Lysophosphatidylcholine acyltransferase 2 (LPCAT2) Regulates The Expression of Macrophage Phenotype Markers in RAW264.7 Cells

Running Title: LPCAT2 Regulates Macrophage Phenotype Markers

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1 Abstract:

Macrophages are key antigen presenting cells that also secrete cytokines during inflammation. They can be polarised to M1 or M2 phenotypes. Molecules such as CD206 and inducible Nitric Oxide Synthase are considered macrophage phenotype markers because they are highly expressed in either M1 or M2 macrophages. LPCAT2 is a phospholipid modifying enzyme that influences inflammatory responses in macrophages. However, how LPCAT2 influences inflammation is not fully understood.

In this study, we have used genetic technology to study the influence of LPCAT2 on macrophage phenotype markers.

Our results show for the first time that overexpression of LPCAT2 promotes the expression of M1 macrophage phenotype markers, and attenuates the expression of M2 macrophage markers.

2 Introduction:

Macrophages are primary effector cells of the innate immune system, that differentiate from monocytes. They belong to a group of phagocytic cells that stem from Myeloid progenitors. These cells have

30 a professional function of antigen-presentation and are characterised
31 with the ability to secrete cytokines that attract other immune cells
32 and initiate inflammation in response to pathogens at the site of
33 infection [1]. Macrophages can be polarized into classically activated or
34 alternatively activated phenotypes. Classically activated macrophages
35 are also known as M1 or M1-like macrophages because they
36 preferentially promote Th1 (T Helper 1) cell responses; thus they are
37 pro-inflammatory and release anti-microbial molecules such as nitric
38 oxide [1, 2]. On the other hand, alternatively activated macrophages
39 preferentially promote Th2 (T Helper 2) cell responses, therefore they
40 are often referred to as M2 or M2-like macrophages. M2 macrophages
41 mainly phagocytose pathogens, and often have a higher expression of
42 mannose and scavenger receptors compare to M1 macrophages. They
43 also promote cell proliferation and tissue repair [3, 4].

44 Certain molecules have been classed as markers of macrophage
45 polarization due to their consistently high expression in either
46 phenotypes of macrophages. In M1 macrophages, molecules such as
47 inducible Nitrogen Oxide Synthase (iNOS), CXCL10, Type I Interferons,
48 Tumour Necrosis Factor Alpha (TNF). Whereas, in M2 macrophages,
49 molecular markers include CD206, CD36, Interleukin 10. M2

50 macrophages also exhibit increased fatty acid oxidation, and
51 adipogenesis, and a higher expression of molecules involved in fatty
52 acid metabolism such as carnitine palmitoyltransferase, (PPAR) [5].

53 LPCAT2 (Lysophosphatidylcholine Acyltransferase) also known as
54 LysoPAF acetyltransferase localises in macrophage membranes and
55 lipid droplets [6, 7]. It preferentially uses arachidonic-CoA as a donor
56 for the acylation of fatty acids, thereby producing several varieties of
57 arachidonic acid. Arachidonic acid is a key precursor for the synthesis
58 of lipid mediators such as prostaglandins, leukotrienes, and lipoxins.
59 Therefore, LPCAT2 influences inflammation by regulating the
60 synthesis of fatty acids and lipid mediators such as PAF, Leukotriene
61 B4, and Prostaglandin E2, and the expression of proteins involved in
62 macrophage inflammatory responses to lipopolysaccharide (LPS) [8,
63 9]. Further research is needed to understand the mechanism of
64 LPCAT2 involvement in macrophage inflammatory responses. In this
65 paper, we propose that LPCAT2 regulates macrophage inflammatory
66 responses by influencing the expression of molecular markers of
67 macrophage polarization.

3 Materials and Methods:

Chemicals reagents used to prepare buffers and BCA Assay kit were purchased from Sigma Aldrich, UK and Fisher Scientific, UK. Buffers used include RIPA buffer, Phosphate-buffered saline (PBS), Tris-buffered saline (TBS), Blocking Buffer, Cell lysis buffer, elution buffer, SDS sample buffer, and ECL detection reagent [23]. PolyPlus Lipofectamine 2000 was purchased from Source Bioscience, UK. Opti-MEM, Power SYBR Green, RNa to cDNA kit, and gel casting materials were purchased from Life Technologies, UK. Antibodies, Protein A/G agarose gel beads, and protein ladders were obtained from Santa Cruz Biotechnology, UK and Cell Signalling Technologies, UK. DMEM culture medium and other cell culture materials were purchased from Lonza, UK. PCR primers were designed with Primer3 Plus Bioinformatics Softwaew and NCBI BLAST, and purchased from Eurofins Genomics.

3.1 Culturing of RAW264.7 Cell Line:

RAW264.7 cell line was obtained from the European Collection of Cell Cultures (ECACC) through Public Health England, UK. RAW264.7 macrophages were maintained in Dulbecco's Modified Eagle Medium (DMEM) [Lonza, BE12-914F] supplemented with 10%(v/v) Foetal Bovine Serum (FBS) [Labtech.com,

86 BS-110] and 1%(v/v) 0.2M L-Glutamine [BE17-605E], and incubated at 37ºC,
87 5% CO_2. Lipopolysaccharide (*E. coli* O111:B4) [Sigma-Aldrich, L2630] was
88 resuspended in LAL reagent water (<0.005EU/ml endotoxin levels) [Lonza,
89 W50-640]. Cell culture medium was used to dilute all ligands to the needed
90 concentration before stimulation.

91 *3.2 Expansion and Purification of Plasmids:*

92 Kanamycin-resistant TrueORF Gold plasmid was expanded by transformation
93 of 1 g/ml pCMV6 plasmid vector [4.9kb; OriGene, PS100001] with or without
94 murine LPCAT2 recombinant DNA [Open Reading Frame; Forward 5–CAG
95 ACT GTT ACG GGCTTT GCA - 3; Reverse 5– ACC TGA TGT CGC TCG CTTTT -
96 3] in 10 l Alpha-Gold competent *E. coli* cells [Insight Biotechnology, UK] using
97 GenElute™ plasmid MiniPrep Kit [Sigma-Aldrich, PLN10].

98 *3.3 Transfection of RAW264.7 Cells with Plasmid Containing LPCAT2 Insert:*

99 2.5μg of pCMV6 plasmid vector with or without murine LPCAT2
100 recombinant DNA was transfected into RAW264.7 macrophages using
101 Lipofectamine 2000 transfection reagent [PolyPlus transfection, 114-
102 07]. Macrophages were cultured in DMEM 24 hours before gene
103 silencing, then in Opti-MEM (Reduced Serum Medium) for 24 hours

104 during gene silencing and incubated at 37°C, 5% CO_2. Prior to cell
105 stimulation or any further experiments, Opti-MEM (Reduced Serum
106 Medium) was replaced with DMEM cell culture medium for 24 hours
107 and incubated at 37ºC, 5% CO_2. Transfection mixture was prepared
108 using Opti-MEM.

109 *3.4 Reverse Transcription and Real Time Quantitative PCR (RT-qPCR):*

110 The reaction mastermix contained 37%(v/v) nuclease-free, 230nM of
111 target primers (a mixture of both forward and reverse primers),
112 60%(v/v) Power SYBR Green, and 3μl of ≥ 100ng/μl cDNA. The reaction
113 was initiated at 95ºC for 10 minutes, then up to 40 repeated cycles of
114 denaturing (15 seconds, 95ºC), annealing, and extension (60 seconds,
115 60ºC). GAPDH was used as an endogenous control. GAPDH [forward
116 sequence, 5–CCT CGT CCC GTA GAC AAA ATG; reverse sequence, 5–
117 TCT CCA CTT TGC CAC TGC AA]; LPCAT2 [forward sequence, 5–TGG
118 AAG CGC CCA TTT TTG TC; reverse sequence, 5–GGG ATG AAG GCT
119 CTG CCA AC]; iNOS [forward sequence, 5 – CGC CTT CAA CAC CAA
120 GGT TG; reverse sequence, 5 – TCA GAG TCT GCC CAT TGC TG];
121 COX2 [forward sequence, 5–CCA CAG TCA AAG ACA CTC AGG TAG A;
122 reverse sequence, 5– CCA GGC ACC AGA CCA AAG AC]; PPAR

123 [forward sequence, 5–CCA CTC GCA TTC GTT TGA CA; reverse
124 sequence, 5 – TCG CTC AGC TCT TCC GAA GTG]; CD206 [forward
125 sequence, 5– AAA TGG AGC CGT CTG TGC AT; reverse sequence, 5 –
126 AAG TGC AAT GGA CAA AAT CCAA].

127 *3.5 Immunoblotting:*

128 Equal amounts of whole cell lysates and eluates were separated on pre-cast
129 SDS-PAGE gels and blotted on to a PVDF membrane using a blot module. The
130 blots were blocked with 0.1% bovine serum albumin in PBS-0.1% Tween 20,
131 probed with primary and then HRP-conjugated secondary antibodies. The
132 target proteins separated on the PVDF membrane were detected and
133 analysed using ImageJ.

134 *3.6 Data Analysis:*

135 Statistical Analysis was carried out in R Statistical Programming
136 Software and graphs were plotted using ggplot2 package. All
137 independent experiments were repeated at least 3 times. Data
138 represent Mean ± Standard Error of Mean. Paired T-Test with
139 Dunnetts T3 multiple comparison test were used for statistical analysis.

140 All statistical tests were significant at 95% confidence interval, $p \leq$
141 0.05.

142 4 Results:

143 *4.1 Cloning of RAW264.7 Cells Over-expressing LPCAT2:*

144 LPCAT2 was cloned in E. coli and over-expressed in RAW264.7 cells using
145 vector plasmid carrying a neomycin-resistant gene. After 3 days, RAW264.7
146 cells transfected with empty vector pasmid (pCMV6) and treated with
147 700µg/ml of geneticin, however, RAW264.7 cells transfected with neomycin-
148 resistant vector plasmid over-expressing LPCAT2 survived for up to 7 days
149 [data not shown]. Thus, cells not over-expressing LPCAT2 were killed off,
150 allowing for cloning of cells over-expressing LPCAT2.

151 *4.2 Expression of LPCAT2 in RAW264.7 Cell Transfected with LPCAT2 siRNA or*
152 *Vector Plasmid Over-Expressing LPCAT2:*

153 The gene and protein expression of LPCAT2 were analysed to confirm the
154 over-expression of LPCAT2. Figure 1A shows that LPCAT2 gene is
155 significantly higher in cells over-expressing LPCAT2 (4.18 ± 1.1, $p = 0.05$), and

156 LPCAT2 protein increased (6-fold) in cells over-expressing LPCAT2 (Figure

157 1B&C).

158

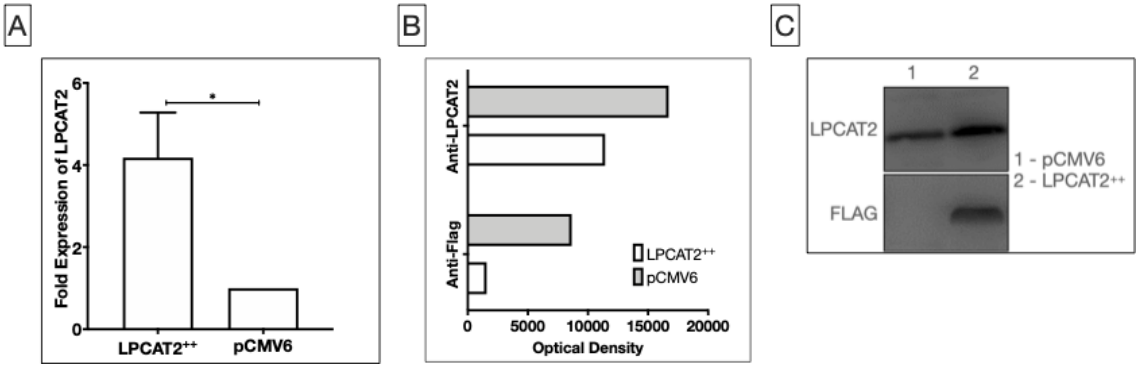

Figure 1: Over-expression of LPCAT2 in RAW264.7 cells caused ≥70 increase in LPCAT2 gene expression **(A)** and ≥50% increase in LPCAT2 protein expression **(B & C)**. pCMV6 is an empty vector plasmid, whereas LPCAT2^{++} indicates cells over-expressing LPCAT2. *p≤0.05. Data represents mean of at least 3 independent experiments (n≥3) ± standard error (A), and optical densities (B) of western blots (C).

159

160 *4.3 LPCAT2 Regulates the Expression of CD206, Inducible Nitric Oxide Synthase*

161 *(iNOS), PPARγ, and Cyclo-oxygenase 2 (COX2):*

162 Here, the effect of LPCAT2 over-expression on the gene expression of these

163 macrophage phenotype markers was examined. Figure 2 shows that in

164 RAW264.7 cells over-expressing LPCAT2, there was a significant increase in

165 iNOS (281.43 ± 13.33, $p < 0.0001$) and COX2 (2659.39 ± 253.09, $p = 0.0005$)

166 after treating the cells with 100ng/ml LPS. On the other hand, LPS

167 significantly downregulated the gene expression of CD206 (0.89 ± 0.07, $p = 0.03$) and PPARγ (0.4 ± 0.004, $p = 0.0009$), and over-expression of LPCAT2
168
169 further decreased the gene expression of CD206 (0.7 ± 0.07, $p = 0.1$) and
170 PPARγ (0.16 ± 0.003, $p = 0.3$). In non-treated cells, LPCAT2 over-expression
171 significantly increased the gene expression of CD206 (3.22 ± 0.38, $p = 0.0012$)
172 and iNOS (44.48 ± 8.46, $p = 0.0021$), but significantly decreased the gene
173 expression of PPARγ (0.19 ± 0.03, $p < 0.0001$).

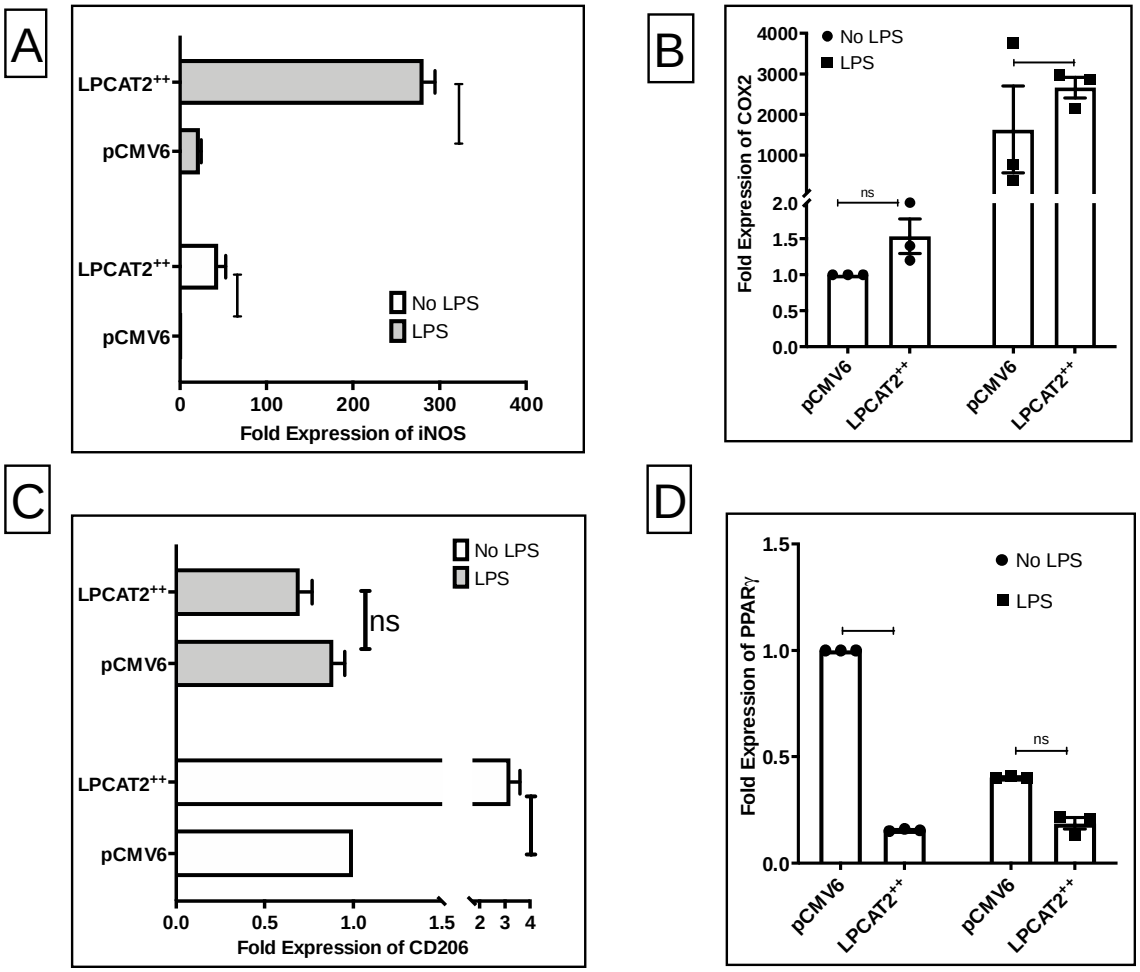

Figure 2: Over-expression of LPCAT2 increases the gene expression of inducible Nitric Oxide Synthase, iNOS (**A**) and Cyclo-oxygenase 2, COX2 (**B**). On the other hand, over-expression of LPCAT2 results in a significant decrease in the gene expression of CD206 (**C**) and peroxisome proliferator-activated receptor gamma, PPAR γ (**D**). TLR4 ligand is 100ng/ml E. coli O111:B4 lipopolysaccharide. pCMV6 represents RAW264.7 cells transfected with empty vector plasmid and LPCAT2++ represents RAW264.7 cells over-expressing LPCAT2. *$p \leq 0.05$. Data represents the mean of 3 independent experiments (n=3) $\pm$ standard error.

4.4 *LPCAT2 Expression is Significantly Increased in Primed RAW264.7 Cells:*

177 Figure 3 shows that priming RAW264.7 cells with 25ng/ml IFNγ for 24 hours
178 and treating the cells for 2 hours with TLR4 ligand, LPS, significantly increases
179 the gene expression of LPCAT2 (2.71 ± 0.63, $p = 0.03$). Likewise, priming
180 RAW264.7 cells with a PPARγ antagonist, T0070907, and then treating the
181 cells for 6 hours with LPS results in a dose-dependent increase in LPCAT2
182 gene expression (3.37 ± 0.84, $p = 0.03$; 5.38 ± 1.45, $p = 0.002$; 9.77 ± 1.72, $p =$
183 0.02) as seen in Figure 3B.

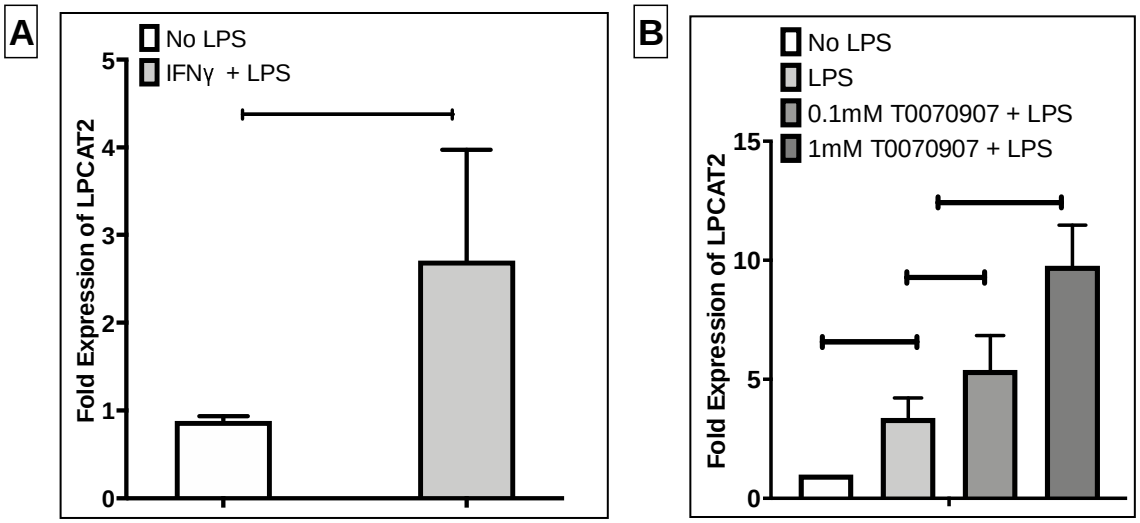

Figure 3: LPCAT2 gene expression is significantly increased in primed RAW264.7 cells. RAW264.7 cells were primed with either 25ng/ml of Interferon gamma (IFNγ) or varying concentrations of PPARγ antagonist (T0070907) and then stimulated with either 100ng/ml **(A)** or 1000ng/ml **(B)** of TLR4 ligand (*E. coli* O111:B4 lipopolysaccharide). *p≤0.05. Data represents mean of 3 independent experiments (n=3) ± standard error.

5 Discussion:

COX2 and iNOS [5, 10] are common markers of M1 macrophages, and CD206 [11] and PPARγ [12] are markers of M2 macrophage. PPARγ receptor and TLR4 are some of the key receptors responsible for regulating macrophage activation to either M1 or M2 phenotypes [13]. During inflammation there is an increased release of chemokines and cytokines which can be regulated by various factors including Nitric oxide and prostaglandins [14]. COX2

193 synthesises prostaglandins by breaking down arachidonic acids – a catabolic
194 process [15]. On the other hand, PPAR mainly store fatty acids as
195 triglycerides in adipocytes – an anabolic process [16]. Anabolic processes are
196 increased in M2 macrophages, whereas catabolic processes are increased in
197 M1 macrophages [5]. Therefore, our results imply that LPCAT2 promotes
198 catabolism and synthesis of lipid mediators, while inhibiting anabolism or
199 adipogenesis (Figures 2 & 3). The inflammatory influence of LPCAT2 stems
200 from its anabolic activity which builds up inflammatory mediator – PAF, or
201 Arachidonic acid – a precursor for lipid mediators.

202 Pro-inflammatory conditions induce an increased expression of LPCAT2 as
203 seen in Figure 3, implying a possible increase in its enzymatic activity which
204 would lead to higher amounts of arachidonic acid [18]. Accumulation of
205 arachidonic acid increases the expression and activity of COX2 [19, 20]. This is
206 in line with our results which shows that LPCAT2 over-expression induces an
207 increased expression of COX2 after stimulation with LPS. Furthermore, Over-
208 expression of LPCAT2 significantly increased iNOS expression, both in LPS-
209 stimulated and nonstimulated macrophages. The synthesis of nitric oxide by
210 nitric oxide synthases and its release are hallmarks of inflammation [8], and
211 inducible nitric oxide synthase (iNOS also known as NOS2), is classed as an M1

212 marker because of its consistently high expression in pro-inflammatory
213 macrophages [5].

214 Cluster of Differentiation molecules are widely used to identify
215 immune cell phenotypes and function, CD206 belongs to this class of
216 molecules [23]. CD206 expression was not induced in LPS-stimulated
217 macrophages over-expressing). CD206 is expressed in both M1 and M2
218 macrophages. CD206 (a mannose receptor) is commonly known as an
219 M2 macrophage marker because it is highly expressed in M2
220 macrophages, and is induced predominantly by interleukin 4 (which
221 induces M2 macrophage polarization) [5, 22]. A scientific publication
222 claimed that there is no difference in CD206 mRNA expression in M1
223 and M2 macrophage phenotypes, however, the significant difference
224 may have been masked by the variability of CD206 mRNA expression
225 [2].

226 In conclusion, it is evident from the results that the expression of
227 some macrophage phenotype markers are dependent on LPCAT2.
228 Moreover, LPCAT2 promotes the expression of M1 markers while
229 attenuating the expression of M2 macrophage markers. Therefore, this
230 paper is further evidence that LPCAT2 is pro-inflammatory and
231 regulates inflammation by inducing a higher expression of M1 markers.

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