Title: Possible Regulation of Toll-Like Receptor 4 By Lysine Acetylation Through LPCAT2 Activity in RAW264.7 Cells

Running Title: Possible Regulation of TLR4 By Lysine Acetylation Through LPCAT2 Activity

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1 Abstract:

Inflammation is central to several diseases. TLR4 mediates inflammatory signals, however, there are gaps in the understanding of its mechanisms. Recently, TLR4 was found to co-localise with LPCAT2, a lysophospholipid acetyltransferase. This interaction influenced TLR4 subcellular localisation through an unknown mechanism.

In this study, we have combined computational analysis, RNA interference technology, and biochemical analysis to investigate the possibility of TLR4 lysine acetylation and the influence of LPCAT2 on the detected lysine acetylation.

The results suggest for the first time that TLR4 can undergo lysine acetylation and LPCAT2 can influence TLR4 lysine acetylation. This lays a foundation for further research on the role of lysine acetylation on TLR4 and characterisation of LPCAT2 as a protein acetyltransferase.

2 Introduction:

Inflammation is central to many diseases such as cancer, asthma, sepsis, and cardiovascular diseases [1]. It can be caused by infection from various micro-

organisms, or by cell damage [2]. During bacterial infections, TLR4 plays a major role in mediating inflammatory signals after it recognises bacterial lipopolysaccharide bound to co-receptor CD14 [3,4]. Although there is scientifically established information on TLR4 mechanisms of signalling and protein-protein interactions, a lot is yet to be understood about TLR4 mechanisms. Recently, TLR4 was found to co-localise with LPCAT2; this led to a change of its subcellular localisation [5]. Nonetheless, how LPCAT2 affects the subcellular localisation of TLR4 is not known.

LPCAT2 is known as a lipid acyltransferase and acetyltransferase [6]. It is highly expressed in inflammatory cells such as peritoneal macrophages, microglia, and neutrophils [7]. In experimental allergic encephalomyelitis [8,9] and peripheral nerve injury [10–12], LPCAT2 is significantly expressed. Furthermore, LPCAT2 is suggested as a biomarker for sepsis and allergic asthma [13–15]. On the other hand, experimental conditions where LPCAT2 is silenced or inhibited, results in the resolution of inflammation via decreased production of cytokines and LPCAT2 metabolites [5,10,16]. During inflammatory conditions, LPCAT2 and TLR4 expression increases in the lipid raft domain of RAW264.7 macrophage cell line, which serves as a platform for mediating inflammatory signals [5]. Acetylation is a post-translational

49 modification that can influence the subcellular localisation and function of a
50 protein [17,18]. Although it commonly occurs on histones, there are several
51 recent scientific publications that have identified acetylation on non-histone
52 proteins [19]. Lysine acetylation can regulate protein function, interaction,
53 and localisation [20,21]. LPCAT1 which has a very similar structure and
54 function to LPCAT2 is known to palmitoylate histone 4, a protein [22]. This
55 allows for the theory that LPCAT2 could carry out either of its enzymatic
56 activities; acyltransferase or acetyltransferase on proteins such as TLR4.

57 Since several scientific publications suggest that LPCAT2 participates in
58 inflammation, understanding the molecular mechanisms of LPCAT2 will
59 contribute new knowledge on inflammation and could lead to new therapies
60 for inflammatory disorders.

61 This study uses computational analysis, RNA interference technology, and
62 biochemical analysis to detect lysine acetylation on TLR4 and the possibility
63 of LPCAT2 influencing the detected TLR4 lysine acetylation.

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 INTERFERin was purchased from Source Bioscience, UK. Pre-designed siRNA, 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 Cell line and Culture:

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

3.2 Transfection of RAW264.7 Cells with LPCAT2 siRNA:

RAW264.7 cells were cultured 24 hours before gene silencing. Then using Opti-MEM (Reduced Serum Medium) as a diluent, a transfection mixture containing 7nM of siRNA was prepared and added to cells. The cells were incubated at 37ºC, 5% CO_2 with Opti-MEM for 24 hours for efficient gene silencing.

3.3 Reverse Transcription and Real-Time Quantitative PCR:

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 3μl of ≥ 100ng/μl cDNA. The reaction was initiated at 95ºC for 10 minutes, then up to 40 repeated cycles of denaturing (15 seconds, 95ºC),

98 annealing, and extension (60 seconds, 60ºC). GAPDH and ATP5B were used
99 as an endogenous controls.

100 *3.4 Immunoprecipitation:*

101 Equal amounts of whole cell lysates were pre-cleared, and incubated with
102 target antibodies overnight at 4ºC. Then protein A/G agarose gel beads were
103 rinsed with PBS, added to the lysate-antibody mixture, and incubated
104 overnight at 4ºC. The mixture was centrifuged to separate the supernatant
105 from protein A/G agarose beads. The beads were rinsed with PBS, then eluates
106 were made with mild to harsh elution buffers [23].

107 *3.5 Immunoblotting:*

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

3.6 Computational Analysis:

Computational analysis of mouse protein sequences obtained from Uniprot database was carried out using GPS-PAIL 2.0 [24], and R Programming Software (packages used were: Peptides, Biostrings, phangorn, tidyverse, ape, seqinr, rentrez, msa, and ASEB). Both gene (rentrez) and protein sequences were aligned using multiple sequence alignment (msa). The physicochemical properties of peptides were analysed using Peptides package. Phylogenetic trees were made using maximum parsimony with SeaView version 4 [25] and phangorn package. Sequence IDs: LPCAT2- Q8BYI6, NM173014; LPCAT1- Q3TFD2, NM145376; KAT2A- Q9JHD2, NM020004; KAT2B- Q9JHD1, NM020005; CREBBP- NM001025432; ELP3- NM001253812; EP300- NM177821; KAT5- NM001362372; KAT6A- NM001081149; KAT6B- NM017479; KAT8- NM026370.

3.7 Data Analysis:

Statistical Analysis was carried out in R Statistical Programming Software and graphs were plotted using ggplot2 package. Independent experiments were repeated at least 3 times. Data represent Mean ± Standard Error of Mean unless stated otherwise. 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 Transfection of RAW264.7 Cells with LPCAT2 siRNA Does Not Affect the Basal Function of RAW264.7 Cells:

The gene and protein expression of LPCAT2 were analysed to confirm the knockdown of LPCAT2. Figure 1A shows that LPCAT2 gene is significantly lower in cells transfected with LPCAT2 siRNA (0.25 ± 0.02, $p = 0.0015$). Likewise, LPCAT2 protein decreased (4-fold) in cells transfected with LPCAT2 siRNA (Figure 1B&C).

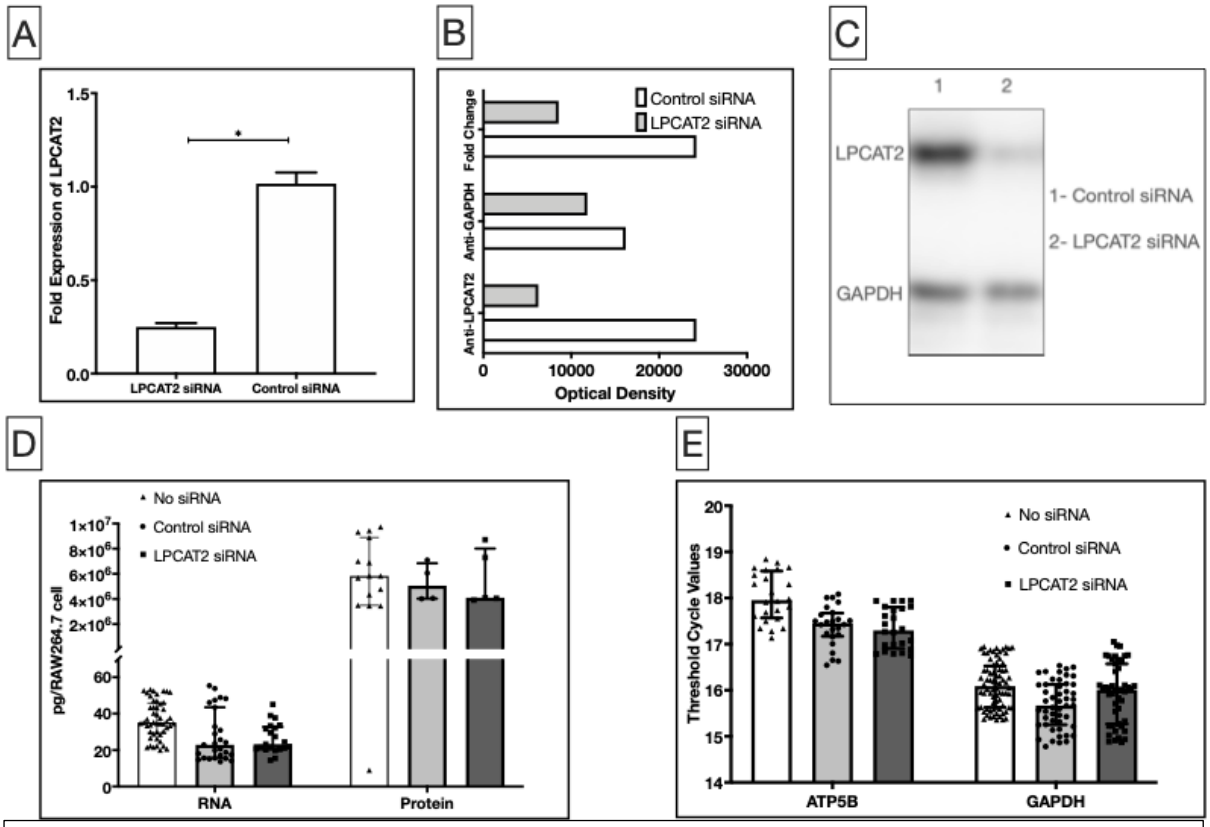

Figure 1: Transfection of RAW264.7 cells with LPCAT2 siRNA causes ≥70% decrease in LPCAT2 gene expression **(A)** and ≥50% decrease in LPCAT2 protein expression **(B&C)**. *p≤0.05. Fold change shows the optical density of LPCAT2 normalised to GAPDH. Data represents mean of at least 3 independent experiments (n≥3) ± standard error (A), and optical densities (B) of western blots (C). Transfection of siRNA into RAW264.7 cells does not cause a significant difference in total RNA and total protein **(D)**, and in the expression of housekeeping genes ATP5B and GAPDH **(E)** when compared to cells with no transfected siRNA. Data represents the median of at least 3 independent experiments (n≥3) ± interquartile range.

The basal function of RAW264.7 cells after transfecting the cells with LPCAT2 siRNA was analysed by measuring the total amount of RNA and Protein, and the gene expression of housekeeping genes ATP5B and GAPDH. Figure 1C shows that transfection of RAW264.7 cells with LPCAT2 siRNA does not significantly affect the total amount of RNA (23.25pg/RAW264.7 cell; 20.56pg/RAW264.7 cell to 32.78 pg/RAW264.7 cell) when compared with non-transfected cells (35.2 pg/RAW264.7 cell; 29.39pg/RAW264.7 cell

to 45.84pg/RAW264.7 cell, p = 0.98) and the total amount of protein (4.09 x 10^6pg/RAW264.7 cell; 3.98 x 10^6 pg/RAW264.7 cell to 8.01 x 10^6pg/RAW264.7 cell) when compared with non-transfected cells (5.82 x 10^6pg/RAW264.7 cell; 3.5 x 10^6pg/RAW264.7 cell to 8.89 x 10^6pg/RAW264.7 cell, p = 0.98). Likewise, Figure 1D shows that transfection of RAW264.7 cells with LPCAT2 siRNA does not significantly affect the gene expression of housekeeping gene ATP5B (17.3; 16.9 to 17.8) when compared with non-transfected cells (17.96; 17.57 to 18.58, p = 0.95) and GAPDH (16.01; 15.27 to 16.57) when compared with non-transfected cells (16.09; 16.52 to 15.63, p = 0.95).

4.2 Analysis of The Relatedness of LPCAT2 to Commonly Known Lysine Acetyltransferases (KATs)

Using R Statistical Programming Software, the genetic relatedness of LPCAT2 to other KATs was analysed. As LPCAT1 is a very similar protein to LPCAT2, it was included as a positive control. Indeed, Figure 2A shows that LPCAT2 and LPCAT1 belong to the same family. Moreover, it suggests that LPCAT2 and LPCAT1 belong to the same superfamily as KAT2A and KAT2B. Therefore, the similarity of the protein sequences was analysed by aligning LPCAT2 to each protein from node 7 in Figure 2A. Figure 2B-D shows that

LPCAT2 vs LPCAT1 has less than 5% gaps in alignment (B), whereas, LPCAT2 vs KAT2A (C) and LPCAT2 vs KAT2B (D) have less than or equal to 35% gaps in alignment.

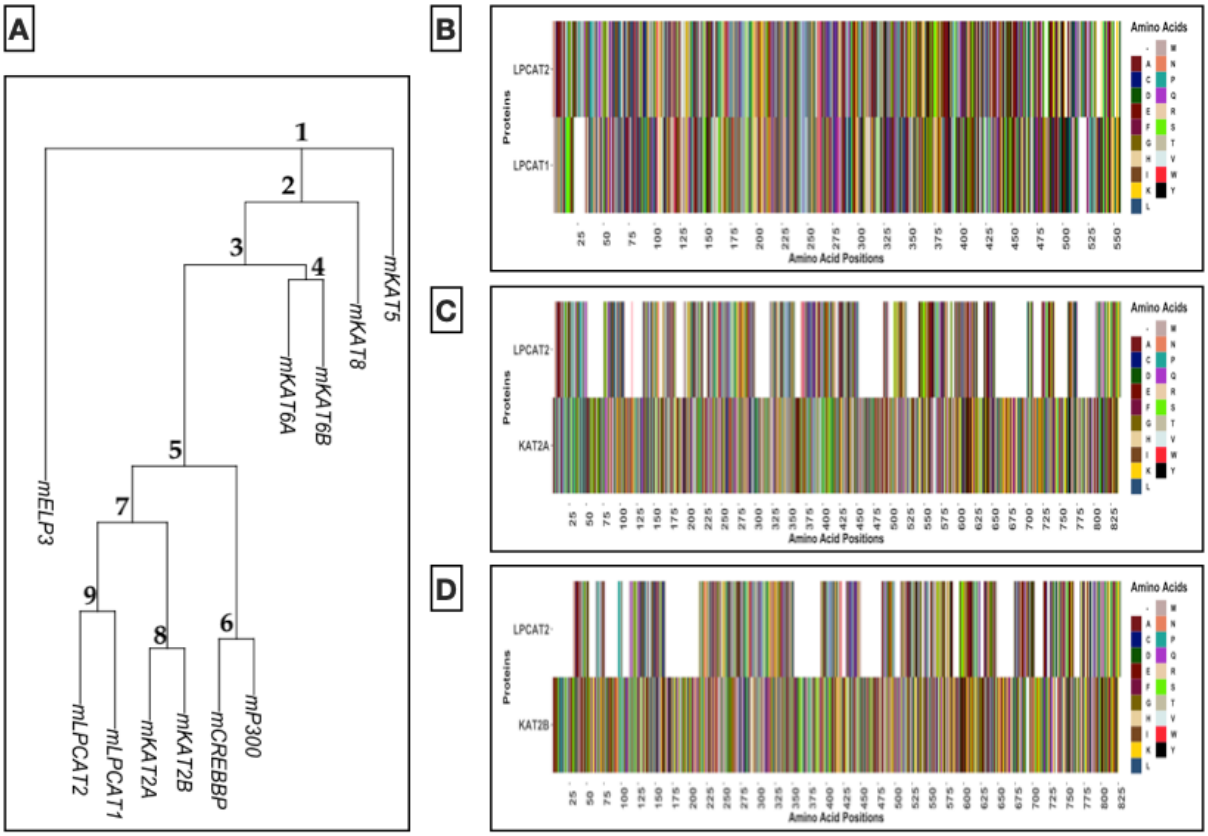

Figure 2: Analysis of The Relatedness of LPCAT2 To Other Lysine Acetyltransferases in Mice. **A** – Phylogenetic tree showing the degree of relatedness of LPCAT1 gene, LPCAT2 gene, and genes of other KATs. Numbers indicate node positions. **B to D** – Sequence alignment of proteins in node 7, white space indicates gaps in alignment. Letters symbolising amino acids are IUPAC standards.

4.3 LPCAT2 Influences Pan-Lysine Acetylation in RAW264.7 Cells

Acetylated lysine residues were detected using acetylated lysine antibodies. Figure 3 shows that stimulating RAW264.7 cells with LPS increased the density of some acetylated lysine protein bands, especially around 10kDa. The

179 bars show fold change after normalising optical densities to acetylated alpha
180 tubulin densities. Some protein bands (100kDa and ~5kDa) showed ≥50%
181 reduction after knockdown of LPCAT2 with or without LPS (Figure 3C).
182 Although the median is very similar across all treatment, the upper quartile is
183 lower when LPCAT2 is silenced (Figure 3B). This indicates lower band
184 intensities of lysine acetylated proteins after LPCAT2 knockdown.

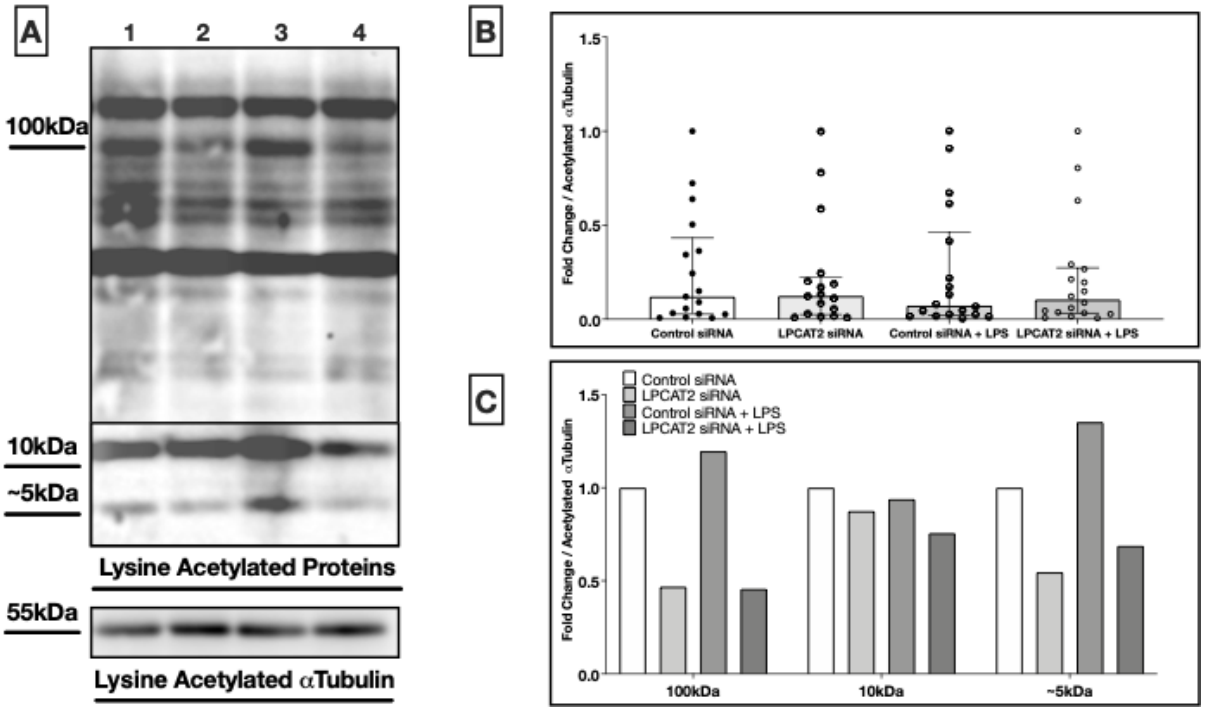

Figure 3: Analysis of Pan-Lysine Acetylation in Lipopolysaccharide-Stimulated RAW264.7 Cells. **(A)** – Western blot image of lysine acetylated proteins. Acetylated Alpha Tubulin was used as a positive control. Lane 1 - Control siRNA, Lane 2 - LPCAT2 siRNA, Lane 3 - Control siRNA + LPS, Lane 4 - LPCAT2 siRNA + LPS. **(B)** – Fold change in optical density of lysine acetylated proteins normalised to acetylated αTubulin. Bars represent median and error bars represent interquartile range. **(C)** – Fold change in optical density of bands at 100kDa, 10kDa, and ~5kDa normalised to acetylated αTubulin and control siRNA.

4.4 In Silico Prediction of Lysine Acetylation of Lipopolysaccharide Receptors

Due to the presence of acetylated lysine which can be induced by LPS and decreased by silencing LPCAT2 at 100kDa, the protein sequences of mouse TLR4 which is about 100kDa in size and its co-LPS-receptors; MD2 and CD14 were analysed for the possible presence of lysine residues that can be acetylated. Two software were used for this analysis; GPS-PAIL version 2.0 and ASEB. Table 1 shows that GPS-PAIL version 2.0 software predicted that

195 TLR4 is acetylated on the lysine residue at position 817 by CREBBP, but CD14
196 and MD2 did not show any possibility for lysine acetylation. RelA was used as
197 a positive control, as it is already experimentally proven to undergo lysine
198 acetylation [26].

Table 1: *In Silico* Prediction of Lysine Acetylation in lipopolysaccharide Receptors Using GPS-PAIL Version 2.0.

Protein	Position	Peptide	Score	Cut-off	Acetyltransferase
CD14	NA	NA	NA	NA	NA
MD2	NA	NA	NA	NA	NA
TLR4	817	KNALLDGKASNPEQ	2.09	1.79	CREBBP
RelA	310	KRTYETFKSIMKKS	2.32	1.79	CREBBP
RelA	122	NLGIQCVKKRDLEQ	2.08	1.69	KAT2B

NA indicates that no sites of lysine acetylation was detected. Position indicates the position of the lysine with predicted acetylation. The acetyltransferases were also predicted based on known information about binding sites of these enzymes. The score indicates the probability of acetylation. The higher the score, the higher the chance of acetylation. The cut-off is the number under the threshold. This analysis was carried out using a high threshold. Red letters signify the supposed position of lysine acetylation.

199 *Table 1*

Further analysis of mouse TLR4 protein sequence using ASEB revealed the possibiility of more than 1 lysine residue undergoing lysine acetylation. In Table 2, the lysine residue at position 817 was predicted to undergo lysine acetylation by CREBBP along with lysine residues at positions 367 and 503. The lysine residue at position 152 was predicted to undergo lysine acetylation by KAT2A and or KAT2B.

Table 2: *In Silico* Prediction of Lysine Acetylation of TLR4 ASEB

Position	Peptide	Acetyltransferase
152	PIGQLITLKKLNVAHNF	KAT2A, KAT2B
367	NKGSISFKKVALPSLSY	CREBBP
503	NLTFLDLSKCQLEQISW	CREBBP
817	KNALLDGKASNPEQ	CREBBP

ASEB predicted more peptides to undergo lysine acetylation in TLR4. However, the peptides selected and shown in this table all have a p-value of p≤0.1.

Table 2

To understand the properties of the predicted peptides and predict the potential roles of the acetylated lysine residues, analysis of each peptide predicted to contain acetylated lysine residue was carried. As shown in Table 3, all peptides were globular, suggesting that the peptides are situated either

211 on the extracellular or the intracellular end of TLR4, but not the
212 transmembrane region. The high aliphatic index values also predict that these
213 peptides are thermostable.

Table 3: Properties of Predicted Lysine Acetylated Peptides in TLR4

Position	Peptide	HI	AI	Membrane Position
152	PIGQLITLKKLNVAHNF	0.29	137.7	Globular
367	NKGSISFKKVALPSLSY	-0.05	91.8	Globular
503	NLTFLDLSKCQLEQISW	0.02	114.7	Globular
817	KNALLDGKASNPEQ	-1.17	65.3	Globular

Hydrophobicity index (HI) indicates a lipophilic or hydrophilic peptide. The aliphatic index (AI) indicates thermostability. All peptides are predicted to be globular because of their low hydrophobicity index.

214 *Table 3*

215 *4.5 LPCAT2 Influences the Lysine Acetylation of Toll-like Receptor 4*

216 Further analysis to confirm the presence of lysine acetylation in TLR4 was
217 carried out by immunoprecipitating TLR4 and blotting for acetylated lysine
218 residues. Figure 4A shows that at 100kDa, TLR4 was detected in both blots
219 using TLR4 antibodies and acetylated lysine antibodies after
220 immunoprecipitation of TLR4. In Figure 4B, the optical densities of TLR4 in
221 both TLR4 blots and acetylated lysine blots were both within the range of

222 3000 to 4500. Treatment of RAW264.7 cells with LPS did not show any
223 difference.

224 In an attempt to ensure that the protein being detected is TLR4, we silenced
225 the expression of TLR4 in RAW246.7 cells and repeated the analysis. In this
226 case, acetylated lysine proteins were immunoprecipitated and eluates were
227 analysed for the presence of TLR4. The postulation was that if the detected
228 protein was not TLR4, then it will not decrease when TLR4 is silenced and it
229 will not be detected by TLR4 antibody. In Figure 4C, we see again that TLR4
230 was detected from eluate of lysine acetylated proteins by TLR4 antibody.
231 Figure 4D shows again that knockdown of TLR4 reduced the presence of
232 lysine acetylation on TLR4 by ≥50%. This is further evidence of the presence
233 of lysine acetylation on TLR4. TLR4 expression was also reduced in cells not
234 treated with LPS but transfected with TLR4 siRNA (Poloamina, 2021).

235 As the initial study was to understand the role of LPCAT2 on lysine
236 acetylation, we sought to determine the influence of LPCAT2 on the detected
237 lysine acetylation on TLR4. Figure 4E, shows a TLR4 blot of acetylated lysine
238 eluates from RAW264.7 cells. The optical densities in Figure 4F shows ≥50%
239 decrease in acetylated TLR4 lysine after knockdown of LPCAT2.

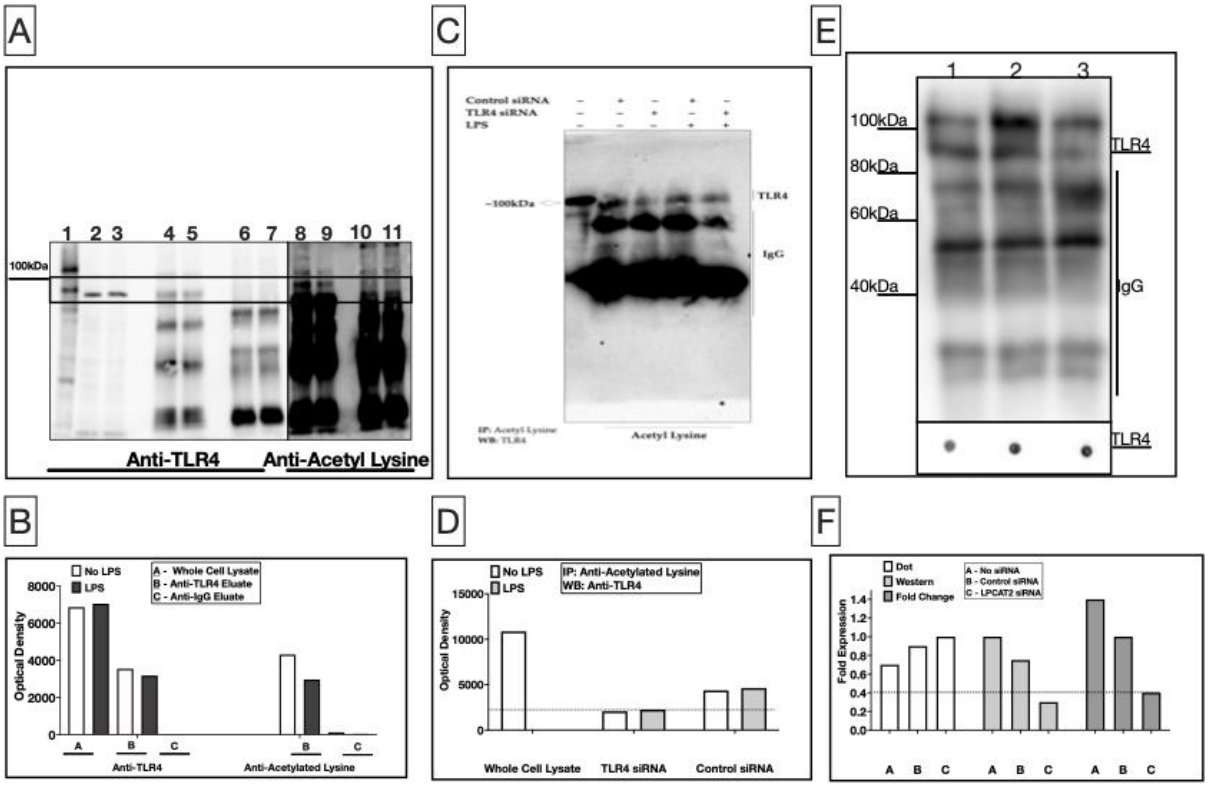

Figure 4: **A–** Acetylated Lysine Detected on Precipitated TLR4. Lane 1- biotinylated protein ladder; Lanes 2 & 3 - TLR4 blots of whole cell lysates; Lanes 4 & 5 -TLR4 blots of TLR4 eluate; Lanes 6 & 7 - TLR4 blots of IgG eluate; Lanes 8 & 9 -acetylated lysine blots of TLR4 eluate; Lanes 10 & 11 - acetylated lysine blots of IgG eluate. Lanes 2, 4, 6, 8, and 10 are samples from RAW264.7 cells not treated with LPS, whereas lanes 3, 5, 7, 9, and 11 are samples from RAW264.7 cells treated with 100ng/ml LPS. **B–** Optical densities of protein bands at 100kDa. Anti-IgG elution was used as isotype control. **C–** TLR4 blot (WB) of acetylated lysine precipitates (IP). The first lane is a blot of lysate of RAW264.7 cells not transfected with siRNA or treated with LPS. The other lanes are blots of acetylated lysine eluates. - indicates absence, and + indicates presence. **D–** Optical densities of protein bands at 100kDa. **E–** Acetylated lysine blots (WB) of TLR4 precipitates (IP). Lane 1 - No siRNA, Lane 2 - Control siRNA, Lane 3 - LPCAT2 siRNA. TLR4 dot blots of uniform amounts of proteins from TLR4 eluates were used as controls of protein amounts. **F–** Optical density of protein bands at ~90kDa. Fold change refers to values normalised to dot blot of TLR4 in eluates and to control siRNA.

4.6 LPCAT2 Regulates the Expression of Interferon Beta and Interferon-Inducible Protein 10 (IP10):

A recent publication has shown that LPCAT2 can regulate the expression of pro-inflammatory cytokines; TNFα and IL6 (Abate et al, 2020), however, interferon-beta (IFNβ) and interferon-inducible protein 10 (IP10) which are

247 dependent on TLR4-Trif signalling pathway, were not analysed. Figure 5A
248 shows that knockdown of LPCAT2 results in a significant decrease (5.38 ±
249 0.69, p = 0.00025) in the gene expression of IFNβ in cells stimulated with
250 100ng/ml lipopolysaccharide. IP10 which can be also be induced by IFNβ, was
251 analysed. Figure 5B shows that knockdown of LPCAT2 significantly decreased
252 the gene expression (168.7 ± 32.27, p = 0.021). In non-treated cells,
253 knockdown of LPCAT2 did not effect a significant change in IFNβ gene (p =
254 0.46), and IP10 gene (p = 0.97) expression.

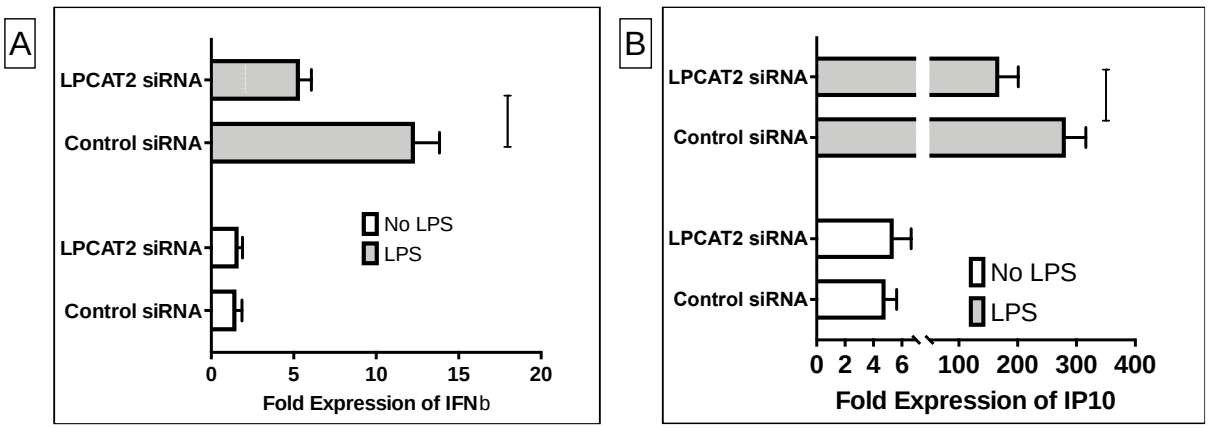

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Figure 5: Knockdown of LPCAT2 affects the gene expression of Interferon beta, IFNβ **(A)** and interferon-inducible protein 10, IP10 **(B)** in RAW264.7 cells stimulated with TLR4 ligand (100ng/ml of E. coli O111:B4 lipopolysaccharide). Non-treated RAW264.7 cells did not show any significant difference. *p≤0.05. Data represents the mean of at least 3 independent experiments (n≥3) ± standard error.

256

257 # 5 Discussions:

258 LPCAT2 is commonly known as a lipid-modifying enzyme. However, there is
259 scientific evidence that LPCAT1, which is similar in structure and function to

LPCAT2, palmitoylates histone 4 [22]. This implies that LPCAT2 may modify proteins. Moreover, LPCAT2 co-localises with TLR4 and modifies its subcellular localisation and hence its function, through an unknown mechanism [5]. Addition of acyl groups to proteins can regulate its subcellular localisation and or function [27]. Using both computational and biochemical analysis, our results suggest that TLR4-LPCAT2 co-localising may result in the lysine acetylation of TLR4 which may influence its subcellular localisation.

5.1 LPCAT2 Gene and Protein Sequence is homologous to other lysine acetyltransferases:

50% to 70% homology is required for conservation of enzyme functions [28]. Figure 2 shows that LPCAT2 shares at least 65% similarity with KAT2A and KAT2B, which are established lysine acetyltransferases. Moreover, analysis of their gene sequences showed that LPCAT2, LPCAT1, KAT2A, and KAT2B evolved from the same superfamily. The results from this computational analysis lays a foundation for further scientific experiments that will be required to classify LPCAT2 as a lysine acetyltransferase.

5.2 LPCAT2 Influences Lysine Acetylation Detected on TLR4 Protein:

The housekeeping genes– ATP5B and GAPDH and the total RNA and protein amount did not undergo any significant changes when RAW264.7 cells were transfected with siRNA (Figure 1). This eliminates the possibility that the observed gene expression or protein expression is as a result of overall changes in the cell.

Palmitoylation can regulate the subcellular localisation and signalling of TLR2. It is also present on other TLRs like TLR5 and TLR10, but not on TLR4 [29]. As TLR4 was not found amongst palmitoylated proteins, we explored the next possibility which is acetylation. LPCAT2 has both acyltransferase and acetyltransferase [6], however, unlike LPCAT1 there is no previously published evidence that suggests that LPCAT2 can carry out its enzymatic activities on proteins. Our results show that when LPCAT2 is knocked down in RAW264.7 cells, the band intensities of some lysine acetylated proteins reduces especially at 100kDa and 5kDa (Figure 3). Except TLR4, there are many other proteins with an approximate mass of 100kDa. On the UniProt database [30], there are about 3500 proteins between 90kDa and 110kDa. Only about 25 of these proteins have published evidence of lipidation. Therefore, a

294 more TLR4-specific study is needed to confirm its lysine acetylation.
295 Computational analysis to predict TLR4 lysine acetylation suggests that TLR4
296 may undergo lysine acetylation on lysines 152, 367, 503, and 817 cataysed by
297 KAT2A, KAT2B, or CREBBP (Table 1 & Table 2). Biochemical analysis shows
298 the presence of lysine acetylation on TLR4 (Figure 4), however, further
299 experiments will be required to know the specific lysine residues that are
300 acetylated on TLR4 and their role in regulating TLR4 function. In Table 3, we
301 predicted that the TLR4 peptides that have acetylated lysine residues are
302 hydrophilic and globular. Although this is not confirmatory, it suggests that
303 lysine acetylation may be occuring on the extracellular domain of TLR4 where
304 it binds to its ligands, or the intracellular domain of TLR4 where it binds to
305 other proteins and transmits signals. Indeed, a thesis suggested that TLR4
306 lysine acetylation influences its interaction with its adaptor proteins MyD88,
307 TRAM, and TRIF; which eventually affects the production of inflammatory
308 cytokines [31]. We have previously shown that knockdown of LPCAT2
309 reduces MyD88-dependent cytokines after LPS-TLR4 interaction [5]
310 Likewise, Figure 5 shows that TRIF-dependent cytokines are influenced by
311 knockdown of LPCAT2. TLR4 protein or gene expression is not affected by

312 silencing LPCAT2 (data not shown), however, Figure 4 suggests for the first
313 time that LPCAT2 may influence lysine acetylation detected on TLR4.

314 In conclusion, the results from this study suggest that TLR4 undergoes lysine
315 acetylation and LPCAT2 influences the detected lysine acetylation. This lays
316 a foundation for further research on the role of lysine acetylation on TLR4,
317 and characterisation of LPCAT2 as a protein acetyltransferase.

318 6 References:

319 [1] Chen, L. et al. Inflammatory responses and inflammation-associated
320 diseases in organs. Oncotarget 9, 7204–7218 (2018).

321 [2] Pahwa, R., Goyal, A., Bansal, P. & Jialal, I. Chronic Inflammation
322 (StatPearls Publishing, PMID: 29630225, 2020).

323 [3] Poltorak, A. et al. Defective lps signaling in c3h/hej and c57bl/10sccr mice:
324 mutations in tlr4 gene. Science 282, 2085–8 (1998).

325 [4] Beutler, B. & Rietschel, E. T. Innate immune sensing and its roots: the story
326 of endotoxin. Nat Rev Immunol 3, 169–76 (2003).

[5] Abate, W., Alrammah, H., Kiernan, M., Tonks, A. J. & Jackson, S. K. Lysophosphatidyl- choline acyltransferase 2 (lpcat2) co-localises with tlr4 and regulates macrophage inflam- matory gene expression in response to lps. Sci Rep 10, 10355 (2020).

[6] Shindou, H. et al. A single enzyme catalyzes both platelet-activating factor production and membrane biogenesis of inflammatory cells. cloning and characterization of acetyl-coa:lyso- paf acetyltransferase. J Biol Chem 282, 6532–9 (2007).

[7] Shindou, H., Hishikawa, D., Harayama, T., Eto, M. & Shimizu, T. Generation of membrane diversity by lysophospholipid acyltransferases. J Biochem 154, 21–8 (2013).

[8] Kihara, Y. et al. Platelet-activating factor production in the spinal cord of experimental allergic encephalomyelitis mice via the group iva cytosolic phospholipase a2-lyso-pafat axis. J Immunol 181, 5008–14 (2008).

[9] Kihara, Y. Systematic understanding of bioactive lipids in neuro-immune interactions: Lessons from an animal model of multiple sclerosis. Adv Exp Med Biol 1161, 133–148 (2019).

344 [10] Shindou, H. et al. Relief from neuropathic pain by blocking of the
345 platelet-activating factor- pain loop. FASEB J 31, 2973–2980 (2017).

346 [11] Hishikawa, D., Hashidate, T., Shimizu, T. & Shindou, H. Diversity and
347 function of mem- brane glycerophospholipids generated by the remodeling
348 pathway in mammalian cells. J Lipid Res 55, 799–807 (2014).

349 [12] Inoue, K. & Tsuda, M. Microglia in neuropathic pain: cellular and
350 molecular mechanisms and therapeutic potential. Nat Rev Neurosci 19, 138–
351 152 (2018).

352 [13] Davenport, E. E. et al. Genomic landscape of the individual host response
353 and outcomes in sepsis: a prospective cohort study. Lancet Respir Med 4, 259–
354 71 (2016).

355 [14] Valentine, W. J., Hashidate-Yoshida, T., Yamamoto, S. & Shindou, H.
356 Biosynthetic en- zymes of membrane glycerophospholipid diversity as
357 therapeutic targets for drug develop- ment. Adv Exp Med Biol 1274, 5–27
358 (2020).

359 [15] Watanabe, H. et al. Lpcat2 methylation, a novel biomarker for the
360 severity of cedar pollen allergic rhinitis in japan. Am J Rhinol Allergy
361 1945892420983646 (2020).

362 [16] Xiao, X. et al. Robo4 deletion ameliorates paf-mediated skin inflammation
363 via regulating the mrna translation efficiency of lpcat1/lpcat2 and the
364 expression of paf receptor. Int J Biol Sci 16, 1086–1095 (2020).

365 [17] Spange, S., Wagner, T., Heinzel, T. & Krämer, O. H. Acetylation of non-
366 histone proteins modulates cellular signalling at multiple levels. Int J Biochem
367 Cell Biol 41, 185–98 (2009).

368 [18] Thevenet, L. et al. Regulation of human sry subcellular distribution by its
369 acetyla- tion/deacetylation. EMBO J 23, 3336–45 (2004).

370 [19] Glozak, M. A., Sengupta, N., Zhang, X. & Seto, E. Acetylation and
371 deacetylation of non- histone proteins. Gene 363, 15–23 (2005).

372 [20] Choudhary, C. et al. Lysine acetylation targets protein complexes and co-
373 regulates major cellular functions. Science 325, 834–40 (2009).

374 [21] Close, P. et al. The emerging role of lysine acetylation of non-nuclear
375 proteins. Cell Mol Life Sci 67, 1255–64 (2010).

376 [22] Zou, C. et al. Acyl-coa:lysophosphatidylcholine acyltransferase i (lpcat1)
377 catalyzes histone protein o-palmitoylation to regulate mrna synthesis. J Biol
378 Chem 286, 28019–25 (2011).

379 [23] Poloamina, V. I. Regulation of The Expression and Lysine Acetylation of
380 Pro-Inflammatory Molecules By Lipid-Modifying Enzyme (LPCAT2) in
381 RAW264.7 Cells. Ph.D. thesis, Uni- versity of Plymouth (2021).

382 [24] Deng, W. et al. Gps-pail: prediction of lysine acetyltransferase-specific
383 modification sites from protein sequences. Sci Rep 6, 39787 (2016).

384 [25] Gouy, M., Guindon, S. & Gascuel, O. Seaview version 4: A multiplatform
385 graphical user interface for sequence alignment and phylogenetic tree
386 building. Mol Biol Evol 27, 221–4 (2010).

387 [26] Li, H. et al. Regulation of nf-b activity by competition between rela
388 acetylation and ubiq- uitination. Oncogene 31, 611–23 (2012).

389 [27] Bijlmakers, M.-J. Protein acylation and localization in t cell signaling
390 (review). Molecular Membrane Biology 26, 93–103 (2009). URL

391 [28] Addou, S., Rentzsch, R., Lee, D. & Orengo, C. A. Domain-based and
392 family-specific se- quence identity thresholds increase the levels of reliable
393 protein function transfer. J Mol Biol 387, 416–30 (2009).

394 [29] Chesarino, N. M. et al. Chemoproteomics reveals toll-like receptor fatty
395 acylation. BMC Biol 12, 91 (2014).

396 [30] UniProt Consortium. Uniprot: the universal protein knowledgebase in
397 2021. Nucleic Acids Res 49, D480–D489 (2021).

398 [31] Ping, W. J. TLR4 Acetylation/Methylation Modification Modulates LPS-
399 Activated Inflam- mation. Ph.D. thesis, Zhejiang University (2011).