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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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

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

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

49 inducible Nitrogen Oxide Synthase (iNOS), CXCL10, Type I
50 Interferons, Tumour Necrosis Factor Alpha (TNF α). 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, (PPAR γ) [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 Software
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 37°C, 5% CO₂. 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].

3.3 Transfection of RAW264.7 Cells with Plasmid Containing LPCAT2

Insert:

2.5 μ g of pCMV6 plasmid vector with or without murine LPCAT2 recombinant DNA was transfected into RAW264.7 macrophages using Lipofectamine 2000 transfection reagent [PolyPlus transfection, 114-07]. Macrophages were cultured in DMEM 24 hours before gene silencing, then in Opti-MEM (Reduced Serum Medium) for 24 hours during gene silencing and incubated at 37°C, 5% CO₂. Prior to cell stimulation or any further experiments, Opti-MEM (Reduced Serum Medium) was replaced with DMEM cell culture medium for 24 hours and incubated at 37°C, 5% CO₂. Transfection mixture was prepared using Opti-MEM.

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

The reaction mastermix contained 37%(v/v) nuclease-free, 230nM of target primers (a mixture of both forward and reverse primers), 60%(v/v) Power SYBR Green, and 3 μ l of $\geq$ 100ng/ μ l cDNA. The reaction was initiated at 95°C for 10 minutes, then up to 40 repeated cycles of denaturing (15 seconds, 95°C), annealing, and extension (60

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

3.5 Immunoblotting:

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

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

3.6 Data Analysis:

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

4 Results:

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

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

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

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

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

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

4.3 *LPCAT2 Regulates the Expression of CD206, Inducible Nitric Oxide Synthase (iNOS), PPAR γ , and Cyclo-oxygenase 2 (COX2):*

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

Figure 3 shows that priming RAW264.7 cells with 25ng/ml IFN γ for 24 hours and treating the cells for 2 hours with TLR4 ligand, LPS, significantly increases the gene expression of *LPCAT2* (2.71 ± 0.63 , $p = 0.03$). Likewise, priming RAW264.7 cells with a PPAR γ antagonist, T0070907, and then treating the cells for 6 hours with LPS results in a dose-dependent increase in *LPCAT2* gene expression (3.37 ± 0.84 , $p = 0.03$; 5.38 ± 1.45 , $p = 0.002$; 9.77 ± 1.72 , $p = 0.02$) 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 (IFN γ) or varying concentrations of PPAR γ 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) $\pm$ standard error.

5 Discussion:

COX2 and iNOS [5, 10] are common markers of M1 macrophages, and CD206 [11] and PPAR γ [12] are markers of M2 macrophage. PPAR γ 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 synthesises prostaglandins by breaking down arachidonic acids – a catabolic process [15]. On the other hand, PPAR γ mainly store fatty acids as triglycerides in adipocytes – an anabolic process [16]. Anabolic processes are increased in M2 macrophages, whereas catabolic processes are increased in M1 macrophages [5]. Therefore, our results imply that LPCAT2 promotes catabolism and synthesis of lipid mediators, while inhibiting anabolism or adipogenesis (Figures 2 & 3). The inflammatory influence of LPCAT2 stems from its anabolic activity which builds up inflammatory mediator – PAF, or Arachidonic acid – a precursor for lipid mediators.

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

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

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