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

Running Title: LPCAT2 Regulates Macrophage Phenotype Markers

Authors: Victory Ibigo Poloamina[1], Hanaa Alrammah[1], Wondwossen Abate[2], Gyorgy Fejer[1], Simon K. Jackson[3].

[1]University of Plymouth, Faculty of Health, Plymouth, UK PL4 8AA

[2]University of Exeter, College of Medicine and Health, Exeter, UK EX1 2HZ

[3]MolEndoTech Ltd, Brixham, UK TQ5 8BA

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 lipid mediator 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

30 phagocytic cells that stem from Myeloid progenitors. These cells have
31 a professional function of antigen-presentation and are characterised
32 with the ability to secrete cytokines that attract other immune cells
33 and initiate inflammation in response to pathogens at the site of
34 infection [1]. Macrophages can be polarized into classically activated
35 or alternatively activated phenotypes. Classically activated
36 macrophages are also known as M1 or M1-like macrophages because
37 they preferentially promote Th1 (T Helper 1) cell responses; thus they
38 are pro-inflammatory and release anti-microbial molecules such as
39 nitric oxide [1, 2]. On the other hand, alternatively activated
40 macrophages preferentially promote Th2 (T Helper 2) cell responses,
41 therefore they are often referred to as M2 or M2-like macrophages. M2
42 macrophages mainly phagocytose pathogens, and often have a higher
43 expression of mannose and scavenger receptors compare to M1
44 macrophages. They also promote cell proliferation and tissue repair [3,
45 4].

46 Certain molecules have been classed as markers of macrophage
47 polarization due to their consistently high expression in either
48 phenotypes of macrophages. In M1 macrophages, molecules such as

49 inducible Nitrogen Oxide Synthase (iNOS), CXCL10, Type I
50 Interferons, Tumour Necrosis Factor Alpha (TNFα). Whereas, in M2
51 macrophages, molecular markers include CD206, CD36, Interleukin
52 10. M2 macrophages also exhibit increased fatty acid oxidation, and
53 adipogenesis, and a higher expression of molecules involved in fatty
54 acid metabolism such as carnitine palmitoyltransferase, (PPARγ) [5].

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

68 by influencing the expression of molecular markers of macrophage
69 polarization.

70 3 Materials and Methods:

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

83 *3.1 Culturing of RAW264.7 Cell Line:*

84 RAW264.7 cell line was obtained from the European Collection of Cell
85 Cultures (ECACC) through Public Health England, UK. RAW264.7
86 macrophages were maintained in Dulbecco's Modified Eagle Medium
87 (DMEM) [Lonza, BE12-914F] supplemented with 10%(v/v) Foetal Bovine
88 Serum (FBS) [Labtech.com, BS-110] and 1%(v/v) 0.2M L-Glutamine [BE17-
89 605E], and incubated at 37ºC, 5% CO_2. Lipopolysaccharide (*E. coli* O111:B4)
90 [Sigma-Aldrich, L2630] was resuspended in LAL reagent water (<0.005EU/ml
91 endotoxin levels) [Lonza, W50-640]. Cell culture medium was used to dilute
92 all ligands to the needed concentration before stimulation.

93 *3.2 Expansion and Purification of Plasmids:*

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

100 *3.3 Transfection of RAW264.7 Cells with Plasmid Containing LPCAT2*

101 *Insert:*

102 2.5μg of pCMV6 plasmid vector with or without murine LPCAT2

103 recombinant DNA was transfected into RAW264.7 macrophages using

104 Lipofectamine 2000 transfection reagent [PolyPlus transfection, 114-

105 07]. Macrophages were cultured in DMEM 24 hours before gene

106 silencing, then in Opti-MEM (Reduced Serum Medium) for 24 hours

107 during gene silencing and incubated at 37ºC, 5% CO_2. Prior to cell

108 stimulation or any further experiments, Opti-MEM (Reduced Serum

109 Medium) was replaced with DMEM cell culture medium for 24 hours

110 and incubated at 37ºC, 5% CO_2. Transfection mixture was prepared

111 using Opti-MEM.

112 *3.4 Reverse Transcription and Real Time Quantitative PCR (RT-qPCR):*

113 The reaction mastermix contained 37%(v/v) nuclease-free, 230nM of

114 target primers (a mixture of both forward and reverse primers),

115 60%(v/v) Power SYBR Green, and 3μl of ≥ 100ng/μl cDNA. The

116 reaction was initiated at 95ºC for 10 minutes, then up to 40 repeated

117 cycles of denaturing (15 seconds, 95ºC), annealing, and extension (60

118 seconds, 60ºC). GAPDH was used as an endogenous control. GAPDH
119 [forward sequence, 5–CCT CGT CCC GTA GAC AAA ATG; reverse
120 sequence, 5–TCT CCA CTT TGC CAC TGC AA]; LPCAT2 [forward
121 sequence, 5–TGG AAG CGC CCA TTT TTG TC; reverse sequence, 5–
122 GGG ATG AAG GCT CTG CCA AC]; iNOS [forward sequence, 5 –
123 CGC CTT CAA CAC CAA GGT TG; reverse sequence, 5 – TCA GAG
124 TCT GCC CAT TGC TG]; COX2 [forward sequence, 5–CCA CAG TCA
125 AAG ACA CTC AGG TAG A; reverse sequence, 5– CCA GGC ACC
126 AGA CCA AAG AC]; PPARγ [forward sequence, 5–CCA CTC GCA
127 TTC GTT TGA CA; reverse sequence, 5 – TCG CTC AGC TCT TCC
128 GAA GTG]; CD206 [forward sequence, 5– AAA TGG AGC CGT CTG
129 TGC AT; reverse sequence, 5 – AAG TGC AAT GGA CAA AAT
130 CCAA].

131 *3.5 Immunoblotting:*

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

136 target proteins separated on the PVDF membrane were detected and analysed
137 using ImageJ.

138 *3.6 Data Analysis:*

139 Statistical Analysis was carried out in R Statistical Programming
140 Software and graphs were plotted using ggplot2 package. All
141 independent experiments were repeated at least 3 times. Data
142 represent Mean ± Standard Error of Mean. Paired T-Test with
143 Dunnetts T3 multiple comparison test were used for statistical analysis.
144 All statistical tests were significant at 95% confidence interval, $p \leq$
145 0.05.

146 4 Results:

147 *4.1 Cloning of RAW264.7 Cells Over-expressing LPCAT2:*

148 LPCAT2 was cloned in E. coli and over-expressed in RAW264.7 cells using
149 vector plasmid carrying a neomycin-resistant gene. After 3 days, RAW264.7
150 cells transfected with empty vector pasmid (pCMV6) and treated with
151 700µg/ml of geneticin, however, RAW264.7 cells transfected with neomycin-

152 resistant vector plasmid over-expressing LPCAT2 survived for up to 7 days
153 [data not shown]. Thus, cells not over-expressing LPCAT2 were killed off,
154 allowing for cloning of cells over-expressing LPCAT2.

155 *4.2 Expression of LPCAT2 in RAW264.7 Cell Transfected with LPCAT2*
156 *siRNA or Vector Plasmid Over-Expressing LPCAT2:*

157 The gene and protein expression of LPCAT2 were analysed to confirm the
158 over-expression of LPCAT2. Figure 1A shows that LPCAT2 gene is
159 significantly higher in cells over-expressing LPCAT2 (4.18 ± 1.1, p = 0.05),
160 and LPCAT2 protein increased (6-fold) in cells over-expressing LPCAT2
161 (Figure 1B&C).

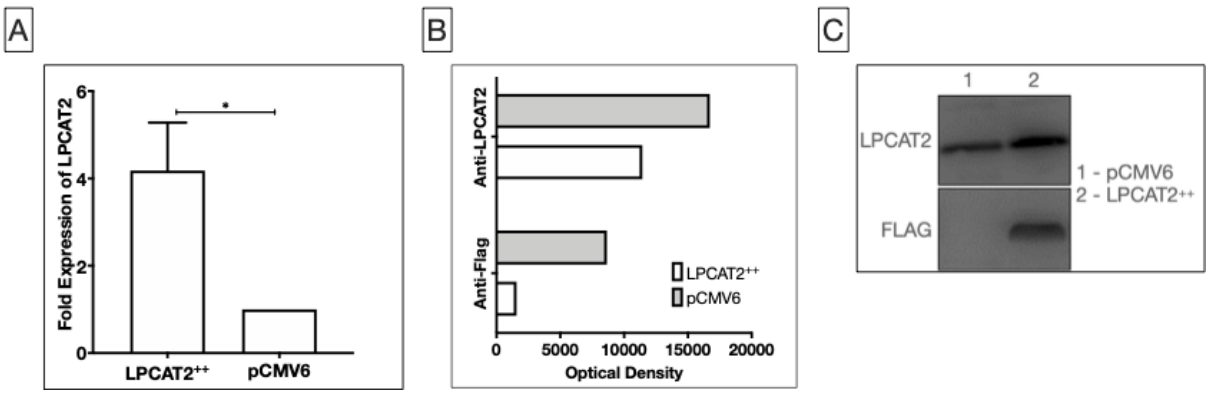
162 

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).

163

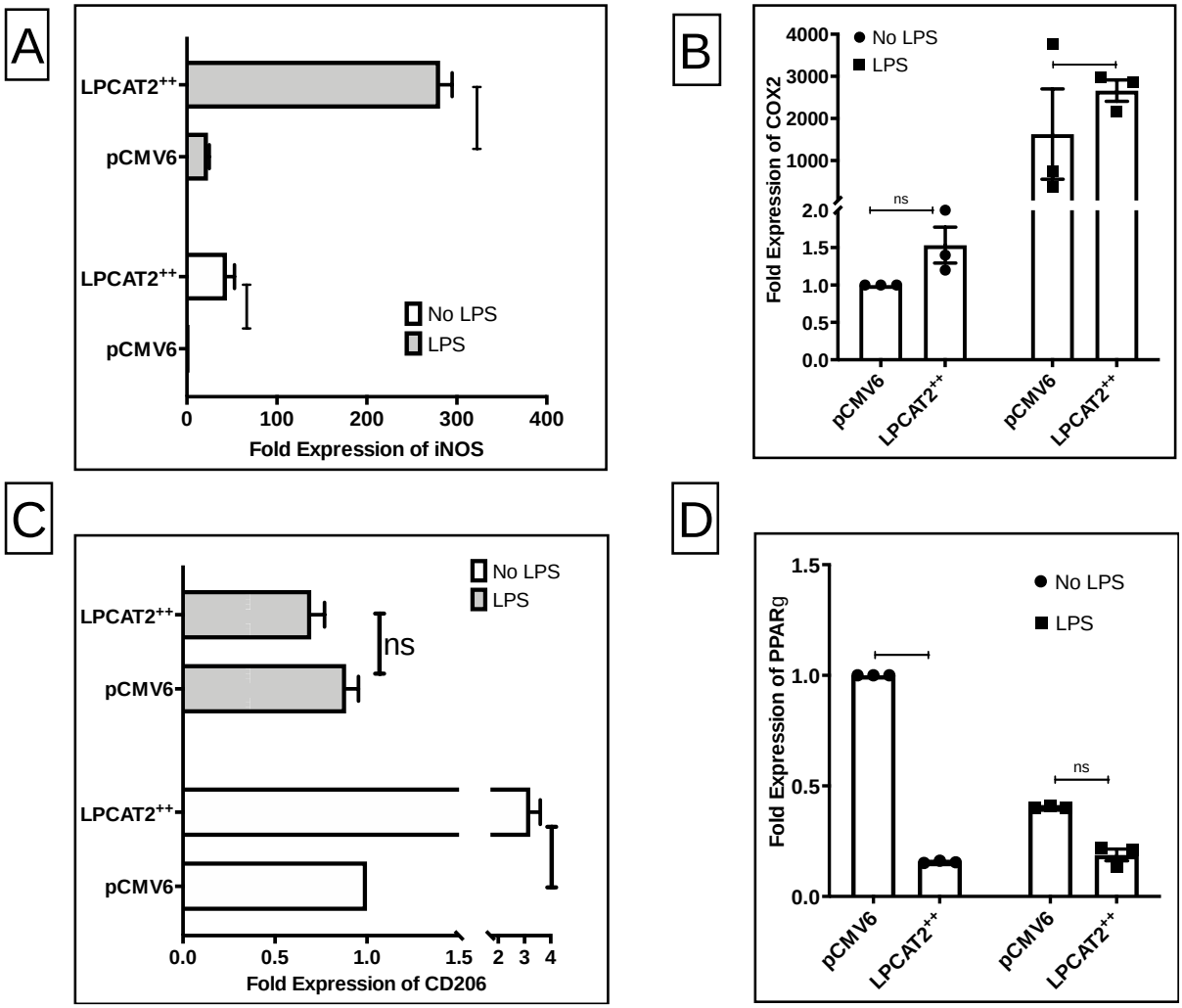
164 *4.3 LPCAT2 Regulates the Expression of CD206, Inducible Nitric Oxide*

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

166 Here, the effect of LPCAT2 over-expression on the gene expression of these
167 macrophage phenotype markers was examined. Figure 2 shows that in
168 RAW264.7 cells over-expressing LPCAT2, there was a significant increase in
169 iNOS (281.43 ± 13.33, $p < 0.0001$) and COX2 (2659.39 ± 253.09, $p = 0.0005$)
170 after treating the cells with 100ng/ml LPS. On the other hand, LPS
171 significantly downregulated the gene expression of CD206 (0.89 ± 0.07, p =
172 0.03) and PPARγ (0.4 ± 0.004, $p = 0.0009$), and over-expression of LPCAT2
173 further decreased the gene expression of CD206 (0.7 ± 0.07, $p = 0.1$) and
174 PPARγ (0.16 ± 0.003, $p = 0.3$). In non-treated cells, LPCAT2 over-expression
175 significantly increased the gene expression of CD206 (3.22 ± 0.38, $p = 0.0012$)

176 and iNOS (44.48 ± 8.46, $p = 0.0021$), but significantly decreased the gene

177 expression of PPARγ (0.19 ± 0.03, $p < 0.0001$).



178

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, PPARγ (**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.

179

180 *4.4 LPCAT2 Expression is Significantly Increased in Primed RAW264.7*

181 *Cells:*

182 Figure 3 shows that priming RAW264.7 cells with 25ng/ml IFNγ for 24 hours

183 and treating the cells for 2 hours with TLR4 ligand, LPS, significantly increases

184 the gene expression of LPCAT2 (2.71 ± 0.63, $p = 0.03$). Likewise, priming

185 RAW264.7 cells with a PPARγ antagonist, T0070907, and then treating the

186 cells for 6 hours with LPS results in a dose-dependent increase in LPCAT2

187 gene expression (3.37 ± 0.84, $p = 0.03$; 5.38 ± 1.45, $p = 0.002$; 9.77 ± 1.72, $p =$

188 0.02) as seen in Figure 3B.

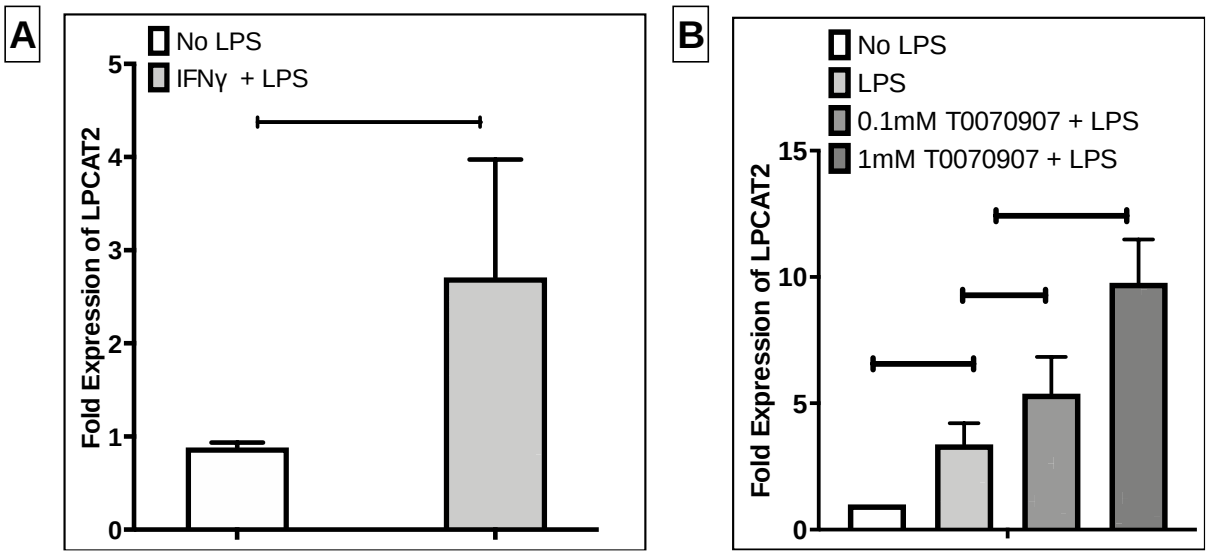


Figure 3: LPCAT2 gene expression is significantly increased in primed RAW264.7 cells. RAW264.7 cells were primed with either 25ng/ml of Interferon gamma (IFNγ) or varying concentrations of PPARγ antagonist (T0070907) and then stimulated with either 100ng/ml **(A)** or 1000ng/ml **(B)** of TLR4 ligand (*E. coli* O111:B4 lipopolysaccharide). *$p \leq 0.05$. 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

197 various factors including Nitric oxide and prostaglandins [14]. COX2
198 synthesises prostaglandins by breaking down arachidonic acids – a catabolic
199 process [15]. On the other hand, PPARγ mainly store fatty acids as
200 triglycerides in adipocytes – an anabolic process [16]. Anabolic processes are
201 increased in M2 macrophages, whereas catabolic processes are increased in
202 M1 macrophages [5]. Therefore, our results imply that LPCAT2 promotes
203 catabolism and synthesis of lipid mediators, while inhibiting anabolism or
204 adipogenesis (Figures 2 & 3). The inflammatory influence of LPCAT2 stems
205 from its anabolic activity which builds up inflammatory mediator – PAF, or
206 Arachidonic acid – a precursor for lipid mediators.

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

216 inducible nitric oxide synthase (iNOS also known as NOS2), is classed as an
217 M1 marker because of its consistently high expression in pro-inflammatory
218 macrophages [5].

219 Cluster of Differentiation molecules are widely used to identify
220 immune cell phenotypes and function, CD206 belongs to this class of
221 molecules [23]. CD206 expression was not induced in LPS-stimulated
222 macrophages over-expressing). CD206 is expressed in both M1 and M2
223 macrophages. CD206 (a mannose receptor) is commonly known as an
224 M2 macrophage marker because it is highly expressed in M2
225 macrophages, and is induced predominantly by interleukin 4 (which
226 induces M2 macrophage polarization) [5, 22]. A scientific publication
227 claimed that there is no difference in CD206 mRNA expression in M1
228 and M2 macrophage phenotypes, however, the significant difference
229 may have been masked by the variability of CD206 mRNA expression
230 [2].

231 In conclusion, it is evident from the results that the expression of
232 some macrophage phenotype markers are dependent on LPCAT2.
233 Moreover, LPCAT2 promotes the expression of M1 markers while

attenuating the expression of M2 macrophage markers. Therefore, this paper is further evidence that LPCAT2 is pro-inflammatory and regulates inflammation by inducing a higher expression of M1 markers.

6 References:

[1] Owen, J. A., Punt, J., Stranford, S. A., Jones, P. P. & Kuby, J. Kuby immunology (W.H. Freeman, New York, 2013), 7th ed edn. URL http://www.loc.gov/catdir/enhancements/fy1502/2012950797-d.html.

[2] Jaguin, M., Houlbert, N., Fardel, O. & Lecureur, V. Polarization profiles of human m-csfgenerated macrophages and comparison of m1-markers in classically activated macrophages from gm-csf and m-csf origin. Cell Immunol 281, 51–61 (2013).

[3] Mosser, D. M. The many faces of macrophage activation. J Leukoc Biol 73, 209–12 (2003).

[4] Nikonova, A. et al. M1-like macrophages are potent producers of anti-viral interferons and m1-associated marker-positive lung macrophages are

250 decreased during rhinovirus-induced asthma exacerbations. EBioMedicine 54,
251 102734 (2020).

252 [5] Orecchioni, M., Ghosheh, Y., Pramod, A. B. & Ley, K. Macrophage
253 polarization: Different gene signatures in m1(lps+) vs. classically and m2(lps-
254) vs. alternatively activated macrophages. Front Immunol 10, 1084 (2019).

255 [6] Moessinger, C. et al. Two different pathways of phosphatidylcholine
256 synthesis, the kennedy pathway and the lands cycle, differentially regulate
257 cellular triacylglycerol storage. BMC Cell Biol 15, 43 (2014).

258 [7] Abate, W., Alrammah, H., Kiernan, M., Tonks, A. J. & Jackson, S. K.
259 Lysophosphatidylcholine acyltransferase 2 (lpcat2) co-localises with tlr4 and
260 regulates macrophage inflammatory gene expression in response to lps. Sci
261 Rep 10, 10355 (2020).

262 [8] Harayama, T., Shindou, H., Ogasawara, R., Suwabe, A. & Shimizu, T.
263 Identification of a novel noninflammatory biosynthetic pathway of platelet-
264 activating factor. J Biol Chem 283, 11097–106 (2008).

265 [9] Serhan, C. N., Ward, P. A. & Gilroy, D. W. Fundamentals of inflammation
266 (Cambridge University Press, Cambridge, 2010).

267 [10] Giroux, M. & Descoteaux, A. Cyclooxygenase-2 expression in
268 macrophages: modulation by protein kinase c-alpha. J Immunol 165, 3985–91
269 (2000).

270 [11] Bertani, F. R. et al. Classification of m1/m2-polarized human
271 macrophages by label-free hyperspectral reflectance confocal microscopy and
272 multivariate analysis. Sci Rep 7, 8965 (2017).

273 [12] Bouhlel, M. A. et al. Ppargamma activation primes human monocytes into
274 alternative m2 macrophages with anti-inflammatory properties. Cell Metab 6,
275 137–43 (2007).

276 [13] Glass, C. K. & Natoli, G. Molecular control of activation and priming in
277 macrophages. Nat Immunol 17, 26–33 (2016).

278 [14] Kobayashi, Y. The regulatory role of nitric oxide in proinflammatory
279 cytokine expression during the induction and resolution of inflammation. J
280 Leukoc Biol 88, 1157–62 (2010).

281 [15] Rafiee, L., Hajhashemi, V. & Javanmard, S. H. Maprotiline inhibits cox2
282 and inos gene expression in lipopolysaccharide-stimulated u937 macrophages

283 and carrageenan-induced paw edema in rats. Cent Eur J Immunol 44, 15–22
284 (2019).

285 [16] Trümper, V. et al. Redox regulation of ppar in polarized macrophages.
286 PPAR Res 2020, 8253831 (2020).

287 [17] Shimakata, T. & Stumpf, P. K. The purification and function of acetyl
288 coenzyme a:acyl carrier protein transacylase. J Biol Chem 258, 3592–8 (1983).

289 [18] Morimoto, R., Shindou, H., Tarui, M. & Shimizu, T. Rapid production of
290 platelet-activating factor is induced by protein kinase c-mediated
291 phosphorylation of lysophosphatidylcholine acyltransferase 2 protein. J Biol
292 Chem 289, 15566–76 (2014).

293 [19] Nakatsuka, A., Mizuno, R., Ono, N., Nakayama, J. & Ohhashi, T.
294 Arachidonic acid-induced cox-1 and cox-2-mediated vasodilation in rat
295 gingival arterioles in vivo. Jpn J Physiol 55, 293–302 (2005).

296 [20] Hayek, M. G. et al. Enhanced expression of inducible cyclooxygenase
297 with age in murine macrophages. J Immunol 159, 2445–51 (1997).

298 [21] Jiang, Z. et al. Cd14 is required for myd88-independent lps signaling. Nat
299 Immunol 6,565–70 (2005).

300 [22] Schulz, D., Severin, Y., Zanotelli, V. R. T. & Bodenmiller, B. In-depth
301 characterization of monocyte-derived macrophages using a mass cytometry-
302 based phagocytosis assay. Sci Rep 9, 1925 (2019).

303 [23] Cruse, J., Lewis, R. & Wang, H. Immunology Guidebook (Elsevier, 2004).