

Jiang *et al.*, (2004) in their investigation of the possible mechanisms of activation of macrophages (RAW264.7 cells) via transfected mammalian DNA, used calf thymus (CT) DNA, FuGENE 6 transfection reagent, RAW264.7 cells, *Escherichia coli* DNA (bacterial DNA) and LPS (*Escherichia coli*, strain 0111:B4, Sigma).

Both Liu *et al.*, (2007) and Iyer *et al.*, (2010) investigated the mechanisms by which the gene expression of IL-10 could be regulated in macrophages, by using RAW264.7 cells and murine bone marrow- derived macrophages (BMDMs) as cell models and LPS (*Escherichia coli*, strain 0217:B8 or *Escherichia coli*, strain 055:B5, Sigma-Aldrich) (1 µg/ml) as a stimulus.

Xu *et al.*, (2007) used RAW264.7 to investigate TLR4 as a sensor for autophagy associated with innate immunity while Giang *et al.*, (2009) utilised RAW264.7 cells in a comparative study to demonstrate the inhibitory activity of Zeumbone and Zerumbone 2,3-epoxide on NF-κB, and nitric oxide (NO). In studies on LPCAT characterisation, Morimoto *et al.*, (2010) utilised RAW264.7 cells to investigate the LPCAT-2 phosphorylation at Ser34 to enhance PAF production in endotoxin–stimulated murine macrophages. Tarui *et al.*, (2014) utilised RAW264.7 cells and CHO cells stably expressing murine PAFR to investigate the effect of selective inhibition of the PAF biosynthetic enzyme LPCAT-2.

Moreover, many of these studies have shown that RAW264 cells can be used to study in vitro effects that are also seen in vivo in the corresponding murine models. This is an important linkage for in vitro cell models. Shiojiri *et al.*, (2002); Morimoto *et al.*, (2010); Iyer *et al.*, (2010); Luu *et al.*, (2014) have all used mice as an animal model for the in vivo investigation as well as using RAW264.7 cell line as a model for the in vitro. However, showing similar effects in human leukocytes would be required before

extrapolating results to human conditions. However, it is difficult to transfect primary cells in addition to requiring ethical approval (Wilding & Bodmer, 2014).

However, there are well-validated models of primary human leukocytes. For example, Mono Mac 6 (MM6) cells maintain many features of human monocytes and are continuously growing, but they are difficult to transfect (Moesby *et al.*, 1999). Due to difficulty in transfecting MM6 cells and using siRNA techniques we developed for RAW264.7 cells, the current project did not repeat findings using this cell line.

Previous work that used an activity assay and a pharmacological inhibitor of LPCAT, showed the presence of LPCAT in MM6 cells, and that it has an immuno-regulatory role (Jackson *et al.*, 2008).

Very recently, Abate & Jackson, (2015) have used ShRNA techniques to show the same effects of LPCAT-2 knockdown in MM6 cells as occur in RAW264.7 cells. This result suggests that the results obtained in the current study would also occur in other monocyte and macrophage cells and probably *in vivo*.

1.8. Rationale for the study.

Traditional treatments for sepsis have focused on source control, antimicrobials, vasopressors, and fluid revival with minimal success at saving the lives of sepsis patients. Research has shown that severely septic patients continuously experience undue morbidity and mortality despite recent progress in critical care (Iwashyna *et al.*, 2014; Kaukonen *et al.*, 2014; Peake *et al.*, 2014; The Global Sepsis Alliance, 2015; Page *et al.*, 2015; The Global Sepsis Alliance, 2016; Kempker *et al.*, 2016; Papali *et al.*, 2017(a,b); Patel *et al.*, 2017; Rhodes *et al.*, 2017).

Despite, the extensive resources that have been invested in clinical trials of new treatments; almost all have failed to improve outcomes i.e. no specific therapy for sepsis (Rice, 2006; Shorr *et al.*, 2007; Osuchowski *et al.*, 2007; Daniels, 2011; Phua *et al.*, 2011; Reichel *et al.*, 2011; Levy *et al.*, 2012; Perner *et al.*, 2012; Reinhart *et al.*, 2013; McPherson *et al.*, 2013; Liu *et al.*, 2014; ProCESS Investigators *et al.*, 2014; Global Sepsis Alliance, 2016; Kim *et al.*, 2017; Patel *et al.*, 2017; Rhodes *et al.*, 2017; Safiri *et al.*, 2017).

In addition, the injurious effects of sepsis last beyond the acute process; patients who survive the early episode experience higher rates of death in the first year after hospital discharge compared with age-matched controls (Weycker *et al.*, 2003; Shen *et al.*, 2010; Daniels, 2011; Levy *et al.*, 2012; Ranieri *et al.*, 2012; Seymour *et al.*, 2012; Stronati *et al.*, 2013; Liu *et al.*, 2014; Rhee *et al.*, 2014; Rogers *et al.*, 2014; Schorr *et al.*, 2014; Singer, 2014; The Global Sepsis Alliance, 2015; Kishore *et al.*, 2015; Rhee *et al.*, 2015(a,b) ; The Global Sepsis Alliance, 2016; Seymour, *et al.*, 2016; Stoller *et al.*, 2016; Kim *et al.*, 2017; Rhodes *et al.*, 2017; Rannikko *et al.*, 2017).

The achievement of anti-sepsis therapies clearly depends on sufficiently modulating the immune response (with the use of specific treatments) with the least probable 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 repressed immune and inflammatory responses (Riedemann *et al.*, 2003; von Knethen *et al.*, 2007; Rittirsch *et al.*, 2007; Wang *et al.*, 2010; Svanes *et al.*, 2010; Sze *et al.*, 2012; Wang *et al.*, 2012 (a,b); Suresh *et al.*, 2013; Wu *et al.*, 2013; Steling *et al.*, 2013; Stronati *et al.*, 2013; Vallés *et al.*, 2013; Vincent *et al.*, 2013; Vincent *et al.*, 2014; Henriksen *et al.*, 2015; Wang *et al.*, 2015; Wong *et al.*, 2015; Papali *et al.*, 2017; Patel *et al.*, 2017).

Due to the importance to find a novel therapeutic agent in sepsis; Daniels, (2011) has summarized the potential target sites for the development of new therapies as pathogen recognition such as; lipopolysaccharide, TLRs, neutrophil depletion, cell adhesion, for developing novel therapeutic agents in sepsis such as; anti-endotoxin, TLR antagonists-TAK-242, granulocyte colony-stimulating factor and leucocyte–endothelial interactions respectively. There have been also numerous trials of agents that block specific inflammatory mediators (eg cytokines) with no outcome on mortality in clinical sepsis trials (Vincent 2006; Angus 2011).

The LPCAT enzymes offer novel potential of specific therapies for the overwhelming inflammatory responses of sepsis; specifically, the control of the PC/LysoPC (LPC) balance. In addition, recent studies suggest that LPCAT may regulate inflammatory responses to different bacterial ligands involving many inflammatory mediators (Shindou *et al.*, 2007; Harayama *et al.*, 2008; Jackson *et al.*, 2008a; Morimoto *et al.*, 2010; Shindou *et al.*, 2013; Morimoto *et al.*, 2014).

However, to exploit LPCAT as a possible target for novel therapies, more information on the fundamental role of LPCAT in macrophage responses to bacterial infection need to be understood. Moreover, it has been only recently, with the sequencing and cloning of LPCAT that different members of this enzyme family have been identified (Chen *et al.*, 2006; Nakanishi *et al.*, 2006; Shindou *et al.*, 2007; Zhao *et al.*, 2008; Hishikawa *et al.*, 2008). Identification of the specific LPCAT that regulates macrophage responses and

therefore would be the best target for anti-inflammatory or anti-sepsis therapy, also needs to be confirmed.

Work from the Jackson laboratory and others have shown that the phospholipid metabolizing enzyme Lysophosphatidylcholine acyltransferase (LPCAT) can control inflammatory responses to LPS in macrophages (Jackson *et al.*, 2008; Sevastou *et al.*, 2013). It was previously shown that LPCAT has a role in inflammatory responses using pharmacological inhibitors, although the precise LPCAT species responsible could not be determined (Jackson *et al.*, 2008).

Availability of the gene sequences and cloning of LPCAT species allowed the identification of four major family members (LPCAT1-4) each with different but overlapping activities. (Chen *et al.*, 2006; Shindou *et al.*, 2007; Edwards & Constantinescu, 2009; Hishikawa *et al.*, 2008; Morimoto *et al.*, 2010; Zheng *et al.*, 2012; Shindou *et al.*, 2013; Morimoto *et al.*, 2014). The challenge is therefore to determine which LPCAT species is responsible for regulating the inflammatory response to LPS and other bacterial factors. This knowledge will allow new insights in to the mechanisms of inflammation and will be required for new therapies aimed at these targets to be developed.

Recent studies indicate that the stimulation of the isoform LPCAT-2, which is localized in cell endoplasmic reticulum and located in lipid droplets where they catalyse the formation of PC, and are important in inducing an inflammatory immune response (Morimoto *et al.*, 2010; Moessinger *et al.*, 2010; Li *et al.*, 2012; Stanca *et al.*, 2013; Morimoto *et al.*, 2014; Abate & Jackson, 2015).

The priming of macrophages releases inflammatory mediators (Adib-Conquy *et al.*, 2012; Wang *et al.*, 2010 (a,b); Arroyo-Caro *et al.*, 2013; Schulte *et al.*, 2013; Hishikawa *et al.*, 2014; Wiersinga *et al.*, 2014; Wang *et al.*, 2014 (b); Płóciennikowska *et al.*, 2015; Walkey *et al.*, 2015; Papali *et al.*, 2017; Patel *et al.*, 2017; Kim *et al.*, 2017). Therefore,

the research in this thesis was conducted based on the hypotheses that the LPCAT-2 enzyme is involved in the inflammatory response of macrophages to inflammatory stimuli such as LPS, through regulating inflammatory pathways and potentially also by regulating the activation state of the macrophage.

To answer the primary research questions concerning the role of LPCAT-2 in the macrophage inflammatory response, the effect of LPCAT-2 overexpression and knockdown on the expression of cytokines and the most suitable reference gene for the analysis of these results will be explored.

In addition, many infections are polymicrobial or involve molecules that can stimulate several TLR pathways and the activation of these pathways will be important in dysregulated responses leading to sepsis (Harbarth *et al.*, 2003; Weycker *et al.*, 2003; Maier *et al.*, 2004; Corrêa *et al.*, 2007; Rittirsch *et al.*, 2007; Wang *et al.*, 2007; Puneet *et al.*, 2010; Retamar *et al.*, 2012; Pavlaki *et al.*, 2013; Packiriswamy *et al.*, 2013; Lozano *et al.*, 2013; Bloos *et al.*, 2014; Boomer *et al.*, 2014; Liu *et al.*, 2014; Lizza *et al.*, 2014; Ramachandran *et al.*, 2014; Yealy *et al.*, 2014). Therefore, this project aims to enhance our knowledge of the role of LPCAT-2 in response to different bacterial stimuli by using molecular patterns such as lipopolysaccharide as an example of Gram negative bacteria stimuli and the synthetic triacylated lipoprotein-TLR1/2 ligand (Pam3CSK4) for Gram positive bacteria.

Knowledge gained from this research will not only improve our understanding of the role of phospholipid metabolism in regulating the inflammatory response in macrophages but may identify novel molecular targets for the development of new therapies for sepsis.

1.9. Hypothesis, Aims and objectives of this project.

1.9.1. Hypothesis.

The hypothesis to be tested in this research, is that LPCAT-2 plays a key role in the macrophage inflammatory response to infectious stimuli.

1.9.2. Aims.

To answer the hypothesis, this project aims to study the effect of LPCAT-2 gene inhibition and overexpression on macrophage inflammatory responses and explore the mechanisms by which LPCAT-2 regulates the inflammatory response in macrophages.

1.9.2. Objectives.

1. To Investigate the effect of LPCAT-2 on LPS- induced inflammatory response using RAW264.7 cells transiently over-expressing LPCAT-2.
2. To study the effect of LPCAT-2 on Pam3CSK4-inflammatory response using RAW264.7 cells transiently over-expressing LPCAT-2.
3. To investigate the mechanisms by which LPCAT-2 regulates the inflammatory response in these cells.
4. To study the effect of LPCAT-2 on the LPS -induced inflammatory response using specific siRNA in RAW264.7 cells to knock down the over-expressed LPCAT-2.
5. To establish a stably transfected RAW264.7 cell line with plasmid carrying murine or human LPCAT-2.
6. To investigate the effect of LPCAT-2 on LPS-induced inflammatory responses using RAW264.7 cell line that stably over-express LPCAT-2.

Chapter 2: Materials and Methods

2.1. Materials.

2.1. Cell culture.

RAW264.7 cell line was obtained from the European Collection of Cell Cultures (ECACC) through the Health Protection Agency (HPA), UK) and Dimethyl Sulfoxide (DMSO) was obtained from Fisher Scientific UK Ltd., UK. Dulbecco modified Eagle's medium (DMEM) culture media with 25 mM HEPES buffer and 4.5 g/L glucose, L-glutamine were obtained from Lonza Group Ltd., UK. Foetal bovine serum (FBS) was obtained from Biosera supplied by Labtech International Ltd, UK.

Dulbecco's phosphate buffered saline (PBS) and *E coli* lipopolysaccharide (LPS) O111:B4 were obtained from Sigma-Aldrich Co. LLC., UK. OPTI-MEM® (reduced serum medium), Geneticin antibiotic, Lipofectamine 2000 transfection reagent were obtained from Life Technologies Ltd., UK while the transfection reagents: JetPrime and Interferin were obtained from (Polyplus-transfection Inc., UK). Silencer® select Negative control #1 SiRNA and LPCAT-2 SiRNA were obtained from Life Technology Ltd, UK.

2.1.1. Plastic and Glassware.

25 cm² tissue culture flasks, 75 cm² tissue culture flasks, 10 ml serological tips, scrapers, 7 ml or 30 ml Universal containers, 50 ml falcon tubes, 1.5 ml Eppendorf tubes, (10, 200, 1000) µl pipette tips with or without filters, 6 well plates, 12 well plate, 96 well plates, 50 ml syringe were obtained from Greiner Scientific Laboratory Supplies Ltd., UK while 10 µl pipette tips without filters, 2 ml Eppendorf tubes, 0.2 µm pore size sartorius, squirt bottles, 6 cm diameter single cell culture dishes were obtained from (Fisher Scientific UK Ltd., UK).

2.1.2 Total RNA extraction.

Guanidium thiocyanate, water-saturated phenol, sodium acetate (anhydrous), sodium citrate, Glacial acetic acid, N-laurosylsarcosine (Sarkosyl), RNase- and DNase-free water, Tris-base, 2-mercaptoethanol were obtained from Fisher Scientific UK Ltd., UK. 1-Bromo-3-chloropropane (1-Bromo-3-chloropropane, I-BCP, Trimethylene bromochloride, Trimethylene chlorobromide), 2-isopropanol, ethanol was obtained from Sigma-Aldrich Co. LLC., UK. DNase-1 amplification kit was obtained from Sigma-Aldrich Co. LLC., UK.

2.1.3 Quantitative or real time PCR (qPCR or RT-PCR).

High Capacity RNA - to - cDNA Kit, Real time PCR kit and Applied Biosystems™ MicroAmp™ Fast Optical 96-Well Reaction Plate, 0.1 mL were obtained from Life Technologies Ltd. the primers were purchased from Eurofins MWG Operon, Germany.

2.1.4. Agarose gel electrophoresis.

Electrophoresis grade agarose was obtained from (Fisher Scientific UK Ltd., UK), EDTA, 10000x SyBr Safe and acetic acid were obtained from (Sigma-Aldrich Co. LLC., UK).

2.1.5 Cloning and purification of plasmid carrying the human or murine LPCAT-2 inserts.

pCMV6 -entry vector (4.9 kb) plasmids with or without cDNA clones of the murine LPCAT- 2 or human LPCAT- 2 (Appendix 1 Materials, Figure 1), Alpha-gold competent cells were obtained from Insight Biotechnology Ltd, UK. GenElute™ plasmid MiniPrep Kit (MiniPrep Kit, PLN70 SIGMA) was obtained from Sigma-Aldrich Co.

LLC., UK. Luria Britani (LB) broth and Agar –Agar were obtained from Sigma-Aldrich Co. LLC., UK.

2.1.6. Western blotting (WB).

Molecular weight marker and Luminata™ Crescendo Western HRP Substrate were obtained from Millipore Corporation Billerica, MA, UK. NuPAGE4-12% Bis-Tris Gel 1.0 mm x 10 well, Lithium dodecyl sulphate (LDS) sample buffer, Anti-flag Ab/ Goat - Anti Mouse Secondary Ab and SeeBlue® Pre-Stained standard were obtained from Life Technologies Ltd., UK. LPCAT-2 Antibody was obtained from Novus Biologicals Ltd, UK, Bovine serum Albumin(BSA) were obtained from Sigma-Aldrich Co. LLC., UK.

2.1.6.1. Estimation of protein concentration.

Micro BCA assay kit was obtained from Thermo Scientific Pierce, UK.

2.1. 7. ELISA assay.

Mouse TNF-alpha ELISA duo set-kit (catalogue number: DY410), Mouse IL-6 ELISA duo set kit (catalogue number: DY406) were obtained from R&D Systems, Inc., UK. ELISA TMB Substrate was obtained from Insight Biotechnology Ltd., UK. Maxisorp 96 well ELISA plates, Plate sealers and Tween-20 were obtained from Fisher Scientific UK Ltd., UK.

2.1.8. All instruments in this project.

Mini plate spinner centrifuge, Mini centrifuge (Labnet International.Inc, USA), Nanovue systems (GE Healthcare Life Sciences, USA) and Nanodrop 2000 spectrophotometer (Thermo-Fisher-Scientific, Waltham, MA, USA). Veriti 96 well Thermal Cycler, Primer

express software and StepOnePlus Real- Time PCR System (Thermo-cycler 96 well plate Real time-PCR) were obtained from Life Technologies Ltd., UK. EC3 imaging system, Ultra Violet Products (UVP) image gel documentation system (UVP ultra violet product, Tech, Japan), ImageQuant LAS4000 system device and software were sourced from (GE Mini plate spinner centrifuge, Mini centrifuge (Labnet International.Inc, USA), Nanovue systems (GE Healthcare Life Sciences, USA) and Nanodrop 2000 spectrophotometer (Thermo-Fisher-Scientific, Waltham, MA, USA). Veriti 96 well Thermal Cycler, Primer express software and StepOnePlus Real- Time PCR System (Thermo-cycler 96 well plate Real time-PCR) were obtained from Life Technologies Ltd., UK. EC3 imaging system, Ultra Violet Products (UVP) image gel documentation system (UVP ultra violet product, Tech, Japan), ImageQuant LAS4000 system device and software were sourced from (GE Healthcare Bio-Sciences AB, Sweden). Spectrafuge 24D (Labnet International.Inc, USA), Stuart® orbital Incubator | S150 | (Geneflow, UK) and Sorvall Legend Micro 17R Centrifuge, Refrigerated (Thermo-Scientific, UK). Ultrasound Sonicator, Plate shaker (Thermo-Fisher-Scientific, UK), Techne DRI-BLOCK® (Techne(Cambridge) Ltd. Duxford Cambridge, UK), Jenway 1000 Hotplate and Stirrer (Jenway, UK). SNAP-id. ZOOM® Dual power back and XCell SureLock™ Minin-cell was obtained from Life Technologies Ltd., UK. Versa Max Micro-Plate Reader with SoftMax Pro Software was sourced from Molecular devices, USA.

2.2. Methods.

2.2.1. Cell culture.

RAW264.7 cells (Adherent murine macrophage cell line which is derived from an Abelson murine leukaemia virus-induced tumour and isolated in 1978) were maintained in DMEM media with 25 hepes and 4.5 g/l glucose supplemented with 200 mM L- glutamine, 10 % Foetal bovine serum (FBS) as suggested by the supplier.

The cells were allowed to grow up to 50 -70 % confluence at 37 °C in a humidified atmosphere in 5 % carbon dioxide (CO₂). The cells were sub-cultured in a class 2 cabinet twice a week under sterile conditions with a media changing to feed the cells in between.

2.2.1.1. Cell Viability Determination.

Cell viability was determined by the Trypan blue dye exclusion method. This method was carried out by a 1 : 8 serial dilution of the cells and then, cell stained with an equal volume of the Trypan blue dye.

The percentage of unstained cells (viable cells) vs the stained cells (dead cells) (viewed and counted under the inverted Microscope at X10 magnification) were used to calculate the percentage of viable cells. The acceptable percentage of cell viability test to set any experiment was 95 % and above.

2.2.1.2. Freezing and Storage cells.

Upon delivery, RAW264.7 cells were stored in liquid nitrogen. The cryovial were taken out from the liquid nitrogen tank following the standard operating procedure (SOP) for Liquid nitrogen handling /sample storage. The liquid Nitrogen tanks (Mr frosty) were also used for the long-term storage of the cells, which were previously kept in cryopreservation tubes for 24 hours at -80 °C until it was ready to be used.

2.2.1.3. RAW 264.7 cells Cryopreservation and thawing.

Adherent RAW264.7 cells of early passage were scraped, centrifuged at speed 160 g for 5 minutes at room temperature and then counted using a haemocytometer. Cell viability was assessed as described in section 2.2.1.1. The cells were cryopreserved by re-suspended the cells with 500 µL of complete DMEM medium and 500 µL of 20 % dimethyl sulphoxide (DMSO) solution added drop wise while shaking in cryo-

preservation tubes. The cells were immediately stored at -80 °C for 24 hours before they were transferred to liquid nitrogen (Mr frosty) for long term storage.

Recovering of the frozen cells, the cryovial was removed from liquid nitrogen and rapidly thawed, and then immediately transferred into a universal container containing 10ml of supplemented growth cell culture media (DMEM). They were further centrifuged at a speed of 160 g for 5 minutes at room temperature (RT) to remove DMSO.

Then, the cell pellets were re-suspended again in 10 ml of fresh DMEM medium and re-centrifuged at a speed of 160 g for 5 minutes at room temperature (RT), and then transferred into a 25 cm² tissue culture flask until 80 % confluence was reached. The cells were then cultured as stated in section 2.2.1 above.

2.2.1.4. Transfection RAW264.7 cell culture using Lipofectamine 2000 transfection reagent.

RAW264.7 cells were plated in 6 wells plate (RT-PCR or WB) at a concentration of 1×10^6 cell/ well (2.5 ml of DMEM medium) and were incubated at 37 °C for 24 hours prior to transfection with 2.5 µg of purified pCMV6 -entry vector plasmid carrying the murine LPCAT-2 (mLPCAT-2) recombinant gene into 150 µL of reduced serum media (OPTI-MEM medium) in separate sterile eppendorf tubes.

Then, 6, 9, 12, and 15 µL of lipofectamine 2000 reagent were respectively added to 150 µl of reduced serum media (OPTI-MEM medium) in separate sterile eppendorf tubes. Both eppendorf tubes were incubated for 5 minutes at room temperature.

Following this, 150 µl of mLPCAT-2 DNA were combined with 150 µl of the previously diluted transfection reagent were incubated for 20 minutes at room temperature. Then, 250 µl of this mixture was added drop wise to each well to transfect RAW264.7 cells using low serum medium (OPTI-MEM) for 24 hours at 37 °C.

Next day, the low serum medium for all wells were replaced with the supplemented growth medium (DMEM medium) for 24 hours extra at 37 °C in order to transfect RAW264.7 cells for 48 hours as an optimisation establishment. RAW264.7 cells that transfected with purified empty vector (pCMV6 -entry vector) plasmid was used as negative control until the end of this research to study the effect of LPCAT-2 on inflammatory responses.

2.2.1.5. Transfection RAW264.7 cell culture using JetPrime.

RAW264.7 cells were plated at a cell concentration of 2×10^5 cell/well (2.5 ml of DMEM medium) on 6 wells plate were incubated at 37 °C for 24 hours prior to transfection with 2.5 µg of purified pCMV6 -entry vector plasmid carrying the murine LPCAT-2 (mLPCAT-2) recombinant gene into 150 µL of reduced serum media (OPTI-MEM medium) in separate sterile eppendorf tubes.

Then, 6, 9, 12, and 15 µL of JetPrime transfection reagent were respectively added into 150 µl of reduced serum media (OPTI-MEM medium) in separate sterile eppendorf tubes.

Next, both eppendorf tubes were incubated for 5 minutes at room temperature.

Then, 150 µl of mLPCAT-2 DNA was combined with 150 µl of the previously diluted JetPrime transfection reagent. All these eppendorf tubes were incubated for 20 minutes at room temperature. Then, 250 µl of this mixture was added drop wise into each well to transfect RAW264.7 cells using 2 ml of low serum medium (OPTI-MEM)/ well for 24 hours at 37 °C. The next day, the low serum medium for all wells were replaced with the supplemented growth medium (DMEM medium), then incubated at 37 °C for 24 hours extra in order to transfect RAW264.7 cells for 48 hours to establish the transfection optimisation. RAW264.7 cells that transfected with purified empty vector (pCMV6 -entry vector) plasmid was used as negative control.

2.2.1.6. LPCAT-2 gene silencing.

RAW 264.7 cells were plated in a 6 well plate at a concentration of 1×10^6 cells/well (2.5 ml of DMEM medium) to overexpress the murine LPCAT-2 gene for 48 hours (see section 2.2.1.4).

Then, the cells were scraped and counted using trypan blue cell viability method (see section 2.2.1.1). Then the cells were seeded at a cell concentration of 0.2×10^6 cells/ well (2.5 ml of DMEM medium) in DMEM culture medium (complete medium) supplemented. RAW 264.7 cells were incubated for 24 hours at 37 °C prior the LPCAT-2 gene silencing (2.5 ml of DMEM medium).

The cells were then transfected in reduced serum OPTI-MEM medium with a control negative siRNA (Ambion SiRNA, cat#: 4390843) or siRNA directed against LPCAT-2 (Ambion SiRNA ID s114512) at final concentration of 5 nM using interferin transfection reagent (a non-liposomal cationic amphiphile transfection reagent which is developed for the delivery of SiRNA into the cells in culture). After 48 hours, the medium was changed and a complete DMEM medium added and then, the cells were stimulated with 1 µg/ml of LPS for 6 hours. RAW264.7 over-expressing LPCAT-2 gene is termed 'LPCAT-2⁺'.

2.3. Stimulation of RAW 264.7 cells.

2.3.1. Stimulation of RAW 264.7 cells with LPS.

RAW264.7 were plated at a concentration of 0.5×10^6 cell / well (2.5 ml of complete DMEM medium) in a 6 well plate and incubated in a standard culture condition at 37 °C (please see section 2.2.1 and 2.2.1.1, 2.2.1.4.). The cells were stimulated with 1 µg /ml of Lipopoly-saccharide (LPS) for 6 hours for all the experiment sets. Supernatant were collected and stored at -80 °C to be ready for ELISA assay. While the cells were treated with RNA lysis buffer and stored at -20 °C to be ready for total RNA extraction (as described in section 2.4.1).

2.3.2. Stimulation of RAW 264.7 cells with Pam3CSK4 (Pam3C).

RAW264.7 were plated at a concentration of 0.5×10^6 cell /well (2.5 ml of DMEM medium) in a 6 well plate (qPCR) and incubated in a standard culture condition at 37 °C (please see section 2.2.1 and 2.2.1.1, 2.2.1.4). The cells were stimulated with 0.5 µg/ml Pam3CSK4 (Pam3C) for 6 hours.

2.3.3. The effect of selective peroxisome proliferator-activated receptors –gamma (PPAR-γ) antagonist T0070907 on LPCAT2 expression.

A concentration of (0.1 and 1) mM of the selective PPAR-γ antagonist T0070907 (2-chloro-5-nitro-N-4-pyridinylbenzamide) and vehicle (DMSO) is added into a 6 well plate RAW264.7 cell line at a concentration of 1×10^6 cell/well into 2.5 ml of complete DMEM medium. Then, incubated at 37 °C for two hours prior LPS stimulation in a concentration of 1 µg /ml was added and incubated for 6 hours at 37 °C. Then, the RNA samples were analysed using RT-PCR analysis system.

2.4. Reverse transcription coupled - Real Time polymerase chain reaction (RT-PCR).

2.4.1. Total RNA extraction.

2.4.1.1. Denaturing solution preparation.

Stock denaturing solution was prepared by dissolving 25 g of guanidium thiocyanate in 29.3 ml of water at 65 °C, followed by 1.76 ml of 0.75 M sodium citrate, pH 7.0, and 2.64 ml of 10 % sarkosyl. Denaturing solution was activated before use by addition of 72 µl of 2- mercaptoethanol to each 10 ml of the stock solution.

2.4.1. 2. 2 M sodium acetate, pH 4.0 preparation.

A 16.42 g of sodium acetate (anhydrous) was added to 35 ml of water and 40 ml glacial acetic acid. And then, the pH was adjusted to 4.0 with glacial acetic acid and the final volume was brought to 100 ml with water.

2.4.1. 3. Total RNA extraction.

Total RNA was extracted using acid guanidium thiocyanate – phenol- no chloroform extraction method that described by Chomczynski Sacchi (1987) with minor modifications which is included using the bromochloropropane instead of chloroform in order to introduce the liquid interphase. RNA pellets were re-suspended with 75 % ethanol to dissolve the residual guanidinium thiocyanate, twice against its re-suspension. However, it was re-suspended only once with 75 % ethanol as described in the original extraction method.

The RNA denaturation was done using a guanidinium thiocyanate denaturing solution (containing 2-Mercaptoethanol). Next, sodium acetate (pH 4) was used for acidification. Isopropanol was also used twice to precipitate RNA.

The final step of RNA extraction was air-drying of all RNA pellets and then, subsequently was dissolved in RNase and DNase-free water. Then, treated with DNase -1 kit to remove any genomic DNA contamination during the procedure of total RNA extraction method and then, all RNA samples were stored at -80 °C (Chomczynski and Sacchi, 1987). The method was done briefly, following the experiments, culture supernatants were removed from RAW264.7 cells (were stored at -80 °C for ELISA) and 500 µl of denaturing solution was added directly to the wells. Cells were rested in denaturing solution for 1 - 2 minutes to allow complete lysis and the solution was pipetted up and down 10 times to ensure all the lysate was collected.

The samples were allowed to sit for 5 minutes before being frozen at -20 °C for later analysis but not exceeded 30 minutes.

To extract RNA, 50 µl of 2 M sodium acetate, pH 4.0 was added to the cell lysates respectively and mixed thoroughly by inversion. Then, 500 µl water saturated phenol was added, mixed by inversion, followed by 100 µl of 1-bromo-3-chloropropane, and shaken vigorously by hand to ensure proper mixing. Samples were cooled on ice for 15 minutes then centrifuged for 20 minutes, 15000 g, at 4 °C. And then, the upper aqueous phase containing mostly RNA was transferred to RNase- and DNase free Eppendorf tube and 500 µl of isopropanol was added to precipitate the RNA. Samples were incubated at -20 °C until needed for the next step.

Next, samples were centrifuged at 15000 g for 20 minutes at 4 °C. The supernatant was discarded and the gel-like precipitates (RNA) were retained then dissolved in 300 µl of denaturing solution. And then, 300 µl of isopropanol were added and the samples were incubated at -20 °C for at least 30 minutes (1 hour).

Samples were incubated at -20 °C until needed for the next step or after which they were centrifuged for at 15000 g for 10 minutes at 4 °C. The supernatants were discarded and the pellets were re-suspended in 500 µl of 75 % ethanol and vortexed for 10 seconds to mix, and then incubated for 15 minutes at room temperature to dissolve any residual guanidinium thiocyanate. At this point, samples were stored at -20 °C until RNA extraction could be completed.

Samples were centrifuged for at 15000 g for 10 minutes at 4 °C, and the supernatant was discarded. The pellets were air dried for around 15 - 30 minutes in a laminar flow hood at room temperature, and then the RNA was dissolved in 30 µl of RNase- and DNase-free water and incubated for 15 minutes at 60 °C to ensure complete solubilisation of the RNA.

To remove any residual genomic DNA, samples were treated with the DNase 1 kit (Sigma, UK). To the solubilised RNA, 3 µl of 10 x reaction buffer and 3 µl amplification grade (Deoxy-ribonuclease I) DNase 1 (1 unit/µl) were added and samples were left to stand at room temperature for 15 minutes. To stop the reaction, 3 µl stop solution was added to each tube. Samples were then incubated at 70 °C for 10 minutes, and then placed on ice ready to quantify the total RNA concentrations using nanodrop 2000 ready for reverse transcription (please see section 2.4.2). RNA purity was assessed by observing the ratio of absorbance at 260 nm and 280 nm. The accepted RNA purification ratio was 1.8 - 2.1 for any RNA sample to be considered as a pure.

2.4. 2. Reverse transcription.

Total RNA samples were first diluted using RNase or DNase free water grade by taking 0.5 µg of each RNA sample to be diluted up to 9 µl in a thin walled PCR tubes. And then, all samples were reverse transcript to complementary deoxyribonucleic acid (cDNA) using high capacity RNA to cDNA reverse transcriptase kit following the supplier's instructions (Life Technologies Ltd) by preparing the master mix accordingly to the number of RNA samples that were needed to be reverse transcript for each reaction by adding 10 µl/ sample of the reverse transcriptase buffer.

Then, 1 µl / sample of the reverse transcriptase enzyme were added to the master mix. Then, 11 µl/ sample of the master mix were added in equal amount into each diluted RNA sample. The reverse transcription run conditions were 37 °C for 1 hour (60 minutes) stopped by heating at 95 °C for 5 min, and then the run was held at 4 °C using 96 well thermal cycler machine (Vertti). All samples were stored at -20 °C until used.

The qPCR primers for murine LPCAT-2, LPCAT-2 open reading frame (ORF), TNF- α , IL-10, IL-6, TLR-4, TLR-2, CD14, COX-2, PPAR- γ , iNOS, CD206 and all the reference gene (GAPDH) were designed using Primer Express 6 software version 6.1.10 (Life

technologies Ltd, UK) to amplify 100 bp product for all the targets (please see table 1 for primer sequences).

The specificity of each sequence was checked by NCBI nucleotide basic local alignment search tool (BLAST) (Altschul *et al.*, 1990).

The sequences for hairpin, self-dimer and heterodimer were also checked using oligoanalyser 3.1 (Integrated DNA Technologies). The sequences are given in table 1.

2.4.4. Real time –Polymerase chain reaction (PCR).

The cDNA from the reverse transcription reaction (please see section 2.4.2) was amplified using Power SYBR Green kit according to the supplier's instructions. Briefly, the cDNA (0.5 µl) from RT reaction was used for each PCR reaction.

The qPCR (Real time PCR) reaction conditions were 95 °C for 10 minutes at the holding stage, followed by 40 cycles in which including the denaturation at 95 °C for 30 seconds, annealing at 53 °C for 60 seconds, and then, the extension at 72 °C for 120 seconds followed by a final 6 minutes extension at 72 °C. All primers were designed and checked using primer express software 'Primer Express 6 software version 6.1.10'. All Primers that have been used are shown in Table1.

Fold increase values were calculated as $(2^{-(\Delta\Delta Ct)})$ i.e. $RQ = 2^{\text{power}-(\Delta\Delta Ct)}$; pCMV6 without LPS or Pam3CSK4 is represented as 1 fold increase ($2^{-(0)} = 1$). All data were normally distributed (please see table 2 in Appendix 1, material and methods supplements).

Reference & target Genes	Sequence	Melting Temperature (°C)
Human LPCAT-2 (ORF)* insert	Forward 5' –GCT GCC CTC ATT CAA CATT - 3' (18)	60
	Reverse 5' –CAA CTA GAG GGA CTT GTG CAT TC - 3' (23)	
Murine LPCAT-2 ORF* insert	Forward 5' –CAG ACT GTT ACG GGC TTT GCA - 3' (21)	60
	Reverse 5' –ACC TGA TGT CGC TCG CTT TT - 3' (20)	
Human LPCAT-2 **	Forward 5' –GCA CAC GCT CCC CTT GACT - 3' (19)	60
	Reverse 5' –TTA TGG CCC ACT GCA ATC GT - 3' (20)	
GAPDH **	Forward 5' –CCT CGT CCC GTA GAC AAA ATG - 3' (21)	60
	Reverse 5' –TCT CCA CTT TGC CAC TGC AA - 3' (20)	
Murine LPCAT-2 **	Forward 5' –GCA CAC GCT CCC CTT GACT - 3' (19)	60
	Reverse 5' –TTA TGG CCC ACT GCA ATC GT- 3' (20)	
Murine IL-10**	Forward 5' –CTT GCA CTA CCA AAG CCA CAA G- 3' (22)	60.
	Reverse 5' –GGA AGT GGG TGC AGT TAT TGT CT- 3' (23)	
Murine IL-6**	Forward 5' –AGA AGG AGT GGC TAA GGA CCA A - 3' (22)	60
	Reverse 5' –ACG CAC TAG GTT TGC CGA GTA - 3' (21)	
Murine TNF- α **	Forward 5' –AGG ACC CAG TGT GGG AAG CT - 3' (20)	60
	Reverse 5' –AAA GAG GAG GCA ACA AGG TAG AGA- 3' (24)	
Murine TLR-4**	Forward 5' –ACT CTG ATC ATG GCA CTG TTC ATC T - 3(25)	60
	Reverse 5' –TCA GCA AAG TCC CTG ATG ACA TTC C - 3'(25)	
Murine TLR2**	Forward 5' –AGG GAT CCG GGT GGT AAA AA - 3'(20)	60
	Reverse 5' –CAG CCG AGG CAA GAA CAA AG - 3' (20)	
Murine CD206**	Forward 5' –AAA TGG AGC CGT CTG TGC AT- 3' (20)	60

	Reverse 5' – AAG TGC AAT GGA CAA AAT CCA A - 3' (22)	
Murine iNOS **	Forward 5' – CGC CTT CAA CAC CAA GGT TG- 3' (20)	59.4
	Reverse 5' – TCA GAG TCT GCC CAT TGC TG - 3' (20)	
Murine CD14**	Forward 5' –GCA GTG GCC TTG TCA AGA- 3'(18)	60
	Reverse 5' – <u>GCA</u> TGA CGA GGA CCC GTA A - 3' (19)	
Murine PPAR γ **	Forward 5' –CCA CTC GCA TTC GTT TGA CA - 3' (20)	60
	Reverse 5' – TCG CTC AGC TCT TCC GAA GTG- 3' (21)	
Murine COX- 2**	Forward 5'- CCA CAG TCA AAG ACA CTC AGG TAG A-3'(25)	60
	Reverse 5'- CCA GGC ACC AGA CCA AAG AC -3'(20)	

Table 1. All primers sequences for Real time –Polymerase chain reaction (RT-PCR).

*This primer was used to check the overexpression of LPCAT2 insert gene in mammalian cells.

Note: Human or murine LPCAT2 Open Reading Frame insert (ORF)* insert

** These primers were used for the routine daily work RT-PCR work.

2.7. Agarose gel electrophoresis.

Product size and primer specificity was confirmed using agarose gel electrophoresis. PCR samples had 2.5 μ l of loading buffer (orange G dye) added and then were loaded into a 1 % (w/v) Tris-acetic acid EDTA (TAE) agarose gel.

Gels were made by dissolving agarose within an appropriate volume of TAE buffer (40 mM Tris-base, 10 mM EDTA and 0.1 % acetic acid) which was heated in a microwave for 1 minute and then cooled to 50 °C, and then 4 μ L SyBer safe (10000 x) was added to the gel to enable visualization of DNA under UV light (Appendix 1, materials, 1.3.2 and 1.3.2).

Gels were run at 60 – 100 V according to the size of the product for an appropriate time. Bands were checked for presence and size using Ultra Violet Products (UVP) image gel documentation system linked to a PC.

2.8. Cloning and expanding a Plasmid carrying the murine LPCAT-2 inserts.

The True ORF Gold plasmid which has kanamycin resistance for colonies selection on Luria Britani Agar (LB-Agar) containing 25 µg/ml kanamycin (Appendix 1, figure 3, (1.1) Material support information Figures) were expanded by transformation of 1 µg/ml of plasmids (pCMV6 -entry vector plasmid (4.9 kb)) with or without the inserts of the murine LPCAT-2 by cloning into 10 µL of Alpha-gold competent cells in Eppendorf tubes incubated on ice for 30 minutes (optimum temperature to mix the DNA plasmid with *E. coli* competent cells).

Then, heat shock using heat block at 42 °C for 45 seconds (special temperature to let the membrane of *E. coli* become porous and for the Plasmid DNA clone having the kanamycin resistance gene for selection in *E. coli* colonies grown on Luria Britani (LB)-plate containing 25 µg/ml of kanamycin (KAN); to enter the cells). Followed by this was the cooling on ice for 2 minutes to close the pore in the cell membrane of *E. coli* competent cells. Incubation in a shaking incubator for 1 hour at 37 °C (optimum temperature for *E. coli* growth on KAN-LB-plate) followed.

Then, the mix was diluted (Transformed competent) into (1 %, 10 %, 50 %, 100 %) using SOC medium (appendix 1). 100 µL was transferred onto an LB plate with kanamycin concentration of 25 µg/ml (Appendix 1, (1.2.1), culture media, material and methods), and incubated at 37 °C overnight no more than 18 hours.

The next day one single colony of the transformed cells was transferred into LB broth with kanamycin concentration of 60 µg/ml (Appendix 1, (1.2.2 and 1.2.3), culture media,

material and methods), and then incubated overnight for the best yield of plasmid at 37 °C in shaking incubator.

The transformed kanamycin resistant *E. coli* growth was confirmed by the obvious turbidity with in the LB-Broth tubes in comparison with no growth of competent cells alone that showed clear LB-broth.

2.8.1. Purification of the Plasmid with or without the murine LPCAT-2 inserts.

Alpha- gold competent cells (*E. coli*) culture, incubated overnight at 37 °C in a shaking incubator were harvested by transferring 2 ml of it into 2 ml micro-centrifuge tubes and then spun at 12,000 g for 1 minute. And then, the cell pellets which are contained the Plasmids with or without the murine LPCAT-2 inserts were kept to be purified using the MiniPrep Kit following the supplier instructions.

Briefly; the MiniPrep purification procedure was applied in a sterile environment using benzyl flame. All cell pellets were re-suspended by adding 200 µl of Re-suspension solution that containing RNase enzyme with a mild vortex until homogeneous (to ensure good recovery i.e. incomplete resuspension would result in poor recovery). And then followed by adding 200 µl of cell lysis solution was added into all tubes with gentle mixing (6-8 times) immediately and incubated for no more than 5 minutes at room temperature (RT) in order to avoid permanent supercoiled plasmid denaturing as a result of prolong alkaline lysis.

Then, 350 µl of neutralised/Binding solution were added into all Eppendorf tubes with gentle inverted (4 - 6 times). Then all samples were centrifuged at 12000 xg at 4 °C for 10 minutes (the cell debris, protein, lipids, SDS, and chromosomal DNA would precipitate and fallen out of the solution as viscous cloudy precipitate).

In addition, if the supernatant was contained large amount of floating particulates after centrifugation, then the supernatant was re-centrifuged before proceeding to the next steps.

Meanwhile the silica- column tubes were prepared by placed it into a new fresh Eppendorf tubes which is provided within the kit by adding 500 μ l of column solution. Then column tubes were centrifuged at 12000 x g for 1 minute.

And then, the clear supernatant from the previous tubes were transferred into column tubes respectively and then the samples were centrifuged at 12000 xg at 4 °C for 1 minute.

Then, 500 μ l of the optional wash solution were added into each sample after discarding the liquid of the previous step (it is necessary to avoid nuclease contamination of the final plasmid product), were re-centrifuged at 12000 xg at 4 °C for 1 minute.

And then, 750 μ l of the wash solution were added into each sample, and then were re-centrifuged within the same speed and time. And then, all samples were re-centrifuged at high speed for 2 minutes without adding any washing solution to remove the excess ethanol.

Finally, 100 μ l of elute solution were added into each sample after transferring the silica column into new fresh tubes with clear labelling, then centrifuged at 12000 xg for 1 minutes at 4 °C. The MiniPrep purified plasmid DNA concentration for all samples were quantified using nanodrop 2000 and were stored at -80 °C until used.

2.8.2. Western blotting (WB).

2.8.2.1. Total lysate preparation and protein quantification.

The cells lysate of transfected RAW264.7 were prepared using the radioimmunoprecipitation assay buffer (RIPA buffer) (Collett & Erikson, 1978; Abate & Jackson, 2015) (Appendix 1, material and methods, 1.5) to lyse the cells into protein from the transfected and non-transfected cells, then keep all samples on ice for 30 minutes and

then the samples were sonicated using an ultrasound probe to have high concentration of protein. And then the protein in each sample was quantified using micro BCA Assay Kit following the supplier instructions.

A sterile phosphate buffer saline (PBS), pH (7.2 - 7.4) was used as a reagent diluent to prepare the dilutions within this assay for both the standard and protein samples as well. The total volume that added to each well of the 96 wells plate was 200 including 100 μ l of each standard or a protein sample respectively. And then 100 μ l of the working solution of the detection reagent which is prepared immediately in a ratio of (25: 24: 1) to three components (A: B: C).

The micro- BCA standard reagent stock was 2 mg/ml of bovine serum albumin (BSA) was used to prepare the working solution started with the highest concentration 200 μ g/ml by adding 50 μ l from the micro-BCA standard reagent stock into 450 μ l of PBS followed by 6 serial dilutions in 6 sterile 1.5 ml Eppendorf tubes that had 250 μ l of PBS and the blank tube had PBS only. All protein samples (1: 25 or 1: 50) were prepared by adding (96 or 98) μ l of PBS respectively into each well, and then (4 or 2) μ l of each sample were added respectively at room temperature. Then 100 μ l of micro-BCA detection reagent was added into all wells including the standard. The 96 well was sealed using plate sealer.

The plate was shaken gently for 1 minute using plate shaker and was subsequently incubated at 37 °C for 2 hours. The protein concentration was assessed using versa max microplate reader with SoftMax Pro 2.4.1 Software which is depended on the absorbance in a micro-plate reader at 595 nm. A standard curve was produced between absorbance value and protein concentrations, and the test sample was calculated based on comparison to the standard curve using 5 - parameter statistical model.

2.8.2.2. Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE).

The protein samples in a concentration of (10 – 20) µg within a 20 µL of each sample as a maximum volume to be loaded into each well of the precast-gel (NuPAGE® Bis-Tris Gels), 10 µL of SeeBlue, and 10 µl of biotin marker were loaded into gel. The gel electrophoresis was run using XCell SureLock™ Mini-cell following the supplier instructions.

Briefly; protein samples were prepared using 12.5 µl of NuPAGE® loading sample buffer (4x) and 5 µl of NuPAGE® reducing reagent (10 x) and deionised water up to 50 µl, and 10 µL of biotin marker were vortex and incubated in dry heat block at 70 °C for 10 minutes. And then, the samples were placed on ice prior loading into precast-gel.

Then, NuPAGE® Western blotting running buffer (SDS running buffer) stock (10 x). The (1 x) NuPAGE® was prepared by adding 100 ml of the stock NuPAGE running buffer (10 x) (Appendix 1, Materials, 1.4) into 900 ml of deionised water. Then, the NuPAGE gel (10 % polyacrylamide Bis-Tris-Cl buffered gel (pH 6.4) was washed using deionised water and running buffer prior inserting into the XCell SureLock™ and sealed using the wedge. And then, 200 ml of running buffer were added into upper buffer chamber which were covering the wells.

Then, 20 µl of each sample, 10 µL of SeeBlue, 10 µl of biotin marker were loaded into gel, the 600 ml of the running buffer were added into outer buffer chamber. And then, the XCell SureLock™ Mini-cell Novex system was attached into the power supply and run at 200 V for an hour at room temperature.

2.8.2.3. Western immuno-blotting transfer.

After the NuPAGE® SDS-PAGE electrophoresis and prior to NuPAGE® Western blotting transfer buffer, and the NuPAGE® transfer blot using the XCell SureLock™

Mini-cell Novex system apparatus. The SDS-PAGE protein samples were transferred into polyvinylidene difluoride (PVDF) membrane following the supplier instructions.

It runs at 30 V for 90 minutes. Briefly; NuPAGE® Transfer buffer (20 x) as a stock was used to prepare the working solution in a concentration of (1x) by adding 50 ml of the NuPAGE® Transfer buffer, 100 ml of Methanol (200 ml for two gels), and 849 ml of deionised water (dd-H₂O) (749 ml for two gels). The gel was removed from the electrophoresis system tank (as described in section 2.8.2.2).

Then, the plates were removed and the gel were placed between 2 pieces; first one of filter papers were placed on the top of the gel (filter papers were previously cut in the same size of the gel prior the soaking in 1 x transfer buffer). And then, the PVDF membrane was placed facing the gel surface (it was cut in the same size of the gel and pre-soaked into methanol for 15 seconds and in deionised water for 2 minutes and then, placed in the transfer buffer for 55 minutes).

Next, the second filter paper was placed on the PVDF membrane and any trapped air bubbles were removed using a roller. Then, two pre-soaked blotting pads were placed into the cathode (-) core of the Blot, and then the gel/membrane assembly were carefully pickup and were placed on blotting pads in the current orientation (please see figure 1), so that the gel was closed to the cathode core, then two more blotting pads were placed over the gel/membrane assembly.

Then, the anode (+) were placed on top of the pads and the blot was firmly hold together and slide it into the guide rails on the lower buffer chamber. The gel tension wedge was inserted into the lower buffer chamber and was locked in position, then the blot modulate was filled with 1 x transfer buffer until the gel/membrane assembly covered, and then the 600 ml was added into outer buffer chamber. And then, the lid was placed on the unit and connected into electrical lead to the power supply. The transfer process was run using 30 Volt (V) constant for 90 minutes at room temperature.

2.8.2.4. Western blotting ECL detection.

ECL substrate is an enhanced chemiluminescent substrate that used to detect horseradish peroxidase (HRP) activity from antibodies and other western blot probes.

Prior to the ECL detection, WB membrane were blocked using the blocking buffer which consisted of 0.1 % or 0.05 % of skimmed milk (due to its low-fat content and cheaper price) in PBS tween (PBS-T20).

Then the detection steps were subsequently done using the purified anti Ddk mouse mAb clone 4C5 (Anti-flag Ab/ Goat-anti-mouse) or Anti-LPCAT2 Antibody as a primary Abs with a dilution (1: 1000) and Goat -Anti Mouse-IgG or Goat -Anti Rabbit -IgG respectively as a secondary Ab (1: 1000).

And then, incubated for 10 minutes at room temperature using the snap system apparatus or for at 1 hour at room temperature or overnight at 4 °C for the ordinary WB on a rolling shaking machine. Subsequently, were followed by three times washes using 30 ml of 0.1 PBS-T 20 for Snap system or 0.05 % PBS-T 20 for ordinary WB on shaker machine post adding the primary or the secondary Ab.

And then, the bands were checked according to their molecular weight using UVP gel documentation system linked to a PC. Developing colour for protein detection was performed by using enhanced chemiluminescence (ECL) to detect peroxidase activity from HRP-conjugated antibody.

According to the manufacturer's instructions, the membrane was removed after washing with TBS, dry on a paper towel and laid out on a plastic sheet. And then, the detection substrate solution was carefully pipetted over the entire membrane inside the tray of imaging system. The protein bands were visualized by a gel documentation system using EC3 imaging system.

2.8.2.5. Stripping and Re-probing buffer for WB membrane.

50 ml of re-probing solution was prepared by adding 28.65 g guanidium HCl (Gn-HCl) / 50 ml, (0.2 %) i.e. 100 μ l of Triton x - 100 (Tx - 100) / 50 ml, (0.1 M) i.e. 358 μ L of β -mercaptoethanol (BME) was prepared by dissolving (Gn-HCl) with 20 mM Tris-Cl (pH= 7.5) until 50 ml in hot water.

And then, Tx-100 (100 μ l) was added followed by adding 358 μ l of BME and the re-probing procedure was applied inside the fume cabinet by adding 25 ml of the re-probing buffer for the WB membrane, incubate for 5 minutes. It was followed by 4 washes using 0.05 % PBS-T20 for 5 minutes each wash.

Then incubate the membrane with extra 25 ml of re-probing buffer followed by 4 times washes for 5 minutes each using 0.05 % PBS-T20 on shaker for all washes and re-probing steps. This procedure was useful to get rid of the previous Antibodies (Abs) without affecting the protein bands which were remained within their position on the PVFD membrane.

2.9. Establishing a stable transfected RAW264.7 cell line culture.

RAW264.7 cells were plated in a concentration of 1×10^6 cell/ well (2.5 ml complete DMEM medium) incubated at 37 °C for 24 hours. Then, the murine LPCAT-2 was over-expressed (as descried in section 2.2.1.4).

Further, RAW264.7 cells with over-expressed murine LPCAT2, and untreated RAW264.7 cells were plated in a concentration of 5×10^5 cell /well (500 μ L of DMEM medium) in a 12 well plate and were respectively treated with DMEM medium with a concentration of (0, 500, 600, 700) μ g/ml/well (500 μ L DMEM medium) of the G418 antibiotic, in duplicates (2 wells for each treatment).

Then, the plate was incubated at 37 °C within 5 % CO₂ humidity atmosphere. The wells also were checked daily under inverted microscope and the medium containing G418

were changed daily for 5 - 6 days. And then, a concentration of 200 µg/ ml of G418 were used as maintaining medium of the stable transfected cells clone.

The maintaining media were changed every 3 days for extra 9 days in order to check the stability. Finally, the stable cell line was frozen and stored at -80 °C after adding 10% DMSO (freezing medium) as a cell stock storage after analysing the overexpression of the LPCAT2 in RAW264.7 cells using western blotting (WB) and real time quantitative polymerase chain reaction (RT-PCR). RAW264.7 that stably over-expressed the murine LPCAT-2 will be termed 'LPCAT-2⁺⁺' in chapter 5, and transfected RAW26.7 cells with empty vector will be termed 'pCMV6' in chapter 5.

2.9.1. Killing curve of RAW264.7 cell culture.

RAW264.7 cells (passage 9) were plated in two plates of 96 well plates, started in a concentration of (10^5 cells/ml)/ well (150 µL) in duplicate dilution.

Different concentrations of the antibiotic Geneticin (G418) (800,700, 600, 500, 400, 200, 0) µg/ml were applied and then, the medium containing the antibiotic were changed every three days for the first week, and then, daily for the second week and the confluence of the cells were checked daily using the inverted microscope until the whole cells were dead in all wells.

This procedure was done in order to optimise the convenient concentration of the G418 antibiotic to establish a stable overexpression of murine LPCAT-2 in mammalian cell line that used to select the cells that has the resistant gene to neomycin antibiotic as well as its ability to kill the murine RAW264.7 cells which were lacked with this gene.

2.9.2. Transient transfected RAW264.7 cell line culture.

RAW264.7 cells were plated in a concentration of 1×10^6 cell/ well (2.5 ml DMEM medium) (WB) or 0.5×10^6 cell/ well (2.5 ml DMEM medium) (ELISA or qPCR)

Incubated for 24 hours at 37 °C. And then, 2.5 µg of DNA plasmid with or without cDNA clone of murine LPCAT-2 was transfected into RAW264.7 using 9 µl of lipofectamine 2000 transfected reagent respectively.

A non-transfected RAW264.7 cells were kept as a negative control in duplicate for 48 hours transfection to be analysed with the over-expressed LPCAT-2 respectively using western immune-blotting and real time qPCR.

2.11. Enzyme Linked Immune-Absorbent Assay (ELISA).

2.11. Mouse TNF- α or IL-6 cytokines enzyme linked immune-absorbent assay (ELISA).

ELISA experiments were performed to assay the protein expression of TNF-alpha, or IL-6 following the manufacturer's instructions. Briefly, capture antibody; anti-TNF- α (0.8 µg/ml) or anti-IL-6 (2 µg/ml). Capture antibody was made to bind to the wells of a Maxisorp 96 wells ELISA plate (The wells were coated with diluted capture antibody using PBS as a diluent, sealed and incubated overnight at 4 °C).

Then IL-6 or TNF- α plates were washed three times with PBS/0.05 % v/v Tween-20. Then, any non-specific binding sites on the surface were blocked using 1 % w/v BSA in PBS for 1 hour at room RT. Further, the plates were washed three times.

Then, the plates were incubated with serially diluted recombinant cytokine standards, test samples for 2 hours at RT or overnight at 4 °C. The plates were then washed to remove unbound antigen. And then, a 50 ng/mL or 150 ng/ml of the detection antibody was added and were incubated at RT for 2 hours with a subsequent washing to remove the unbound antibody-enzyme conjugates.

This was followed by the addition of 100 µl/well of streptavidin horseradish peroxidase (Streptavidin-HRP) at dilutions ratio (1 : 40) using the diluent reagent (1 % BSA in PBS) and then, the plates were incubated for 30 minutes at RT.

The plates were washed for three times and then, 100 μ l of the substrate solution which is the colour reagent Tetramethylbenzidine (TMB) for 30 minutes at RT.

Finally, 50 μ l of the stop solution; 2 M sulphuric acid (2 M H₂SO₄) were added to each well.

The absorbance of the plate wells was measured using the versa max micro-plate reader with SoftMax pro version 2.4.1 software to determine the presence and quantity of antigen 5-parameter statistical model.

2.12. Data analysis and statistics.

At least, three independent experiments for qPCR and for ELISA were done in duplicate. qPCR results were analysed using a number of algorithms. The PC-Miner software (Zhao *et al.*, 2005) was used to calculate the Cycle Threshold and the Efficiency values of the genes of interest.

The ELISA protein was normalised against the total RNA concentration (Muratore *et al.*, 2014) and re-analysed due to the cytotoxicity of the lipofectamine 2000 transfection reagent.

The one-way analysis of variance (ANOVA) including Tuckey as a multiple comparison for all pairs columns and t-test which is provided by the GraphPad Prism 5 software was used to statistically analyse the data of both RT-PCR and ELISA and the P-value < 0.05 was considered significant. Normal distribution was determined using Sigma Plot 13.0 as well as SPSS® software according to (Shapiro and Wilk, 1965), and all data were found to be normally distributed (for an e.g. Please see table 5 in Appendix 1).

Chapter 3:
Transient over-expression of LPCAT-2 in
RAW264.7 cells

Introduction.

As described in chapter 1, sepsis is a life-threatening organ dysfunction caused by a dysregulated host response to infection. This dysregulated response results in an overwhelming inflammatory response to infection, especially to bacterial infection. No specific therapy for sepsis exists and the morbidity and mortality from this condition therefore remains high with a consequent huge economical and healthcare impact (Angus *et al.*, 2006; Cheng & Shi, 2009; Guzzo *et al.*, 2011; The Global Sepsis Alliance, 2016). Lipopolysaccharide (LPS) the endotoxin of Gram-negative bacteria, is an important trigger of sepsis and recognition of LPS by macrophages produce the inflammatory response to infection that can elicit sepsis. (Cohen, 2002; Strassheim *et al.*, 2002; Hoesel & Ward, 2004; Płóciennikowska *et al.*, 2015). Therefore, understanding the mechanisms and molecular pathways by which macrophages respond to signals such as LPS, will be key to deriving therapies to combat the uncontrolled inflammatory response which is the hallmark of sepsis.

Work from the Jackson laboratory and others have shown that the phospholipid metabolizing enzyme Lysophosphatidylcholine acyltransferase (LPCAT) can control inflammatory responses to LPS in macrophages (Jackson *et al.*, 2008; Sevastou *et al.*, 2013). It was previously shown that LPCAT has a role in inflammatory responses using pharmacological inhibitors, although the precise LPCAT species responsible could not be determined (Jackson *et al.*, 2008). Availability of the gene sequences and cloning of LPCAT species allowed the identification of four major family members (LPCAT1-4) each with different but overlapping activities. (Chen *et al.*, 2006; Shindou *et al.*, 2007; Edwards & Constantinescu, 2009; Hishikawa *et al.*, 2008; Morimoto *et al.*, 2010; Zheng *et al.*, 2012; Shindou *et al.*, 2013; Morimoto *et al.*, 2014). The challenge is therefore to determine which LPCAT species is responsible for regulating the inflammatory response to LPS and other bacterial factors. This knowledge will allow new insights in to the

mechanisms of inflammation and will be required for new therapies aimed at these targets to be developed.

Recent work has suggested that LPCAT-2 is the major species responsible for facilitating inflammatory responses in macrophages (Abate *et al.*, 2016, Al-Rammah *et al.*, 2016). In addition, it has been shown that LPCAT-2 is inducible in response to LPS and other bacterial ligands (Shindou *et al.*, 2007; Morimoto *et al.*, 2010, 2014) Mounting evidence suggests therefore, that LPCAT-2 is induced in macrophages in response to bacterial infection and can drive the inflammatory response in these cells.

To determine the importance of LPCAT-2 is response to infection, the current chapter explores the role of LPCAT-2 in the inflammatory response of macrophages by transfecting and overexpressing the gene for LPCAT-2 in these cells. Overexpression of LPCAT-2 would be hypothesized to enhance the inflammatory response to LPS and other bacterial ligands. The cellular readouts for inflammatory responses in overexpression of LPCAT-2 are cytokines known to be produced by macrophages in TLR4-dependent responses to LPS. These include TNF α , IL-6, IL-10 and IFN- γ , and they have been shown to be predominantly important in the development of septic shock (Doherty *et al.*, 1992; Silva & Cohen, 1992; Rothe *et al.*, 1993; Dellinger *et al.*, 2004; Daniel, 2010). In addition, as there are many bacterial causes of sepsis, including Gram positive organisms, other molecules that can stimulate different TLR pathways were used. To explore other TLR ligands, this project used the synthetic triacylated lipoprotein Pam3CSK4 which is a TLR1/2 ligand with similar structure to lipoprotein cell wall components found in both Gram positive and Gram negative bacteria.

This chapter explores the gene expression profiles of RAW264.7 cells transfected with LPCAT-2 (LPCAT-2⁺) and using pCMV6 empty vector plasmid as control. Transient transfection of RAW264.7 cells using Lipofectamine 2000 and a plasmid carrying the murine LPCAT-2 was used to study the overexpression of (LPCAT-2). In addition,

silencing of the LPCAT-2 gene using specific siRNA sequences, is a convenient method of proving the role of LPCAT-2 in the inflammatory responses. Therefore, experiments were also performed to evaluate the effect of silencing of the over-expression of LPCAT-2 in the inflammatory readouts.

3.1. Cloning of murine and human Lysophosphatidylcholine acyltransferase – 2 (LPCAT -2).

A single colony of transformed competent bacteria i.e. transformed with pCMV6 plasmid with or without the murine (mLPCAT-2) was inserted onto separate Luria Britania (LB) plates with kanamycin (KAN) 25 µg/ml and diluted in four different dilutions: (1 %, 10 %, 50 % and 100 %) (Please see section 2.8. in materials and methods).

The plate with 50 % or 100 % diluent transformed cells showed many colonies compared with 1% transformed cells which showed few colonies. In addition, non-transformed competent bacteria (i.e. bacteria not possessing the gene for kanamycin resistance) showed no growth while the non-transformed competent cell colonies on a plate without kanamycin showed full growth of colonies. This is shown in figure 3.1.

Purification of the plasmid with or without the mLPCAT-2 clones was performed after the transformed colonies were selected by transferring 1 single colony from the LB Plate with 25 µg/ml of kanamycin into LB-Broth with 60 µg/ml of kanamycin overnight at 37 °C in a shaking incubator (for more details, please see 2.8. materials and methods.).

Next, the purified miniprep plasmid and the mLPCAT-2 recombinant gene was checked by traditional PCR and agarose gel electrophoresis to confirm the existence of the inserted genes (1730 bp) within the pCMV6-Entry vector (4919 bp) Plasmid as shown in figure 3.2.

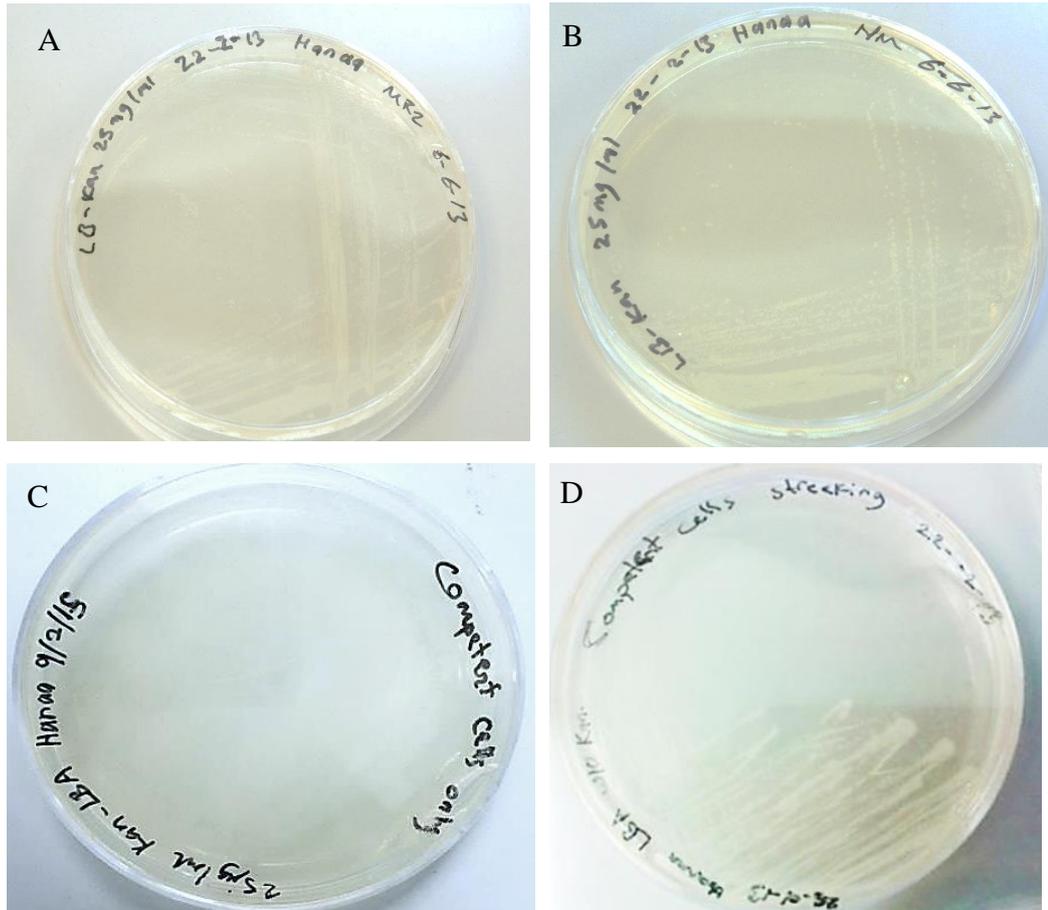


Figure 3.1. Competent bacteria transformed with or without Plasmid containing murine LPCAT-2 (mLPCAT-2 and hLPCAT-2).

(A): Transformed competent cells with the murine LPCAT-2 gene with SOC medium (a nutrient- rich microbial growth medium) mixture incubated at 37 °C in a shaking incubator for 1 hour prior to inoculation on Louriia Britania (LB) agar plates with 25µg/mL Kanamycin (KAN) overnight at 37 °C in a shaking incubator that showed the single colonies.

(B): Transformed competent cells with the human LPCAT-2 gene with SOC medium mixture incubated at 37 °C in a shaking incubator for 1 hour prior to inoculation on Louriia Britania (LB) agar plates with 25 µg/mL Kanamycin (KAN) overnight at 37 °C in a shaking incubator that showed the single colonies.

(C): Non-transformed competent cells with no colonies.

(D): Non-transformed competent cells on LB plate without Kanamycin with bacterial colonies.

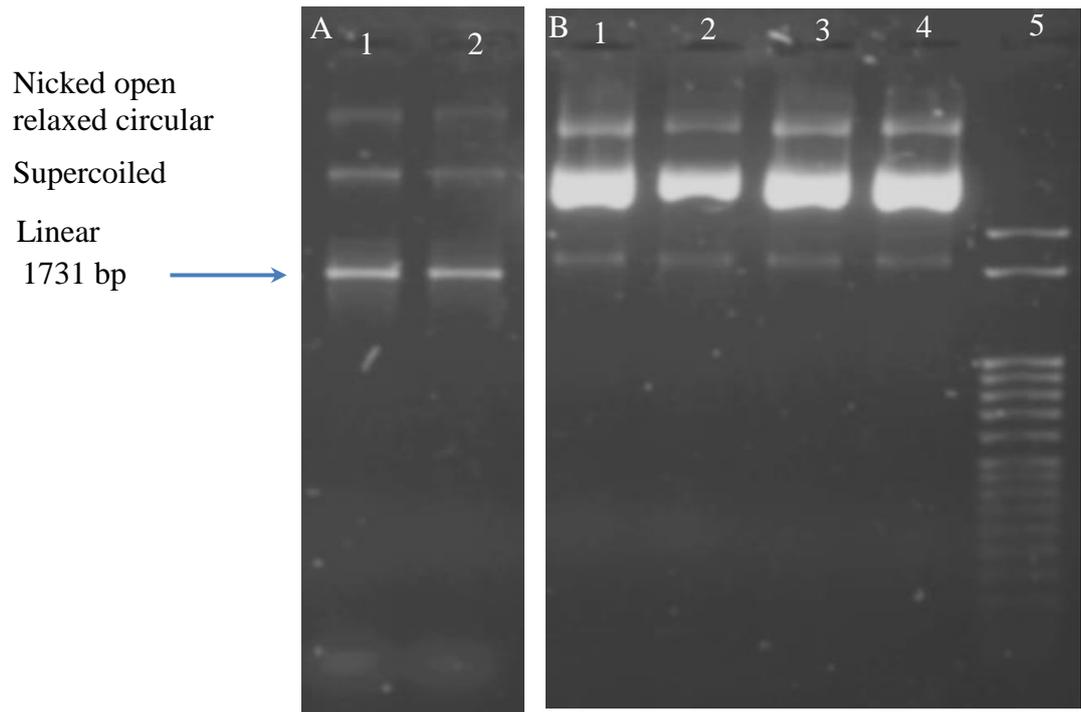


Figure 3.2. Agarose gel electrophoresis confirmation of miniprep purification and cloning of the PCR product from murine LPCAT-2 recombinant gene in pCMV6 - entry vector (4.919 kb) Plasmid.

(A) PCR product performed with conventional PCR format on agarose gel electrophoresis which shows the murine (Lane 1 and 2) LPCAT-2 recombinant gene (1731 bp) in pCMV6 - Entry vector (4.919 kb) Plasmid.

(B) MiniPrep Plasmid (pCMV6 - Entry vector (4.919 kb)) carrying the murine LPCAT-2 (Lane 1 - 4) recombinant gene which shows three bands: Linear, more super coiled and nicked open circular.

Lane 5: Molecular DNA Ladder.

3.2. Transient expression of Murine Lysophosphatidylcholine acyltransferase – 2 (LPCAT -2) in RAW264.7 cells.

To optimise the transfection, two different transfection reagents (Lipofectamine 2000 and Jet Prime) at four different volumes (6, 9, 12, 15) μ l, (please see section (2.2.1.4) and (2.2.1.5), materials and methods), were used to express LPCAT-2 in RAW264.7 for 48 hours. Transfection was transient due to the cytotoxicity of the transfection reagent. Figure 3.3, shows an obvious reduction in the number of the viable transfected cells with increasing concentration of the transfection reagent.

This also shows that the number of cells treated with Lipofectamine 2000 was greater than the number of cells treated with JetPrime. This was due to the difference in the number of RAW264.7 cells per well in each experiment set; 1×10^6 cells/well for Lipofectamine 2000 as against 2×10^5 cells/well for JetPrime transfection reagent.

However, Lipofectamine 2000 reagent was toxic to the transiently transfected RAW264.7 cells with a miniprep purified plasmid carrying the murine LPCAT-2 genes but not the JetPrime, although its efficiency is better than the efficiency of JetPrime.

Therefore, Lipofectamine 2000 transfection reagent was selected for use for all the future experiments. To confirm the LPCAT-2 expression, the protein samples were checked by western blot using anti-Flag antibody as shown in Figure 3.4.

western blot results show protein expression bands of LPCAT-2 which shows two bands at a molecular weight of 56 and 35 KD in the transfected RAW264.7 using mouse Anti-flag antibody. The band at 35KD is suggested to be another isoform of LPCAT-2.

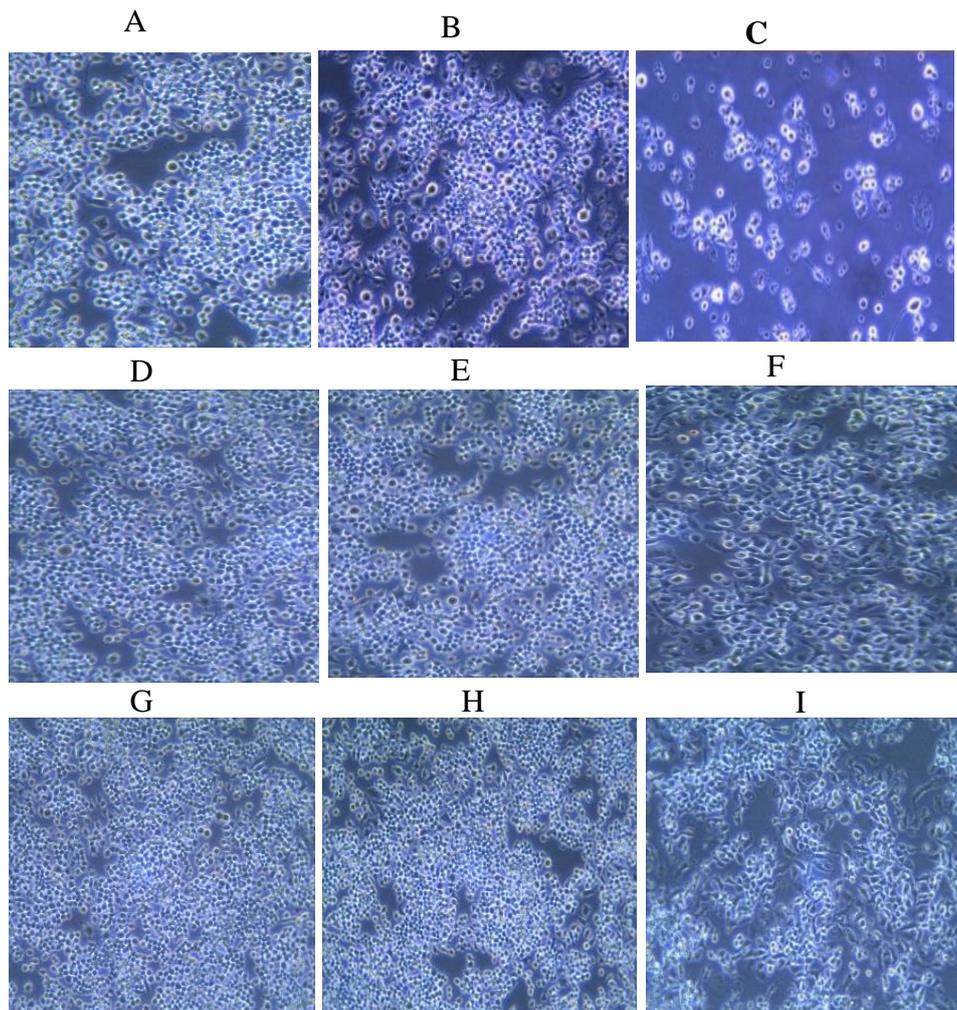


Figure 3.3. Transfection of RAW264.7 cells using JetPrime and Lipofectamine 2000 transfection reagents after 48 hours (Using inverted microscope, X10).

Cell proliferation is reduced with the type of transfection reagents and their concentrations related to the starting concentration of RAW264.7 cells (2×10^5) and (1×10^6) cells/ 2.5 mL complete DMEM medium for JetPrime and Lipofectamine 2000 transfection respectively as below:

(A) RAW264.7 cells alone as a control without Jet Prime transfection reagent after 48 hours. (B) RAW264.7 cells with 6 μ L of Jet Prime after 48 hours. (C) Transfected RAW264.7 cells with 2 μ g of purified plasmid carrying murine LPCAT-2 insert and 6 μ L JetPrime showing a reduction in cell number after 48 hours. (D) **Control**, non-transfected RAW264.7 cells without Lipofecamine2000 (E) RAW264.7 cells with 6 μ L of Lipofecamine2000 transfection reagent after 48 hours. (F) Transfected RAW264.7 cells with 2.5 μ g of a purified plasmid carrying murine LPCAT-2 insert and 6 μ L Lipofectamine 2000 transfection reagent after 48 hours. (G) Control, non-transfected

RAW264.7 cells without Lipofecamine2000 transfection reagent after 48 hours. **(H)** RAW264.7 cells with 9 μ L of Lipofecamine2000 after 48 hours. **(I)** Transfected RAW264.7 cells with 2.5 μ g of a purified plasmid carrying murine LPCAT-2. recombinant gene and 9 μ L Lipofectamine 2000 after 48 hours.

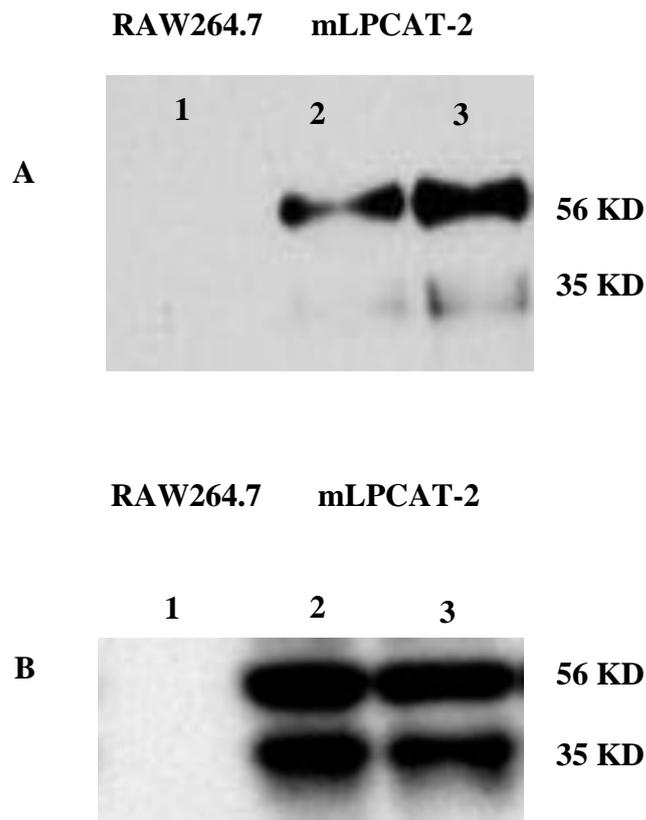


Figure: 3.4. Western blotting confirmation of murine LPCAT-2 expression in RAW264.7 cells using JetPrime.

(A) or Lipofectamine 2000 **(B)** transfection reagents, detected using Anti-Flag Ab. Lane 1 – control untransfected RAW 264.7 cells, Lane 2 – RAW cells transfected with 6 μ L of transfection reagent, Lane 3 – RAW cells transfected with 9 μ L transfection reagent.

3.3. Expression of murine LPCAT-2 in transiently transfected RAW264.7 murine macrophages.

A transient transfection is an important step to ensure inserting a single LPCAT-2 isoform; through a transfection process using the MiniPrep purified pCMV6 - entry vector (4.919 kb) plasmid carrying the recombinant murine LPCAT-2 into mammalian cells. RAW264.7 cells were used as a model of murine macrophages to investigate the role of the LPCAT-2 enzyme in the immune response in these cells.

Murine LPCAT-2 gene was transiently transfected into RAW264.7 cells using Lipofectamine 2000 transfection reagent and the MiniPrep purified pCMV6 - entry vector (4.919 kb) plasmid carrying the recombinant murine LPCAT-2 gene. The plasmid was previously amplified by transformation of competent bacteria (*E. coli*). The LPCAT-2 overexpression was confirmed using RT-PCR (see Figures 3.4 and 3.5). The mean values in fold increase is shown in table 2.

The achievement of satisfactory gene over-expression (as shown by the fold increase) relative to Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression was important as this would determine the reliability of subsequent results.

First, transiently transfected RAW264.7 cells (LPCAT-2⁺) were plated in 6 well plates at a cell concentration of 5×10^5 cells/ well in 2.5 ml of complete DMEM. Transiently transfected cells showed a higher expression of LPCAT-2 (6.46 -fold increase; $P < 0.03$) compared to control RAW264.7 cells with empty vector. This is shown in Figure 3.5 below. RAW264.7 cells that transiently over-expressed the murine LPCAT-2 will be termed 'LPCAT-2⁺', RAW264.7 cells with empty vector will be termed 'pCMV6', Increased LPCAT-2 protein expression was confirmed by Western blotting using specific Anti-Flag antibody (Figure 3.5).

LPCAT-2 gene expression in RAW264.7.

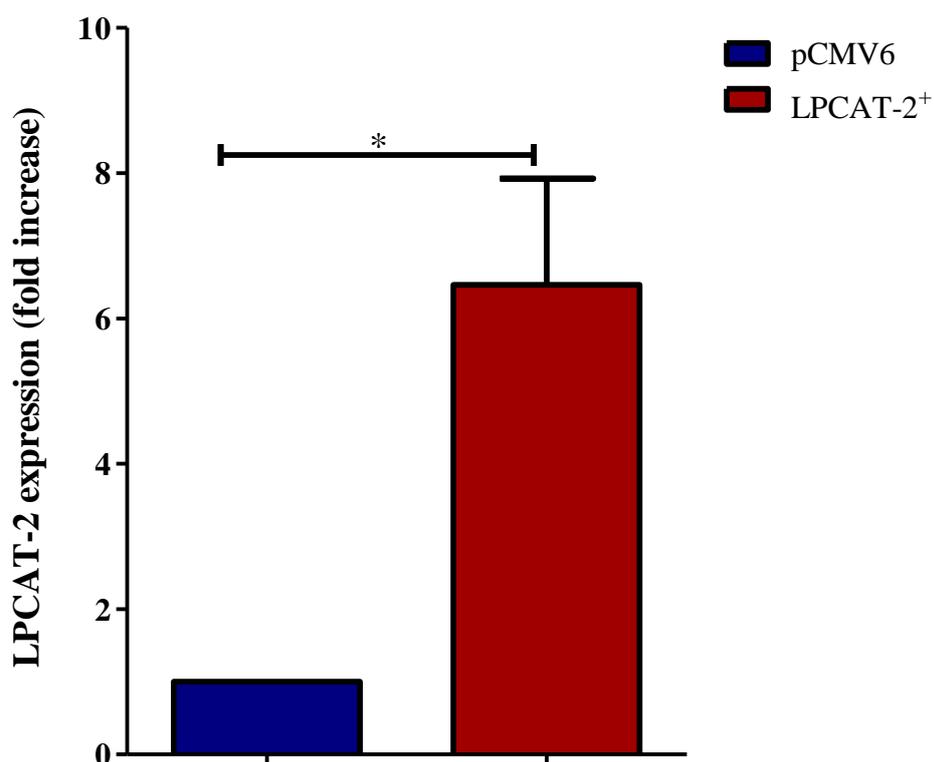


Figure 3.5. Confirmation of Murine LPCAT-2 gene over expression in RAW264.7 cells by RT-PCR.

This shows a significant over-expression of LPCAT-2 in the LPCAT-2 transfected cells (LPCAT-2⁺) (Red Bar) after 24 hours incubation at 37 °C compared to cells transfected with empty vector (pCMV6) (Blue Bar) (Fold increase = 6.46; * $P = 0.0264$; $n = 4$), pCMV6 = RAW264.7 cells transfected with empty vector plasmid; LPCAT-2⁺ = RAW264.7 cells transfected with murine LPCAT-2 gene.

3.4. LPS-stimulated induction of LPCAT-2 in RAW264.7 cells transiently over-expressed murine LPCAT-2.

To explore the successful over-expression of the LPCAT-2 gene, further experiments were conducted to compare the gene expression of LPCAT-2 and its effects on immune responses. Stimulation of RAW264.7 cells with LPS produced a significant increase in LPCAT gene expression in cells over-expressing the LPCAT-2 gene (Figure 3.6).

RAW264.7 cells were transiently transfected by plasmid with or without murine LPCAT-2 insert using Lipofectamine 2000 transfection reagent.

These cells were subsequently stimulated with LPS for 6 hours at 37 °C. As shown in Figure 3.6, LPS treatment stimulates LPCAT-2 expression but this is significantly upregulated (P= 0.0001) in cells transfected with LPCAT-2 gene (LPCAT-2⁺) compared to pCMV6 cells. The mean values in fold increase is shown in table 2.

LPCAT-2 gene expression in RAW264.7 stimulated with LPS

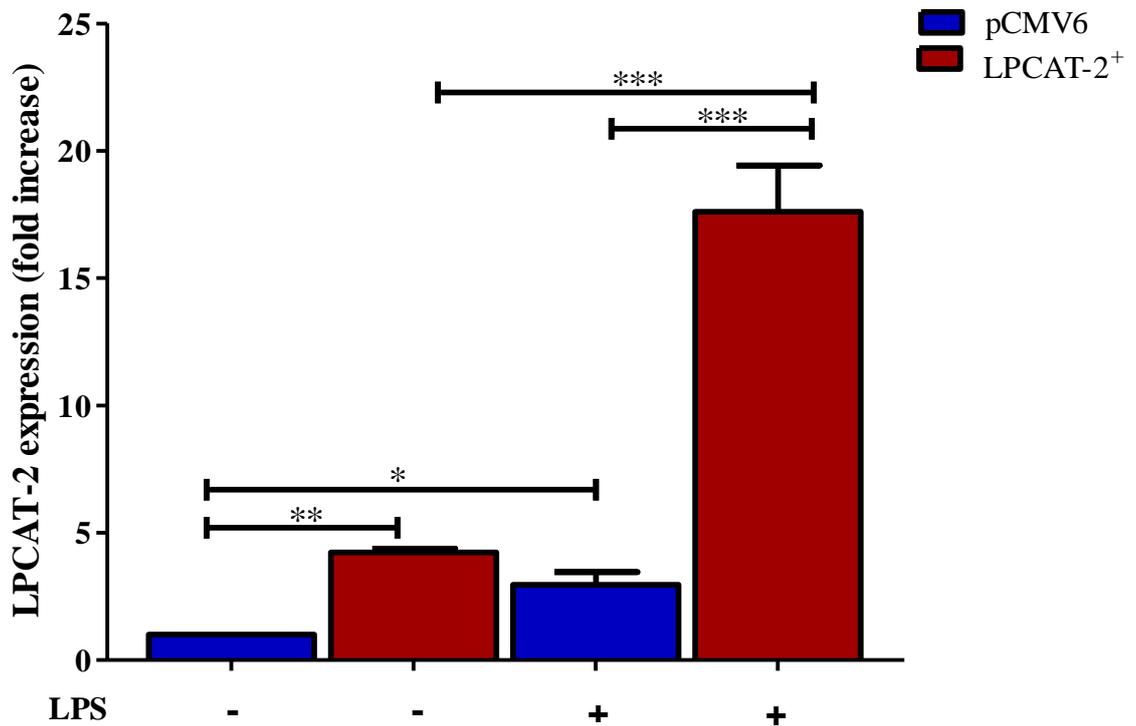


Figure 3.6. RT-PCR confirmation of LPCAT-2 gene expression in RAW264.7 cells stimulated with LPS

LPCAT-2 was significantly over-expressed in LPCAT-2⁺ cells (Red Bar) without LPS compared to pCMV6 (Blue Bar) (4.4 fold increase) and this was equivalent to the LPS-induced expression of LPCAT-2 in pCMV6 cells. However, LPCAT-2 expression was much higher when LPCAT over-expressing cells (LPCAT-2⁺) were stimulated with LPS (*E coli* lipopolysaccharide (LPS) O111:B4) (19.9 fold increase). Data represented the mean of four independent experiments (n = 4) ± standard error. * = P < 0.01, ** = P < 0.001, *** = P < 0.0001, pCMV6 = RAW264.7 cells transfected with empty vector plasmid; LPCAT-2⁺ = RAW264.7 cells transiently transfected with murine LPCAT-2 gene.

3.5. Induction of TNF- α and IL-6 gene expression in RAW264.7 cells transiently transfected with murine LPCAT-2.

Following the successful over-expression of mLPCAT-2 in RAW264.7 cells, further experiments were conducted to investigate the effects of this overexpression on innate immune responses using expression of the pro-inflammatory cytokines tumour necrosis factor (mTNF- α) and interleukin-6 (mIL-6).

When LPCAT-2⁺ and pCMV6 cells were stimulated with LPS for 6 hours at 37 °C, mTNF- α and mIL-6 mRNA were significantly up-regulated ($P = 0.0001$, $n = 4$) compared with LPS-stimulated pCMV6 cells (Figure 3.7A and B). The mean values in fold increase is shown in table 2.

TNF- α gene expression in RAW264.7 cells stimulated with LPS.

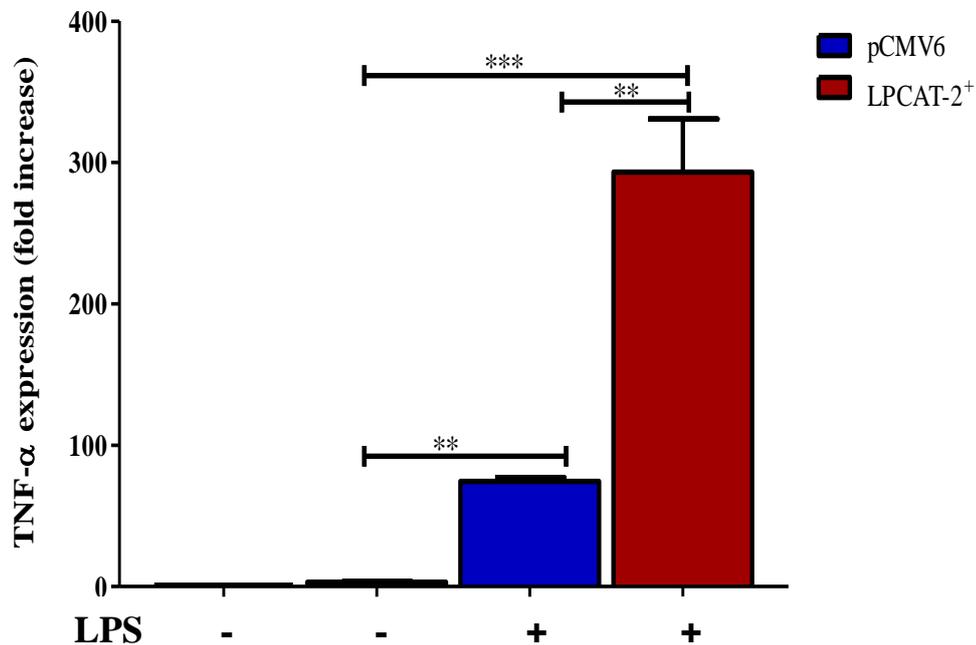


Figure 3.7A. LPCAT-2 overexpression up-regulates pro-inflammatory cytokine, TNF- α in RAW264.7 that Transiently over-expressed LPCAT-2 stimulated with lipopolysaccharide (LPS).

pCMV6 and LPCAT-2⁺ cells were stimulated with 1 μ g/ml LPS for 6 hours at 37 °C and analysed by RT-PCR normalised against the CT values of GAPDH. Overexpression of LPCAT-2 led to significantly increased gene expression of TNF-alpha compared to the pCMV6 cells. Data represented the mean of four independent experiments (n = 4) \pm standard error, ** = $P < 0.001$, *** = $P < 0.0001$, pCMV6 = RAW264.7 cells transfected with empty vector plasmid (Blue Bar), LPCAT-2⁺ = RAW264.7 cells transfected cells with murine LPCAT-2 (Red Bar).

IL- 6 gene expression in RAW264.7 cells stimulated with LPS.

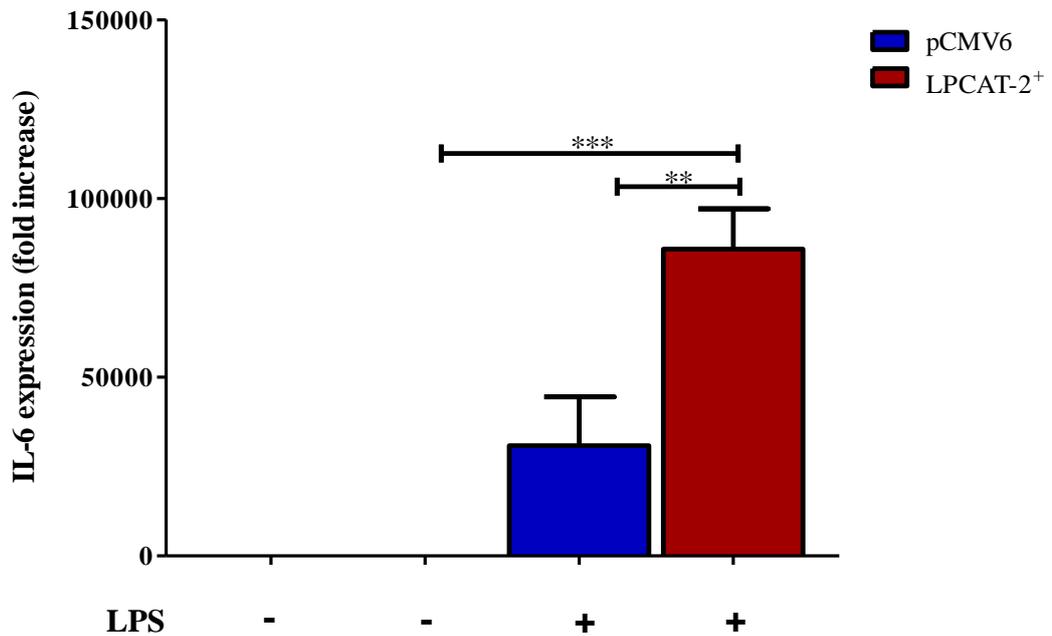


Figure 3.7B. LPCAT-2 overexpression up-regulates pro-inflammatory cytokines (IL-6) in transiently transfected RAW264.7 cells stimulated with lipopolysaccharide (LPS).

pCMV6 and LPCAT-2⁺ cells were stimulated with 1 µg/ml LPS for 6 hours at 37 °C and analysed by RT-PCR normalised against the CT values of GAPDH. Overexpression of LPCAT-2 led to significantly increased gene expression of IL-6 compared to the pCMV6 cells.

Data represented the mean of four independent experiments (n = 4) ± standard error.

* = $P < 0.05$, *** = $P < 0.0001$, pCMV6 = RAW264.7 cells transfected with empty vector plasmid (Blue Bar); LPCAT-2⁺ = RAW264.7 cells transfected with murine LPCAT-2 gene (Red Bar).

3.6. Induction of IL-10 gene expression in RAW264.7 cells transiently transfected with murine LPCAT-2.

Experiments were also conducted to investigate the expression of the anti-inflammatory cytokine IL-10 in cells over expressing LPCAT-2. LPS stimulation of RAW264.7 cells transfected with an empty vector plasmid (pCMV6) produced a significant induction of IL-10 (mIL-10) gene expression (Figure 3.8). The mean values in fold increase is shown in table 2.

However, in comparison, LPS- stimulation of RAW264.7 cells transfected with the LPCAT-2 plasmid (LPCAT-2⁺) induced much less IL-10 (Figure 3.8). Interestingly, transfection of RAW264.7 cells with LPCAT-2 led to an increase in IL-10 gene induction in unstimulated cells (Figure 3.8).

IL-10 gene expression in RAW264.7 cells stimulated with LPS.

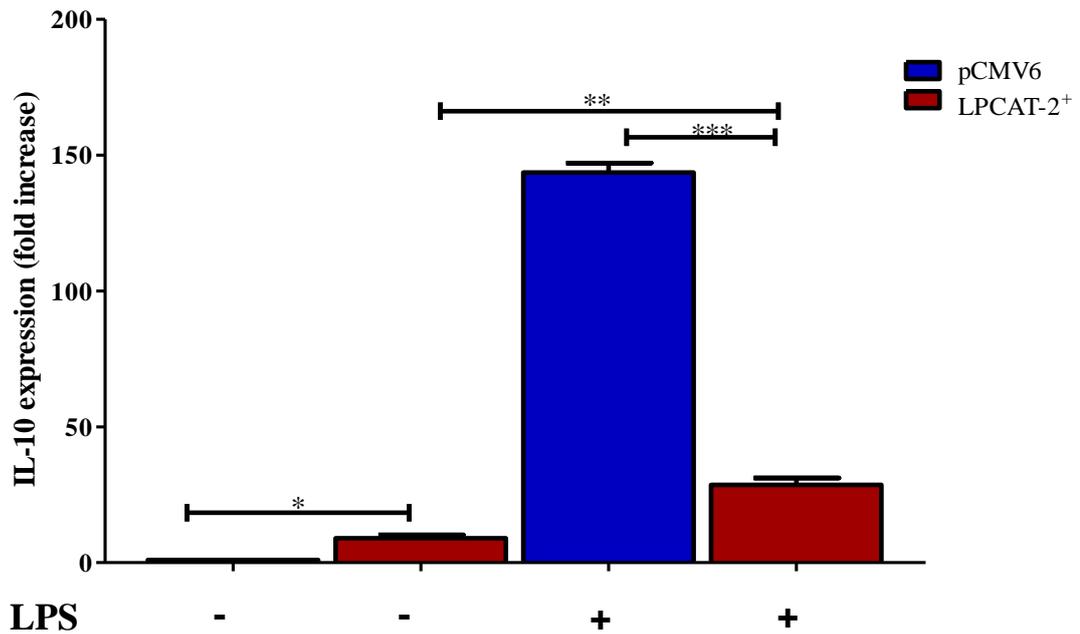


Figure 3.8. LPCAT-2 over-expression down-regulates IL-10 in LPS-stimulated RAW264.7 cells.

RT-PCR analyses showing LPCAT-2 significantly blunts IL-10 mRNA expression in LPCAT-2⁺ cells (Red Bar) compared to pCMV6 cells (Blue Bar) ($P < 0.0001$). In the absence of LPS stimulation LPCAT-2⁺ cells showed a higher induction of IL-10 mRNA compared to the pCMV6 cells. ($P < 0.0003$). Data represented the mean of four independent experiments ($n = 4$) \pm standard error. * = $P < 0.01$, ** = $P < 0.001$, *** = $P < 0.0001$, pCMV6 = RAW264.7 cells transfected with empty vector plasmid; LPCAT-2⁺ = RAW264.7 cells transfected with murine LPCAT-2 gene.

3.7. Enzyme-Linked Immunosorbent Assay (ELISA) for TNF-alpha and IL-6 protein in RAW264.7 cells stimulated with LPS.

To confirm the effects of LPCAT over expression on macrophage responses, cytokine protein expression was investigated by ELISA. RAW264.7 cells transfected with LPCAT-2 (LPCAT-2⁺) and RAW264.7 cells transfected with empty vector (pCMV6) were treated with LPS and supernatants were collected after 24 hours and analysed for TNF-alpha and IL-6 protein concentration by ELISA (Figure 3.9 A and B). The mean values in pg/ml is shown in table 2.

The cytokine protein concentration values were normalised against total RNA concentrations (Muratore *et al.*, 2014) (see section 2.11, materials and methods). Normalisation against the RNA concentrations was done because this provided a suitable yardstick against which the protein concentrations can be measured.

The toxic effect of the combination of the plasmid with the LPCAT-2 recombinant protein and the Lipofectamine transfection reagent reduces the number of viable cells in the transfected RAW264.7 cells.

The results (Figure 3.9) show that there is significant upregulation of TNF- α and IL-6 protein production ($P = 0.0001$) in LPCAT-2⁺ cells treated with LPS.

Enzyme-linked immunosorbent assay (ELISA) for TNF- α in RAW264.7 stimulated with LPS normalised against total RNA concentration

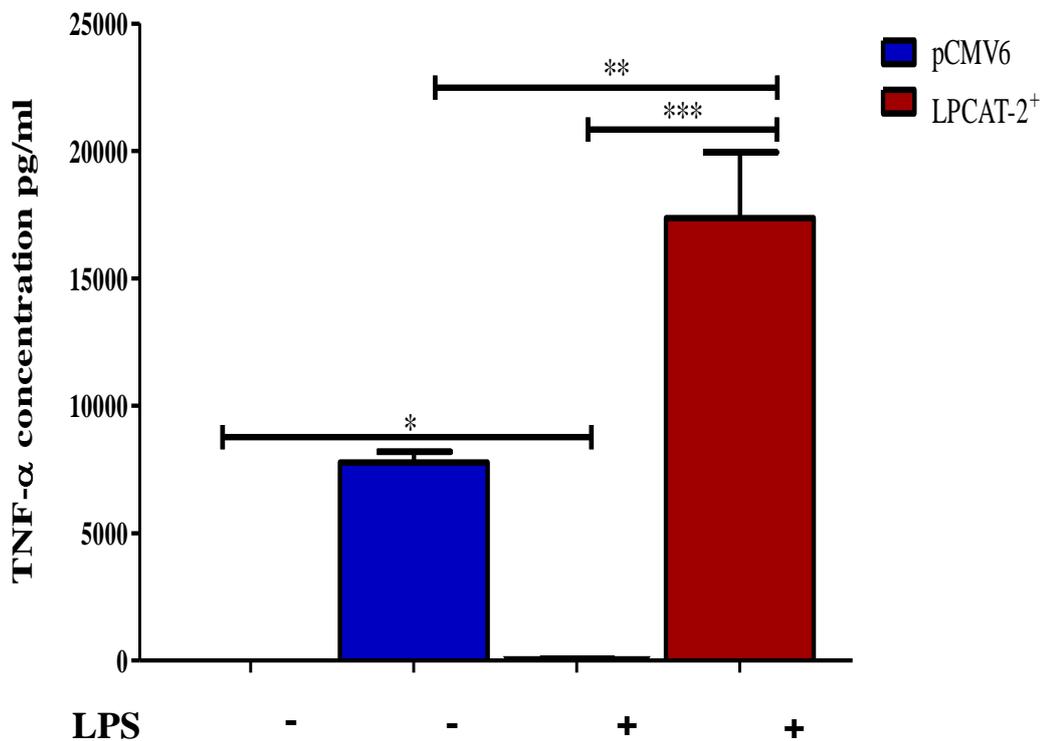


Figure 3.9A. Enzyme-Linked Immunosorbent Assay (ELISA) for TNF- α in in RAW264.7 cells stimulated with LPS

LPCAT-2 over-expression up-regulates pro-inflammatory cytokine, TNF- α in cells stimulated with LPS. TNF- α protein concentrations were calculated after normalisation of the ELISA values against the total RNA concentrations (please see 2.11, material and methods). Results are presented as Mean \pm Standard error (SE) from four independent experiments (n = 4), * = $P < 0.01$, ** = $P < 0.001$, *** = $P < 0.0001$, pCMV6 = RAW264.7 cells transfected with empty vector plasmid (Blue Bar); LPCAT-2⁺ = RAW264.7 cells transfected with murine LPCAT-2 gene (Red Bar).

Enzyme-linked immunosorbent assay (ELISA) for IL-6 in RAW264.7 stimulated with LPS normalised against total RNA concentration

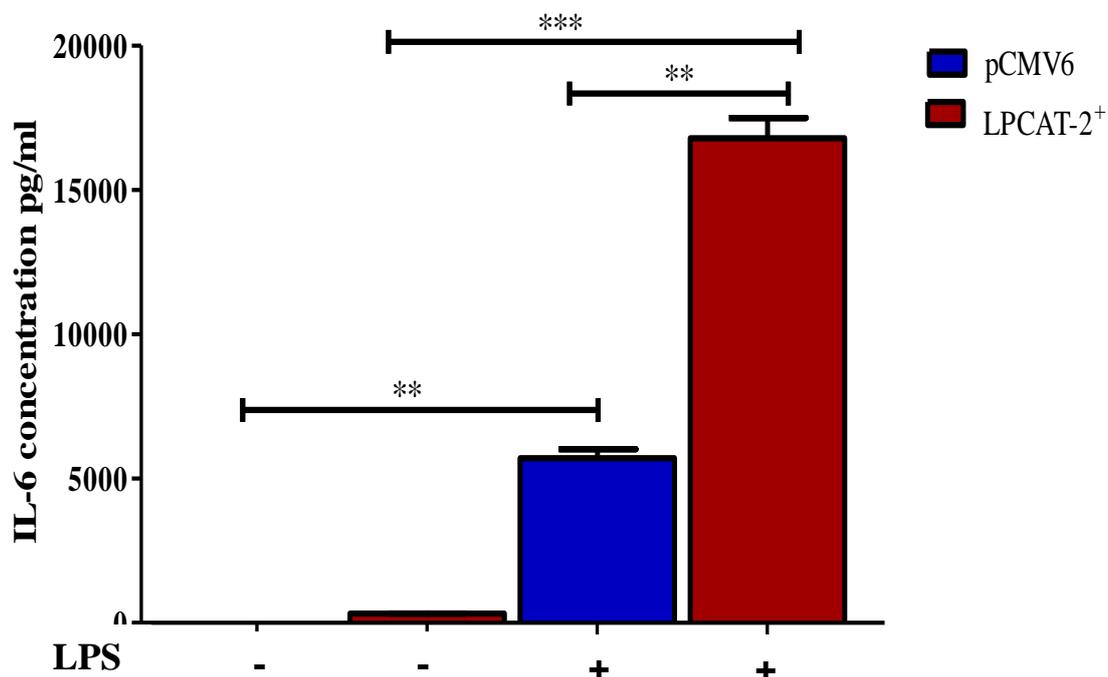


Figure 3.9B. Enzyme-Linked Immunosorbent Assay (ELISA) for IL-6 in in RAW264.7 cells stimulated with LPS.

LPCAT-2 over-expression up-regulates IL-6 cytokines in cells stimulated with LPS. IL-6 protein concentrations were calculated after normalisation of the ELISA values against the total RNA concentrations (please see 2.11, material and methods). Results are presented as Mean \pm Standard error (SE) from four independent experiments ($n = 4$), ** = $P < 0.001$, *** = $P < 0.0001$, pCMV6 = RAW264.7 cells transfected with empty vector plasmid (Blue Bar), LPCAT-2⁺ = transiently transfected RAW264.7 cells with murine LPCAT-2 gene (Red Bar).

3.8. TLR2 ligand-stimulated induction of LPCAT-2 in RAW264.7 cells transiently transfected with LPCAT-2.

Previous results (Figures 3.7 - 3.9) showed that LPCAT-2 over expression in RAW264.7 cells significantly up-regulates inflammatory cell responses to LPS. TLR4 is the macrophage cell membrane surface receptor for LPS. However, it is important to find out if LPCAT may also affect responses to other TLR ligands as this will help to elucidate mechanistic approaches to the regulation of the inflammatory response.

The effect of Pam3CSK4 stimulation on LPCAT-2 gene expression is shown in Figure 3.10, The mean values as a fold increase is shown in table 2.

It can be seen that transfection with LPCAT-2, as expected, results in increased LPCAT-2 expression but this expression is significantly further increased when cells are stimulated with Pam3CSK4 for 6 hours at 37 °C.

LPCAT-2 gene expression in RAW264.7 cells stimulated with Pam3CSK4 .

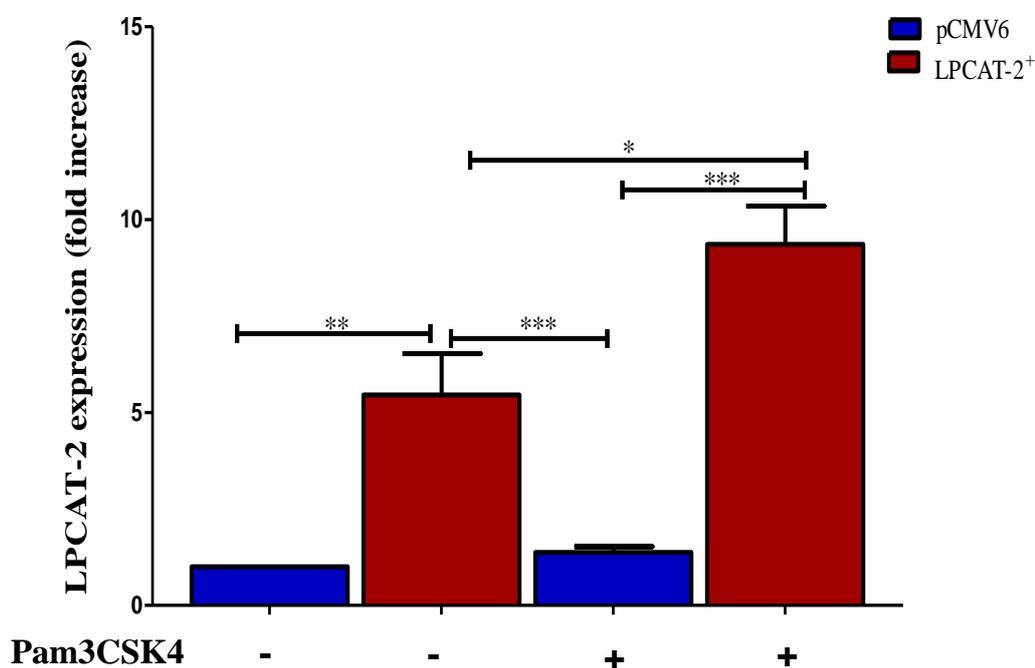


Figure 3.10. Over-expression of murine LPCAT-2 in untreated and Pam3CSK4-stimulated murine macrophages using RT-PCR.

This shows a significant over-expression of LPCAT-2 in the LPCAT-2⁺ (Red Bar) after 24 hours' incubation at 37 °C compared to pCMV6 (Blue Bar) (Fold increase = 5.5; * $P = 0.0001$; $n = 4$), *** = $P < 0.0001$; Results are presented the mean \pm Standard error (SE) from four independent experiments ($n = 4$), * = $P < 0.01$, ** = $P < 0.001$, *** = $P < 0.0001$. pCMV6 = RAW264.7 cells transfected with empty vector plasmid; LPCAT-2⁺ cells = RAW264.7 cells transfected with murine LPCAT-2 gene.

3.9. Expression of TNF- α and IL-6 in transiently transfected and non-transfected RAW264.7 cells stimulated with Pam3CSK4.

Experiments were conducted to study if the LPCAT-2 gene over-expression would affect inflammatory cell responses to the TLR2 ligand Pam3CSK4. This was done by measuring gene expression of the pro-inflammatory cytokines TNF- α and IL-6. RAW264.7 cells were transiently transfected by plasmid with or without murine LPCAT-2 recombinant gene, using Lipofectamine 2000 transfection reagent for 48 hours at 37 °C.

Then both transfected and RAW264.7 cells with empty vector plasmid (pCMV6), were stimulated with Pam3CSK4 for 6 hours at 37 °C. As can be seen in Figure 3.11, overexpression of LPCAT-2 significantly upregulated TNF- α and IL-6 gene expression ($P < 0.0002$ and < 0.0001 respectively).

Interestingly, as can be seen in Figure 3.11, The mean values as a fold increase is shown in table 2. LPCAT-2⁺ (over-expression of LPCAT-2) cells alone, without Pam3CSK4 stimulation, induced a significant induction of TNF- α gene expression compared to pCMV6 cells (RAW264.7 transfected with empty vector plasmid). However, this effect was not so evident for the induction of IL-6.

TNF- α gene expression in RAW264.7 cells stimulated with Pam3CSK4.

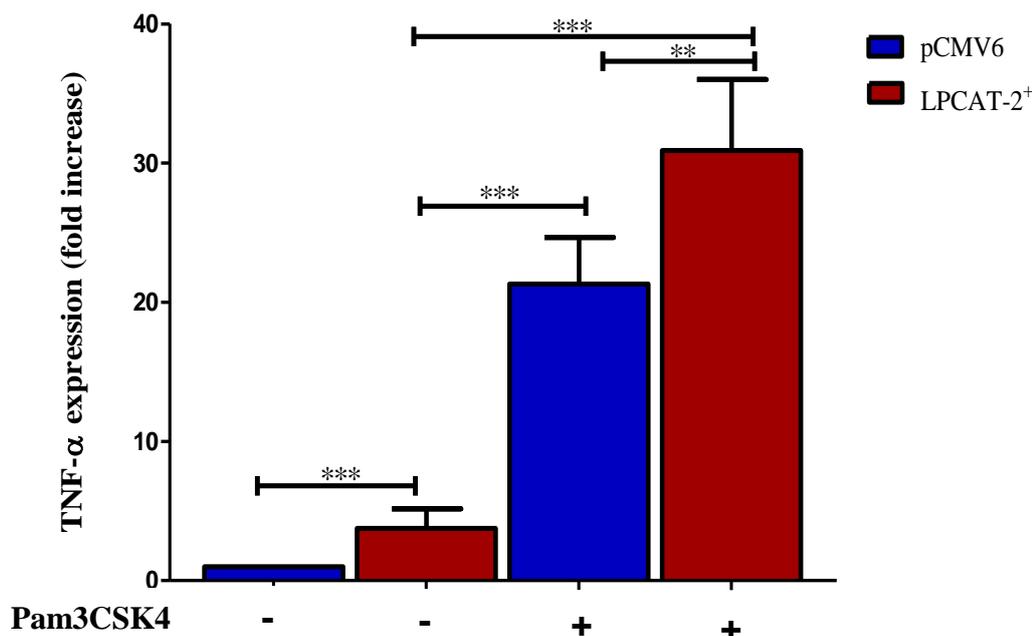


Figure 3.11A. LPCAT-2 overexpression up-regulates pro-inflammatory cytokine, TNF- α in RAW264.7 cells stimulated with Pam3CSK4 using RT-PCR analysis system.

pCMV6 and LPCAT-2⁺ cells were stimulated with 0.5 μ g/ml Pam3CSK4 for 6 hours at 37 °C and analysed by RT-PCR normalised, against the CT values of GAPDH. Overexpression of LPCAT-2 led to significantly increased gene expression of TNF-alpha (3.75 fold increases) in untreated pCMV6 cells and LPCAT-2⁺ respectively. However, it shows significantly up-regulated TNF- α in both cells, which is significantly higher in Pam3CSK4 stimulated LPCAT-2⁺ cells (**Red Bar**) compared to stimulated pCMV6 cells (**Blue Bar**) (21.3 vs 30.9) fold increase. Data represented the mean of four independent experiments (n = 4) \pm standard error. ** = $P < 0.001$, *** = $P < 0.0001$, pCMV6 = RAW264.7 cells transfected with empty vector plasmid; LPCAT-2⁺ cells = RAW264.7 cells transfected with murine LPCAT-2 gene.

IL-6 gene expression in RAW264.7 cells stimulated with Pam3CSK4.

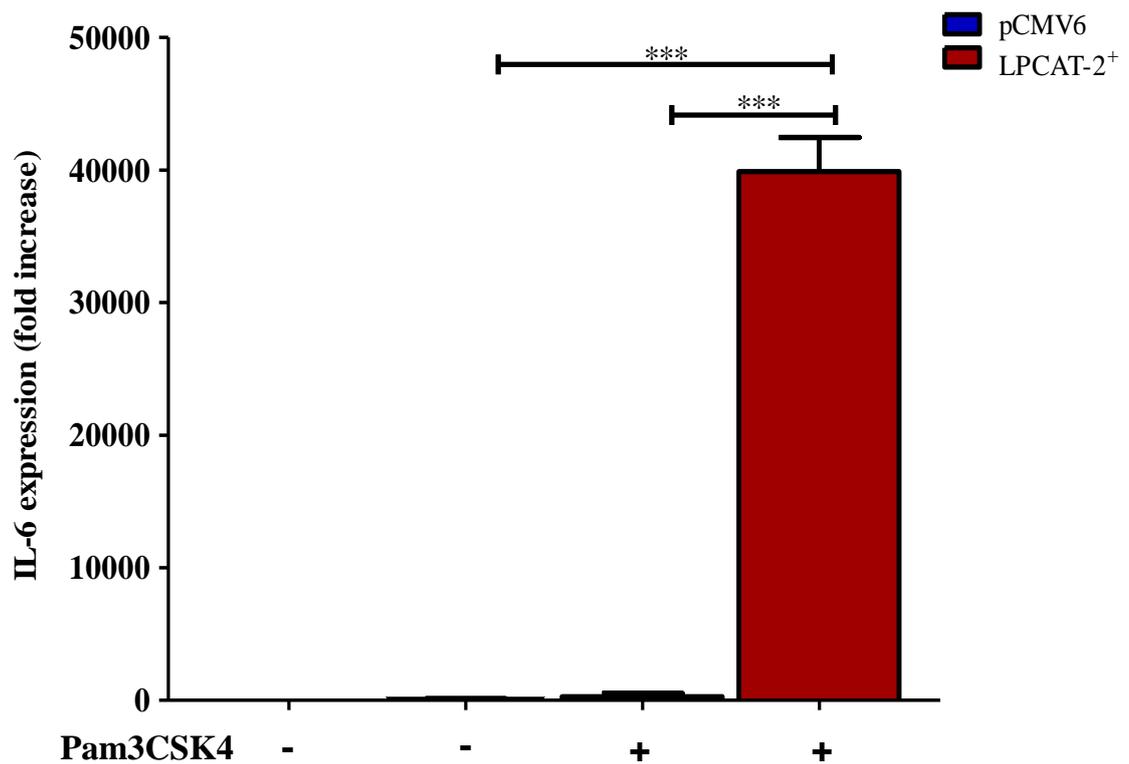


Figure 3.11B. LPCAT-2 overexpression up-regulates pro-inflammatory cytokine, IL-6 in RAW264.7 cells stimulated with Pam3CSK4 using RT-PCR analysis system.

pCMV6 and LPCAT-2⁺ cells were stimulated with 0.5 μ g/ml Pam3CSK4 for 6 hours at 37 °C and analysed by RT-PCR normalised, against the CT values of GAPDH. Overexpression of LPCAT-2 led to significantly increased gene expression of TNF-alpha (1 fold increases) and (45.9) in untreated pCMV6 cells and LPCAT-2⁺ respectively. However, it shows significantly up-regulated IL-6 in both cells, which is significantly higher in Pam3CSK4 stimulated LPCAT-2⁺ cells (**Red Bar**) compared to stimulated pCMV6 cells (**Blue Bar**) (114.9 vs 40228.9) fold increase. Data represents The mean of four independent experiments (n = 4) \pm standard error., *** = $P < 0.0001$. pCMV6 = RAW264.7 cells transfected with empty vector plasmid; LPCAT-2⁺ cells = RAW264.7 cells transfected with empty murine LPCAT-2 gene.

3.10. IL-10 expression in transiently transfected and non-transfected RAW264.7 cells with Pam3CSK4.

Experiments were also conducted to compare the induction of the anti-inflammatory cytokine IL-10, between transient RAW264.7, with or without, LPCAT-2 recombinant protein on plasmid vector, in four independent experiments, that the overexpression of LPCAT-2 gene has already checked and confirmed using RT-PCR analysis. The results show that RAW264.7, with or without Pam3CSK4 stimulation, is inducing IL-10 (45.9 vs 14.4) and (1 vs 0.8) fold increase respectively (pCMV6 vs LPCAT-2⁺). The results of IL-10 induction are shown in Figure 3.12, The mean values as a fold increase is shown in table 2.

IL- 10 gene expression in RAW264.7 cells stimulated with Pam3CSK4.

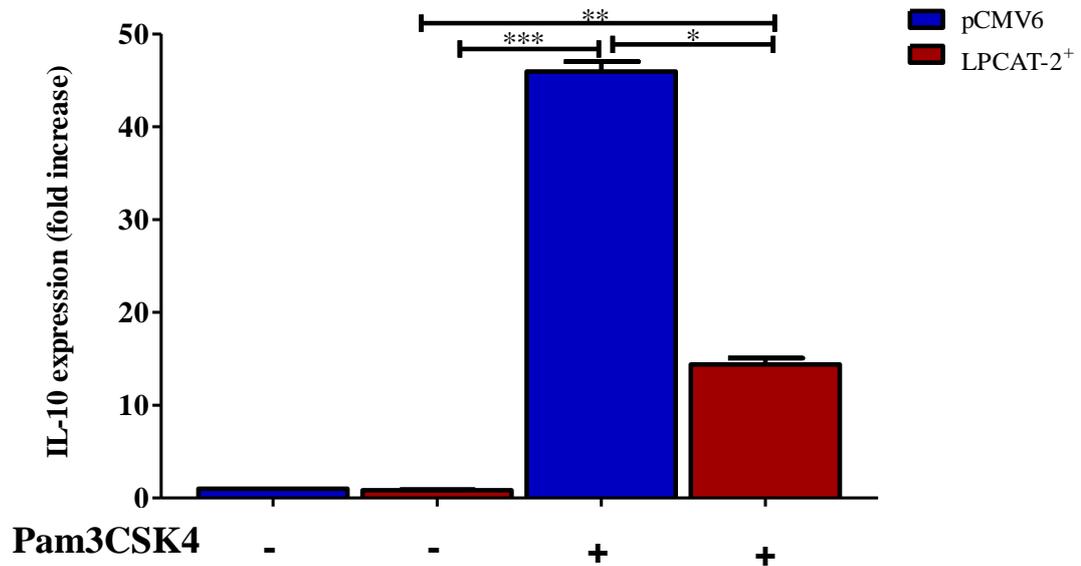


Figure 3.12. IL-10 gene expression in RAW264.7 cells stimulated with Pam3CSK4 using RT-PCR analysis.

RT-PCR analysis shows LPCAT-2 significantly reduces IL-10 mRNA expression in LPCAT-2⁺ cells (0.8 fold increase) (Red Bar) compared to pCMV6 cells (Blue Bar). ($P = 0.0001$). In the Pam3CSK4 stimulation for both cells; pCMV6 cells showed a significant higher induction of mL-10 mRNA compared to the LPCAT-2⁺ cells (45.9 vs 14.4, $P = 0.027$). There is significant induction of IL-10 gene expression in both LPCAT-2⁺ and pCMV6, with significant differences, and much higher IL-10 induction in Pam3CSK4 induced pCMV6 cells. LPCAT-2 overexpression significantly down-regulated IL-10 in LPCAT-2⁺ cells compared to pCMV6 cells which induced significantly higher IL-10 by Pam3CSK4 stimulation. Data represented the mean of four independent experiments ($n = 4$) \pm standard error. * = $P < 0.03$, ** = $P < 0.001$, *** = $P < 0.0001$. pCMV6 = RAW264.7 cells transfected with empty vector plasmid; LPCAT-2⁺ cells = RAW264.7 cells transfected with empty murine LPCAT-2 gene.

3.11. Investigation the effect of LPCAT-2 silencing in the inflammatory response in LPS- induced RAW264.7 cells.

Having established that over-expression of LPCAT-2 in macrophages increases the inflammatory response to TLR stimulation, we wanted to determine if the opposite was also true i.e. if inhibiting LPCAT-2 expression would decrease the inflammatory responses of the macrophages. We hypothesised therefore, that if LPCAT-2 were relevant in the inflammatory response of macrophages, successfully silencing the gene encoding this enzyme would result in a significant decrease in the expression of inflammatory cytokines such as TNF- α and IL-6. To inhibit LPCAT-2 expression, the technique of small interfering RNA (siRNA) was used (Hamilton & Baulcombe, 1999) (see materials and methods section 2.2.1.6. LPCAT-2 gene silencing).

3.12. LPCAT-2 gene silencing in RAW264.7 stimulated with LPS.

To establish the involvement of LPCAT-2 in the inflammatory responses of macrophages, the over-expressed LPCAT-2 gene was silenced and cell responses to subsequent stimulation by LPS was investigated. The success of a satisfactory gene silencing efficiency is vital, as this will determine the reliability of subsequent findings.

In this project, approximately 88 % LPCAT-2 gene silencing was achieved, the mean values as a fold increase is shown in table 2. This is shown in Figure 3.13. RAW264.7 over-expressing LPCAT-2 gene is termed 'LPCAT-2⁺'.

The effect of silencing overexpressed LPCAT-2 on LPCAT-2 gene expression in RAW264.7 cells stimulated with LPS.

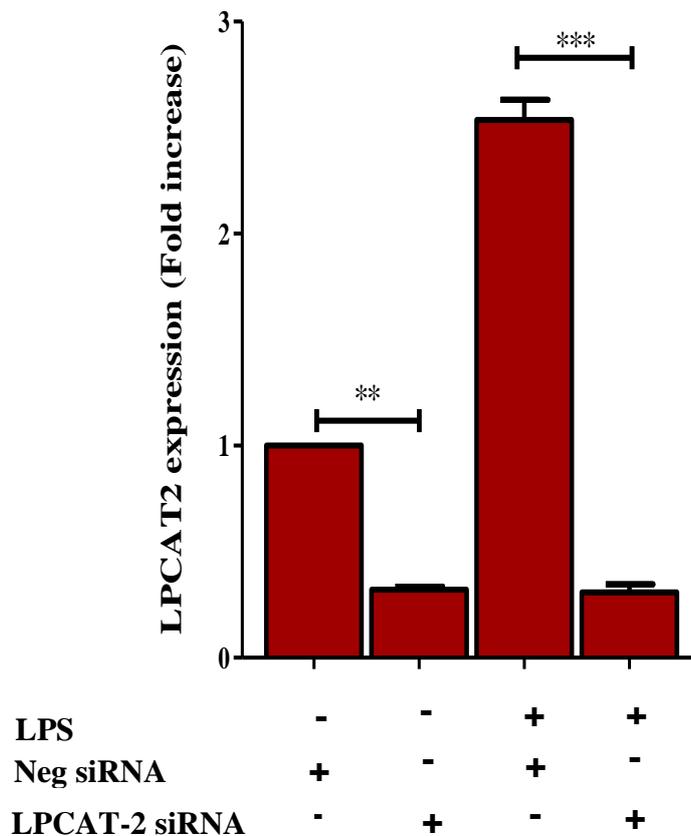


Figure 3.13. LPCAT-2 expression in RAW 264.7 cells transiently over-expressing LPCAT-2 and treated with siRNA to knockdown LPCAT-2 gene expression in the absence and presence of LPS stimulation.

The data was normalised against the CT values of GAPDH using RT-PCR analysis. RAW264.7 cells over-expressing the LPCAT-2 gene were treated with siRNA to knock-down LPCAT-2 gene expression. LPCAT-2 was down-regulated (silenced) by a mean factor of (70 - 80 %). siRNA LPCAT-2 gene silencing knocked down LPCAT-2 expression by 70% in the absence of LPS and by 88 % in LPS-stimulated cells.

** = $P < 0.01$, *** = $P < 0.0001$, Data represented as Mean \pm SE, from three independent experiments (n = 3). LPCAT-2⁺ = RAW264.7 transiently overexpressing LPCAT-2 gene.

3.13. Expression of TNF- α and IL-10 in silencing transfected RAW264.7 cells with LPCAT-2.

After the successful silencing of the over-expressed LPCAT-2 gene, further experiments were conducted to compare the gene expression of the pro-inflammatory cytokine TNF- α , and the anti-inflammatory cytokine IL-10, between samples containing the negative siRNA and LPS, LPCAT-2 siRNA and LPS (Silenced over-expressed LPCAT-2 genes).

The findings of the comparison are illustrated in figure 3.14. There is an increase in IL-10 when the RAW264.7 cells with the silenced LPCAT-2 are stimulated with LPS. See figure 3.14(A & B), the mean values as a fold increase is shown in table 2.

The effect of silencing overexpressed LPCAT-2 on TNF- α gene expression in RAW264.7 stimulated with LPS.

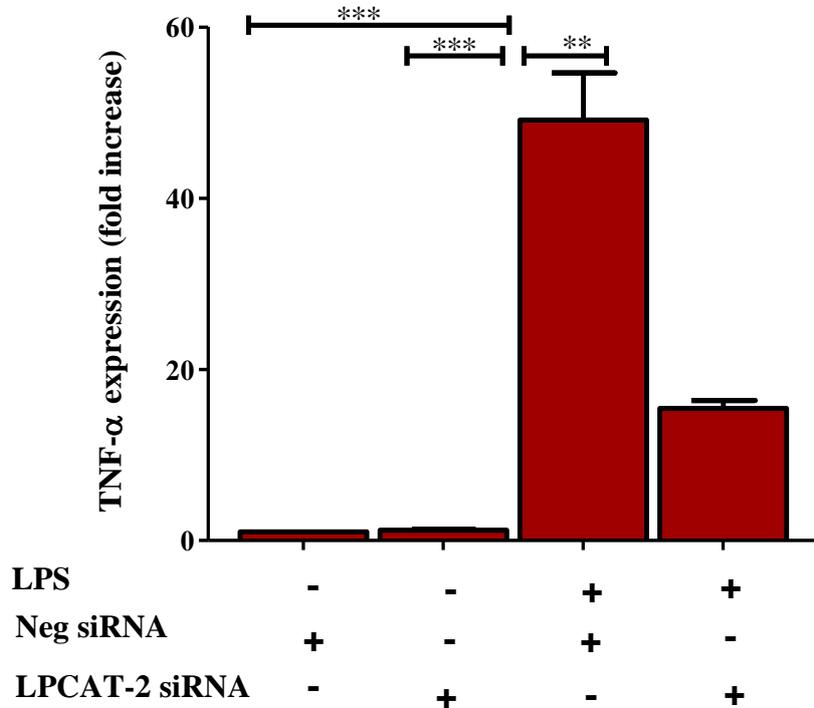


Figure 3.14A. The effect of silencing LPCAT-2 on TNF- α expression in stimulated and non-stimulated RAW264.7 cells.

The data was normalised against the CT values of GAPDH using RT-PCR analysis. The silencing LPCAT-2 was significantly down-regulated TNF- α in the silencing over-expressed LPCAT-2 is decreasing the TNF- α 1 vs (1.2; LPCAT-2⁺ with negative siRNA), and, it when the cells are stimulated with LPS, the TNF- α gene expression is significantly reduced due to the effect of silencing LPCAT-2 with fold increased, 15.4 vs (49.2; LPCAT-2⁺ with negative SiRNA + LPS) ($P = 0.0001$, $n = 3$).

** = $P < 0.01$, *** = $P < 0.0001$, Data represented as Mean \pm SE, from three independent experiments ($n = 3$). LPCAT-2⁺ = RAW264.7 transiently overexpressing LPCAT-2 gene.

The effect of silencing overexpressed LPCAT-2 on IL-10 gene expression in RAW264.7.

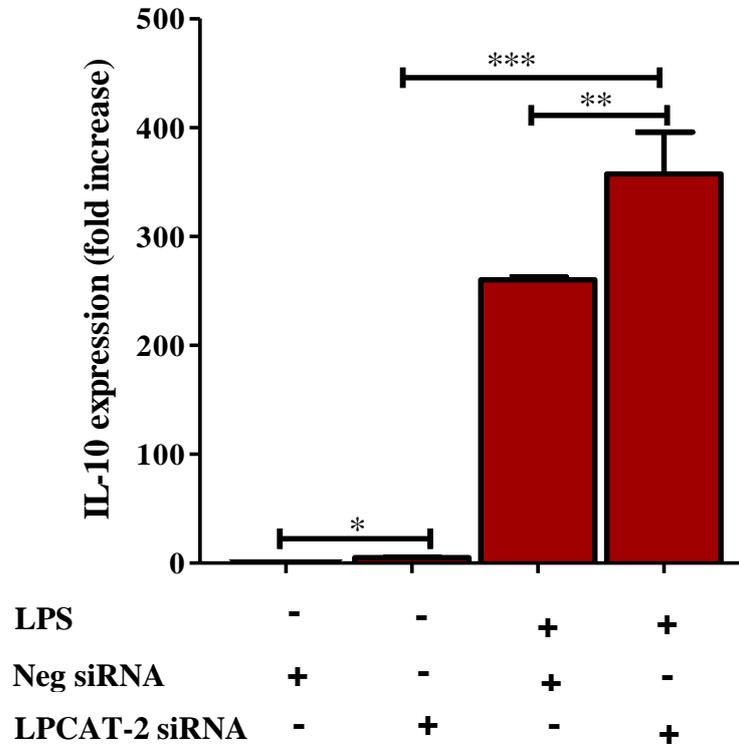


Figure 3.14B. The effect of silencing LPCAT-2 on IL-10 expression in stimulated and non stimulated RAW264.7 cells.

The data was normalised against the CT values of GAPDH using RT-PCR analysis. The silencing over-expressed LPCAT-2 was significantly up-regulated the inflammatory cytokine (IL-10) in the non-stimulated silencing; LPCAT-2⁺ cells as a fold increase, 1 vs 4.9; LPCAT-2⁺ with negative siRNA and it is increased more when the both cells were stimulated with LPS, but higher in the silencing overexpressing LPCAT-2 in a fold increase; 357.3 vs (260.2; LPCAT-2⁺ cells with negative siRNA, ($P= 0.0001$, $n = 3$).

* = $P < 0.01$, *** = $P < 0.0001$, Data represented as Mean \pm SE, $n = 3$. LPCAT-2⁺ = RAW264.7 cells transiently overexpressing LPCAT-2.

Summary of results.

Table 2 summarised the results of chapter 3; successful overexpression of LPCAT-2 in murine macrophages, RAW264.7 cells using Lipo-fectamine and JetPrime transfection reagents prior LPS stimulation, showed that Lipofectamine superior to JetPrime. Therefore, this project has used 9 µl of Lipofectamine transfection reagent as a protocol for all experiments. Overexpressing cells produce greater inflammatory gene expression (TNF- α , IL-6 and IL-10) LPS or Pam3CSK4 induced RAW264.7 cells, and TNF- α , IL-6 protein production in LPS induced RAW264.7 cells (Please see table 2), these results have agreed with Abate *et al.*, (2016), Alrammah *et al.*, (2016) that LPCAT-2 is inducible and a key role in regulating inflammatory responses, while (Shindou *et al.*, (2007); Morimoto *et al.*, (2010, 2014) revealed to the inducible properties of LPCAT-2.

Silencing the over-expressed LPCAT-2 in cells causes significant reduction in inflammatory gene expression (TNF- α) when stimulated with LPS. This agreed with recent results obtained by our laboratory in which LPCAT-2 silenced in MM6 cells using shRNA also caused a reduction in inflammatory cytokine production (Abate & Jackson, 2015). However this project used siRNA and RAW264.7 cell line to silence over-expressed LPCAT-2 and shows clear evidence of the role of LPCAT-2 in macrophage inflammatory responses i.e. these findings together revealed that LPCAT-2 has a vital role in regulating the induction of proinflammatory cytokines (Mayr *et al.*, 2014; Kumar, 2014; Lozano *et al.*, 2014; Ogura *et al.*, 2014; Singer *et al.*, 2014; Henriksen *et al.*, 2015; Singer *et al.*, 2016; Jeganathan *et al.*, 2017).

The molecular mechanisms by which LPCAT-2 induces these effects should now be investigated and this is performed in Chapter 4.

Mean (fold increase vs empty vector pCMV6 control)					
A	Target	pCMV6	LPCAT-2 ⁺	pCMV6 + LPS	LPCAT-2 ⁺ +LPS
	LPCAT-2	1	6.46	-	-
	LPCAT-2	1	4.4	2.9	17.6
	TNF- α	1	3.2	74.4	259.8
	IL-6	1	6.6	30863.8	85800.2
	IL-10	1	8.9	143.6	28.7
B	LPCAT-2	1	5.5	1.4	9.4
	TNF- α	1	3.75	21.3	30.9
	IL-6	1	45.9	114.9	40228.9
	IL-10	1	0.8	45.9	14.4
Mean (pg/ml vs empty vector pCMV6 control)					
C	TNF- α	4.6	45.6	7777	17371.7
	IL-6	3.1	315.8	5705.5	16785.8
Mean (fold increase vs empty vector pCMV6 control)					
D	Target	LPCAT-2 ⁺ + Neg siRNA	LPCAT-2 ⁺ + LPCAT-2 siRNA	LPCAT-2 ⁺ + Neg siRNA + LPS	LPCAT-2 ⁺ + LPCAT-2 siRNA +LPS
	LPCAT-2	1	0.3	2.54	0.31
	TNF- α	1	1.2	49.2	15.4
	IL-10	1	4.9	260.3	357.3

Table 2. The effect of over-expressed LPCAT-2 and silencing overexpressing LPCAT-2 on inflammatory responses in LPS or Pam3CSK4 induced murine macrophages, RAW264.7 cells.

A: Gene expression of over-expressed LPCAT-2 in LPS stimulated RAW264.7 cells (gene expression). Mean (fold increase vs empty vector pCMV6 control). **B:** The effect of over-expressed LPCAT-2 in Pam3CSK4 - stimulated RAW264.7 cells (gene expression). Mean (fold increase vs empty vector pCMV6 control). **C:** The effect of over-expressed LPCAT-2 on protein release (pg/ml) in LPS-induced RAW264.7 cells. Mean (pg/ml vs empty vector pCMV6 control).

D: The effect of silencing over-expressed LPCAT-2 in LPS- stimulated RAW264.7 cells (gene expression). Mean (fold increase vs empty vector pCMV6 control). pCMV6 = RAW264.7 + empty vector, LPCAT-2⁺ = RAW264.7 cells transiently transfected with LPCAT-2, Mean (fold increase vs empty vector pCMV6 control).

Chapter 4:
***Mechanism of action of LPCAT-2 in the
inflammatory response to bacterial ligands***

4.1. Mechanism of action of LPCAT-2 in the inflammatory response to bacterial ligands.

A fundamental reason for the investigation of LPCAT and inflammation has been the lack of specific treatments for sepsis (Angus *et al.*, 1997; Daniels, 2011; Global Sepsis Alliance, 2016). Sepsis is regarded, at least initially, as an overwhelming inflammatory response to infection (Adib-Conquy *et al.*, 2000; Adib-Conquy *et al.*, 2012; Boomer *et al.*, 2014; Global Sepsis Alliance, 2016). Triggering infections include Gram-negative and Gram-positive bacteria and their associated molecular patterns (Schorr *et al.*, 2007; Daniels, 2011; Adhikari *et al.*, 2010; Global Sepsis Alliance, 2015; Moskowitz *et al.*, 2017; Minasyan *et al.*, 2017). The excessive, sustained and uncontrolled activation of macrophages in sepsis leads to a vicious cascade of inflammatory mediator release that amplifies the inflammation to the detriment of the host (Rangel-Frausto *et al.*, 1995; Płóciennikowska *et al.*, 2015).

As part of the first line of host defence during infection the PRR are a family of receptors present on the cell membrane or in the cytosol of cell such as monocytes, macrophages and dendritic cells that recognise pathogen-associated molecular patterns (PAMPs) (Kim *et al.*, 2007; Płóciennikowska *et al.*, 2015). As a result of recognition of the PAMPs, TLRs trigger the induction of pro-inflammatory mediators helping to eradicate infection (Chen *et al.*, 2011; Płóciennikowska *et al.*, 2015).

LPS is recognised by TLR4 on the cell surface facilitated through LPS-binding protein(LBP) to form complexes, which are transferred and bind to soluble CD14 (sCD14) or membrane-bound CD14 which binds to the extracellular domain of TLR4-MD2 complexes. The CD14 and MD2 act as accessory proteins for LPS/TLR4 binding (Ulevitch & Tobias 1999; Heumann & Roger 2002; Płóciennikowska *et al.*, 2015), followed by TLR4 oligomerisation and activation of the down stream signaling cascade (Płóciennikowska *et al.*, 2015).

As the previous results (Chapter 3) showed that LPCAT-2 has a key role in macrophage inflammatory responses to LPS and other bacterial ligands, we now sought to elucidate the mechanism of action of LPCAT-2 in the inflammatory response. As TLR recognition of bacterial PAMPs is key for the signaling and responses of macrophages, this chapter explored the role of LPCAT-2 in the expression of the LPS receptors TLR4 and CD14. In addition, we investigated the effect of LPCAT-2 on the expression of TLR2, the receptor for the synthetic triacylated lipoprotein Pam3CSK4 which is a TLR1/2 ligand with similar structure to lipoprotein cell wall components found in both Gram positive and Gram negative bacteria.

To further elucidate the mechanisms of LPCAT-2 action in macrophages, the role of LPCAT-2 on lipid mediators was also studied. LPS is identified to stimulate COX-2 (Funk, 2001), and this suggested that the overexpression of LPCAT-2 would also significantly increase inducible COX-2 expression. Recent studies have confirmed that the peroxisome proliferator-activated receptors (PPARs) and in particular (PPAR- γ) have important roles in the regulation of macrophage phenotype (Karp *et al.*, 1991; Mueller *et al.*, 1998; von Knethen *et al.*, 2007; Chawla, 2010). This, coupled with the role of PPAR- γ in lipid metabolism, suggested that LPCAT-2 might have effects on PPAR- γ and this was therefore investigated in the current project.

We hypothesise that PPAR- γ upregulates fatty acid oxidation and supports an M2 macrophage phenotype whereas LPCAT-2 utilises fatty acids for phospholipid synthesis and would be associated with an M1 inflammatory phenotype of macrophage activation. This was examined by determining the expression of iNOS as a molecular marker of M1 macrophages and CD206 as a marker of M2 macrophages (Mandal *et al.*, 2011) in cells over expressing LPCAT-2 or where LPCAT-2 gene expression was silenced.

TLR4 gene expression in RAW 264.7 cells stimulated with LPS.

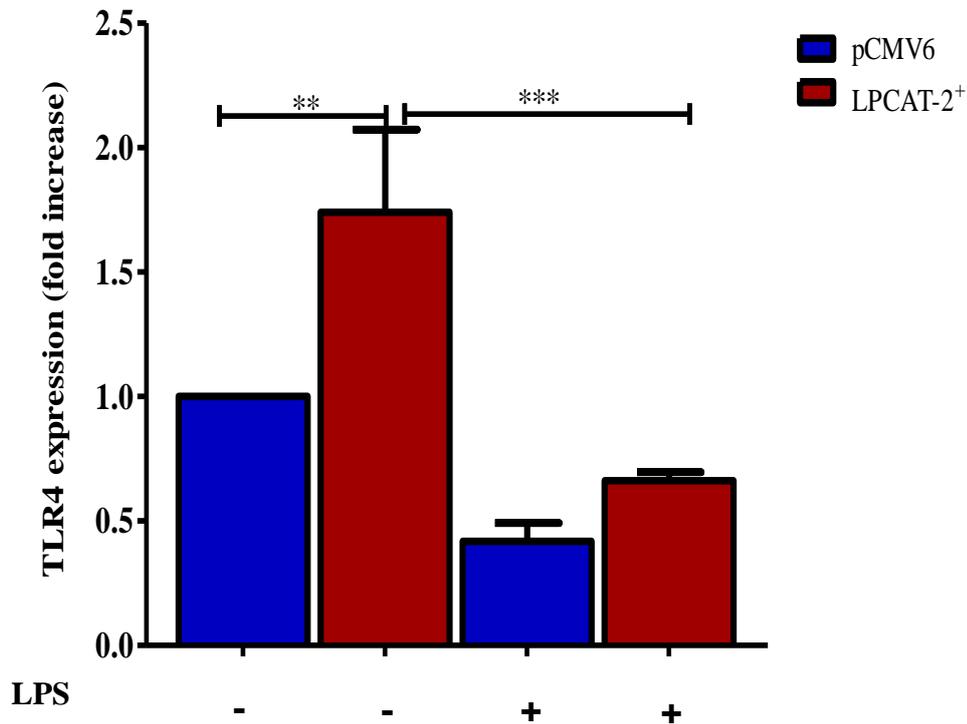


Figure 4.1A. The effect of over-expression of LPCAT-2 on the expression of TLR4, in RAW264.7 cells.

RAW264.7 cells were transfected with LPCAT-2 (LPCAT-2⁺; Red Bar) or untransfected (pCMV6; Blue Bar) and stimulated with LPS or without as controls. LPCAT-2⁺ cells show increased expression of TLR4 in unstimulated cells ($p < 0.005$ vs pCMV6). Treatment with LPS decreased TLR4 expression in both pCMV6 and LPCAT-2⁺ cells. Data represents the mean of 4 independent experiments ($n = 4$) \pm standard error, ** = $P < 0.01$, *** = $P < 0.0001$ versus pCMV6. pCMV6= RAW264.7 + empty vector plasmid, LPCAT-2⁺ = LPCAT-2 transient transfected RAW264.7 cells.

CD14 gene expression in RAW264.7 stimulated with LPS.

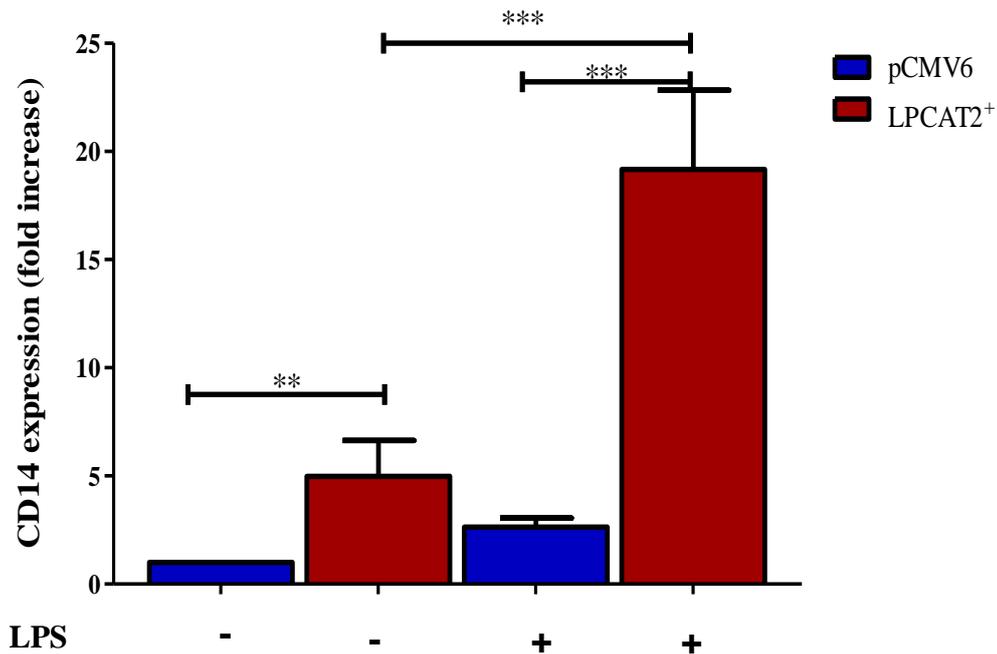


Figure 4.1B. The effect of over-expression of LPCAT-2 on the expression of CD14 in RAW264.7 cells.

RAW264.7 cells were transfected with LPCAT-2 (LPCAT-2⁺; Red Bar) or untransfected (pCMV6; Blue Bar) and stimulated with LPS or without as controls. LPCAT-2 transfection induced significant CD14 expression and this was further significantly increased after LPS treatment ($p < 0.0001$ vs pCMV6). Data represented the mean of 4 independent experiments ($n = 4$) \pm standard error, ** = $P < 0.01$, *** = $P < 0.0001$ versus pCMV6. pCMV6 = RAW264.7 + empty vector plasmid; LPCAT-2⁺ = LPCAT-2 transient transfected RAW264.7 cells.

TLR2 gene expression in RAW264.7 cells stimulated with LPS.

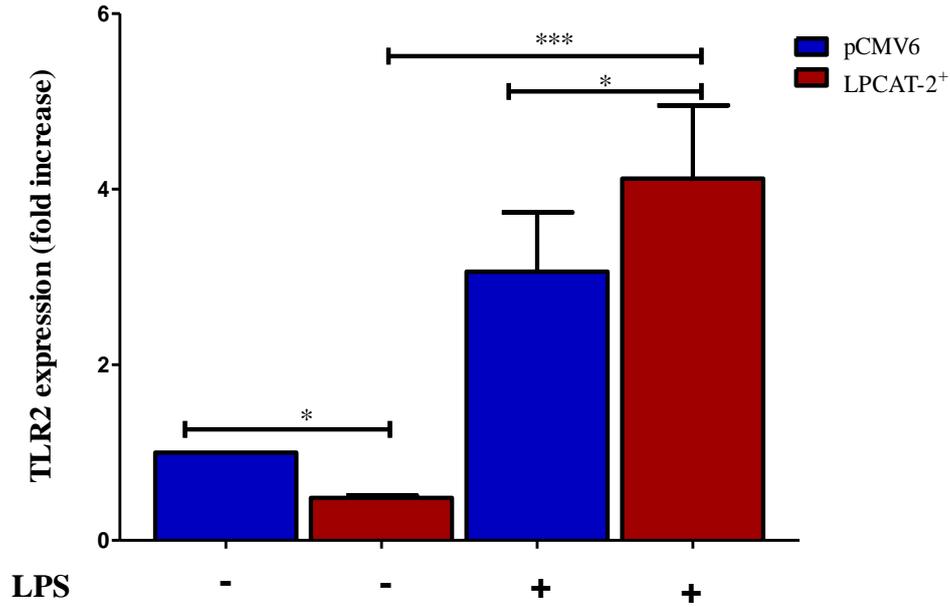


Figure 4.1C. The effect of over-expression of LPCAT-2 on the expression of TLR2 in RAW264.7 cells.

RAW264.7 cells were transfected with LPCAT-2 (LPCAT-2⁺; Red Bar) or untransfected (pCMV6; Blue Bar) and stimulated with LPS or without as controls. LPCAT-2 transfection significantly increased the expression of TLR2 in response to LPS in both unstimulated and LPS-stimulated cells. ($P=0.0052$ vs pCMV6). Data represents the mean of 4 independent experiments ($n = 4$) \pm standard error, * = $P < 0.1$, *** = $P < 0.001$ versus pCMV6. pCMV6 = RAW264.7 + empty vector plasmid; LPCAT-2⁺ = LPCAT-2 transient transfected RAW264.7 cells.

TLR2 gene expression in RAW264.7 cells stimulated with Pam3CSK4 .

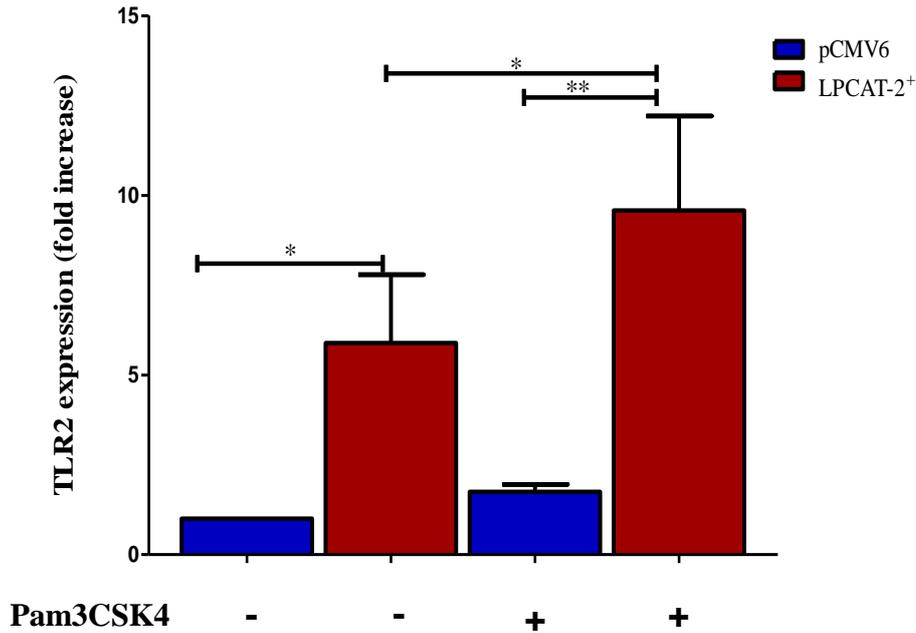


Figure 4.1D. The effect of over-expression of LPCAT-2 on the expression of TLR2 in RAW264.7 cells.

RAW264.7 cells were transfected with LPCAT-2 (LPCAT-2⁺; Red Bar) or empty vector plasmid (pCMV6; Blue Bar) and stimulated with Pam3CSK4 or without as controls. LPCAT-2 transfection significantly increased the expression of TLR2 in response to Pam3CSK4 in both unstimulated and LPS-stimulated cells. ($P=0.0062$ vs pCMV6). Data represents the mean of 3 independent experiments ($n = 4$) \pm standard error, * = $P < 0.01$, ** = $P < 0.001$ versus pCMV6. pCMV6 = RAW264.7 + empty vector plasmid; LPCAT-2⁺ = LPCAT-2 transiently transfected RAW264.7 cells.

4.2. Effect of overexpression of LPCAT-2 on cyclo-oxygenase enzymes (COX-2) and peroxisome proliferator-activated receptor–gamma (PPAR- γ) expression in RAW264.7 macrophages.

To further elucidate the mechanisms of LPCAT-2 action in macrophages, the role of LPCAT-2 on lipid mediators was also studied. LPS is identified to stimulate COX-2 (Funk, 2001), and this project is suggested that the overexpression of LPCAT-2 would also significantly increase inducible COX-2 expression.

As the previous experiments showed that LPCAT-2 can promote the expression of molecules in the TLR-mediated inflammatory pathways while suppressing PPAR- γ , a molecule associated with anti-inflammatory phenotype we next investigated if LPCAT-2 can regulate macrophage phenotypes.

Recent studies have shown that inhibition of COX-2 using Meloxicam inactivates the MAPK pathway and regulates p53 and cell apoptosis, both of which are stimulated via TLR4 signalling, which is critical for LPS-induced signalling cascades (Park *et al.*, 2016). Several studies have reported that COX-2 regulates p53 activity and inhibits p53-dependent apoptosis (Choi *et al.*, 2005; Chen *et al.*, 2009) which suggests that LPCAT-2 might have a role to play in cell cycle and apoptosis.

RAW264.7 cells with (LPCAT-2⁺) and without pCMV6 (WT) murine LPCAT-2 insert were stimulated with LPS for 6 hours and COX-2 expression was measured by RT-PCR. COX-2 was not expressed in cells without LPS treatment (Fig 4.2) confirming this is an LPS inducible gene. LPS treatment induced COX-2 expression in RAW264.7 cells and this was significantly increased ($p < 0.0001$) in LPCAT-2⁺ cells (Fig 4.2).

The opposite effect was seen with PPAR- γ expression in RAW264.7 cells (Fig 4.3). LPS treatment caused a down-regulation in PPAR- γ gene expression pCMV6 (WT) cells while LPCAT-2⁺ caused a complete reducing of PPAR- γ expression in cells with or without

LPS treatment (Fig 4.3), table 3 is shown the mean values as a fold increase vs pCMV6 cells.

Indeed, that the results of the current work suggested that LPCAT-2 has a vital role in regulation other enzymes within the macrophages cells, more studies need to be applied.

COX-2 gene expression in RAW264.7 cells stimulated with LPS.

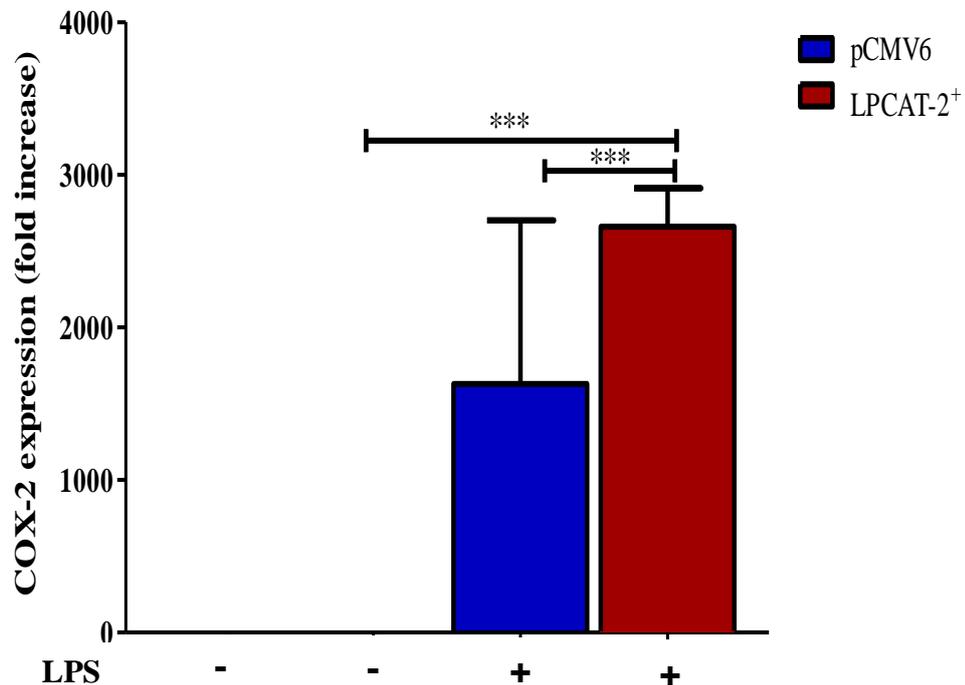


Figure 4.2. The effects of LPCAT-2 over-expression on COX-2 expression in RAW264.7 macrophages.

RAW 264.7 cells either transfected with LPCAT-2 (LPCAT-2⁺) (Red Bar) or with empty vector (control, WT) (Blue Bar) were stimulated with LPS for 6 hours, COX-2 gene expression was determined by RT-PCR. LPS treatment is required for COX-2 expression (inducible) and COX-2 expression was significantly increased in LPCAT-2⁺ cells. Data represents the mean of three independent experiments (n=3) \pm standard error.

***= $P < 0.0001$. pCMV6 = RAW264.7 + empty vector plasmid, LPCAT-2⁺ = RAW264.7 cells transiently transfected with murine LPCAT-2.

PPAR- γ gene expression in RAW264.7 cells stimulated with LPS.

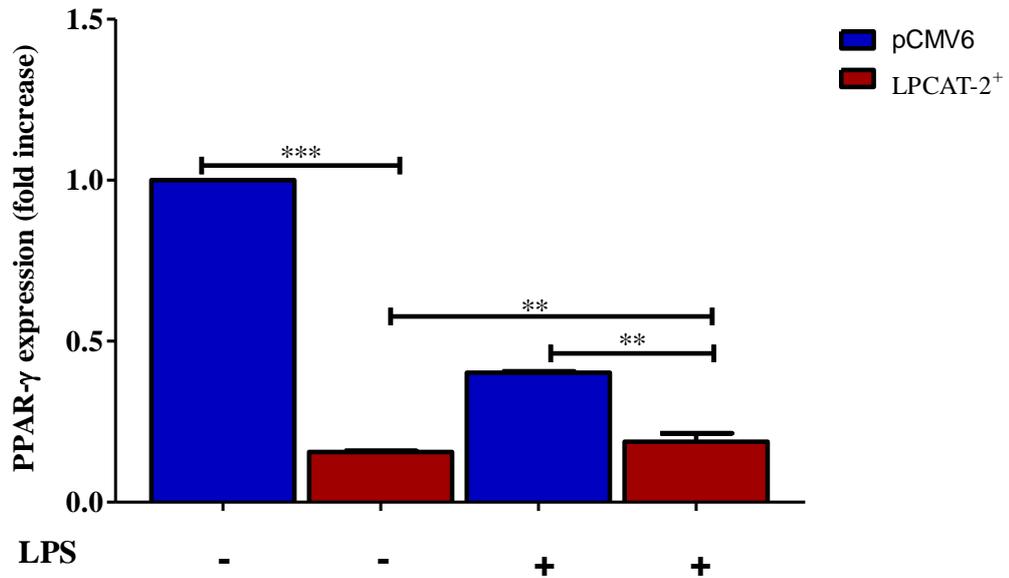


Figure 4.3. The effects of LPCAT-2 over-expression on PPAR- γ expression in RAW264.7 macrophages.

RAW 264.7 cells either transfected with LPCAT-2 (LPCAT-2⁺) (Red Bar) or with empty vector (control, WT) (Blue Bar) were treated with LPS for 6 hours or untreated controls and PPAR- γ gene expression was determined by RT-PCR. LPS treatment decreased PPAR- γ gene expression in pCMV6 cells. LPCAT-2 overexpression (LPCAT-2⁺) significantly down-regulated PPAR- γ gene expression in both LPS stimulated and unstimulated pCMV6 cells.

Data represents the mean of three independent experiments (n=3) \pm standard error.

= $P < 0.001$, *= $P < 0.0001$. pCMV6 = RAW264.7 + empty vector plasmid, LPCAT-2⁺ = RAW264.7 cells transiently transfected with murine LPCAT-2.

4.3. The effect of the selective peroxisome proliferator-activated receptor- γ (PPAR- γ) antagonist T0070907 on LPCAT-2 expression.

Recent studies have confirmed that the peroxisome proliferator-activated receptors (PPARs) and in particular (PPAR- γ) have important roles in the regulation of macrophage phenotype (Karp *et al.*, 1991; Mueller *et al.*, 1998; von Knethen *et al.*, 2007; Chawla, 2010; Assunção *et al.*, 2017). This, coupled with the role of PPAR- γ in lipid metabolism, suggested that LPCAT-2 might have effects on PPAR- γ .

The results in Figure 4.3 show that transfection of RAW264.7 macrophages with LPCAT-2 inhibits PPAR- γ expression. It was therefore important to examine if inhibition of PPAR- γ could also affect LPCAT-2 expression. For this, the selective PPAR- γ antagonist T0070907 (2-Cloro-5-nitro-N-4-pyridinylbenzaminde) at a concentration of (0.1 and 1 mM) or vehicle (DMSO) were added to RAW264.7 cells.

The cells were then treated with LPS (1 μ g/ml) for 6 hours at 37 °C. Expression of LPCAT-2 gene was determined by RT-PCR.

Interestingly, when PPAR- γ was inhibited by treating the cells with the selective -gamma antagonist, T0070907, the level of LPCAT-2 gene expression increased. This increase in LPCAT-2 expression was found to be dependent on the concentration of the PPAR- γ antagonist and therefore a dose-response relationship linking the two molecules was established (Figure 4.4), Table 3 is shown the mean values as a fold increase vs control RAW264,7.

Effect of selective PPAR- γ antagonist on LPCAT-2 expression using Real time polymerase chain reaction(RT-PCR) analysis.

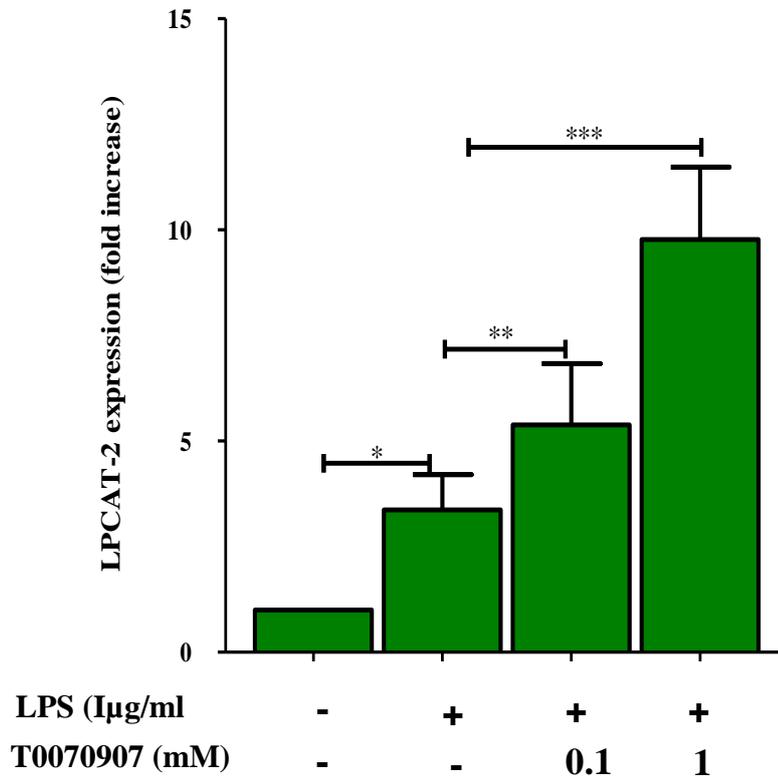


Figure 4.4. RT-PCR analysis of the effect of selective peroxisome PPAR- γ antagonist T0070907 on LPCAT-2 expression.

RAW264.7 cells were either untreated (control) or treated with or without the selective PPAR- γ antagonist T0070907. Addition of PPAR- γ antagonist T0070907 was found to enhance expression of LPCAT-2 in a dose-dependent manner in response to LPS. Data represented the mean of three independent experiments ($n=3$) \pm standard error.

*= $P < 0.05$ vs control, **= $P < 0.001$ ***= $P < 0.0001$. Control= RAW264.7 cells.

4.4. The effect of LPCAT-2 overexpression on the expression of M1 and M2 macrophage markers inducible nitric oxide synthase (iNOS) and CD206.

As the previous experiments showed that LPCAT-2 can promote the expression of signalling molecules in the TLR-mediated inflammatory pathways while suppressing PPAR- γ , a molecule associated with anti-inflammatory phenotype we next investigated if LPCAT-2 can regulate macrophage phenotypes.

Macrophage M2 or alternatively activated phenotype is promoted by several transcription factors, including PPAR γ (Bouhrel *et al.*, 2007). Toll-like receptor signaling, particularly TLR4 stimulated by LPS and other microbial ligands, drives macrophages to a preferentially M1 or classically activated phenotype (Stein *et al.*, 1992).

PPAR- γ has been shown to promote an anti-inflammatory (M2), alternatively activated macrophage phenotype by reprogramming macrophages for oxidative metabolism by increasing fatty acid oxidation and mitochondrial biogenesis (Chawla, 2010).

This project hypothesis, PPAR- γ upregulates fatty acid oxidation and supports an M2 macrophage phenotype where LPCAT-2 utilises fatty acids for phospholipid synthesis and would be associated with M1 inflammatory phenotype of macrophage activation.

This was examined by determining the expression of iNOS as a molecular marker of M1 macrophages and CD206 as a marker of M2 macrophages (Mandal *et al.*, 2011).

Therefore, this project has investigated the possible role of LPCAT-2 in macrophages polarisation i.e. M1 or M2 like phenotype by using specific markers (iNOS for M1 and CD206 for M2).

RAW264.7 cells with either the transfected murine LPCAT-2 insert (LPCAT-2⁺) or vector pCMV6 (WT) were stimulated with Pam3CSK4 (TLR2 ligand) or LPS (TLR4 ligand) for 6 hours at 37 °C and the expression of iNOS and CD206 was measured by RT-PCR. As shown in Figure 4.5, overexpression of LPCAT-2 leads to a significant

expression of iNOS in unstimulated cells and this was further significantly increased in cells stimulated with either LPS (Fig 4.5a) or Pam3CSK4 (Fig. 4.5b). Furthermore, cells transfected with LPCAT-2 were seen to have a significant decrease in the expression of the M2 marker CD206 when stimulated with either LPS or Pam3CSK4 (Fig 4.5 c, d), table 3 is shown the mean values as a fold increase vs pCMV6 cells.

It is becoming recognised that lipid metabolism may be fundamental to the metabolic regulation of the inflammatory response (Croasdell *et al.*, 2015; Assunção *et al.*, 2017). In this regulation, lipid catabolism and fatty acid oxidation are associated with oxidative phosphorylation, M2 phenotype and lipid synthesis is linked to glycolysis and the M1 phenotype (Croasdell *et al.*, 2015).

Therefore, this suggests that it appears from the previous project results, that LPCAT-2 can antagonize this function of PPAR- γ by which the two molecules have opposite functions. Consequently, PPAR- γ may suppress LPCAT-2 expression and promote an M2 macrophage phenotype, whereas activation of LPCAT-2 can suppress the activation of PPAR- γ and would promote an M1 phenotype, i.e. these results suggested that the LPCAT-2 might have a vital role in Macrophages polarisation (M1-M2 polarisation).

Therefore, all these results of this project in this chapter and in chapter 3 together, suggested that the overexpression of LPCAT-2 has promote the M1 like phenotype but not M2.

iNOS gene expression in RAW264.7 cells stimulated with LPS.

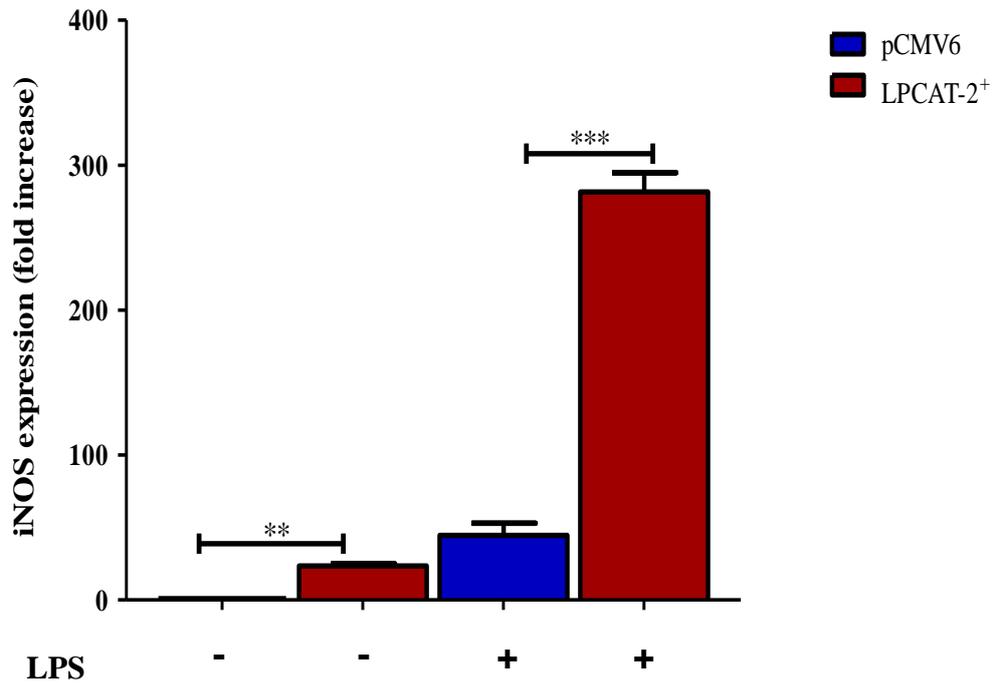


Figure 4.5A. The effect of LPCAT-2 over expression on iNOS expression in RAW264.7 stimulated by LPS using RT-PCR analysis.

Transfection of RAW264.7 cells with LPCAT-2 (LPCAT-2⁺) results in significant iNOS expression in untreated pCMV6 cells and significantly increased iNOS expression after treatment with LPS.

Data represented the mean of three independent experiments (n=3) ± standard error.

** = $P < 0.005$, *** = $P < 0.0001$, pCMV6 = RAW264.7 + empty vector plasmid (Blue Bar); LPCAT-2⁺ = RAW264.7 cells transiently transfected with LPCAT-2 (Red Bar).

iNOS gene expression in RAW264.7 cells stimulated with Pam3CSK4.

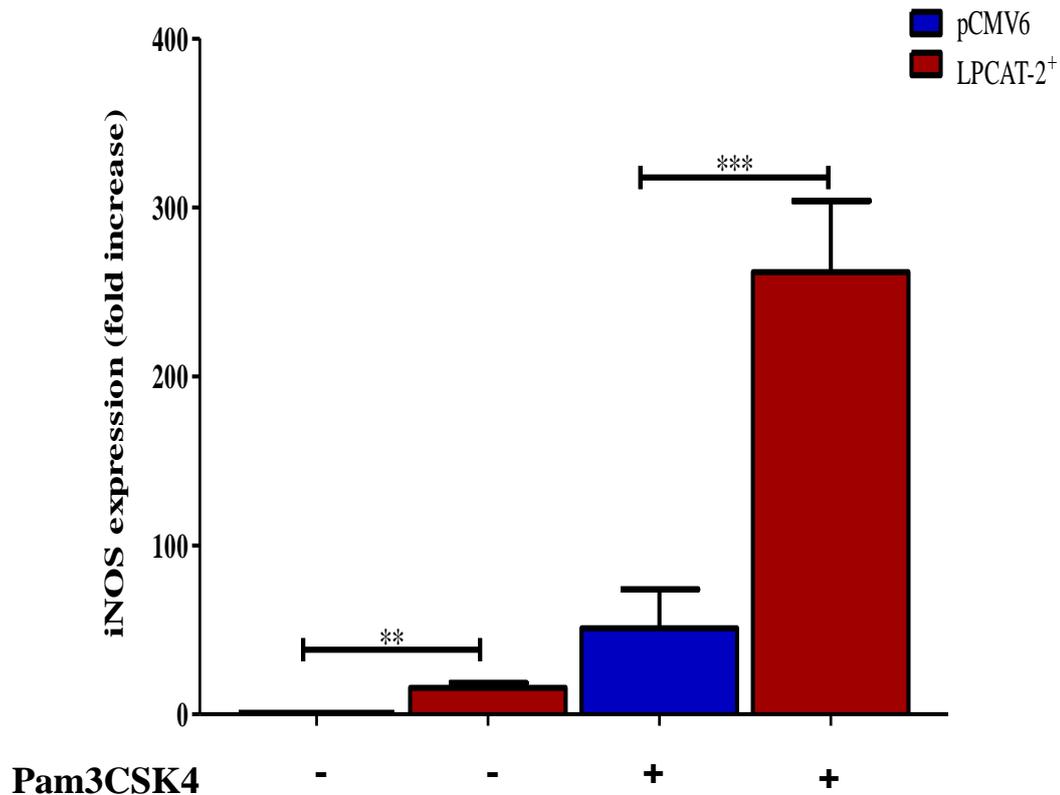


Figure 4.5B. The effect of LPCAT-2 over expression on iNOS expression in RAW264.7 stimulated by Pam3CSK4 using RT-PCR analysis.

Transfection of RAW264.7 cells with LPCAT-2 (LPCAT-2⁺) results in significant iNOS expression in untreated pCMV6 cells and significantly increased iNOS expression after treatment with Pam3CSK4.

Data represented the mean of three independent experiments (n=3) ± standard error.

** = $P < 0.05$ *** = $P < 0.005$. pCMV6 = RAW264.7 + empty vector plasmid (Blue Bar); LPCAT-2⁺ = RAW264.7 cells transiently transfected with LPCAT-2 (Red Bar).

CD206 gene expression in RAW264.7 cells stimulated with LPS.

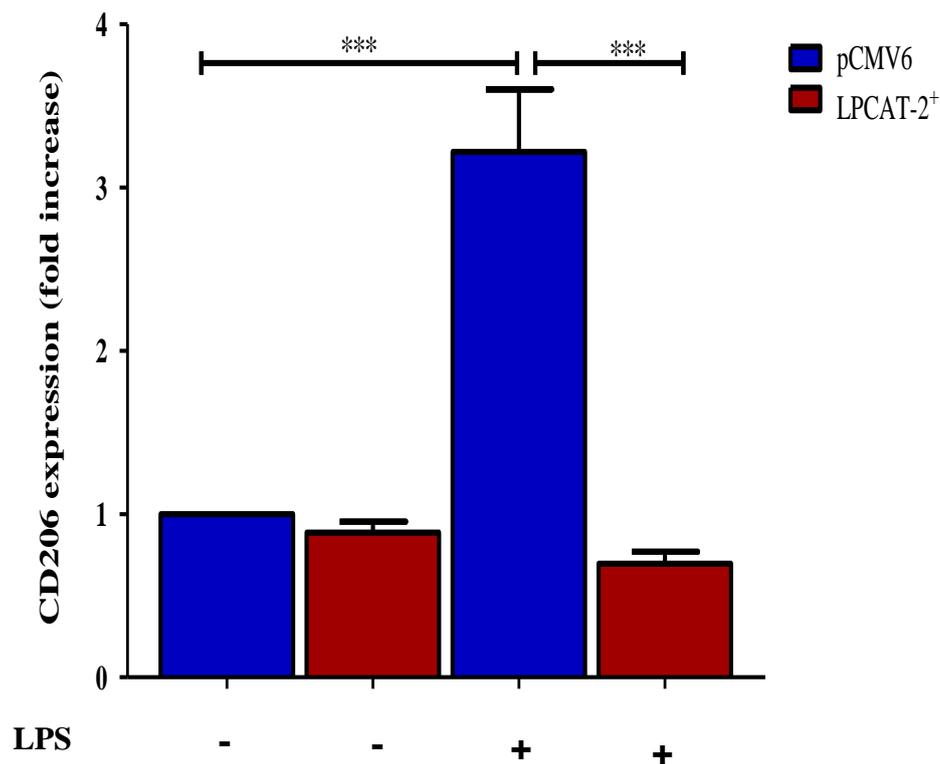


Figure 4.5C. The effect of LPCAT-2 over expression on CD206 expression in RAW264.7 stimulated by LPS using RT-PCR analysis.

LPCAT-2 overexpression resulted in significant inhibition of CD206 expression in cells treated with LPS, while it is significantly increased in pCMV6 treated with LPS. Data represent the mean of 4 independent experiments ($n = 4$) \pm standard error.

*** = $P < 0.0005$, pCMV6 = RAW264.7 + empty vector plasmid (Blue Bar); LPCAT-2⁺ = RAW264.7 cells transiently transfected with LPCAT-2 (Red Bar).

CD206 gene expression in RAW264.7 cells stimulated with Pam3CSK4.

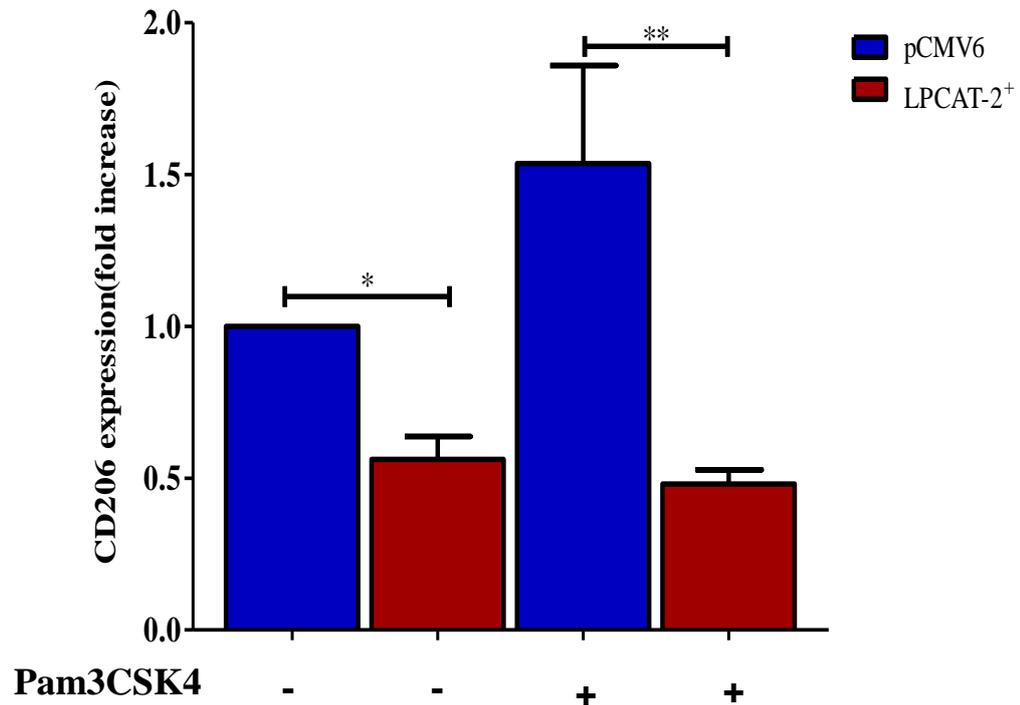


Figure 4.5D. The effect of LPCAT-2 over expression on CD206 expression in RAW264.7 stimulated by Pam3CSK4 using RT-PCR analysis.

LPCAT-2 overexpression resulted in significant inhibition of CD206 expression in cells treated with LPS, while it is significantly increased in pCMV6 treated with Pam3CSK4. Data represent the mean of three independent experiments (n=3) \pm standard error.

* = $P < 0.05$, ** = $P < 0.005$, pCMV6 = RAW264.7 + empty vector plasmid (Blue Bar); LPCAT-2⁺ = RAW264.7 cells transiently transfected with LPCAT-2 (Red Bar).

Summary of results.

Potential mechanisms, the over-expressed LPCAT-2 shows a key regulatory role in the inflammatory responses in chapter 3 as well as its effect in this chapter on the expression of receptors after 6 hours stimulation with LPS in RAW264.7 cells by down regulation of TLR4 and upregulation of TLR2 and CD14, and when the cells were stimulated with Pam3CSK4, over-expressed shows highly increasing in TLR2 gene expression after 6 hours stimulation, the current results suggest that LPCAT-2 could have a controlling role in polymicrobial sepsis (Schorr *et al.*, 2007; Adhikari *et al.*, 2010; Global Sepsis Alliance, 2015; Moskowitz *et al.*, 2017; Minasyan *et al.*, 2017). The implications of over-expressed LPCAT-2 played antagonised properties to PPAR- γ function (PPAR- γ has been shown to promote an anti-inflammatory (M2)), interestingly, when PPAR- γ inhibited by adding the selective -gamma antagonist, T0070907 to RAW264.7 cells, the level of LPCAT-2 gene expression upregulated. This increase in LPCAT-2 expression was found to be dependent on the PPAR- γ antagonist concentration and therefore a dose-response relationship connecting the two molecules was established, the other evidence that over-expressed LPCAT-2 induced COX-2 which is inducible enzyme as well and has an important role in prostaglandin synthesis, these results were coupled with iNOS (marker for M1) and CD206 (marker for M2) gene expression revealed the possible role of of LPCAT-2 in macrophages polarisation i.e. the current findings (Please see table 3) coupled with chapter 3 results suggested that the LPCAT-2 might have a vital role in macrophages polarisation by promoting M1 like phenotype but not M2. (M1-M2 polarisation) (Chawla, 2010; Mandal *et al.*, 2011).

Thus the successful transient overexpressing of LPCAT-2 would allowed this project to explore the role of LPCAT-2 in a stably over-expressed cell line with murine LPCAT-2 in chapter 5.

Mean (fold increase vs empty vector pCMV6 control)					
	Target	pCMV6	LPCAT-2 ⁺	pCMV6 + LPS	LPCAT-2 ⁺ +LPS
A	TLR4	1	1.8	0.3	0.7
	CD14	1	4.9	2.6	19.2
	TLR2	1	0.5	3.1	4.1
	COX-2	1	1.5	1628	2659.4
	PPAR- γ	1	0.2	0.4	0.19
	iNOS	1	23.4	44.5	281.4
	CD206	1	0.89	3.2	0.7
B	TLR2	1	5.9	1.8	9.6
	iNOS	1	15.7	50.9	261.8
	CD206	1	0.56	1.5	0.5
C	Control (RAW264.7 cells)	RAW264.7 + LPS	RAW264.7 + LPS + 0.1 mM T0070907 (mM)	RAW264.7 + LPS + 1 mM T0070907	
	1	3.37	5.38	9.76	

Table 3. The effect of over-expressed LPCAT-2 on gene expression using RT-PCR analysis.

A: TLR4, TLR2, CD14, COX-2, PPAR- γ , iNOS and CD206 in LPS induced murine macrophages, RAW264.7 cells.

B: TLR2, iNOS and CD206 in Pam3CSK4 induced murine macrophages, RAW264.7 cells. pCMV6 = RAW264.7 + empty vector, LPCAT-2⁺ = RAW264.7 cells transiently transfected with LPCAT-2, Mean (fold increase vs empty vector pCMV6 control).

C: RT-PCR analysis of the effect of selective peroxisome PPAR- γ antagonist T0070907 on LPCAT-2 expression. Control = RAW264.7 cells, PPAR- γ antagonist (T0070907).

Chapter 5:

Stable overexpression of LPCAT-2

Establishing a stably transfected RAW264.7 cells with a plasmid containing a murine LPCAT-2 gene (LPCAT-2⁺⁺).

Previous work in our laboratory (Jackson *et al.*, 2008a) and from other workers (Shindou *et al.*, (2007), suggested that LPCAT-2 might regulate the inflammatory responses to LPS in macrophages. In addition, Moromoto and co-researchers (2010 & 2014) over-expressed the LPCAT-2 gene to study the early production of PAF and found that LPCAT-2 was highly highly expressed in inflammatory cells activated by LPS stimulation through TLR4 (Moromoto *et al.*, 2010 & 2014), but they did not explore its effect on more general inflammatory responses including cytokine production. Having previously, successfully achieved transient over-expression of LPCAT-2 (see chapter 3), this project aimed to establish a stable cell line overexpressing the LPCAT-2 gene in RAW264.7 cells to study its role in the inflammatory response. A macrophage cell line consistently overexpressing LPCAT-2 would clearly offer many advantages in studying the role of LPCAT-2 in the inflammatory response. These include avoiding the cytotoxicity of repeated use of the transfection reagents, the high variability in the cell number between the control (RAW264.7 cells + empty vector plasmid) and the transient over-expressed LPCAT-2 cells (LPCAT-2⁺) as well as better economy in the in vitro laboratory work.

Stable transfection of the cells, would avoid the need for repeated transfection experiments, which require more reagents, repeated cloning of the plasmid with and without the murine LPCAT-2 gene inserts, then purifying these plasmids, shorteing the physical efforts and the period of time to apply the experiments, the period of time between repeated experiments. It would also offer more uniform cell concentrations for each independent experiment.

To achieve stable over-expression of LPCAT-2 required achieving three challenging steps as following:

The first crucial step was to establish the optimum concentration of Genticin antibiotic (G418) that killed the RAW264.7 cells which lacked the neomycin antibiotic resistance gene by using the killing curve method (please see section 2.9.1). The second step was to insert the LPCAT-2 gene into RAW264.7 cells using the transient transfection method using lipofectamine 2000 transfection reagent (please see section 2.9.2).

The third challenging step was establishing stably transfected RAW264.7 cells with plasmid carrying the LPCAT-2 gene inserts using G418 (please see section 2.9), then using RT-PCR (please see section 2.4) and wetern bloting (please see section 2.8.2) to confirm the expression of the LPCAT-2 gene and protein respectively.

Using the stably over-expressing LPCAT-2 cell line established in this chapter has shown that LPCAT-2 might have a vital role in the regulation of inflammatory cytokines gene expression and protein release. These results confirm that, LPCAT-2 might be a useful target for the development of therapies aimed at controlling the inflammatory response.

5.1. Killing curve of RAW264.7 cells.

The first critical step for establishing a stable cell line is selection based on antibiotic resistance and therefore, determining the optimal antibiotic concentration for selecting the stable cell lines (the mammalian cell colonies of RAW264.7 cells) that would be transfected with the plasmid carrying the murine LPCAT-2 (LPCAT-2 clones). The transfection process (please see 2.2.1.5 in material and methods) changed the RAW264.7 cells to Geneticin antibiotic (G418) resistant cells by taking the Neomycin resistance gene within the plasmid that is carrying the murine LPCAT-2 clones into the cells.

A kill curve is a dose-response experiment whereby cells are subjected to increasing amounts of antibiotic to determine the minimum antibiotic concentration that is needed

to kill all the cells over the course of one week (Davies & Jimenez,1980). In order to determine the optimal antibiotic concentration (killing curve), RAW264.7 cells were plated at cell concentration of 10^5 cells /well (150 μ l) in the first well, and then a doubling dilution of the antibiotic was done for the rest of the wells (until well number 12 in rows). The cells were then treated with different concentrations of the antibiotic G418 (0 - 800 μ g/ml) in each well (150 μ l). The killing curve was observed for 13 days of the experiment in the non-transfected RAW264.7 cells. Low dose was defined as the antibiotic concentration at which minimal visual toxicity was apparent after 7 days of antibiotic treatment, and this was observed at 200 μ g/ml of G418; optimal dose was defined as the lowest antibiotic concentration at which all cells were killed after one week of antibiotic treatment, and this were 500 μ g/ml of G418. High dose was taken as the antibiotic concentration at which visual toxicity was evident within the first 2 - 3 days of antibiotic treatment, and this were 800 μ g/ml of G418.

It was observed, as expected, that increasing doses of G418 were directly related to reduced numbers of viable RAW264.7 cells lacking the neomycin resistance gene as shown in Figure 5.1.

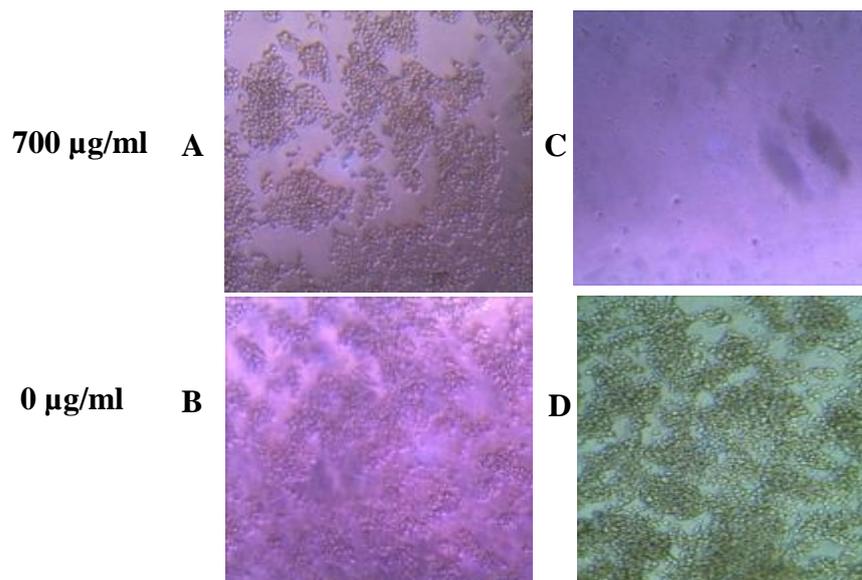


Figure 5.1. RAW264.7 cell killing using G418 antibiotic for three days to select stable cell colonies.

(A) Cells incubated with the highest antibiotic concentrations (700 µg /ml) were all dead (C) after 2-3 days compared with RAW264.7 cells without G418 (B & D). Therefore, 700 µg /mL of G418 was chosen to be the highest safe dose to start establishing stable transfected cells. The experiment was performed in 96 well plates, in duplicate, at cell concentration of 10^5 cells /well (150 µL).

5.2. Transfection of the LPCAT-2 gene into the RAW264.7 cell line.

Having established the optimum concentrations of Geneticin (G418) that will kill untransfected RAW264.7 cells, we next needed to transfect the cells with plasmid containing the LPCAT-2 and the neomycin resistance genes. Figure 5.2 shows the micrographs of the differences in the concentration of the viable stable transfected RAW264.7 cells (LPCAT-2⁺⁺).

The highest viable cell concentration was in the wells that treated with DMEM medium containing 500 µg/mL of G418 within different periods of times compared with the cell that treated with 600 or 700 µg/mL of G418. The negative control for each treatment was RAW264.7 (control).

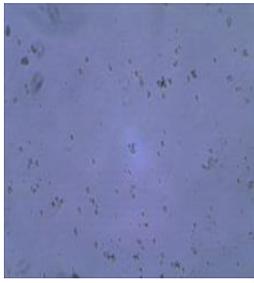
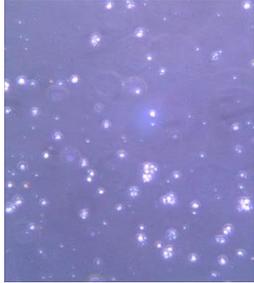
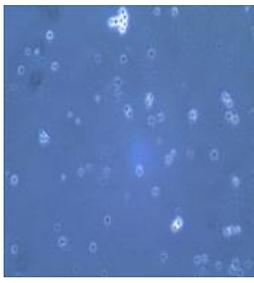
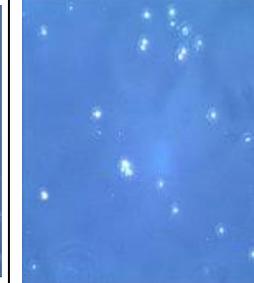
Transfected RAW264.7 cells with 9 µl of lipofectamine 2000 transfection reagent and 2.5 µg of pCMV6 control- entry vector (4.9 kb) plasmid, carrying the clones of murine LPCAT-2 which have the Neomycin resistant gene, and treated with DMEM medium without G418, were viable, and proliferated during the experiment days.

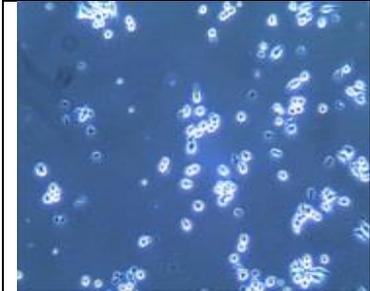
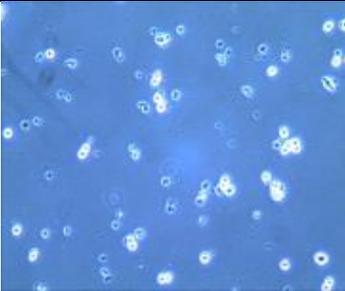
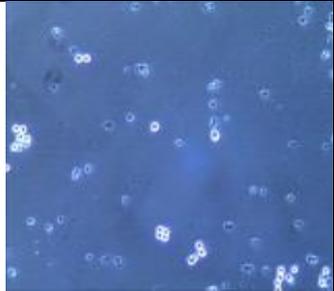
Furthermore, on the ninth day of treatment with G418 antibiotic, the selected stable cell clones were treated with a maintaining medium (DMEM medium containing 200 µg/mL of G418) for 10 extra days.

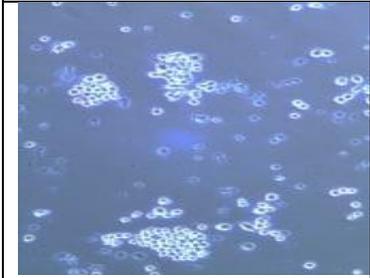
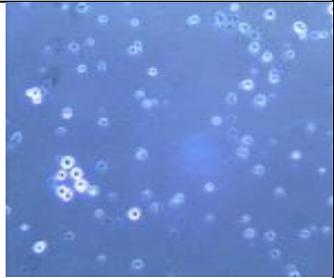
Furthermore, on the ninth day of treatment with the maintaining medium (DMEM medium containing 200 µg/mL of G418), the stable cell line clones were ready to be frozen, although they were left longer than 10 days, and treated with the maintaining medium to confirm that they were the stable cell line.

These results were confirmed and analysed using RT-PCR and western blotting, which is shown in Figure 5.3.

For the RT-PCR, there was a 3.6 fold increase in the expression of the murine LPCAT-2 gene ($P = 0.0387$).

	pCMV6	LPCAT-2 ⁺ after 5 days treatment with G418		
	500 µg/ml	500 µg/ml	600 µg/ml	700 µg/ml
A				

	LPCAT-2 ⁺ after 7 days treatment with G418		
	500 µg/ml	600 µg/ml	700 µg/ml
B			

	LPCAT-2 ⁺⁺ after 9 and 19 days treatment with G418 respectively		
	200 µg/ml		
C			

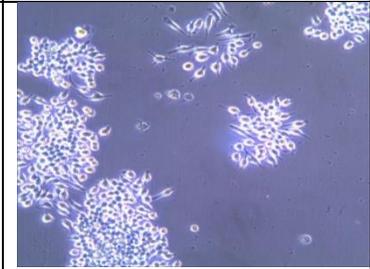
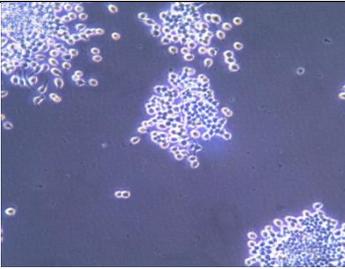
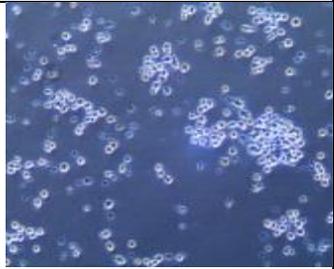
D			
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Figure 5.2. Establishing stable transfected RAW264.7 cells with a murine LPCAT-2 gene insert (LPCAT-2⁺⁺) images taken under the inverted microscope (x10).

Pane A shows pCMV6, LPCAT-2⁺ cells treated with DMEM medium containing (500, 600 & 700) µg /mL of G418 respectively after 5 days.

Pane B shows: LPCAT-2⁺ cells treated with DMEM medium containing (500, 600 & 700) µg /mL of G418 respectively after 7 days which have the Neomycin resistant gene.

Pane C shows: LPCAT-2⁺⁺ cells treated with DMEM medium containing 200 µg /mL of G418 after 9 days.

Pane D shows: LPCAT-2⁺⁺ treated with DMEM medium containing 200 µg /mL of G418 after 19 days.

pCMV6 = RAW264.7 cells +empty vector plasmid, LPCAT-2⁺= RAW264.7 cells transiently transfected with murine LPCAT-2 insert, LPCAT-2⁺⁺= RAW264.7 cells stably transfected with murine LPCAT-2 insert.

5.3. Expression of LPCAT-2 in stably transfected RAW264.7 cells (LPCAT-2⁺⁺).

Expression of murine LPCAT-2 in a cell that are stably transfected with murine LPCAT-2 gene was confirmed by the real time PCR. The stable transfected RAW264.7 cells with murine LPCAT-2 gene insert (LPCAT-2⁺⁺) and empty vector plasmid control (pCMV6) cells were plated in 6 well plates at a concentration of 1×10^6 cells/ well. RNA was extracted and reverse transcribed as detailed in chapter 2. To check for genomic DNA contamination, negative controls consisting of reactions that lacked RT (diluted RNA samples) were run to assess the effectiveness of the DNase treatment (as described in section 2.4.1). This gave negative results indicating there was no genomic DNA contamination of the samples. The qPCR has also no template controls (water samples with each primer mix) in every run and returned undetectable results.

The melting curve analysis was used as indicator to assess whether the PCR assays have produced a single and specific product. Results from this analysis showed that the melting temperatures of the PCR products were clearly single product for each target and the graphs showed no primer dimers present in the reaction plates which indicated the products were specific. All primers were designed and checked (please see section 2.4.3). Results of the RT-PCR experiments for the LPCAT-2 gene over-expression is shown in Figure 5.3. This shows that LPCAT-2 is significantly over-expressed in LPCAT-2⁺⁺ cells compared to pCMV6 cells (RAW264.7 cells) ($P = 0.0378$), table 4 is shown the mean values as a fold increase vs pCMV6 cells.

LPCAT-2 gene expression in RAW264.7.

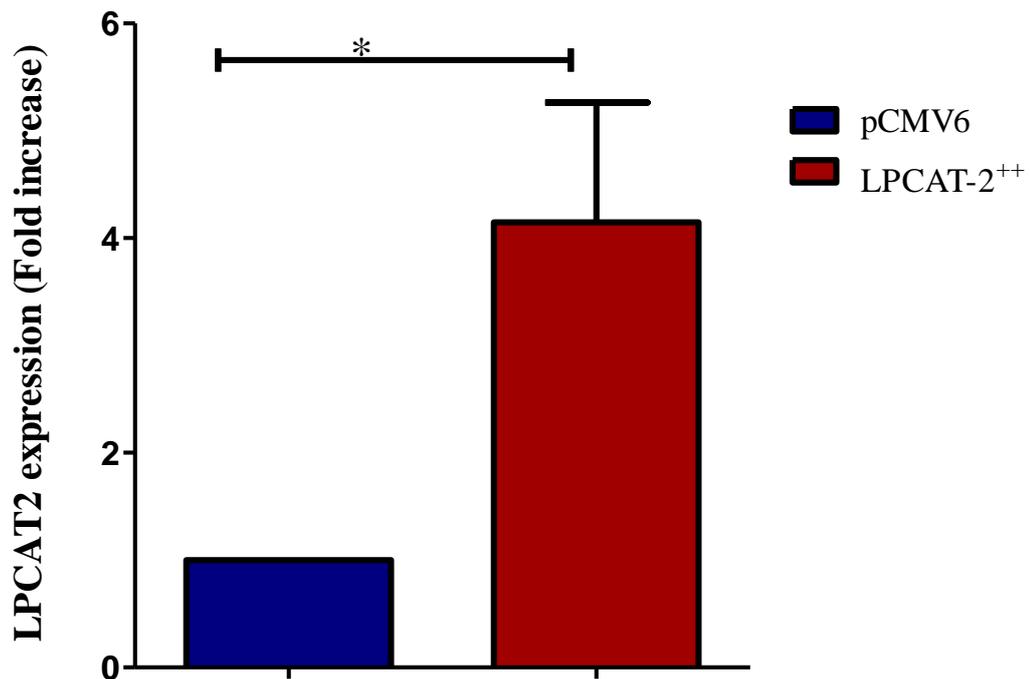


Figure 5.3. LPCAT-2 gene expression in the LPCAT-2⁺⁺ cells and in pCMV6 cells.

LPCAT-2 gene expression by RT-PCR from LPCAT-2⁺⁺ (Red Bar) and pCMV6 cells (Blue Bar). Figure shows a significant over-expression of LPCAT-2 after 24 hours incubation at 37 °C without LPS stimulation ($P = 0.0378$). Data represents the mean of three independent experiments ($n = 3$) \pm standard error. * = $P < 0.05$ vs pCMV6. pCMV6 = RAW264.7 cells + empty vector plasmid, LPCAT-2⁺⁺ = stable transfected RAW264.7 cells with murine LPCAT-2.

In addition to the gene expression of LPCAT-2 in RAW264.7 stably transfected with murine LPCAT-2 (LPCAT-2⁺⁺) and pCMV6 (RAW 264.7 cells + empty vector plasmid) cells as assessed by RT-PCR, it was important to determine if the stably transfected cells also could produce LPCAT-2 protein. Western blotting experiments were used to assess protein production. Figure 5.4 shows the results of western blotting experiments on the LPCAT-2⁺⁺ and pCMV6 cells.

The results show the expression of endogenous LPCAT-2 protein in pCMV6 cells and the over expression of LPCAT-2 protein in LPCAT-2⁺⁺ cells. In addition, when anti-Flag antibody was used to detect the protein, only the stably transfected cells expressed the flag-tagged protein. This confirms the protein expression in the stably transfected cells.

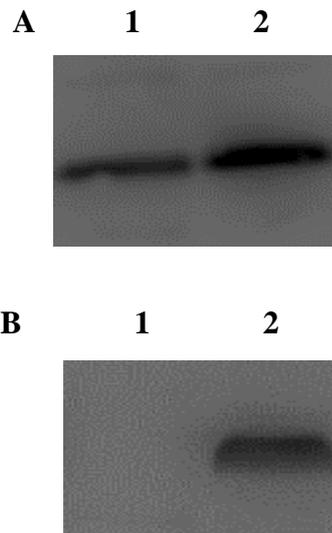


Figure 5.4. Western blotting confirmation of murine LPCAT-2 expression in LPCAT-2⁺⁺ cells and non-transfected RAW264.7 cells without LPS.

- (A) The expression bands of endogenous murine LPCAT-2 protein in control RAW264.7 (pCMV6) cells (lane 1) and LPCAT-2⁺⁺ cells (lane 2) respectively using anti-LPCAT-2 as primary antibody.
- (B) The expression band of flag-tagged protein in control RAW264.6-pCMV6 cells (lane 1), and LPCAT-2⁺⁺ cells (lane 2) respectively using anti-FLAG as primary antibody, pCMV6 = RAW264.7 cells + pCMV6 empty vector plasmid, and LPCAT-2⁺⁺ = RAW264.7 cells stably transfected with murine LPCAT-2.

5. 4. Expression of LPCAT-2 in RAW264.7 cells stimulated with LPS.

LPS can induce the expression of LPCAT-2 in macrophages and LPCAT-2 is thus an LPS inducible gene (Jackson *et al*, 2008). To analyse if the cells stably transfected with LPCAT-2 would show increased expression of LPCAT-2 in response to LPS stimulation stably transfected cells (LPCAT-2⁺⁺ cells) and the pCMV6 cells (RAW264.7 cells + empty vector plasmid) were stimulated with 1 µg/ml of LPS for various times between 0 and 24 hours at 37 °C. RT-PCR analysis showed that cell stimulation with LPS resulted in the induction of the LPCAT-2 gene expression in LPCAT-2⁺⁺ cells and in the pCMV6 cells but LPCAT-2 induction from the LPCAT-2⁺⁺ cells was significantly higher (P=0.0026), table 4 is shown the mean values as a fold increase vs pCMV6 cells.

. The peak of gene expression was at 8 hours post LPS stimulation in both LPCAT-2⁺⁺ and in pCMV6 cells (Figure 5.5), the mean values as a fold increase is shown in table 4. Similar results were obtained for LPCAT-2 protein expression determined by western blotting (Fig 5.6).

LPCAT-2 gene expression in RAW264.7 cells stimulated with LPS

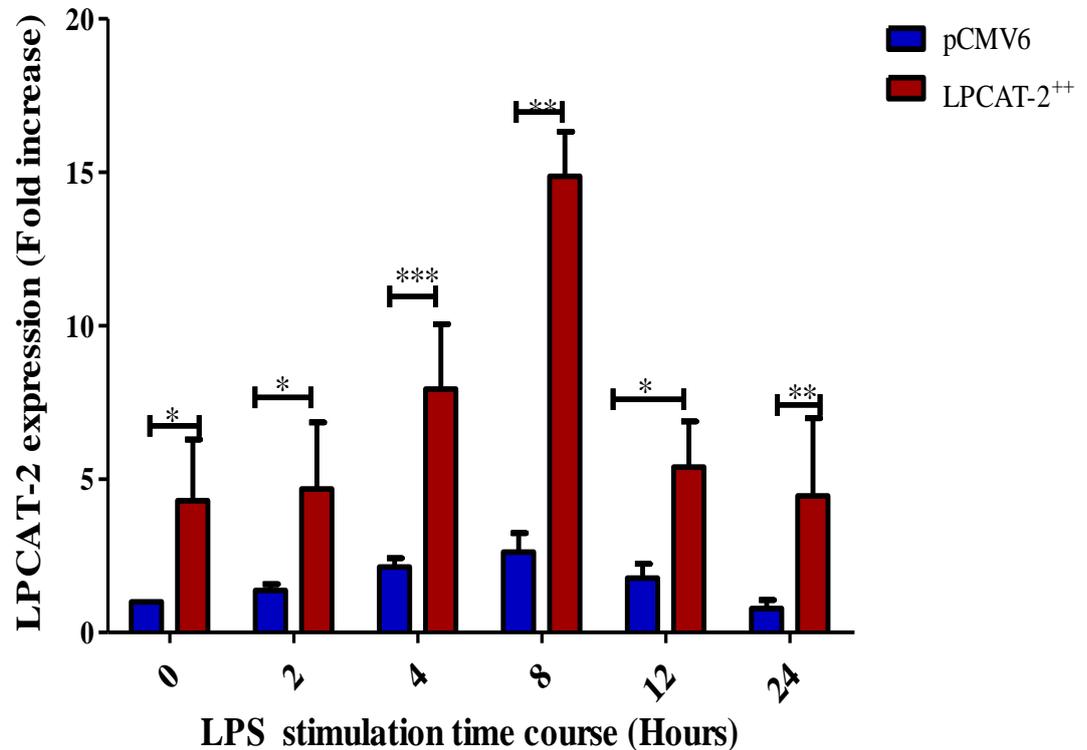


Figure 5.5. LPCAT-2 gene expression in in RAW264.7 cells stimulated with LPS.

The over-expression of murine LPCAT-2 in LPS stimulated murine macrophages; in both LPCAT-2⁺⁺ cells (Red Bar) and in the pCMV6 cells (Blue Bar), but with a higher expression in the former, within an LPS stimulation time course. The peak of LPCAT-2 gene expression of the LPS-stimulated LPCAT-2⁺⁺ cells was at 8 hours post-stimulation with a significant difference between LPCAT-2⁺⁺ cells and the pCMV6 cells. Results were confirmed using RT-PCR analysis in three independent time course experiments. Data represents the mean of three independent experiments (n = 3) ± standard error.

* = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.0001$ vs pCMV6. pCMV6 = RAW264.7 cells + empty vector plasmid, LPCAT-2⁺⁺ = stable transfected RAW264.7 cells with LPCAT-2 gene.

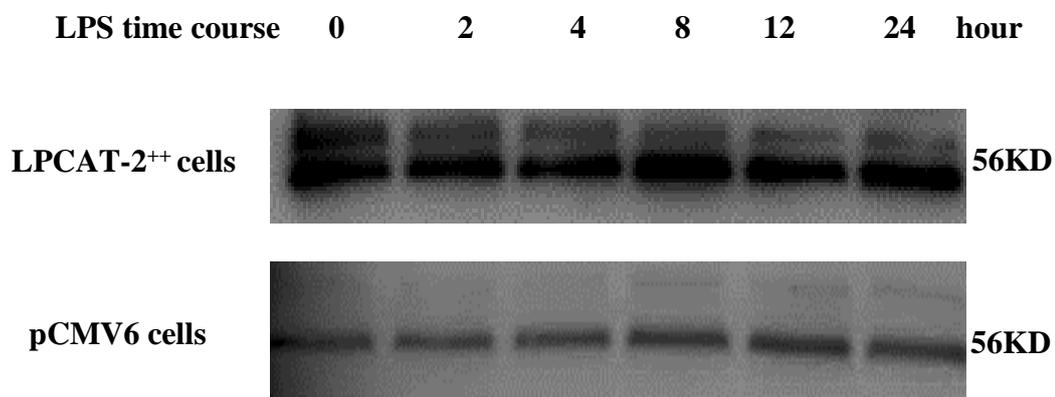


Figure 5.6. Western blot showing expression of LPCAT-2 in LPCAT-2⁺⁺ and pCMV6 (empty vector) cells following treatment with LPS.

The blot shows the increased expression of LPCAT-2 in the stably LPCAT-2⁺⁺ cells stimulated with LPS time-course (0 - 24) hours, compared with endogenous LPCAT-2 expression in pCMV6 cells. Representative blot from three independent experiments using anti-LPCAT-2 as primary antibody.

5.5. The effect of Lysophosphatidylcholine acyltransferase (LPCAT) - 2 on pro-inflammatory cytokines in LPS - stimulated macrophages.

The effect of the stably over-expressed LPCAT-2 on the inflammatory response of RAW264.7 macrophages was determined by analysing inflammatory gene expression in response to LPS. The stable transfected cells (LPCAT-2⁺⁺) and the pCMV6 cells (RAW264.7 cells + empty vector) were plated in 6 well plates at a cell concentration of 1×10^6 cells/ well (2.5 ml DMEM medium), and then stimulated with 1 μ g/ml of LPS for various times (0 and 24 hours at 37 °C. The inflammatory cytokines mTNF- α and IL-6 were determined by RT-PCR. Results for mTNF- α showed an up-regulation in both LPCAT-2⁺⁺ and in pCMV6 cells, with a much higher expression in the former (Figure 5.7). The peak of gene expression was found to be at 8 hours induction in both LPS-induced- LPCAT-2⁺⁺ and in pCMV6 cells, with a significant increase in TNF- α expression in the LPCAT-2⁺⁺ cells (P = 0.005. The mean values as a fold increase is shown in table 4.

TNF- α gene expression in RAW264.7 stimulated with LPS

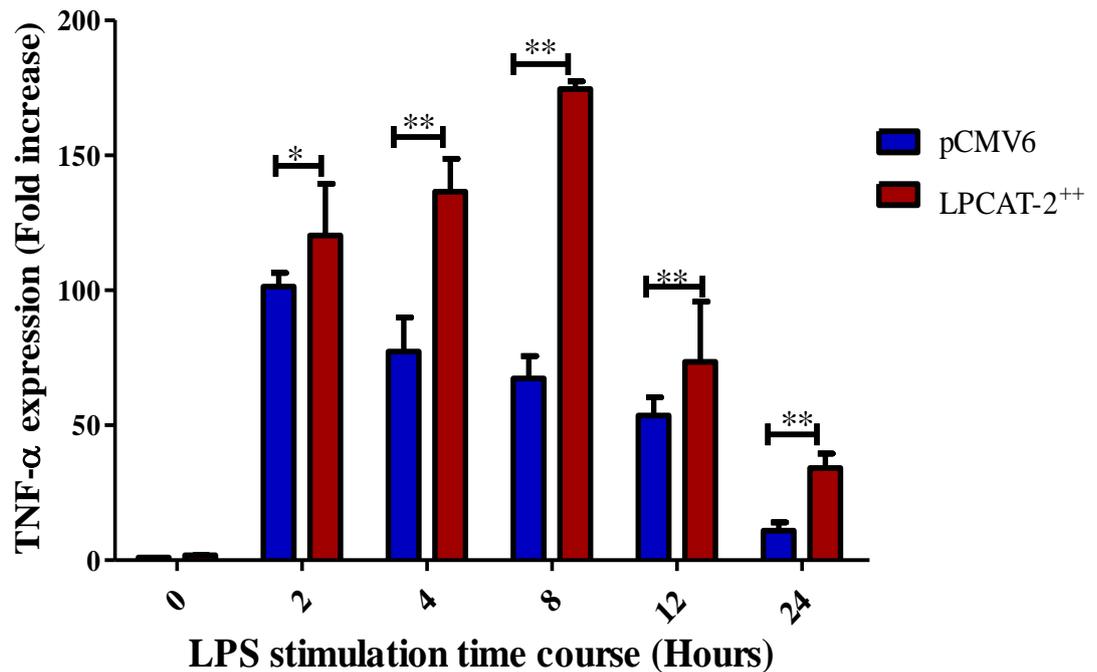


Figure 5.7. Murine Tumour necrosis factor (mTNF- α) gene expression in RAW264.7 cells stimulated with LPS.

These results show that mTNF- α was up-regulated in both LPCAT-2⁺⁺ cells (Red Bar) and in the pCMV6 cells (Blue Bar) within an LPS stimulation time course, but with a much higher expression in LPCAT-2⁺⁺ cells. The peak of mTNF- α gene expression of the LPS-stimulated LPCAT-2⁺⁺ cells were at 8 hours post-induction with a significant difference in the expression levels compared with pCMV6 cells. Results were confirmed using RT-PCR analysis in three independent time course experiments. Data represents the mean of three independent experiments (n = 3) \pm standard error. * = $P < 0.05$, ** = $P < 0.005$. pCMV6 = RAW264.7 cells + empty vector plasmid, LPCAT-2⁺⁺ = stable transfected RAW264.7 cells with LPCAT-2 gene.

Experiments to investigate IL-6 expression followed the procedures for the TNF- α experiments. Results for IL-6 expression showed that LPS significantly up-regulates the cytokine mRNA in both LPCAT-2^{+/+} cells and in pCMV6 cells (Figure 5.8) but with a much higher expression of the cytokine in the LPCAT-2^{+/+} cells (P=0.0078). The peak of IL-6 gene expression was at 8 hours post-induction in both LPS-induced LPCAT-2^{+/+} and in pCMV6 cells, the mean values as a fold increase is shown in table 4.

IL-6 gene expression in RAW264.7 cells stimulated with LPS

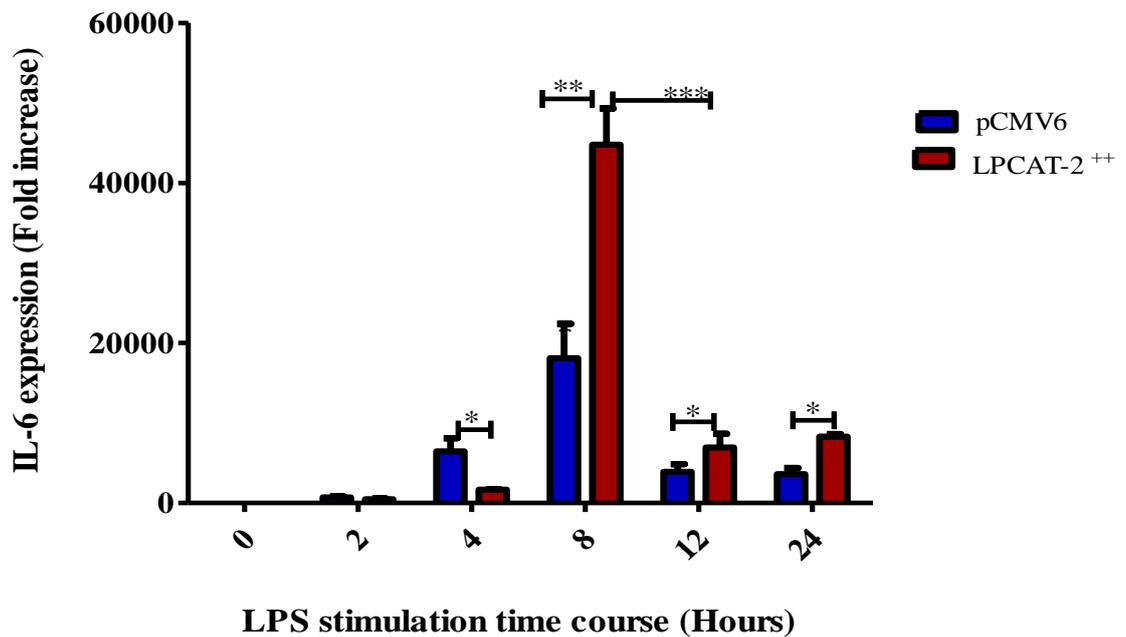


Figure 5.8. Expression of murine interleukine-6 (mIL-6) in RAW264.7 cells stimulated with LPS.

The LPCAT-2⁺⁺ (Red Bar) and the pCMV6 cells (Blue Bar) were stimulated to the time course stimulation with 1 μ g/ml of LPS for 0 - 24 hours. These results show that IL-6 was up-regulated in both LPCAT-2⁺⁺ cells and in the pCMV6 cells within an LPS stimulation time course, but with a much higher expression in the LPCAT-2⁺⁺ cells except at 4 hours post-LPS stimulation where the pCMV6 cells showed a higher mIL-6 expression. The peak of IL-6 gene expression in the LPS-stimulated LPCAT-2⁺⁺ cells was at 8 hours post-induction with a significant difference in gene expression between the LPCAT-2⁺⁺ cells and the pCMV6 cells. Results were confirmed using RT-PCR analysis in three independent time course experiments (n = 3). Data represents the mean of three independent experiments (n = 3) \pm standard error. * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.0001$. pCMV6 = RAW264.7 cells + empty vector plasmid, LPCAT-2⁺⁺ = stable transfected RAW264.7 cells with LPCAT-2 gene.

Summary of the results.

The results from this chapter are summarised in Table 4. This chapter developed a stably transfected murine macrophage cell line that over-expressed LPCAT2. Killing curves of RAW264.7 cells were used to select the lower (200 µg/ml), optimal (500 µg/ml), higher (800 and 700 µg/ml) concentration of G418 antibiotic to select the stable cells that carried the resistant gene of neomycin antibiotic gene within the plasmid carrying mLPCAT-2. Cells transiently transfected with plasmid carrying mLPCAT-2 (LPCAT-2⁺) were treated with (500, 600 and 700) µg/ml of G418 antibiotic to establish the stably over-expressing LPCAT-2 gene insert (stable transfected cells (LPCAT-2⁺⁺)).

RT-PCR results confirmed the LPCAT-2 gene over-expression and the protein expression was confirmed by Western blotting. The expression of LPCAT-2 in LPS-induced stably transfected RAW264.7 is shown as a LPS stimulation time course (0 -24) hours (Table 4), the same expression of LPCAT-2 protein was confirmed using western blotting, then the effect of LPCAT-2 on pro-inflammatory cytokines (TNF- α , IL-6) in LPS- stimulated RAW264.7 cells were confirmed using gene expression (table 4).

Thus, the development of a stably transfected macrophage cell line over-expressing LPCAT2 has been demonstrated and these cells can be used to demonstrate the role of LPCAT2 in the inflammatory response (Alrammah, 2015). Such experiments and approaches suggest that LPCAT-2 might be a useful target in controlling the inflammatory response.

Hour Target	0		2		4		8		12		24	
	pCMV6	LPCAT-2 ⁺⁺	pCMV6	LPCAT-2 ⁺⁺	pCMV6	LPCAT-2 ⁺⁺	pCMV6	LPCAT-2 ⁺⁺	pCMV6	LPCAT-2 ⁺⁺	pCMV6	LPCAT-2 ⁺⁺
LPCAT-2	1	4.3	1.4	4.7	2.1	7.9	2.6	14.9	1.8	5.4	0.8	4.5
mTNF- α	1	1.8	101.3	120	77.2	136.5	67.4	174.5	53.6	73.5	10.9	34.1
IL-6	1	6.5	698.9	481.3	6442	1650.8	18090.9	44797.5	3888.1	6944.9	3603.5	8292
pCMV6						LPCAT-2 ⁺⁺						
1						4.15						

Table 4.

The effect of over-expressed LPCAT-2 on the gene expression of the inflammatory responses in LPS induced murine macrophages, RAW264.7 cells.

This table shows the mean value in fold increase vs empty vector pCMV6 control. pCMV6 and LPCAT-2⁺⁺ cells stimulated with LPS to the time course stimulation with 1 μ g/ml of LPS for 0 - 24 hours. **Row 1:** LPCAT-2 gene expression in RAW264.7 cells stimulated with LPS.

Row 2: Expression of TNF- α in RAW264.7 cells stimulated with LPS. **Row 3:** Expression of mL-6 in RAW264.7 cells stimulated with LPS.

Row 4 and 5: LPCAT-2 gene expression in RAW264.7 cells without LPS. pCMV6 = (RAW264.7 cells + empty vector), LPCAT-2⁺⁺ = stable transfected RAW264.7 cells with LPCAT-2 gene).

Chapter 6:

Discussion, Conclusions &

Future work

6.1. Discussion.

Sepsis is an overwhelming inflammatory response to infection that has proved extremely difficult to treat (Angus *et al.*, 2001; 2011; The Global Sepsis Alliance, 2015 and 2016; Shankar-Hari *et al.*, 2016; Rhodes *et al.*, 2017). Therefore, much research has been devoted to the understanding of the pathophysiological mechanisms underlying sepsis and the identification of potential new therapeutic targets.

In this setting and building on previously established links between LPCAT and inflammatory responses in macrophages (Jackson *et al.*, 2008), this project aimed to detail the role of LPCAT-2 in the inflammatory response with the potential of identifying LPCAT-2 as a novel therapeutic target. In particular, this was achieved using molecular techniques to both over express and to knock down LPCAT-2 in macrophages. The successful application of these techniques has significantly added to knowledge on the role of LPCAT-2 in the inflammatory response and suggests that future research could utilize LPCAT-2 and related proteins as potential new therapeutic targets.

Key findings from the current study are that LPCAT-2 can be successfully over expressed in a murine macrophage cell line and the over expression of LPCAT-2 significantly increases TNF- α and IL-6 gene expressions and protein production, while the anti-inflammatory IL-10 gene expression was significantly decreased. Moreover, over expression of LPCAT-2 significantly decreased the gene expression of TLR4, PPAR- γ and CD206 (a marker of M2 macrophages) while it significantly increased CD14, TLR2, COX-2 and iNOS gene expression (M1 markers).

Importantly, silencing the transient over expression of murine LPCAT-2 resulted in a significant reduction in TNF-alpha and a significant increase in IL-10 gene expression. Taken together, these results firmly establish that LPCAT-2 is pivotal to the inflammatory response in macrophages and the work has also resulted in the RAW264.7 cell line stably

overexpressing LPCAT-2 that will be required for further studies and the development of molecules that target the expression of this gene for sepsis therapies.

6.1.1. Over-expression of LPCAT-2 in RAW264.7 cells induces an inflammatory phenotype.

Previous work has established that inhibition of LPCAT enzymes leads to the down-regulation of a number of inflammatory genes stimulated by bacterial endotoxin (LPS) (Schmid *et al.*, 2003; Jackson *et al.*, 2008). Until recently, when LPCAT enzymes were sequenced and cloned, it was not possible to specifically determine which particular LPCAT member was responsible for these actions. The availability of LPCAT sequence data came from important experiments that identified four major LPCAT enzyme LPCAT1, LPCAT-2, LPCAT3 and LPCAT4 isoforms (Chen *et al.*, 2006; Nakanishi *et al.*, 2006; Shindou *et al.*, 2007; Zhao *et al.*, 2008; Hishikawa *et al.*, 2008).

To prove that a protein causes a specific effect, not only should the effects be measured when that protein is knocked down but also when that protein is overexpressed. An important aim of this project was to determine the role of LPCAT-2 in controlling the macrophage inflammatory response to bacteria by specifically over expressing and knocking-down LPCAT-2.

Shindou *et al.*, (2007) over-expressed the murine LysoPAFAT/LPCAT-2 for the first time to study the role of LPCAT-2 on PAF biosynthesis in inflammatory cells. However, the effect of LPCAT-2 on cellular inflammatory responses has not been investigated. Shindou and co-workers showed not only lysoPAF acetyltransferase (LPAFAT) activity (which converts lysoPAF into PAF), but also LPCAT activity required Ca^{2+} in the LPS induced macrophage activation (Shindou *et al.*, 2007), but this project does not look at

the effects of Calcium ions in the media on LPCAT-2 expression or activity, therefore more studies needed to cover this area.

To confirm the over-expression of LPCAT-2, the protein expression was analysed by western blot using an anti-FLAG antibody. Interestingly, western analysis showed that, the LPCAT-2 protein expression in the transfected cells was seen as two bands at 35 and 56 kd, that were not present in the control RAW264.7 cells (which lacks the Flag-tag within normally expressed LPCAT-2). The second band, 35 Kd might be another isoform of LPCAT-2 generated by alternative splicing. This should be explored in future studies. However, expression of FLAG-tagged protein supports the successful over expression of LPCAT-2 in this work and confirms the use of cells over expressing LPCAT-2 in studies of the inflammatory response.

Ferrario *et al.*, (2016) have reported that LysoPC (LPC) may suppress the host inflammatory responses to LPS, and thus re-acylating LPC to form PC molecules, could reduce the amount of LPC, therefore increase the inflammatory process, and possibly raise both the severity and mortality in inflammatory diseases such as sepsis.

In agreement with this suggestion, research has demonstrated that septic patients have significantly decreased levels of LPC (Hong & Song, 2008; Cho *et al.*, 2011). Activation of RAW264,7 cells by LPCAT was shown previously not to be dependent on LPC and to reduce LPC (Jackson *et al.*, 2008). Thus, the activity of the inducible over-expressed LPCAT-2 in this project would be expected to reduce the level of LPC due to formation of PC molecules by re-acylation of the membrane LPC by the remodeling pathway.

Moreover, the transient over-expression of LPCAT-2 gene expression in RAW264.7 cells was successfully confirmed both before and after LPS stimulation. LPS was seen to increase the expression of the transfected LPCAT-2 gene. A similar effect was also seen

when Pam3CSK4, a TLR2/1 ligand, was used to stimulate the RAW264.7 cells to study the effect of overexpressing LPCAT-2 on the inflammatory responses to different bacterial ligands.

A previous study has reported that LPCAT-2 remains induced at 16 hours post LPS stimulation using 100 ng/ml LPS from *S. minnesota* in CHO-K1 cells (Shindou *et al.*, 2007). In the current study, using RAW264.7 cells stably transfected with LPCAT-2, 1 µg/ml of LPS from *E. coli* was used and the LPCAT-2 gene expression was significantly increased at 4 - 8 hours post LPS and reduced at 12 hours post LPS treatment and completely declined after 24 hours. The difference in LPCAT-2 expression kinetics in these studies is possibly due to the cell type, LPS concentrations used and the different bacterial strain of LPS. Our work did not reveal any toxicity to the RAW264.7 cells when LPS was used at this concentration. In addition, several studies have used 1 µg/ml of LPS as stimulus and over these time points did not report any toxicity to murine macrophage (RAW264.7 cells). (Li *et al.*, (2000) (Liu *et al.*, 2007; Iyer *et al.*, 2010).

TLR4 is activated through LPS, augmented by LPS binding protein (LBP) which cooperates with MD2 and CD14 complexes, and stimulates both MAPKs and NF-KB signaling pathways to induce proinflammatory mediators and cytokines that play a key role in controlling cell proliferation and differentiation as well as the cellular responses regulating cytokines and stresses (Johnson & Lapadat, 2002; Caivano, 1998). The maximal MAPK expression occurs 20 - 30 minutes' post LPS treatment in human and murine monocytes and macrophages (Bian *et al.*, 2003). In agreement with our results, this suggests that the increased gene expression of LPCAT-2 will be early in the response of the cells to LPS.

LPCAT-2 has been seen to be highly expressed in inflammatory cells and to be activated by LPS stimulation through TLR4 (Morimoto *et al.*, 2010). This group showed LPCAT-

2 phosphorylation at Ser-34, after 30 minutes post LPS stimulation led to increasing lyso-PAFAT activity. In agreement with this, the current project did not measure LPCAT-2 expression at less than two hours after LPS stimulation.

However, unlike these other studies, we explored the effect of LPCAT-2 over-expression as well as inhibition, on phenotypic responses of cells stimulated with LPS. Thus, it has shown that over-expression of LPCAT-2 led to enhanced levels of pro-inflammatory cytokine production in RAW 264.7 macrophages while inhibition of this LPCAT-2 expression resulted in significant inhibition of the mediator production.

Morimoto and co-workers also established the rapid PAF biosynthesis in 30 seconds by using protein kinase C α (PKC α) to phosphorylate LPCAT-2 at Ser34 following PAF or ATP-stimulation (Morimoto *et al.*, 2014). However, it is unclear whether incorporation of arachidonic acid in the cellular membrane either elevated or decreased its bioavailability for subsequent production of eicosanoid. This project agreed with their results in relation to LPCAT-2 effects on inflammatory response by up-regulating PAF in the remodeling pathway post LPS stimulation, however this project does not measure PAF, but it explores the cytokines production and release. Although the study of Morimoto *et al.*, (2010; 2014) did not explore cytokine gene expression and protein release, as the current project has demonstrated, it does support the fact that LPCAT-2 is inducible in the inflammatory cells due to extracellular stimuli, unlike, LPCAT1 which is a non-inducible isoform. However, the mechanism of action of LPCAT-2 is still not well understood (Harayama *et al.*, 2009; Morimoto *et al.*, 2010; Shindou *et al.*, 2009; Shindou *et al.*, 2013; Morimoto *et al.*, 2014).

An important result determined during the investigation of the effects of over-expressed LPCAT-2 on the inflammatory response, was that inflammatory mediator production was significantly up regulated in the RAW264.7 cells transiently transfected with the LPCAT-

2 insert. This suggests that LPCAT-2 has a pro-inflammatory role in macrophages by enabling induction of inflammatory molecules such as IL-6 and TNF- α in response to bacterial molecules LPS and Pam3CSK4 (Brunialti *et al.*, 2006).

The current project confirmed that there is a significant increase in pro-inflammatory cytokine gene expression, (eg TNF- α and IL-6), in RAW264.7 cells in response to LPS or Pam3CSk4. However, the novel finding is that this increase in cytokine expression is significantly enhanced in cells transfected with LPCAT-2. Thus, for the first time, we have established that LPCAT-2 gene expression is linked to increased inflammatory gene expression in macrophages.

It is tempting to speculate that continued upregulation of LPCAT-2 gene expression could lead to increased inflammatory gene expression seen in various chronic inflammatory and autoimmune disorders, for instance; thyroiditis, type I diabetes, rheumatoid arthritis (Hirano,1992; Tan *et al.*,1990), systemic sclerosis (Feghali *et al.*,1992), mesangial proliferative glomerulonephritis and psoriasis, and neoplasms for examples, cardiac myxoma, renal cell carcinoma, multiple myeloma, lymphoma, and leukaemia (Hirano,1992). More significantly in the context of the current study is that regulation of LPCAT-2 gene expression could be linked to the dysregulated inflammatory response seen in sepsis.

Results from the over-expression of LPCAT-2 and the expression of LPCAT-2 in inflammatory cells (Shindou *et al.*, (2007), and the previous work in our laboratory (Jackson *et al.*, 2008a) strongly suggest that LPCAT-2 could be responsible for the inflammatory responses to LPS and other bacterial ligands in macrophages.

While the over expression of LPCAT-2 gene in macrophages enhanced a range of pro-inflammatory responses, it was important to demonstrate the pro-inflammatory role of

this LPCAT isoform by also inhibiting the gene expression. LPCAT-2 was silenced in RAW264.7 cells using the specific siRNA technology and with this model a strong level of silencing (80%) LPCAT-2 was achieved, When LPCAT-2 was silenced and the cells were stimulated with LPS, there was a significant reduction in inflammatory cytokine production including TNF- α compared with control cells. This agrees with recent results obtained by our laboratory in which LPCAT-2 was silenced in MM6 cells using shRNA (Abate & Jackson, 2015). Similar results were obtained in cells stimulated with the TLR2 ligand Pam3CSK4.

Furthermore, the results of this work also show that LPCAT-2 influences the inflammatory response at the protein level. Cytokine release was assayed by enzyme-linked immunosorbent assay (ELISA) for TNF- α and IL-6 protein expression in the presence and absence of LPS stimulation. A problem due to the fact that the transfected RAW264.7 cells grow at different rates compared with control cells meant that standardisation against protein amounts (the usual method) was not possible. To overcome this, the ELISA results were normalized against the total RNA concentrations as suggested by (Muratore *et al.*, 2014).

The cytokines, IL-6 and TNF- α release was induced after 6 hours of LPS-stimulation in RAW264.7 cells and the amounts of these proteins was significantly higher in the cells that over-expressed LPCAT-2. These results suggest that LPCAT-2 has a key role in regulating the inflammatory response, to infection and therefore is a potential target for novel anti-inflammatory or anti-sepsis drug development. However, more details are required about the pathways and mechanisms of action of LPCAT-2 in this regard before any therapeutic molecules could be developed.

In this project, the molecular techniques of gene expression and gene silencing were utilised. In particular, the small interfering RNA (siRNA) technique (Hamilton and

Baulcombe, 1999) was used. The excellent sequence-specificity (Deng *et al.*, 2014) of the LPCAT-2 isoform that was used provided a high degree of confidence in the accuracy of specific overexpression of LPCAT-2 and silencing (in contrast to the co-silencing of other LPCAT enzymes). The high efficiency properties (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 over-expressed LPCAT-2 gene in this project.

Although RAW cells transiently over-expressing LPCAT-2 have been extremely useful in determining the potential role of LPCAT-2 in macrophage responses, it would be desirable to produce a stable transfectant for long-term studies. Thus, this project also attempted to create a stably transfected cell line using RAW264.7 murine macrophages transfected with the pCMV6-Entry plasmid carrying the inserts clones of murine LPCAT-2 and Lipofectamine 2000 transfection reagent.

Xu *et al.*, (2007) had used a concentration of 600 µg/mL of Geneticin for establishing a stable transfected RAW264.7 cells to study the role of Toll like receptor in innate immunity. In this project we used the same concentration of Geneticin (600 µg/mL) in both killing curve and establishing a stable transfected RAW264.7 with murine LPCAT-2 to study the role of LPCAT-2 in inflammatory responses. Our experiments have produced a stably transfected macrophage cell line for the development of future studies on LPCAT-2 and the inflammatory response.

6.1.2. Potential mechanisms of action of LPCAT-2 in the inflammatory response to bacterial ligands.

This study has shown through gene over-expression and gene silencing techniques, that LPCAT-2 plays a key role in macrophage inflammatory responses to LPS and other bacterial ligands. Moreover, the inhibition of inflammatory responses when the LPCAT-

2 gene is knocked down in other leukocytes, including the human monocyte cell line MM6, confirms the central role of LPCAT-2 regulation of leukocyte inflammatory responses (Abate and Jackson 2015).

This study has also confirmed that LPCAT-2 is inducible, unlike LPCAT1 which is constitutively expressed, which also suggests a role in the inflammatory response. In these regards, LPCAT-2 might be similar to other inducible enzymes such as COX2 with which it might link in regulating phospholipid metabolism. The control of inflammatory gene responses to bacterial ligands by LPCAT-2 leads to the question of potential mechanisms through which these effects are manifest and whether inducible LPCAT-2 activity has wider implications for macrophage activation. These will be necessary points to consider if LPCAT-2 might become a target for novel anti-inflammatory and anti-sepsis therapies.

Regulation of TLR ligand induced pathways may be at several possible control points. It is well established that LPS signaling via TLR4 proceeds via translocation of TLR4 to membrane lipid raft domains – membrane regions enriched in cholesterol, sphingolipids and certain phospholipids that serve as signaling platforms for the assembly of signaling complexes. (Brown & London 2000; Triantafillou *et al.*, 2002, Jackson & Parton, 2004; Triantafillou *et al.*, 2004).

Soong *et al.*, (2004) has also demonstrated TLR2 is translocated to the lipid raft domain of the plasma membrane following the stimulation of the cells with its ligand. Thus, regulation of TLR translocation to lipid raft domains might be a key process mediated by LPCAT-2. Previous studies have shown that LPCAT regulates LPS-induced translocation of TLR4 into lipid raft domains thereby affecting the subsequent down-stream signaling events (Jackson *et al.*, 2008a).

However, in these previous studies, the sequence and cloning of LPCAT enzymes was not available and the existence of the different family members was not known. Therefore, it was unknown, which of the LPCAT proteins modulated this LPS-induced response (Jackson *et al.*, 2008a). The role of LPCAT on the translocation of the TLR2 has not been investigated (Jackson *et al.*, 2008; Schmid *et al.*, 2003).

LPS-induced LPCAT-2 has been shown to be activated in RAW264.7 cells via MAPK-activated protein kinase 2(MK2) located downstream of p38 MAPK (p38 α and p38 δ), mainly expressed in macrophages (Hale *et al.*, 1999). Whether MK2 is required for the pro-inflammatory responses initiated by LPCAT-2 as seen in the current study has not been investigated but would aid future determination of mechanisms.

Based on these previous studies, the findings from this project suggest that overexpression of the enzyme LPCAT-2 could induce cellular responses to LPS by regulating the translocation of TLR4 into membrane lipid raft domains. It would be expected also LPCAT-2 knocked down with a specific siRNA technique could block the expression of LPCAT-2 and down regulate cellular responses to LPS by preventing the translocation of TLR4 into membrane lipid raft. Results obtained in this study with Pam3Cys suggest that LPCAT-2 can also regulate the inflammatory responses to other bacterial ligands signaling through TLR2 (Ozinsky *et al.*, 2000; Soong *et al.*, 2004) possibly by controlling assembly of their receptor complexes within membrane raft domains in RAW246.7 cells.

Whether LPCAT-2, an enzyme that normally re-acylates LysoPC (LPC) can regulate TLR4 translocation to membrane raft domains via this mechanism or another mechanism remains unknown. However, LPCAT is responsible for forming new PC component species from LPC re-acylation with unsaturated fatty acids (Chilton *et al.*, 1996). The

synthesis of PC has been shown to be crucial for all cell survival (van der Luit *et al.*, 2002; Jackson *et al.*, 2008).

In addition, evidence suggests that the biosynthesis of PC could be controlled in response to the lipid requirements of the vesicular trafficking (McMaster *et al.*, 2001; Bankaitis *et al.*, 2002). It would be expected that monocyte and macrophage cell membrane composition would influence the fluidity as well as enhancing the lipids and protein movement within and about the lipid raft regions (Rouquette-Jazdanian *et al.*, 2002).

Furthermore, LPCAT is responsible for adding the unsaturated fatty acids (arachidonic acids) to Lyso-PC (LPC) to form a new PC molecular species (Yamashita *et al.*, 1997). Subsequently, the balance between LPC and its acylation to PC, which is catalysed by LPCAT, could represent a crucial control point for the survival and functioning of the innate immune system cells.

The dysregulation of this balance could result in inappropriate cellular responses toward infectious stimuli that would allow the progression of systemic inflammation and organ injury (Van der Luit *et al.*, 2002; Fuchs *et al.*, 2005). This suggests that the LPCAT-2 might regulate monocyte and macrophage inflammatory responses through both regulating arachidonate availability for mediator formation as well as facilitating the formation of signaling complex and innate responses to inflammatory stimuli.

Additionally, Rouquette-Jazdanian *et al.*, (2002) have revealed that glycerophospholipids, for instance, phosphatidylcholine (PC) are components of lipid rafts. Any modification of the PC fatty acyl composition within these regions could alter the localization of LPS signaling receptors and thereby effect LPS induced responses. LPCAT-2, through regulating the physical state of the lipid microenvironment in membrane rafts might modulate the signaling receptor response to LPS (Jackson *et al.*,

2008; Yamashita *et al.*, 2014). Through regulating the membrane PC acylation with unsaturated fatty acids, LPCAT might regulate membrane fluidity (Darmani *et al.*, 1993; Jackson *et al.*, 2004).

6.1.3. Effect of LPCAT-2 on macrophage polarisation.

Macrophages can be activated to elicit different functions through interactions with their environment. Macrophages can be ‘polarized’ towards a pro-inflammatory phenotype expressing pro-inflammatory cytokines and antimicrobial factors (‘classical activation’ or M1 polarization) by microbial agents like lipopolysaccharides (LPS). Macrophages can also be polarized toward the generation of anti-inflammatory mediators and promote tissue remodeling factors, critical for resolution of inflammation and tissue repair (‘alternative activation’ or M2 polarization) induced by anti-inflammatory cytokines (IL-13, IL-4) (Shaykhiev *et al.*, 2009).

In research on Microglia/Macrophages, *in vitro* stimulation with LPS and interferon- γ (IFN γ) promoted the differentiation of “classically activated” M1 microglia/macrophages that released destructive proinflammatory mediators (Ding *et al.*, (1988). In contrast, interleukin (IL)-4 and IL-10 induce an “alternatively activated” M2 phenotype that possesses neuroprotective properties (Kigerl *et al.*, 2009; Goerdt *et al.*, 1999; Durafourt *et al.*, 2012).

The dualistic roles of distinctly polarized macrophage populations have been reported in several studies related to the presence of LPS. For example, it was reported that the activation of pattern recognition receptors (PPR) via invading pathogens are induced as part of the M1-like macrophage phenotype (innate immunity activation) (Mukhopadhyay *et al.*, 2006).

In this project, specific phenotypic markers of macrophage activation have been used to confirm the activation phenotype (M1 or M2) of the macrophage cell models used. Thus, induction of iNOS gene expression was used as a classical M1 marker for macrophages that are programmed to release pro-inflammatory mediators including iNOS, TNF- α , IL-6 (Ding *et al.*, 1988).

The inducible nitric oxide synthase (iNOS) is over-expressed in different types of cells including macrophages, hepatocytes and astrocytes, and stimulated in response to varied immune modulating molecules, for examples; lipopolysaccharide (LPS), interleukin (IL)-1, interferon gamma (IFN- γ), tumour growth factor beta (TGF- β) and pro-inflammatory cytokines (Moncada *et al.*, 1991; Li and Verma, 2002; Liu and Malik, 2006) i.e. the iNOS is highly expressed in LPS induced the murine cells, RAW264.7 cells which have been chosen in this project. In contrast, CD206 expression was used as a marker for M2-Like macrophage phenotype that down-regulate the pro-inflammatory cytokines but express the anti-inflammatory cytokine IL-10. Interleukin-10 (IL-10) was chosen in this project because it is a crucial anti-inflammatory cytokine that regulates the immune response of almost all leukocytes (Bonizzi & Karin, 2004). Results from this project also showed that the IL10 is a key regulatory cytokine.

In addition, it was noted that M2-like macrophages showed significant increase in expression of PPAR- γ . Through measurement of these markers, an important finding of the current study was that increased expression of LPCAT-2 induces the polarization of macrophages from M2-like phenotype into M1-phenotype. This property of LPCAT-2 may be important in conditions, such as sepsis, that display an overwhelming M1 phenotype that cannot be regulated.

This project results suggest that LPCAT-2 has a vital role in shifting the macrophage phenotyping into M1 rather than M2 and this would have an outcome on the discovery of

new specific anti-septic therapies, i.e. the activation of macrophage phenotypes as either M1 or M2 depends on the nature of the stimuli. Ding and fellow researchers showed the main targets of IL-10 on 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).

Moreover, IL10 also prevents the function of dendritic cells by reducing the production of the IL-12 and the expressions of MHC class II and co-stimulatory molecules (McBride *et al.*, 2002). In addition, IL-10 promotes Th2- phenotype through preventing the production of IFN- γ of T lymphocytes (Romagnani 1995). Then, directly blocks the proliferation of CD4+ T cells and the production of cytokines, for instance; IL-2, IFN- γ , IL-4, IL-5, and TNF-alpha (Joss *et al.*, 2000), accordingly impairing cellular immune responses, and controls the imbalance of the Th1/Th2 i.e. M1/M2 –phenotype of macrophages. In the current project, overexpression of LPCAT-2 was shown to down-regulate IL-10 expression, suggesting that LPCAT-2 promotes a pro-inflammatory phenotype in macrophages.

Consequently, this project, aimed to understand more about the mechanism of action of the inducible LPCAT-2 isoform. Recent studies have confirmed that the peroxisome proliferator-activated receptors (PPARs) and in particular (PPAR- γ) have important roles in the regulation of macrophage phenotype (Karp *et al.*, 1991; Mueller *et al.*, 1998; von Knethen *et al.*, 2007, Chawla, 2010).

This, coupled with the role of PPAR- γ in lipid metabolism, suggested that LPCAT-2 might have effects on PPAR- γ . Interestingly, when PPAR- γ was inhibited by treating the cells with the selective PPAR- γ antagonist, 2-Chloro-5-nitro-N-4-pyridinyl-benzamide (T0070907), the level of LPCAT-2 gene expression increased. This increase in LPCAT-

2 expression was found to be dependent on the concentration of the PPAR- γ antagonist and therefore a dose-response relationship linking the two molecules was established.

Furthermore, the effect of LPCAT-2 on the LPS-induced PPAR γ expression was investigated and it was found that the over-expression of LPCAT-2 significantly suppressed LPS-induced PPAR- γ expression in macrophages. PPAR- γ has been shown to promote an anti-inflammatory (M2) alternatively activated macrophage phenotype by reprogramming macrophages for oxidative metabolism by upregulating fatty acid oxidation and mitochondrial biogenesis (von Knethen *et al.*, 2007; Chawla, 2010).

It appears from the current results, that LPCAT-2 can antagonize this function of PPAR- γ and that the two molecules have opposite functions. Thus, PPAR- γ may suppress LPCAT-2 expression and promote an M2 macrophage phenotype whereas activation of LPCAT-2 can suppress the activation of PPAR- γ and would promote an M1 phenotype. Moreover, it is becoming recognised that lipid metabolism may be central to the metabolic regulation of the inflammatory response (Assunção *et al.*, 2017; Croasdell *et al.*, 2015).

In this regulation, lipid catabolism and fatty acid oxidation are linked with oxidative phosphorylation and an M2 phenotype and lipid synthesis is linked to glycolysis and an M1 phenotype (Croasdell *et al.*, 2015). In the current hypothesis, PPAR- γ upregulates fatty acid oxidation and supports an M2 macrophage phenotype where LPCAT-2 utilises fatty acids for phospholipid synthesis and would be associated with an M1 inflammatory phenotype of macrophage activation. This picture is somewhat complicated since it has been suggested that other LPCAT isoforms, such as LPCAT3, may have different effects on macrophage activation (Zhao *et al.*, (2008). This may be due to the fatty acid substrate specificities and other differences between the LPCAT isoforms.

To further elucidate the mechanisms of LPCAT-2 action in macrophages, the role of LPCAT on lipid mediators was also studied. LPS is known to induce COX-2, and we suggested that the overexpression of LPCAT-2 would also significantly increase inducible COX-2 expression. Indeed, it was found that, over-expression of LPCAT-2 while significantly down-regulating PPAR- γ gene expressions, significantly up-regulated the expression of COX-2 in transiently transfected RAW264.7 cells.

Recent studies have shown that inhibition of COX-2 using Meloxicam inactivates the MAPK pathway and regulates p53 and cell apoptosis, both of which are stimulated via TLR4 signaling, which is critical for LPS-induced signaling cascades (Park *et al.*, 2016). Several studies have reported that COX-2 regulates p53 activity and inhibits p53-dependent apoptosis (Choi *et al.*, 2005; Chen *et al.*, 2009) which suggests that LPCAT-2 might have a role to play in cell cycle and apoptosis. Recently, evidence has emerged that LPS can be recognized via TLR4-independent mechanisms, leading to inflammasome activation (Kayagaki *et al.*, 2013; Hagar *et al.*, 2013).

Moreover, it was reported that suppressors of cytokine signaling (SOCS3) is involved in suppressing the M1 proinflammatory phenotype (Qin *et al.*, 2012). LysMCre-SOCS3^{fl/fl} mice which lack SOCS3 are vulnerable to developing LPS-induced sepsis linked with enhanced activation of signal transducer and activator of transcription1/3 (STAT1/3) and elevated plasma levels of M1 cytokines/chemokines such as IL-1 β , TNF- α , IL-6 (Ding *et al.*, 1988; Qin *et al.*, 2012).

Rønn, *et al.*, (1982); Li *et al.*, (2006); Chen *et al.*, (2011); Qin *et al.*, (2012) reported that important role of SOCS3 in restricting inflammatory responses in myeloid cells has been demonstrated in a diversity of animal models for multiple sclerosis, arthritis, allograft rejection, lung injury, atherosclerosis, and septic shock.

Taken together, these results suggest that LPCAT-2 activation polarises the macrophage phenotype into an M1 classically activated cell that is important in clearing bacterial infections. Continuous M1 activation however, would allow continuous inflammatory responses that could then result in the development of sepsis. Thus, LPCAT-2 may be a key molecule for the regulation of macrophage activation and thus an attractive target for the development of novel sepsis therapies.

6.1.4. Potential therapeutic strategies.

Sepsis is a life-threatening condition that is estimated to result in as many as 20,000 deaths per day Worldwide (Angus et al. 2001). Despite extensive investigations and numerous clinical trials, there is currently no approved drug for sepsis (Ward 2012). Although much has been learnt about the underlying pathophysiology of sepsis and the inducing infection molecules, such as LPS, that mediate an overwhelming inflammatory response, the approach of blocking inflammatory mediators has not proved successful in clinical sepsis trials (Vincent 2006; Angus 2011).

A better understanding of the mechanisms and control points of the inflammatory response are thus urgently needed to provide new targets for diagnostic and therapeutic interventions. A key aim of the current study therefore, was to increase our understanding of LPCAT-regulated inflammatory responses so that novel therapeutic approaches to sepsis could be developed.

Due to the importance to find a novel therapeutic agent in sepsis, Daniels, (2011) summarized the potential target sites for developing novel therapeutic agents in sepsis including; anti-endotoxin, Toll like receptor (TLR) antagonists- TAK-242, Anti- Tumour necrosis factor (TNF), Interleukin (IL) -6 antagonist, anti-Platelet activating factor.

However, despite many clinical trials, blocking individual inflammatory mediators has proven ineffective in treating sepsis (Peplow & Mikhailidis, 1990; Morimoto *et al.*, 2010 & 2014; Papali *et al.*, 2017; Patel *et al.*, 2017; Rannikko *et al.*, 2017). Some of this disappointment is due to the poor compliance of animal models with human sepsis patients and the differences in underlying immunological responses between animals and humans (Lilley *et al.*, 2015). It also suggests that we do not concentrate on mediators per se but investigate how the inflammatory response is regulated.

TNF- α neutralisation was shown to be useful in reducing the inflammatory reactions in cirrhotic rats with induced bacterial peritonitis where TNF- α is increased (Sanchez *et al.*, 2013). Hence, blocking TNF- α or IL-1 β via TNF-neutralizing antibodies, soluble TNF- α receptors or IL-1 receptor has been suggested as a useful target for the treatment of numerous inflammatory diseases such as inflammatory bowel disease and rheumatoid arthritis (Dinarello, 2000).

Similarly, IL-6 is a fundamental pro-inflammatory cytokine and an endogenous mediator of LPS-induced fever (Roth *et al.*, 1998). Thus, the results of the current project suggest that LPCAT-2 might have a crucial role in regulation of these cytokines and control their gene expressions as well as their release. Thus, LPCAT-2 might also be a useful target in controlling the inflammatory response in a range of conditions.

From the current studies, LPCAT-2 would be an attractive target for the development of novel therapies for sepsis and other inflammatory diseases. LPCAT-2 is seen to control the inflammatory response to different bacterial ligands, and to many different inflammatory mediators. This in itself would make LPCAT-2 an attractive target as different mediators could be targeted at one time. Moreover, the discovery that LPCAT-2 might also play a pivotal role in the polarization of macrophages might allow a different and new approach to the treatment of sepsis. Switching macrophage activation states is a

promising approach in sepsis as it is becoming realized that sepsis represents a fundamental ‘reprogramming’ of the innate immune response to infection.

The most suitable methods that would be employed to inhibit LPCAT-2 present their own problems, however. Utilising siRNA approaches in humans would be impractical and different versions of gene knockdown would have to be used – possibly using CRISPR technology. More promising might be the development of anti-LPCAT-2 chemical inhibitors but these would have a long discovery pipeline before a clinical candidate was ready.

Furthermore, LPCAT-2 is also essential for immune responses and probably has many other functions that simply knocking down all of these would cause additional side effects. Thus, further knowledge of the mechanisms of LPCAT-2 activity in different cells under different conditions is vital for the development of any therapeutic approach. Moreover, other LPCAT family members might also have related functions that would need to be elucidated. These aspects are discussed in the ‘future work’ section.

6.2. Conclusions

1. Overexpression of LPCAT-2 in transiently or stably transfected RAW264.7 cells significantly increase TNF- α and IL-6 gene expression as well as other inflammatory proteins.
2. Overexpression of LPCAT-2 in transiently transfected RAW264.7 cells significantly decrease IL-10, TLR4 and CD206 (M2-like marker) while significantly increasing CD14 and iNOS (M1-like macrophage phenotype marker) suggesting that LPCAT-2 induces an M1-like macrophage phenotype.
3. Overexpression of LPCAT-2 in transiently transfected RAW264.7 cells significantly increase COX-2 while it is significantly down-regulating

PPAR- γ gene expression in transiently transfected RAW264.7 cells. Selective inhibition of peroxisome proliferator-activated receptor-gamma (PPAR- γ) induces LPCAT-2 expression.

4. Silencing LPCAT-2 gene expression using siRNA significantly attenuates the inflammatory response to infection by reducing inflammatory gene expression (TNF- α , IL-6) while significantly increasing IL-10.

6.3. Future work.

1. Further investigate the stably transfected RAW264.7 cells with murine LPCAT-2 recombinant gene using genomics and proteomics techniques to determine the response of LPCAT-2 enzyme in inflammatory response.
2. Further explore the link between LPCAT-2 expression, macrophage activation and polarization.
3. Explore the mechanism of action of LPCAT-2 in inflammatory responses by elucidating its role in responses using other TLRs as well as its proposed network role in cell apoptosis through P53.
4. Analyse the effects of LPCAT-2 expression and knockdown in human cells and animal models.
5. Further studies will be needed to explore the possibility to use the LPCAT-2 enzyme as a combination with cytokines and other target as anti-septic agents.
- 6- Further studies to explore and understand LPCAT-2 functions at different stages of inflammation to discover potential target for medical intervention.

6.4. References

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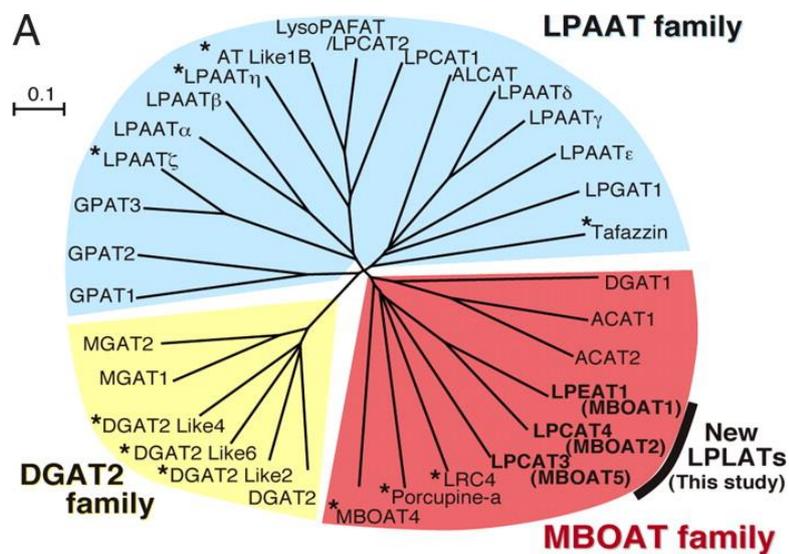
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Appendix 1: general introduction support information(SI), Materials: Material support.

Appendix 1 : General introduction support information(SI)



B

Product	Enzyme	Acyl-CoA								Reference No.			
		2:0	4:0	12:0	14:0	16:0	18:0	18:1	18:2		18:3	20:4	22:6
PC	LPCAT1												(20,43)
	LPCAT2												(21)
	LPCAT3												This Study
	LPCAT4												This Study
	?												
PE	LPCAT3												This Study
	LPCAT4												This Study
	LPEAT1												This Study
	?												
PS	LPCAT3												This Study
	LPEAT1												This Study
	?												
PA	LPCAT1												(43)
	LPAATα												(11,12,13)
	LPAATβ												(13)
	?												
PG	LPCAT1												(20,43)
	LPGAT												(16)
	?												
PI	?												

Figure 10. Phylogenetic tree of mouse LPLAT family and summary of characterized LPLATs. A phylogenetic tree was drawn by using ClustalW (www.ebi.ac.uk/clustalw). Hishikawa *et al.*, PNAS 2008; 105: 2830 - 2835.

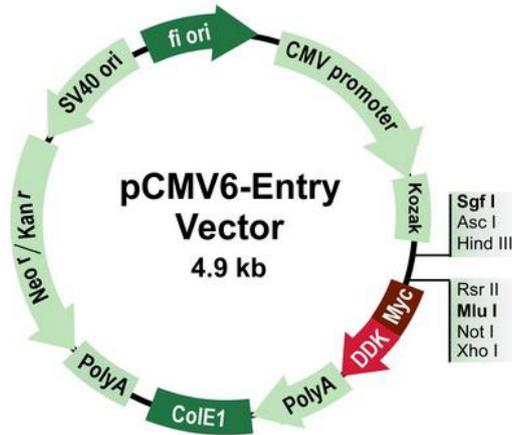
Appendices:

Appendices

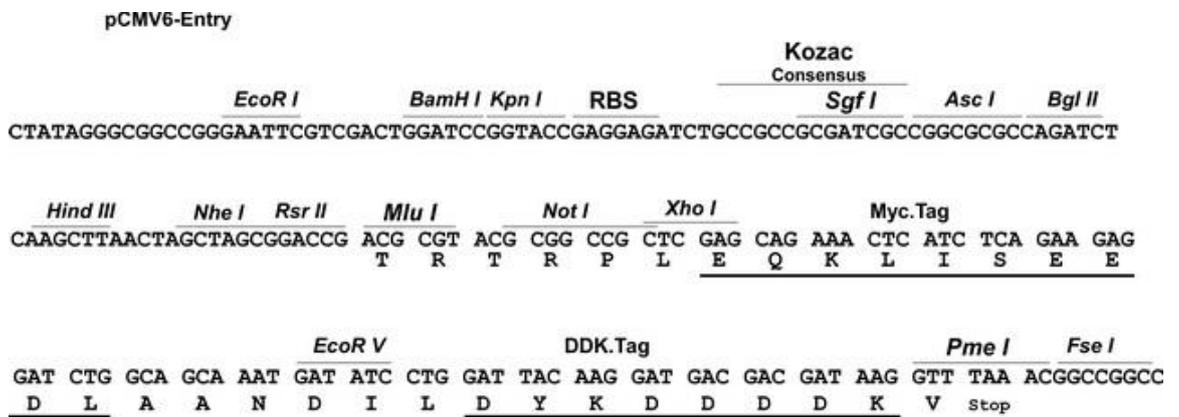
Appendix 1: Materials: Material support.

Materials:

A 1.1 Material support information Figures.

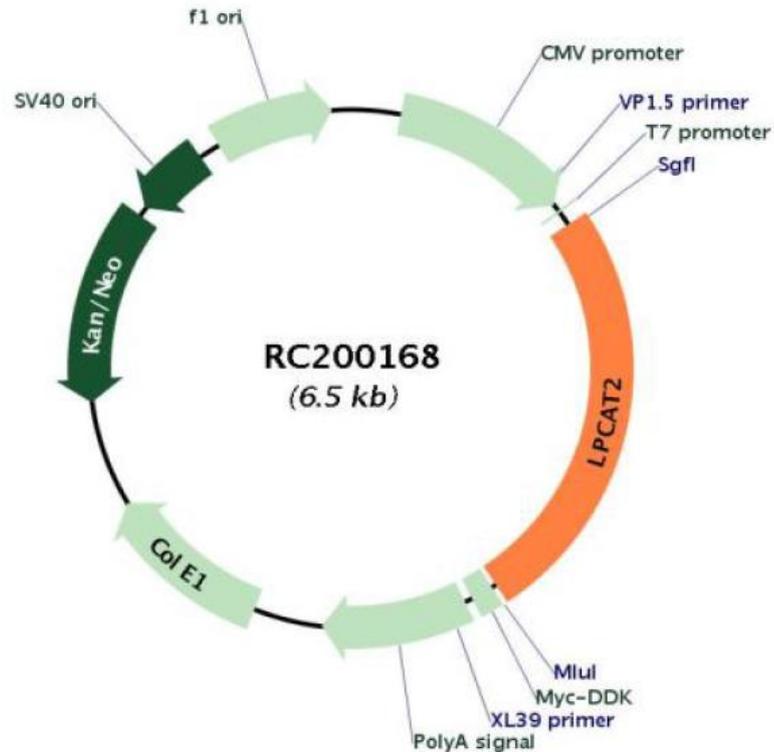


Schematic of the multiple cloning sites:



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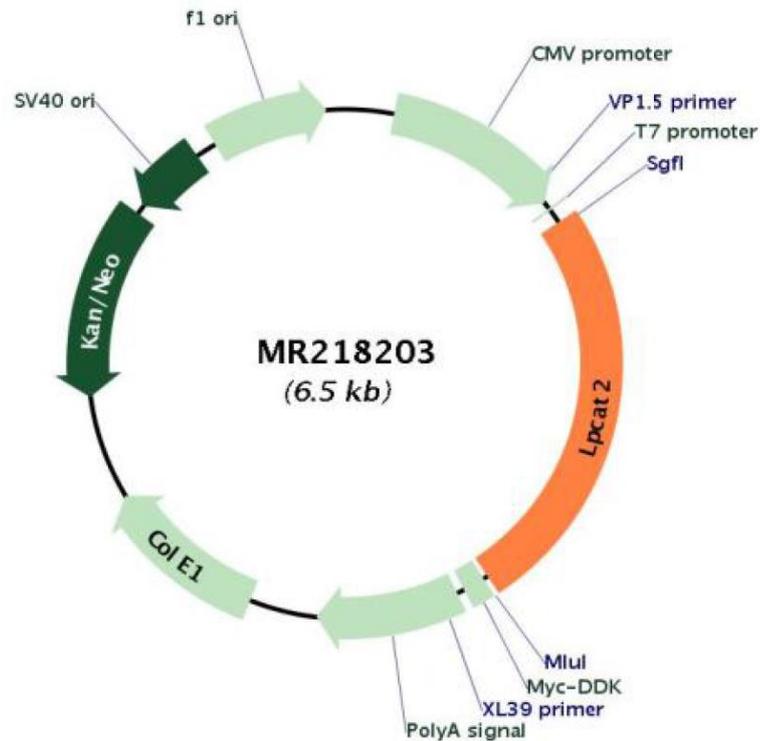
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P V L L R Y P N K L D T V T W T W Q G Y
ACA TTC ATT CAG CTT TGT ATG CTT ACT TTC TGC CAG CTC TTC ACA AAG GTA GAA GTT GAG
T F I Q L C M L T F C Q L F T K V E V E
TTT ATG CCA GTT CAA GTA CCA AAT GAT GAA GAA AAA AAT GAT CCT GTC CTT TTT GCC AAT
F M P V Q V P N D E E K N D P V L F A N
AAA GTC CGG AAT TTA ATG GCA GAA GCT CTG GGA ATA CCA GTA ACA GAT CAT ACC TAT GAA
K V R N L M A E A L G I P V T D H T Y E
GAC TGC AGA TTG ATG ATT TCA GCA GGA CAG CTA ACA TTG CCT ATG GAA GCT GGG CTG GTG
D C R L M I S A G Q L T L P M E A G L V
GAA TTT ACT AAA ATT AGC CGA AAA TTG AAA TTA GAT TGG GAT GGT GTT CGT AAG CAT TTG
E F T K I S R K L K L D W D G V R K H L
GAT GAA TAT GCA TCT ATT GCG AGT TCC TCA AAA GGA GGA AGA ATT GGA ATT GAA GAA TTC
D E Y A S I A S S S K G G R I G I E E F
GCC AAG TAT TTA AAG TTG CCT GTT TCA GAT GTC TTG AGA CAA CTT TTT GCA CTC TTT GAC
A K Y L K L P V S D V L R Q L F A L F D
AGG AAC CAT GAT GGC AGC ATT GAC TTC CGA GAG TAT GTG ATT GGC CTG GCT GTC TTG TGC
R N H D G S I D F R E Y V I G L A V L C
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N P S N T E E I I Q V A F K L F D V D E
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D G Y I T E E E F S T I L Q A S L G V P
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E F K S F A L K H P E Y A K I F T T Y L
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NluI NotI XhoI Myc-tag EcoRV
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T R T R P L E Q K L I S E E D L A A N D
DDK-tag PmeI FseI
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D RC200168 The complete (insert plus vector) sequence of [RC200168] clone

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E Circular map for MR218203:Download sequence: 

Cloning scheme for MR218203:

EcoRI *BamHI* *KpnI* *RBS* *BglII* *Kozac Consensus* *SgfI*

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M N R C

NotI

GCC GAG GCG GCC GCT GTG GCG GCT ACG GTC CCG GGT TCG GGC GTC GGG GAC GCC GGG CTG
A E A A A V A A T V P G S G V G D A G L

KpnI

CGG CCA CCC ATG GTA CCT CGC CAG GCG TCC TTC TTC CCG CCG CCG GTG CCC AAC CCC TTC
R P P M V P R Q A S F F P P P V P N P F

GTC CAG CAG ACT ACG ATC AGT GCC TCC CGA AGG CTG CAG ATG TTT CTT CTT GGA ATT ATT
V Q Q T T I S A S R R L Q M F L L G I I

CTG CTC CCA GTC CGT GCT TTA CTG GTT GGA ATA ATT TTG CTG CTC GCA TGG CCA TTT GCT
L L P V R A L L V G I I L L L A W P F A

GTC ATT TCA ACA GCA TGC TGT CCT GAA AAG CTG ACC CAT CCA ATA AGC AAT TGG AGG AGG
V I S T A C C P E K L T H P I S N W R R

AAG ATC ACT CGG CCC GCT TTG ACA TTT CTG GCG CGT GCC ATG TTC TTC TCC ATG GGG TTT
K I T R P A L T F L A R A M F F S M G F

ACG GTT ACC GTG AAA GGA AAG GTT GCA AGC CCT CTG GAA GCG CCC ATT TTT GTC GTC GCT
T V T V K G K V A S P L E A P I F V V A

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1/2

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 V L V S R V D P D S R K N T I N E I K K
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 N R S C L I T F K P G A F I P G V P V Q
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 P V L L R Y P N K L D T V T W T W Q G Y
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 T F L Q L C V L T F C Q L F T K V E I E
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 F M P V Q A P S E E E K N D P V L F A S
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 R I R N L M A E A L E I P V T D H T Y E
 GAC TGC AGG CTG ATG ATC TCA GCG GGA CAG CTC ACG TTG CCT ATG GAG GCC GGG CTG GTA
 D C R L M I S A G Q L T L P M E A G L V
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 GAT GAA TAT GCG TCT ATC GCA AGC TCT TCC AAA GGA GGC AGA ATT GGA ATC GAG GAG TTT
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 A E Y L K L P V S D V L R Q L F A L F D
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 R N N D G S I D F R E Y V I G L A V L C
 AAC CCT GCC AAT ACA GAA GAG ATC ATC CAG GTG GCA TTT AAG CTC TTT GAT GTT GAT GAG
 N P A N T E E I I Q V A F K L F D V D E
 GAC GGC TAC ATA ACA GAG GAG GAG TTT TGC ACC ATT CTG CAG GCT TCT CTT GGA GTG CCT
 D G Y I T E E F C T I L Q A S L G V P
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 D L N V S G L F R E I A Q R D S V S Y E
EcoRI
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 A S N K V S P E S Q E E G T S D K K V D
MluI NotI XhoI Myc-tag EcoRV
 ACG CGT ACG CCG CCG CTC GAG CAG AAA CTC ATC TCA GAA GAG GAT CTG GCA GCA AAT GAT
 T R T R P L E Q K L I S E E D L A A N D
DDK-tag PmeI FseI
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BIO-SYSTEM

<http://204.9.46.202:8080/NoegenAppExt/DrawMapBySKU?drawMap=drawMap&SKU=MR218203&VECTOR=pCMV6Entry>

F MR218203 The complete (insert plus vector) sequence of [MR218203] clone

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TTTTGGTCATGAGATTATCAAAAAGGATCTTACCTAGATCCTTTTAAATTAATAAATGAAGTTTAA
AATCAATCTAAAGTATATATGAGTAACTGAGGCTATGGCAGGGCCTGCCGCCCCGACGTTGGCT
GCGAGCCCTGGGCCCTTACCCGAACTTGGGGGGTGGGGTGGGGAAAAAGGAAGAAACGCGGGCG
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GTTTATGAACAAAACGACCCAAACCCGTGCGTTTTATTCTGTCTTTTTATTGCCGTATAGCCGGG
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ACCTTTCATAGAAGGCGGCGGTGGAATCGAAATCTCGTGATGGCAGGTTGGGCGTCGCTTGGTC
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CACAGTCGATGAATCCAGAAAAGCGGCCATTTTCCACCATGATATTCCGGCAAGCAGGCATCGCCA
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CACCTGGTTGCTGACTAATTGAGATGCATGCTTTCATACTTCTGCCTGCTGGGGAGCCTGGGGA
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CGTCTATTCTTTGATTTATAAGGGATTTTCCGATTTCCGCCTATTGGTTAAAAAATGAGCTGA
TTTAAACAAAAATTTAACGCGAATTTT

http://www.origene.com/orf_clone/trueclone/NM_017839/RC200168/LPCAT-2.aspx

G Protein sequencing: for Murine LPCAT2

MR218203 representing NM_173014

Red=Cloning site Green=Tags(s)

MNRCAEAAAVAATVPGSGVGDAGLRPPMVPRQASFFPPVPNPFVQQTISASRRLQMFLLGILLPVRALL
VGHILLAWPFAVISTACCPEKLTHPISNWRRKITRPAITFLARAMFFSMGFTVTVKGKVASPLEAPIFVVAPH
STFFDGIACVVAGLPSLVSRNENAQTPLVGRLLRALQPVLVSRVDPDSRKNTDNEIKKRATSGGEWQPQLVFP
EGTCTNRSCLITFKPFAFIPGVVPVQVLLRYPNKLDVTWTWQGYTFLQLCVLTFQCQLFTKVEIEFMPVQAPS
EEEKNDPVLFASTRIRNLMAEALEIPVTDHTYEDCRLMISAGQLTLPMEAGLVEFSKISRKLLDWDGIRKHL
DEYASIASSSKGGRIGIEFAEYLKLPVSDVLRQLFALFDRNNDGSIDFREYVIGLAVLCNPANTEEIIQVAFKL
FDVDEDGYITEEFCTILQASLGVPDLNVSGLFREIAQRDSVSYEEFKSFALKHPEYAKIFTTYLDLQTHVFS
LPVEVQTAPSVASNKVSPESQEETSDDKVDTRTRPLEQKLISEEDLAANDILDYKDDDDDKV

H Murine LPCAT2 INSERT MR218203 representing NM_173014

Red=Cloning site Blue=ORF Green=Tags(s)

TTTTGTAATACGACTCACTATAGGGCGGCCGGGAATTCGTCGACTGGATCCGGTACCGAGGAGATCTGC
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GCATGCTGTCTGAAAAGCTGACCCATCCAATAAGCAATTGGAGGAGGAAGATCACTCGGCCCGCTTT
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TAGTAATAAAGTCAGTCTGAGAGCCAGGAGGAGGGCACCTCAGACAAAAAGGTGGACACCGGTACG
CGGCCGCTCGAGCAGAAACTCATCTCAGAAGAGGATCTGGCAGCAAATGATATCTTGGATTACAAGGA
TGACGACGATAAAGGTTTAA

I Human LPCAT2 INSERT:

>RC200168 representing NM_017839

Red=Cloning site Blue=ORF Green=Tags(s)

TTTTGTAATACGACTCACTATAGGGCGGCCGGGAATTCGTCGACTGGATCCGGTACCGAGGAGAT
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ACCTATGAAGACTGCAGATTGATGATTTACGACGACAGCTAACATTGCCATGGAAGCTGGGCT
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GGAGATCATCCAGGTGGCATTAAAGCTGTTGACGTTGATGAGGATGGCTACATAACGGAGGAA
GAGTCTCCACCATTCTACAGGCTTCCCTTGGAGTGCCTGACCTTGTGTTTCTGGTCTCTTCAA
GAAATAGCCCAAGGGGACTCAATTTCTATGAGGAATTTAAAGTTTTGCTTAAAGCATCCAGA
ATATGCTAAGATATTTACAACATACCTAGACCTCCAGACGTCGCATGTGTTTTTATTACCAAAAGA
AGTCCAGACAACCCCTCCACCAGTAAATAAAGTCAAGCCCTGAAAAGCATGAAGAGATACCT
CAGACAAAAAGATGACACCGGTACCGGCCGCTCGAGCAGAAACTCATCTCAGAAGAGGATCT
GGCAGCAAATGATATCTTGGATTACAAGGATGACGACGATAAAGGTTTAA

Whole =1810
Without tag= 1646
With tags= 1731

J Protein sequencing of human LPCAT2

RC200168 representing NM_017839
 Red=Cloning site Green=Tags(s)

MSRCAQAAEVAATVPGAGVGNVGLRPPMVPRQASFFPPPVPNPFVQQTQIGSARRVQIVLLGILLPIRVLLV
 ALILLAWPFAAIS TVCCPEKLTHPITGWRRKITQTALKFLGRAMFFSMGFIVAVKGGKIASPLEAPVFVAAPH
 STFFDGIACVVAGLPSMVSRENENAQVPLIGRLLRAVQPVLSRVDPDSRKNNTINEIKRTTSGGEWQPQLVFPE
 GICTNRSCLITFKPGAFIPGVPVQPVLLRYPNKLDVTWTWQGYTFIQLCMLTFCQLFTKVEVEFMPVQVFN
 DEEKNDPVL FANKVRNLMAEALGHPVTDHTYEDCRLMISAGQLTLPMEAGLVEFTKISRKLKLDWDGVRK
 HLDEYASIASSSKGGRIGIEEFAKYLKLPVSDVLRQLFALFDRNHDGSDIFREYVIGLAVLCNPSNTEEIQVAF
 KLFVDVDEDDGYITEEEFSTILQASLGVPDLDVSGLFKEIAQGDSISYEEFKSFALKHPEYAKIFTTYLDLQCHV
 FSLPKEVQTPSTASNKVSPKHEESTSDKKDDTRTRPLEQKLISEEDLAANDILDYKDDDDDKV

Figure 11. “pCMV6- Entry Vector 4.9 kb (A) pCMV6-Entry (C-terminal Myc and DDK.

Tagged) that Schematic of the multiple cloning sites: pCMV6- Entry Vector 4.9 kb, (B) full Vector sequence; pCMV6-Entry, PS100001. 4919 bp, (C) circular map of The complete (human LPCAT-2 insert plus vector) .(D) RC200168 The complete (insert plus vector) sequence of [RC200168] clone (human LPCAT-2), (E) circular map of The complete (murine LPCAT-2 insert plus vector). (F) The complete (insert plus vector) sequence of [RC200168] clone (murine LPCAT-2), (G) Protein sequencing: for Murine LPCAT-2 (H) Murine LPCAT-2 insert /(I) Human LPCAT-2 insert (J) protein sequencing of human LPCAT-2. **Updated 2-5-2010, Origene, UK”.**

Tests of normality

	Kolmogorov-Smirnov ^a			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
VAR00001	.219	3	.	.987	3	.783
VAR00002	.297	3	.	.917	3	.441
VAR00003	.310	3	.	.899	3	.383

a. Lilliefors Significance Correction

<https://statistics.laerd.com/spss-tutorials/testing-for-normality-using-spss-statistics.php>

Table 5. The normality test for RT-PCR and ELISA data using SPSS version 22.

<https://statistics.laerd.com/spss-tutorials/testing-for-normality-using-spss-statistics.php>

1.2. Total RNA isolation by acid guanidinium thiocyanate-phenol- no chloroform extraction from RAW264.7 cell line (Adapted from Chomczynski Sacchi (1987)).

Reagents.

- Guanidinium thiocyanate (4 M)
- Sodium citrate (0.75 M, pH 7.0)
- N-laurosarcosine (sarkosyl) (10 %)
- 2-mercaptoethanol (BME)
- Sodium acetate (anhydrous) (2M, pH4.0)
- Glacial acetic acid
- Water saturated phenol
- 1 –bromo-3-chloropropane
- Isopropanol (~100 %)
- Ethanol (70 % or 75 %)
- RNase- and DNase- free water

Denaturing solution:

Stock denaturing solution was prepared by dissolving 25 g of guanidinium thiocyanate in 29.3 ml of water at 65°C, followed by 1.76ml of 0.75 M sodium citrate, pH 7.0, and 2.64 ml of 10 % sarkosyl. Denaturing solution was activated before use by addition of 72 µl of 2- mercaptoethanol to each 10ml of the stock solution.

2 M sodium acetate, pH 4.0:

16.42 g of sodium acetate (anhydrous) was added to 35 ml of water and 40ml glacial acetic acid. And then, the pH was adjusted to 4.0 with glacial acetic acid and the final volume was brought to 100ml with water.

Following the experiments, culture supernatants were removed from RAW264.7 cells and 500 μ l of denaturing solution was added directly to the wells. Cells were rested in denaturing solution for 1 - 2 minutes to allow complete lysis and the solution was pipetted up and down 10 times to ensure all the lysate was collected. The samples were allowed to sit for 5 minutes before being frozen at -20 °C for later analysis.

To extract the RNA, 50 μ l of 2 M sodium acetate, pH 4.0 was added to the cell lysates respectively and mixed thoroughly by inversion. Then, 500 μ l water saturated phenol was added, mixed by inversion, followed by 100 μ l of 1-bromo-3-chloropropane, and shaken vigorously by hand to ensure proper mixing. Samples were cooled on ice for 15 minutes then centrifuged for 20 minutes, 15000 g, at 4 °C.

And then, the upper aqueous phase containing mostly RNA was transferred to an RNase- and DNase free Eppendorf tube and 500 μ l of isopropanol was added to precipitate the RNA. Samples were incubated at -20 °C until needed for the next step.

Next, samples were centrifuged at 15000 g for 20 minutes at 4 °C. The supernatant was discarded and the gel-like precipitates (RNA) were retained then dissolved in 300 μ l of denaturing solution. And then, 300 μ l of isopropanol were added and the samples were incubated at -20 °C for at least 30 minutes (1 hour). Samples were incubated at -20 °C until needed for the next step or after which they were centrifuged for at 15000 g for 10 minutes at 4 °C. The supernatants were discarded and the pellets were re-suspended in 500 μ l of 75 % ethanol and vortexed for 10 seconds to mix, and then incubated for 15 minutes at room temperature to dissolve any residual guanidinium thiocyanate. At this point, samples were stored at -20 °C until RNA extraction could be completed.

Samples were centrifuged for at 15000 g for 10 minutes at 4 °C, and the supernatant was discarded. The pellets were air dried for around 15 - 30 minutes in a laminar flow hood at room temperature, and then the RNA was dissolved in 30 μ l of RNase- and DNase-

free water and incubated for 15 minutes at 60 °C to ensure complete solubilisation of the RNA.

To remove any residual genomic DNA, samples were treated with the DNase 1 kit (Sigma, UK). To the solubilised RNA, 3 µl of 10x reaction buffer and 3 µl amplification grade DNase 1 (1 unit /µl) were added and samples were left to stand at room temperature for 15 minutes. To stop the reaction, 3 µl stop solution was added to each tube. Samples were then incubated at 70 °C for 10 minutes, and then placed on ice ready to quantifying the total RNA concentrations using Nanodrop 2000 and then ready for reverse transcription.

1.2. Culture media:

1.2.1. SOC medium

Components:

20 g/L Tryptone

5 g/L Yeast Extract

4.8 g/L MgSO₄

3.603 g/L dextrose

0.5 g/L NaCl

0.186 g/L KCl

SOC Medium is a nutrient-rich microbial growth medium (a rich broth media) that contains peptides, amino acids, water-soluble vitamins, and glucose in a low-salt formulation used primarily in the recovery step of *Escherichia coli* (*E. coli*) competent cell transformations to maximize the transformation efficiency of competent cells.

1.2.2. LB Agar (250 ml)

LB broth (Sigma-Aldrich, Uk)	5 g
Bacteriological agar (Sigma-Aldrich, Uk)	3.75 g

Molecular water was added to a final volume of 250 ml, sterilized by autoclaving for 15 minutes at 100 kilopascal (kPa) i.e. 15 **pound** per square inch or, **pound**-force per square inch (psi or lb_f/in^2).

1.2.3. LB broth (500 ml) contain: NaCl 5 g, Tryptone 5 g, Yeast extract 2.5 g:

LB broth	10 g
----------	------

Molecular water was added to a final volume of 500ml, sterilized by autoclaving for 15 minutes at 100 kPa i.e. 15 psi (lb_f/in^2).

1.3.1. BCA assay protocol.

Standard series from 2 mg/mL of BSA stock

	A	B	C	D	E	F	G	H	
Required concentration	200	100	50	25	10	5	2.5	0	
Volume of PBS (μL)	450	250	250	250	300	250	250	250	
Volume of BSA (μL)	50	250	250	250	200	250	250	0	
	(Stock)	(A)	(B)	(C)	(D)	(E)	(F)	(G)	(H)

1- Micro BCA wells plate layout (96 well): it depends on the samples number

2- Micro BCA wells plate layout (96 well): it depends on the samples number

	1,2 (standard)	3,4 (Unknown)	5,6 (Unknown)	7,8 (Unknown)
A	200	1	9	17
B	100	2	10	18
C	50	3	11	19
D	25	4	12	20
E	10	5	13	21
F	5	6	14	22
G	2.5	7	15	23
H	0(Blank)	8	16	24

3- Add 100 μ L of each standard to column 1, 2.

4- Add each samples need diluting 1:25 in 100 μ L in all remaining well e.g 98 μ L PBS and 2 μ L of protein sample.

5- Mix the BCA reagents to give a solution of A: B: C of 25:24:1 in 100 μ L for each well.

e.g. 24 samples+8 standards= 32 well X 2= 64 + extra 2 for pipetting mistake. = 66

66 X 100 μ L for each= 6.6 mL

(A) $(6600/50) \times 25 = 3300 \mu\text{L}$

(B) $(6600/50) \times 24 = 3168 \mu\text{L}$

(C) $(6600/50) \times 1 = 132 \mu\text{L}$

6- Add the above amount into a trough, mix to ensure that the crystals are dissolved.

7- Multi- channel pipette, 100 μ L of BCA mix into each well.

8- Vortex plate for 1 minute on plate mixture and then Incubate or 37 $^{\circ}$ C for 2 hrs.

9- Plate reader: save it as protocol the read the plate.

10- Concentration = $\mu\text{g}/\text{mL} \times 50$ (dilution factor) to be ready for western blotting.

1.3.2. Gel electrophoresis using Novex system and NuPAGE® Bis – Tris Gel for western blotting

Pre – cast gels were used. 10 % polyacrylamide Bis-Tris Cl buffered Gel (pH, 6.4)

NuPAGE running buffer stock (10X):

52.3 g Mops

30.3 g Tris Base

5 g SDS

EDTA (0.5 M) = 10 mL

Heat the protein samples after preparation in a heat block at 70 °C for 10 minutes, and then heat the 20 biotin Marker (Millipore, UK).

Use 1X running buffer

Run the gel at 200 V for 90 minutes, Note: keep watching the gel running bands and make sure switch it off when they reach the end even before the 90 minutes.