1
Phospholipid localization implies microglial morphology and function via Cdc42 *in vitro*
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17   **Main Points**

18   ・LysoPS rapidly induces microglia ramification in a receptor independent
19   manner.

20   ・The incorporated LysoPS into microglia is converted to PS and activates
21   Cdc42-signalling.

22   ・LysoPS treatment suppresses the pro-inflammatory microglial phenotype.

23

24   **Key Words:** ramified microglia, lysophospholipid, phosphatidylserine

25

Abstract

Under a quiescent state, microglia exhibit a ramified shape, rather than the amoeboid-like morphology following injury or inflammation. The manipulation of microglial morphology *in vitro* has not been very successful, which has impeded the progress of microglial studies. We demonstrate that lysophosphatidylserine (LysoPS), a kind of lysophospholipids, rapidly and substantially alters the morphology of primary cultured microglia to an *in vivo*-like ramified shape in a receptor independent manner. This mechanism is mediated by Cdc42 activity. LysoPS is incorporated into the plasma membrane and converted to phosphatidylserine (PS) *via* the Lands cycle. The accumulated PS on the membrane recruits Cdc42. Both Cdc42 and PS co-localize predominantly in primary and secondary processes, but not in peripheral branches or tips of microglia. Along with the morphological changes LysoPS suppresses inflammatory cytokine production and NF-kB activity. The present study provides a tool to manipulate a microglial phenotype from an amoeboid to a fully ramified *in vitro*, which certainly contributes to studies exploring microglial physiology and pathology.

1 **Introduction**

2 Resident microglia are assumed to be a specialized population of immune cells

3 in the brain, acting as the first defence in the central nervous system (CNS)

4 (Kreutzberg 1996). Microglia, under a quiescent state, exhibit a ramified shape

5 and are continuously surveying nearby brain conditions (Nimmerjahn et al. 2005;

6 Wake et al. 2009), whereas, in response to injury or inflammatory stimuli, the

7 microglia dynamically alter their morphology: rapid process protrusion, retraction,

8 and thickening, as well as cellular migration (Hanisch and Kettenmann 2007).

9 Activated microglia are capable of returning to a ramified morphology in

10 conjunction with tissue recovery or a reduction in the inflammatory response

11 (Ladeby et al. 2005). Although a greater correlation between microglial

12 morphology and function is assumed, the molecular correlation between

13 morphology and function remains largely unknown. This is partly due to the lack

14 of manipulation techniques of microglial morphology *in vitro*. One of the greatest

15 difficulties in microglial research is the gap between *in vitro* and *in vivo*

16 morphology and function (Butovsky et al. 2014; Rosenstiel et al. 2001). Primary

17 cultured microglia routinely exhibit a flat and/or spindle shaped morphology that

18 is distinct from the typical ramified morphology observed in the steady-state

19 brain. Manipulation of microglial morphology *in vitro* would be an important

20 advancement for addressing the mechanisms underlying microglial dynamic

21 changes of morphology and function.

22 To determine cell polarity, cytokinesis, and morphogenesis, the membrane

23 characteristics implied by phospholipid localization, asymmetry, and composition

24 on the plasma membrane are crucial (Das et al. 2012; Yeung et al. 2008). In

25 yeast, for instance, phosphatidylserine (PS) levels peak during bud emergence,

26 and cells lacking the phosphatidylserine-synthase gene grow more slowly than

27 wild-type (WT), implying that phosphatidylserine localization is important for

28 optimal growth (Fairn et al. 2011). To manipulate phospholipid localization in the

29 plasma membrane, the application of a lyso-form of phospholipids could be an

30 alternative tool, although lysophospholipids (LPLs) serve a wide variety of

31 functions as lipid mediators that are exerted through G protein-coupled

32 receptors specific to each lysophospholipid (Makide et al. 2014). Because

33 lysophosphatidylserine (LysoPS), for instance, appears to be incorporated into

4

the cytoplasm, subsequent acylation results in increased PS in the intracellular membrane domain (Fairn et al. 2011).

In this study, we demonstrate that one of the lysophospholipid members, LysoPS, rapidly and substantially alters the morphology of primary cultured microglia into a ramified shape. The morphological changes take place in a receptor independent manner, but are mediated by Cdc42 Rho GTPase activity. We further reveal that in parallel with the morphological changes LysoPS suppresses lipopolysaccharide (LPS)-induced inflammatory cytokine production and NF-kB activity.

Materials and Methods

Materials

18:1-LysoPS, 18:0-LysoPS, 18:1-PS, di12:0-PS, 17:0-, 18:1-lysophosphatidyl acid (LPA), 18:0-lysophosphatidylethanolamine (LPE), 18:0-lysophosphatidylcholine (LPC), liver (90% 18:0-) lysophosphatidylinositol (LPI) and 18:1-lysophosphatidylglycerol (LPG) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). 18:1-sphingosine-1-phosphate (S1P) was from Cayman Chemical (Ann Arbor, MI, USA). LysoPS with various fatty acids [capric (10:0), lauric (12:0), myristic (14:0), palmitic (16:0) and palmitoleic (16:1)] and LysoPS analogues with oleic acid (18:1) including deoxy-LysoPS were synthesized as previously described (Iwashita et al. 2009; Uwamizu et al. 2015). Eicosatetraenoic (20:4) and docosahexaenoic (22:6) were provided by Ono Pharmaceutical Co., Ltd (Osaka, Japan). High-selectivity agonist for GPR34 (compound No. 12) (Jung et al. 2016), P2Y10 (compound No. 25) (Jung et al. 2016), and GPR174 (LysoPalloT-C3-ph-m-O-C7) (Ikubo et al. 2015) were synthesized as previously described. To prepare liposomes, lipids were dissolved in chloroform in a clean siliconized glass tube, dried under a gentle stream of nitrogen gas, and further dried under a vacuum for 30 min. The dried lipid was hydrated, resuspended in phosphate-buffered saline (PBS) containing 0.1% (w/v) fatty acid-free bovine serum albumin (BSA) (Sigma-Aldrich, St Louis, MO, USA) to a concentration of 2 mM, and sonicated to produce the final solution in accordance with a previous report (Ikubo et al. 2015). We aliquoted

the solutions to avoid freeze-and-thaw cycles and stocked them at −80°C. The purity was at least > 95%. For inhibitors of signalling molecules, a selective inhibitor of geranylgeranylation (GGTI 298: #16176) was purchased from Cayman Chemical. Inhibitors for PKA (KT5720: #420320), PI3K (LY294002: #440202) and MEK (U0126: #662005) were purchased from Calbiochem (La Jolla, CA, USA). Inhibitors for ROCK (Y27632: #257-00511) and Rac1 (NSC23766: #180-02491) were purchased from Wako (Osaka, Japan). Inhibitor for Cdc42 (ML-141: #SML0407) was purchased from Sigma-Aldrich.

Animals

All efforts were made to minimize animal suffering, and to reduce the number of animals used. To generate GPR34 KO mice, the GPR34 locus was targeted using a standard homologous recombination approach in 129-background embryonic stem cells. Briefly, the GPR34 gene was disrupted with a targeting vector, which was designed to replace the GPR34 open reading flame in-flame with a LacZ-neo cassette. GPR34 KO mice were backcrossed to the C57BL/6 background for 12 generations. C57BL/6 WT (SLC, Hamamatsu, Japan) and GPR34 KO mice were housed with food and water available *ad libitum* in a temperature (23 ± 1°C)- and humidity (50%)-controlled environment on a 12/12-h light/dark cycle (lights on at 09.00 h). All mice were maintained in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, 1996). The protocol for the animal experiment was approved by the Animal Ethics Committee of Nagoya University (approval number: 26181, 27204 and 28303).

Primary cultures

Microglia were obtained from a primary mix-culture in accordance with a previous report (Konishi et al. 2006). Briefly, brains were collected from WT and GPR34 KO mice at postnatal day 1 or 2. After removable of the meninges, the tissue was minced with a razor blade and treated with trypsin (Invitrogen, Carlsbad, CA, USA) and DNase I (Roche Applied Science, Indianapolis, IN, USA), and dispersed cells were seeded in a culture flask. After 10–12 d in culture, detached microglia were collected and plated on 24- or 96-well culture

1 dishes. Microglial cells were plated on non-coated glass coverslips for
2 immunostaining and scanning electron microscopy (SEM). The purity was >
3 98%. After 10–12 h, the cultured microglial cells were serum-starved for 4 h, and
4 then stimulated with 10 µM of LysoPS. For quantitative real-time RT-PCR and
5 NF-kB translocation analysis, microglial cells were stimulated with 100 ng/ml of
6 LPS (#L4516; Sigma-Aldrich) for 12 h prior to serum starvation. Cortical neurons
7 were prepared from cerebra of embryonic day (E) 16 embryonic C57BL/6 mice.
8 Neurons were seeded on PLL-coated 12-mm round glass coverslips placed on
9 the bottom of 24-well culture dishes (1.5×10^5 cells/well) and precultured in
10 Neurobasal medium (Invitrogen) containing 0.05 mg/ml penicillin/ streptomycin
11 (Invitrogen), 0.5 mM glutamine, and B27 supplement (Invitrogen) for 4 d. Cortical
12 astrocytes were isolated and cultured from postnatal day 1 to 3 pups from
13 C57BL/6 mice as described (Schildge et al. 2013) and were maintained at 37°C
14 in a CO_2 incubator. The cultured neurons and astrocytes were serum-starved for
15 4 h, and then stimulated with 10 µM of LysoPS.
16
17 *SEM*
18 Microglial cells were collected 60 min after LysoPS application and fixed in 0.1 M
19 phosphate buffer (PB) containing 2.5% glutaraldehyde overnight at 4°C. Cells
20 were post-fixed in 1% OsO_4 in 0.1 M PB for 30 min at room temperature (RT),
21 and dehydrated in an ethanol gradient at RT. After dehydration in absolute
22 ethanol three times, samples were incubated in t-butyl alcohol for 30 min at 30°C
23 three times, and then kept in t-butyl alcohol overnight at 4°C. Specimens were
24 dried using a freeze dryer (VFD-21S; Vacuum Device, Ibaraki, Japan), coated
25 with osmium by ion sputter (HPC-30W; Vacuum Device), and observed with a
26 scanning electron microscope (JSM-7610; JEOL, Tokyo, Japan).
27
28 *Time-lapse imaging of cultured cells*
29 For time-lapse imaging, microglial cells were directly plated onto a glass base
30 dish (#3971-035; Iwaki, Tokyo, Japan). Images were collected at 2 min intervals
31 using an imaging microscope system (LCV110; Olympus, Tokyo, Japan). Cells
32 were maintained at 37°C in 5% CO_2 throughout the experiment. The time-lapse
33 images were used for quantitative analysis of morphologies, as well as dynamics

1 of microglial processes and migration velocity. The speed of microglial migration
2 was determined by tracking microglial cell bodies using MetaMorph 7.5.6.0
3 software (Universal Imaging, Media, PA, USA).

4

5 *Immunocytochemistry and image analysis*
6 Microglial cells cultured on glass coverslips were fixed in 0.1 M PBS, pH 7.4,
7 containing 4% paraformaldehyde (PFA) for 10 min at RT. Then immunostaining
8 was done according to a previous method (Konishi et al. 2010) using rabbit
9 ant-beta III tubulin (1:2000, RRID: AB_444319; Abcam, Cambridge, UK), mouse
10 anti-GFAP (1:1000, RRID: AB_477010; Sigma-Aldrich), rabbit anti-Iba1 (1:1,000,
11 RRID: AB_839504; Wako) and rabbit anti-p65 (1:100, RRID: AB_632037; Santa
12 Cruz Biotech, Santa Cruz, California, USA) as primary antibodies and Alexa
13 Fluor 488 or 594-labelled anti-rabbit IgG (1:1,000; Invitrogen) as a secondary
14 antibody. Actin was visualized by Alexa Fluor 594-labelled phalloidin (1:100;
15 #A12381; Invitrogen). Nuclei were stained with DAPI solution (1:5000;
16 #340-07971; Dojindo Laboratories, Kumamoto, Japan). Images were acquired
17 using fluorescent microscopy (BZ-9000; Keyence, Osaka, Japan). The line-scan
18 intensity distribution analysis was performed using the Image J software (NIH,
19 Bethesda, MD, USA).

20

21 *Determination of cell shape and form factor*
22 To quantitatively assess the shape of microglia, we employed the form factor.
23 Cell circumferences were drawn using the freehand selections tool, and the area
24 and perimeter of each cell was determined using Image J. Then the form factor
25 was calculated using the formula ($4\pi \times$ area/[perimeter]^2) as previously
26 described (Wilms et al. 1997). Form factor indicates complexity of cell shape: 1 =
27 an exactly round cell; value closer to 0 = highly bushy cell. A total of 20 cells
28 were randomly selected from at least three independent experiments and were
29 analyzed.

30

31 *LC-MS/MS analysis*
32 18:1-LysoPS, 17:0-LPA, and di12:0-PS were dissolved in methanol using a
33 water bath sonicator, and stored at −20°C. Microglia cells (1×10^5) were

collected in a test tube with 200 μl of methanol and crushed using a water bath sonicator. After centrifugation at 21,500 × g, the supernatant was added to di12:0-PS (10 μM, final concentration) or 17:0-LPA (100 nM, final concentration). After being passed through a filter (0.2 μm pore size, YMC, Kyoto, Japan), the samples (10 μl for PS analysis or 20 μl for LysoPS analysis) were subjected to LC-MS/MS as previously described with some modifications (Inoue et al. 2011). For LysoPS, LC separation was performed using the UltiMate 3000 HPLC (Thermo Fisher Scientific, Waltham, MA, USA) with C18 CAPCELL PAK ACR columns (100 mm × 1.5 mm, Shiseido, Tokyo, Japan) using a linear gradient of solvent A (5 mM ammonium formate in water) and solvent B (5 mM ammonium formate in 95% (v/v) acetonitrile). MS/MS was carried out on a TSQ Quantiva Triple-Stage Quadrupole mass spectrometer (Thermo Fisher Scientific). LysoPS were monitored in the multiple reaction-monitoring (MRM) mode with neutral loss of the 87 Da fragment of [M-H]- ions. PS analysis was performed similar to the LysoPS analysis, except that the HPLC system consisted of a NANOSPACE SI-II (Shiseido) and C8 CAPCELL PAK UG120 column (150 mm × 1.5 mm, Shiseido), and a TSQ Quantum Ultra triple quadrupole mass spectrometer (Thermo Fisher Scientific) were used. PS analyses were performed in the MRM mode with neutral loss of 185 Da fragment of [M+H]+ ions.

Protein purification
The plasmids of pGEX-evectin2- pleckstrin homology (PH) domain (Uchida et al. 2011) and the pGEX-Pak1-PBD domain were transformed into *Escherichia coli* BL21 (DE3), and protein expressions were induced by addition of 1 mM isopropyl-β-D-thiogalactopyranoside to exponential-phase bacteria. After 4 h at 37°C, the bacteria were harvested and lysed with PBS containing 1% Triton X-100 and protease inhibitor cocktail (Complete EDTA-free; Roche) for 30 min at 4°C. The supernatants were subsequently incubated with glutathione-Sepharose 4B (GE Healthcare Life Sciences, Buckinghamshire, UK) for 1 h at 4°C, and the bound proteins were eluted by adding 10 mM reduced glutathione in 50 mM Tris-HCl, pH 8.0. After removal of glutathione by dialysis against PBS, the proteins were checked by SDS-PAGE, followed by Coomassie Brilliant Blue R-250 staining, and stored at −80°C until further use.

1

Immunolabelling of PS by evectin-2 PH domain-glutathione S-transferase (GST)

probe

The cultured microglia were fixed in PBS, pH 7.4, containing 4% PFA for 10 min

at RT. They were then washed in PBS, pH 6.0, at least three times for 30 min,

and incubated with the evectin-2 PH domain with glutathione S-transferase

(GST-evt-2-PH probe) in PBS, pH 6.0, (3 µg/ml) for 30 min at RT. After washing

in PBS at least three times for 30 min, the cells were blocked with 1% BSA, 0.3%

Triton-X in PBS for 1 h, incubated with rabbit anti-GST (1:1000, RRID:

AB_67419; Bethyl Laboratories, Montgomery, TX, USA) and/or mouse

anti-Cdc42 (1:200, RRID: AB_627233; Santa Cruz) primary antibody overnight,

and washed in PBS at least three times for 30 min. Signals were detected with

Alexa 488- or 647-labelled donkey anti-rabbit or anti-mouse IgG (1:1000 dilution;

Invitrogen). We have examined the use of another antibody recognizing a

different epitope of Cdc42 (anti-rabbit Cdc42, 1:100, RRID: AB_631213; Santa

Cruz) and obtained the same staining as monoclonal mouse antibody.

Additionally, we confirmed the specificity of monoclonal mouse antibody by

using an anti-mouse CD16/CD32 antibody (1:100, RRID: AB_467132;

eBioscience, San Diego, CA, USA) as a blocking system of the FC receptor. We

did not find any non-specific antibody binding with the FC receptor. Actin was

visualized by Alexa Fluor 594-labelled phalloidin. The point mutant K20E of

GST-evt-2-PH probe, in which Lys20 was changed to Glu and lacked the ability

to bind to the PS head group (Uchida et al. 2011), did not show immune-positive

signals in this method.

Adeno-associated virus (AAV) preparation and infection

Rac1 and Cdc42 cDNA were inserted into the pAAV-CAG-EGFP-MCS-WPRE

vector of the AAV Helper-Free System (Stratagene, La Jolla, CA, USA), and

purified according to previous methods (Inutsuka et al. 2014; Lazarus et al.

2011). Briefly, HEK293T cells (Cell BioLabs, Inc., San Diego, CA, USA) were

transfected with the above-described pAAV vector, pHelper (Cell BioLabs Inc.),

and pAAV-DJ (Cell BioLabs Inc.) using a standard calcium phosphate method.

After 3 d, the transfected cells were collected, re-suspended in PBS, and lysed

by four cycles of freeze-thawing. The cell extract was treated with Benzonase nuclease (Merck, Darmstadt, Germany) at 45°C for 15 min and centrifuged twice at 15,000 × g for 10 min, and the supernatant was collected. The virus titre in the supernatant was determined by quantitative PCR (#6233: Takara, Tokyo, Japan), and used for infection. Plated pure microglia were incubated with virus-containing medium at 1.6×10^4 copies/cell for 1 h, and then the medium was replaced with normal culture medium without AAV. Microglial cells at 6 d after infection were used for immunocytochemical analysis.

Rho GTPase activation assays

To determine Rac1 and Cdc42 activation, we performed a GST-pulldown assay using the Rac1 and Cdc42-interactive binding domain of Pak1. Microglial cells were serum-starved for 4 h, and then stimulated with 10 µM LysoPS. Microglia cells were harvested with cold lysis buffer containing 50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.5% Nonidet-P40, 1 mM EGTA, 10 mM $MgCl_2$, 0.1 mM APMSF, 2.5 µg/ml of aprotinin, 2.5 µg/ml of leupeptin and 20 µg of Pak1-PBD GST-fusion protein. The lysates were centrifuged at 15,000 × g for 2 min, and the supernatant was incubated with glutathione-Sepharose 4B (#17-0756-01; GE Healthcare Life Sciences) for 30 min at 4°C. Sepharose was washed with PBS, and the bound proteins were eluted by addition of 18 µl of sample buffer. A total of 15 µl of eluate and total cell lysates were loaded onto SDS-PAGE. After semidry blotting, PVDF membranes (Millipore, Billerica, MA, USA) were blocked with 5% skim milk (Wako) in Tris-buffered saline with 0.1% Tween 20 (TBS-T) and incubated with primary antibodies diluted in blocking solution for 10–12 h at 4°C. Primary antibodies were as follows: rabbit anti-Rac1 (1:200, RRID: AB_2238100; Santa Cruz), mouse anti-Cdc42. Horseradish peroxidase-conjugated secondary antibodies (GE Healthcare Life Sciences) and ECL (GE Healthcare Life Sciences) were used for detection. If necessary, membranes were stripped with stripping buffer (100 mM β-mercaptoethanol, 2% SDS, 62.5 mM Tris, pH 6.8) for 20 min at 55°C.

Quantitative real-time RT-PCR

1　Quantitative real-time RT-PCR (qPCR) was performed as previously described

2　(Tokizane et al. 2013). For primary microglial cells, SYBR Green Cells-to-Ct Kit

3　(Thermo Fisher Scientific) was used to obtain total RNA and synthesize cDNA in

4　accordance with manufacturer instructions. qPCR was performed using SYBR

5　Green (Thermo Fisher Scientific) with the StepOnePlus Real-Time RT-PCR

6　System (Thermo Fisher Scientific). The following primers were used: GPR34

7　forward: 5'-ATATGCTACAACAGCCCGGA-3'; GPR34 reverse:

8　5'-GAACCGAAAGGCATGGTAAG-3'; P2Y10 forward:

9　5'-TAGGTACGATGTGGGCATCA-3'; P2Y10 reverse:

10　5'-CAGCAAAGCGAGAATCTGTG-3'; GPR174 forward:

11　5'-CCATTTGGTCCTGGTCTCTG-3'; GPR174 reverse:

12　5'-CTTCGCACACTGATGCAGAC-3'; IL1-β forward:

13　5'-ACAGAATATCAACCAACAAGTGATATTCTC-3'; IL1-β reverse:

14　5'-GATTCTTTCCTTTGAGGCCCA-3'; IL6 forward:

15　5'-ATCCAGTTGCCTTCTTGGGACTGA-3'; IL6 reverse:

16　5'-TAAGCCTCCGACTTGTGAAGTGGT-3'; TNF-α forward:

17　5'-AGCCGATGGGTTGTACCTTGTCTA-3'; TNF-α reverse:

18　5'-TGAGATAGCAAATCGGCTGACGGT-3'; iNOS forward:

19　5'-GGCAGCCTGTGAGACCTTTG-3'; iNOS reverse:

20　5'-GAAGCGTTTCGGGATCTGAA-3'; IL10 forward:

21　5'-GGTTGCCAAGCCTTATCGGA-3'; IL10 reverse:

22　5'-ACCTGCTCCACTGCCTTGCT-3'; TGF-β forward:

23　5'-CCGCAACAACGCCATCTATG-3'; TGF-β reverse:

24　5'-TGCCGTACAACTCCAGTGAC-3'; IGF-1 forward:

25　5'-GCTGGTGGATGCTCTTCAGT-3'; IGF-1 reverse:

26　5'-TAGGGACGGGGACTTCTGAG-3'; GAPDH forward:

27　5'-CAAGGTCATCCCAGAGCTGA-3'; GAPDH reverse:

28　5'-CGGCACGTCAGATCCACGAC-3'. qPCR reaction conditions were as

29　follows: 1 cycle of 95°C for 20 s, 40 cycles of 95°C for 3 s, and 60°C for 30 s. At

30　the end of the PCR, the samples were subjected to a melting analysis to confirm

31　amplicon specificity. Relative gene expression (ΔCt value) was calculated based

32　on the Threshold Cycle (Ct) of the reference gene (GAPDH) and target gene

1   (ΔCt = Ct$_{\text{target gene}}$ - Ct$_{\text{reference gene}}$). $\Delta\Delta$Ct was calculated as ΔCt$_{\text{LPS treatment}}$ -ΔCt$_{\text{LPS +}}$

2     $_{\text{LysoPS treatment}}$, and $2^{-\Delta\Delta\text{Ct}}$ was determined as the fold change.

3

4   *Statistical analysis*

5   No statistical methods were used to predetermine sample sizes, but the sample

6   sizes used are similar to those generally employed in the field. No data points

7   were removed from statistical analysis. All data were analyzed using the

8   GraphPad Prism 7 software program. The statistical analyses were performed

9   using a one-way analysis of variance (ANOVA) followed by or Dunnett's *post*

10   *hoc* test, a paired *t*-test. For single comparisons between two groups, an

11   unpaired Student's *t*-test was applied. Mann-Whitney U-tests were applied when

12   data were not normally distributed or there was no equal variance. Data were

13   presented as mean ± S.D. or ± S.E.M., and differences were considered to be

14   significant for values at $P < 0.05$.

15

16   **Results**

17   ***LysoPS rapidly induces microglia ramification***

18   We first examined the effect of 18:1-LysoPS on primary cultured microglia in

19   serum-free conditions. A higher dose of LysoPS (10 µM) resulted in rapid and

20   significant changes in microglial morphology, whereas both 0.1 µM and 1 µM did

21   not alter morphology. To observe precise microglial morphology, we employed

22   SEM. Under normal culture conditions, the microglial cells exhibited an

23   amoeboid-like shape with no clear fine processes (Fig. 1A) or a spindle-like

24   shape. Conversely, LysoPS-treated microglia exhibited the typical ramified

25   morphology with a smaller cell body and several long processes whose tips were

26   growth cone-like shape (Fig. 1B). More than 90% of microglia synchronously

27   showed similar changes. In contrast, primary neurons and primary astrocytes did

28   not show any difference in morphological analysis between vehicle and LysoPS

29   treatment (Fig. 1C, D). Time-lapse imaging demonstrated that LysoPS induced

30   morphological changes over a relatively short time period (Fig. 1E and Movie 1).

31   The microglia extended their processes and increased the number of processes

32   during the first 20 min following LysoPS treatment, and the number and length of

33   processes were maintained for more than 2 h (Fig. 1E, F). Intriguingly, the tips of

1　the extended processes kept moving, protruding and retracting in a manner

2　similar to the quiescent state of microglia *in vivo* (Fig. 1G). Next, the microglial

3　migration trajectories were re-plotted by aligning the starting point of each

4　trajectory at the same origin and measuring the velocity of microglial movement.

5　LysoPS treatment dramatically reduced motility and velocity (Fig. 1H, I). These

6　data indicate that LysoPS induced the ramification of cultured microglia and

7　reduced motility of cell bodies, with exception of the tips of processes,

8　suggesting that this morphology and behaviour closely mimicked ramified

9　microglia *in vivo*.

10

11　***Microglial ramification by LysoPS is not mediated through known***

12　***receptors***

13　Recently, some orphan G protein-coupled receptors (GPCRs) have been

14　deorphanized and identified as LysoPS receptors. These include GPR34,

15　P2Y10, and GPR174, all of which belong to the P2Y family. Therefore, we

16　examined expression profile of these LysoPS receptors in microglia through

17　real-time RT-PCR. Among those examined, only GPR34 mRNA was highly

18　expressed by cultured microglia, and mRNA expression levels of other receptors

19　were lower, suggesting that GPR34 is the dominant LysoPS receptor in

20　microglia (Fig. 2A). We further examined the effects of previously identified

21　receptor agonists (Ikubo et al. 2015; Jung et al. 2016), and found that only

22　LysoPS is effective (Fig. 2B). For quantification of morphology, we measured the

23　cell area and perimeter values of individual cells to calculate the form factor

24　(Wilms et al. 1997). The measurement of the form factor clearly indicated that

25　only LysoPS was effective in morphological change (Fig. 2C). To further confirm

26　that morphological change by LysoPS took place in a GPR34 independent

27　manner, we used GPR34-deficient microglial cells. This experiment clearly

28　demonstrated the effect of LysoPS was effective even in GPR34-deficient

29　microglia (Fig. 2D). When LysoPS was applied to GPR34-deficient microglia,

30　quantification by the form factor indicated a value of 0.02 ± 0.002, whereas

31　vehicle-treated GPR34-deficient microglia showed a form factor of 0.33 ± 0.025

32　(Fig. 2D). Additionally, the Gi and Gs inhibitors [pertussis toxin (PTX) and NF449,

33　respectively] did not affect the LysoPS-induced changes (data not shown).

1 These results indicate that the previously identified LysoPS receptors were not
2 involved in microglial ramification induced by LysoPS.
3
4 ***A specific lysophospholipid group is capable of altering microglial***
5 ***morphology***
6 Several LysoPS species with various fatty acids are known, although the
7 endogenous species are restricted *in vivo*. Among various fatty acid lengths,
8 LysoPS with stearic acid (18:0) or oleic acid (18:1) was the most effective in
9 altering the microglial form to a ramified shape. The LysoPS species with shorter
10 or longer fatty acids were less effective (Fig. 3A, B).
11 Under serum-free conditions, we then compared the efficiency of LysoPS with
12 other LPLs, such as 18:1-LPA, 18:1-S1P, 18:0-LPE, 18:0-LPC, 18:0-LPI,
13 18:1-LPG and 18:1-PS. Among them, LysoPS was most effective; LPA, S1P,
14 LPE, and PS did not alter morphologies. Intriguingly, a few lysophospholipids,
15 such as LPC, LPI, and LPG, induced microglial ramification to some extent (Fig.
16 3C, D). This suggests the involvement of head polarity; possibly a negative
17 charge may be important in this function.
18
19 ***LysoPS is rapidly incorporated into microglial cells and converted to PS***
20 LysoPS is rapidly incorporated into the membrane and flipped to its inner
21 monolayer in yeast (Fairn et al. 2011). We then examined the incorporation of
22 exogenous LysoPS into microglia using LC-MS/MS. After 10 min or 1 h of
23 LysoPS (18:1) treatment, microglial cells were quickly washed and harvested to
24 measure the lipid profile by LC-MS/MS. Ten minutes after LysoPS treatment,
25 LysoPS (18:1) in the microglial cells was dramatically increased (more than
26 190-fold), and the level decreased slightly after 1 h. Simultaneously the
27 significant increase in PS (38:5; 18:1 and 20:4) was observed, which was >
28 10-fold after 10 min. Further increase at 1 h (20–40 fold) suggested that PS was
29 synthesized in microglial cells, likely by membrane-bound acyltransferases (Fig
30 4A). To further examine whether LysoPS was converted into PS in microglia, we
31 used LysoPS analogs which did not have the *sn*-2 or *sn*-1 hydroxyl group
32 (2-deoxy-LysoPS, or 1-deoxy-LysoPS). Both deoxy-LysoPSs failed to induce
33 microglial ramification (Fig. 4B), suggesting that exogenously applied LysoPS is

rapidly incorporated and converted to PS by acyltransferase (Lands cycle). The increase in intracellular PS was further examined by using a fusion protein of GST-evt-2-PH, which specifically binds to O-phospho-L-serine, the head group of PS (Uchida et al. 2011). Immunocytochemistry using anti-GST antibody clearly revealed an increase in GST-evt-2-PH in LysoPS-treated microglia (Fig. 4C). These results suggest that microglial PS was highly induced by LysoPS treatment, and the increase was consistent with LC-MS/MS results.

LysoPS induces Cdc42-signalling pathways in microglia

A prominent morphological change in cells is often accompanied by specific intracellular signalling activation. Therefore, we investigated the involvement of major intracellular signal pathways in morphological change induced by LysoPS. We examined the effects of various inhibitors on major signalling in LysoPS-treated microglia. Inhibitors for PKA (KT5720), PI3K (LY294002), ROCK (Y27632), and MEK (U0126) did not inhibit microglial ramification in LysoPS-treated conditions. However, the Cdc42 and Rac1 selective inhibitors, ML141 and NSC23766 respectively, suppressed the effect of LysoPS, although Rac1 inhibitor had less effect than Cdc42 (Fig. 5A).

We then examined the involvement of Cdc42 or Rac1 in microglial ramification using dominant negative (DN) mutants of Cdc42 and Rac1. Although transfection efficiency of cultured primary microglia is extremely low, even with viral vectors, the AAV vector expressing EGFP and either Rac-N17 or Cdc42-N17 were successfully transfected by a minor number of microglial cells. Although EGFP and Rac1-N17 expressing microglia did respond to LysoPS, the EGFP and Cdc42-N17 expressing microglia failed to exhibit the ramification morphology (Fig. 5B, C), suggesting that Cdc42 could be a key molecule in the transformation of microglia by LysoPS.

We next examined Cdc42 activation by LysoPS application using a pulldown system to detect the GTP-bound active form of Rac1 and Cdc42. Concomitant with the DN experiments, LysoPS activated Cdc42 GTPase, but not Rac1 (Fig. 5D, E). These findings suggest that Cdc42 activation was critical for transformation of microglia by LysoPS.

Cdc42 binding to the PS membrane head group is essential for altering
microglial morphology

To reveal alterations in intracellular localizations of Cdc42 and PS after LysoPS treatment, cultured microglia were stained with antibodies specific to Cdc42 and GST-evt-2-PH protein. In the vehicle-treated microglia, evectin-2 expression was very weak and found mainly in recycling endosomes (Uchida et al. 2011) and weakly in the cytoplasmic membrane. LysoPS treatment dramatically increased evectin-2 expression in the cytoplasm and thick primary and secondary processes, but not in further branched peripheral processes and tips. Expression of Cdc42 after LysoPS appeared similar to evectin-2 after LysoPS treatment (Fig. 6A, B). High-power magnification of the primary processes demonstrated a dotty-shaped expression pattern of evectin-2 and Cdc42, with overlap of expression (Fig. 6C).

  Previous results have demonstrated that Rho GTPases, such as Cdc42, interact with PS-containing bilayers through the polybasic motif (PBM) *via* isoprenylation (Finkielstein et al. 2006). Prenylation is an important lipid modification of proteins, and it plays critical roles in regulating protein-membrane interactions. Isoprenylation (geranylgeranylation) of Cdc42 is also necessary for the association with PS (Finkielstein et al. 2006) and subsequent activation (Henry et al. 2006). To determine whether Cdc42 geranylgeranylation was implicated in microglial ramification, we employed a selective inhibitor of geranylgeranylation (GGTI 298), showing that 20 µM GGTI 298 treatment partially suppressed microglial ramification, although localization of both evectin-2 and Cdc42 appeared similar (Fig. 6D). However, a higher dose of GGTI 298 (50 µM) completely suppressed the ramification induced by LysoPS, and localization of evectin-2 and Cdc42 appeared in distinct intracellular membrane structures (Fig. 6E), suggesting that the interaction between the PS head group and Cdc42 by geranylgeranylation was critical for altering microglial morphology from an amoeboid shape to a ramified shape *in vitro*.

LysoPS treatment suppress the LPS-induced pro-inflammatory microglial
phenotype

The functional consequence of ramified microglia treated by LysoPS remains

intriguing. It was previously thought that there was an empirical correlation between microglial morphology and function. Therefore, we examined mRNA expression levels of inflammatory cytokines, such as IL1-β, IL6, TNF-α and iNOS after LysoPS treatment using primary cultured microglia from WT and GPR34-deficient mice. Prior to LysoPS treatment, LPS was pretreated to induce an inflammatory phenotype. mRNA expression of all cytokines, except iNOS, were significantly downregulated by LysoPS treatment compared with vehicle treatment in WT microglia (Fig. 7A). We also examined mRNA expression of the anti-inflammatory cytokine IL-10 and the microglia-derived growth factors TGF-β and IGF-1. Expression levels of IL-10, TGF-β, and IGF-1 mRNA remained unaltered by LysoPS (Fig. 7A). A similar response pattern was observed in microglia from GPR34 knockout mice, with exception to IL-10 (Fig. 7B).

Other inflammatory associated marker, such as NF-kB, was examined. As shown in previous studies (Bonizzi and Karin 2004; May and Ghosh 1998; Ni et al. 2015), the transcription factor NF-kB is an established regulator of expression of numerous proinflammatory cytokine genes. We examined the subcellular localization of the NF-kB RelA (p65) subunit after LysoPS treatment. Because most of the NF-kB p65 subunit was in the microglial cytoplasm following both vehicle and LysoPS treatments under normal conditions, we stimulated the nuclear localization of the p65 subunit by LPS, which induced a clear accumulation of the p65 subunit in microglial nuclei. Under this LPS-activated condition, LysoPS was applied, which suppressed LPS-induced nuclear translocation of the p65 subunit (Fig. 7C). Line-scanning analysis clearly demonstrated the intensity of the intracellular localization of the p65 subunit (Fig. 7D). Under the above-mentioned conditions, the subcellular localization of the p65 was quantified and presented in Fig. 7E. In vehicle- and LysoPS-treated cells, > 90% of microglial cells had p65 subunit localization in the cytoplasm. Conversely, in most LPS-treated cells, the p65 subunit was localized in the nuclei. LysoPS treatment suppressed nuclear localization of the p65 subunit by > 50% under the LPS-treated condition. These data indicate that LysoPS-treatment elicited functional alterations, as well as morphological changes, in microglia.

1   ***Discussion***

2   Results from the present study showed that LysoPS induced fully ramified

3   microglia that accompanied a quiescent function *in vitro*; LysoPS treatment

4   induced a ramified morphology from an amoeboid or spindle (bipolar) shaped

5   morphology, and the function changed from pro-inflammatory to quiescent.

6   Intriguingly, this morphological change was receptor independent, and other

7   cultured neuronal cells, such as neurons and astrocytes, did not respond to

8   LysoPS in terms of morphological changes (Fig. 1C, D), suggesting that LysoPS

9   mediated changes occur specifically in microglia.

10

11   The microglial manipulations including ramification are sought to bring about

12   therapeutic benefits for patients who suffer from neuropathic pain (Tsuda et al.

13   2003), neurodegenerative diseases (Boillee et al. 2006), and even mental

14   disorders (Bayer et al. 1999; Morgan et al. 2010), although some attempts have

15   been made to induce ramified shapes in microglia *in vitro* (Rosenstiel et al.

16   2001). To induce a ramified shape in primary cultured microglia, the stromal

17   cell-derived factor (SDF)-1a, CSF-1 (M-CSF), and a combination of

18   GM-CSF/IL-34 were previously examined (Muessel et al. 2013), (Neubrand et al.

19   2014), (Ohgidani et al. 2014). However, these morphological changes require

20   relatively longer periods: 24 h (SDF-1a), 48 h (CSF-1), and 14 d (GM-CSF/IL-34).

21   The SDF-1a induced actin-dependent cell spreading through CXCR4 in primary

22   cultured microglia, and the morphology of microglial processes was short and

23   relatively flat (Muessel et al. 2013). CSF-1 induced microglial ramification with

24   numerous short and thick processes, and this required activation of PI3K/Akt,

25   Cdc42, and Rac1 (Neubrand et al. 2014). Morphology of the above-mentioned

26   ramified microglia exhibited processes, but precise process morphology was

27   distinct from those observed *in vivo*. In the *in vivo* quiescent state, microglial

28   morphology is characterized by a relatively small soma with several thin long

29   processes, which also have thin branches and the tips of these branches are

30   ruffled and dynamically moving. More recently, Ohgidani et al. (Ohgidani et al.

31   2014) succeeded in inducing ramified microglia-like cells from monocytes, which

32   were more similar to brain microglia in morphology, using GM-CSF and IL-34;

33   the monocytes were incubated with GM-CSF and IL-34 for 14 d, which resulted

in a clear ramified shape, although the precise molecular mechanisms underlying this morphological change were not addressed. The morphology of ramified microglia obtained in the present study resembles *in vivo* microglia, and these microglia closely mimic the dynamics of process tips observed in the brain (Fig. 1 and Movie 1). Importantly, the induced ramified microglia exhibit a non-inflammatory phenotype (Fig. 7A–E), suggesting a greater correlation between microglial morphology and function.

Recently, receptors for LysoPS have been deorphanized, proving that GPR34, P2Y10, and GPR174 are the LysoPS receptors (Makide et al. 2014). Among these receptors, GPR34 expression was shown in microglia (Hickman et al. 2013; Liebscher et al. 2011), and we confirmed that GPR34 was predominantly expressed in primary cultured microglia (Fig. 2A). Previously, GPR-34-deficient mice resulted in slightly enlarged microglial somata and less branches per cell *in vivo* compared with WT mice (Preissler et al. 2015), although our cultured microglia did not show any significant difference in morphology between GPR34-deficient and WT cells (Fig. 2D). Notably, Gi and Gs inhibitors also failed to suppress ramification after LysoPS application. We tested other receptors for LysoPS, such as P2Y10 and GPR174, using their specific agonists (Ikubo et al. 2015; Jung et al. 2016), and the results showed that these ligands had no effect on microglial morphology (Fig. 2B, C). Furthermore, the effective dose of LysoPS (approximately 10 µM) was relatively greater, and dramatic increases in LysoPS and PS were observed inside the microglia after LysoPS treatment (Fig. 4A). It is known that the *lyso*-forms of phospholipids are easily penetrated into cell membrane (Arouri and Mouritsen 2013), and this character of lysolipids is actually used to change localization of intracellular PS in yeast (Fairn 2011). As a genera character of lysolipids, lysolipids are able to be incorporated into membrane and capable of changing lipid composition of membrane. Collectively, these data suggest that the changes observed in microglia following LysoPS treatment was not mediated *via* a receptor, but rather it is more likely that incorporation of LysoPS occurred *via* the plasma membrane and functioned within the microglia.

LysoPS is capable of being incorporated into the membrane (Arouri and Mouritsen 2013), subsequently flipping to the inner monolayer, and rapidly converting to PS *via* membrane-bound lysophospholipid acyltransferases: the deacylation / reacylation reaction is called the Lands cycle (Lands 1958; Shindou and Shimizu 2009). We confirmed increased LysoPS and PS levels in microglial cells using LC-MS/MS (Fig. 4A) and the GST-evt-2-PH probe, which specifically binds to PS head groups (Fig. 4C) (Uchida et al. 2011). Results showed significantly increased LysoPS and PS in microglia. The treatment of LysoPS with shorter or longer fatty acids did not sufficiently alter morphology (Fig. 3A, B), which could be due to differences in permeability depending on the length of acyl chains (Arouri and Mouritsen 2013; Hoyrup et al. 2001). To further address this, we used 1- and 2-deoxy-LysoPS that were not converted to PS by acyltransferases (Fig. 4B). Because these deoxy-LysoPS failed to induce ramification, it could be concluded that LysoPS incorporation and subsequent conversion to PS in microglia is crucial for altering microglial morphology.

Cell morphology is due to the reorganization of actin and microtubule cytoskeletons that are induced by a family of small RhoGTPases. We identified that the small RhoGTPase Cdc42 plays an essential role in LysoPS-induced ramification of microglia (Fig. 5A–E). Cdc42 exists in the plasma membrane and in endomembranes, such as the Golgi complex and endoplasmic reticulum (Farhan and Hsu 2016; Michaelson et al. 2001). In cultured microglia, endogenous Cdc42 was evenly localized in the eminent area of the plasma membrane, and abundantly in the endomembrane (Fig. 6A). Cdc42 associates with PS on the membrane when prenylated. Therefore, prenylation is necessary for Cdc42 to bind to membranes and become activated. When we inhibited Cdc42 prenylation with GGTI 298, a geranylgeranyltransferase inhibitor, Cdc42 translocation to the cytoplasmic membrane, as well as ramification, was totally suppressed (Fig. 6A–E). Cdc42 possesses a carboxy-terminal cationic region in the vicinity of the isoprenylation site, which allows binding to phospholipids with a negative charge, such as PS. This may be why LPLs with a negative charge at polar head groups, such as LPI and LPG, were able to alter microglial morphology to some extent, whereas other non-negatively charged LPLs failed

(Fig. 3C, D). Although LPC does not have a negative charge, LPC slightly altered the morphology. Because PS is made by exchanging serine for the choline head groups of phosphatodylcholine (Vance and Tasseva 2013), LPC application might induce PS level. However, PS application did not alter morphology, which might be due to the lower penetration ability *via* the plasma membrane (Arouri and Mouritsen 2013). Although we do not know why the PS head groups are primarily accumulated in the primary and secondary shafts of microglia and not in further branched processes and tips (Fig. 6C), this restricted accumulation would be a crucial event for recruiting Cdc42 at the restricted process region. Conversely, the lack of PS and Cdc42 in the peripheral branches and tips might be important for maintaining motility. This local regulation of Cdc42 distribution on the plasma membrane could be a critical factor for microglia to change from an amoeboid to a ramified morphology.

As mentioned above, a higher correlation between microglial morphology and function is likely, even in cultured cells. Many results, including the present, indicate that a ramified microglial shape releases less pro-inflammatory cytokines, whereas an amoeboid shape secretes significant levels of pro-inflammatory cytokines (Saijo and Glass 2011). LysoPS has long been known as a signalling phospholipid in mast cell biology, markedly enhancing stimulated histamine release and eicosanoid production (Martin and Lagunoff 1979), and was recently shown to be a mediator in activating clearance of dying cells by macrophages (Nishikawa et al. 2015). These actions are accompanied by pro-inflammatory responses rather than the anti-inflammatory functions mentioned in this paper (Fig. 7A–E). This difference could be due to the involvement of a receptor; the pro-inflammatory effects of LysoPS appear receptor-mediated, although the effect demonstrated in this study was receptor non-mediated. Although further evidence is needed to conclude that re-distributed Cdc42 exerts suppression of inflammatory responses, including the export of NF-kB from the nucleus, a change in Cdc42 intracellular localization could exert different functions (Farhan and Hsu 2016). Alternatively, the PS head groups, rather than Cdc42, may be involved in the suppression of inflammatory responses.

1   In conclusion, the present study addressed a mechanism underlying microglial

2   ramification *in vitro*. The alteration of local phospholipid localization, which

3   recruits the intracellular signalling molecule Cdc42, is pivotal. Composition of

4   lipid species on the plasma membrane could be critical for characterizing cell

5   morphology, as well as function, similar to the raft (Munro 2003); uneven

6   localization of lipids on the plasma membrane locally recruits intracellular

7   signalling molecules and induces local changes in plasma membrane

8   morphology and function. The present study provides a tool to manipulate a

9   microglial phenotype *in vitro*, and these results could promote further research

10   on the microglial multifaceted functions exerted in response to surrounding

11   environments.

12

1 ***Author contributions***

2 K.T. conducted most of the experiments and analyzed the data. H. Konishi

3 performed SEM. K. M., H. Kawana. and J. A. provided the GPR34 KO mice, and

4 performed LC-MS/MS. K. M., T. O. and J. A. provided agonist, and LysoPS

5 analog. S. N. and K. K. provided AAV and performed western blot. K. T., H.

6 Konishi and H. Kiyama designed the study and wrote the manuscript, with help

7 from J. A. and K.K., and H. Kiyama conceived of and led the project.

8

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31
32

Figure Legends

FIGURE 1: Characterization of ramified microglia induced by LysoPS *in vitro*.

(A, B) Images of primary cultured microglia after vehicle or 10 µM of LysoPS treatment. Images were acquired by SEM. Scale bar, 10 µm. (C, D) Images of primary cultured neurons and astrocytes after vehicle or 10 µM of LysoPS treatment. After 1 h of incubation, cells were fixed and immunostained for beta III Tubulin (neuron) and GFAP (astrocyte). Scale bar, 40 µm. (E) A time-lapse image of cultured microglia. Up to 10 µM of LysoPS was added at time point 0'. The number in each panel indicates minutes before and after LysoPS treatment. Scale bar, 20 µm. (F) Representative the number of microglial branch terminal points in each cell is measured at each time-point indicated after LysoPS treatment (n = 5 cells). (G) Representative microglial branch length (µm), from soma to the tip, is measured at each time-point after LysoPS treatment (n = 5 branches). Note that after initial extension, tips exhibit repeated extension and retraction. (H) Representative overlay of individual microglial cell trajectories after either vehicle or LysoPS treatments for 2 h (n = 5 each). (I) The MetaMorph-based quantification of velocity of cell migration. Cells were tracked over a 2 h period. Data represent means ± S.D. of 20 cells per group. [##]$P < 0.01$, Mann-Whitney Rank Sum test.

FIGURE 2: Ramification of microglia induced by LysoPS is not receptor-dependent.

(A) Representative real-time RT-PCR SYBR green fluorescence history vs. cycle number of mRNA expression for GPR34 (red), P2Y10 (purple), GPR174 (blue) and *glyceraldehydes-3-phosphate dehydrogenase* (GAPDH) (green) in microglial cDNA. ΔRn is the signal normalized to fluorescence intensity. (B) Primary cultured microglial cells are incubated with vehicle, 10 µM of LysoPS, 5 µM of GPR34 agonist, 5 µM of P2Y10 agonist, or 5 µM of GPR174 agonist. After 1 h of incubation, cells were fixed and immunostained for phalloidin. Scale bar, 20 µm. (C) Quantification of cell shape was performed by calculating the form factor = $4\pi \times$ area/(perimeter)^2. Data are mean ± S.D. and 20 cells per condition.

Three independent experiments, at least, were quantified. [###]$P < 0.001$, a one-way ANOVA followed by the Dunnett's *post hoc* test. (D) GPR34-deficient (GPR34 KO) microglial cells are incubated with LysoPS. Data are mean ± S.D. and 20 cells per condition. At least three independent experiments were quantified. [###]$P < 0.001$, Mann-Whitney Rank Sum test. Scale bar, 20 µm.

FIGURE 3: Microglial responses to fatty acid chain length of LysoPS and other LPL members

(A) Microglial cells were incubated with vehicle, 10:0-, 12:0-, 14:0-, 16:0-, 16:1-, 18:0-, 18:1-, 20:4-, or 22:6-LysoPS, respectively, at concentration of 10 µM each. (B) Quantification of cell shape was calculated using the form factor. Data are mean ± S.D.; 20 cells per condition were quantified in at least three independent experiments. [##]$P < 0.01$, [###]$P < 0.001$, a one-way ANOVA followed by the Dunnett's *post hoc* test. Scale bar, 20 µm. (C) Primary cultured microglial cells were incubated with vehicle, PS, LysoPS, LPA, S1P, LPE, LPC, LPI, or LPG, respectively, at concentration of 10 µM each, scale bar, 20 µm. (D) The form factor was measured to quantify cell shape. Data are mean ± S.D.. 20 cells per condition were measured in at least three independent experiments. [###]$P < 0.001$, a one-way ANOVA followed by the Dunnett's *post hoc* test.

FIGURE 4: LysoPS is rapidly incorporated into microglial cells and converted to PS. (A) Mass spectrometric determination of the area ratio of PS and LysoPS in microglial cells before and after (10 min and 1 h) LysoPS treatment. Data are mean ± S.D.. Three independent experiments were performed. [#]$P < 0.05$, [###]$P < 0.001$, a one-way ANOVA followed by the Dunnett's *post hoc* test. (B) Primary cultured microglia were incubated with vehicle, 18:1-LysoPS, 2-deoxy-LysoPS, or 1-deoxy-LysoPS, respectively, at 10 µM, and stained with Alexa 594-labelled phalloidin. Scale bar = 20 µm. (C) Microglial cells were incubated with purified recombinant GST-evt-2-PH after fixation and labelled as described in the Materials and Methods. Either vehicle- or LysoPS-treated cells were stained with antibody against GST or Alexa 594-labelled phalloidin. The most right column indicates merged images together with DAPI. Scale bar, 20 µm.

1

FIGURE 5: Cdc42 is involved in LysoPS-induced microglial ramification.

(A) Representative images of microglia incubated with vehicle or 10 μM of LysoPS for 60 min, in the presence of an inhibitor for each signalling pathway: 10 μM of KT5720, 20 μM of LY294002, 10 μM of Y27632, 20 μM of U0126, 50 μM of NSC23766, or 10 μM of ML141. (B, C) EGFP-tagged DN mutants of Rac1 and Cdc42 were infected by AAV. Arrows indicate EGFP-positive microglial cells. The cell shapes were quantified by calculating the form factor. Data are mean ± S.D.. 20 cells were analyzed for each mutant in three independent experiments. $^{###}P < 0.001$, a one-way ANOVA followed by the Dunnett's *post hoc* test. (D, E) After 1 min of incubation, cell lysates were assayed for activation of the small RhoGTPase Rac1 and Cdc42, and normalized to total Rac1 and Cdc42 amount in cell lysates. Data are mean ± S.E.M. of three independent experiments. $^{#}P < 0.05$, Mann-Whitney Rank Sum test.

FIGURE 6: Localization of Cdc42 and GST-evt-2 PH in microglia treated with LysoPS and suppression of ramification by geranylgeranylation inhibition.

(A, B) Confocal images showing localization of GST-evt-2-PH (green), Cdc42 (blue), and phalloidin (red) in microglia after LysoPS treatment. Bottom right of each image is the overlay image of GST-evt-2-PH, Cdc42, and phalloidin (merged). Note that GST-evt-2-PH and Cdc42 are intensely expressed in the soma and primary and secondary processes, but not in the further periphery. Scale bar, 20 μm. (C) The boxed areas in B are shown at a higher magnification. Note the GST-evt-2-PH- and Cdc42-positive signals in similar regions of the primary process. Scale bar, 500 nm. (D, E) A geranylgeranylation inhibitor, GGTI 298 (20 μM: D, 50 μM: E) suppresses LysoPS-induced ramification and alters localization of GST-evt-2-PH and Cdc42 in microglia after 60 min incubation with LysoPS and GGTI 298. Scale bar, 20 μm.

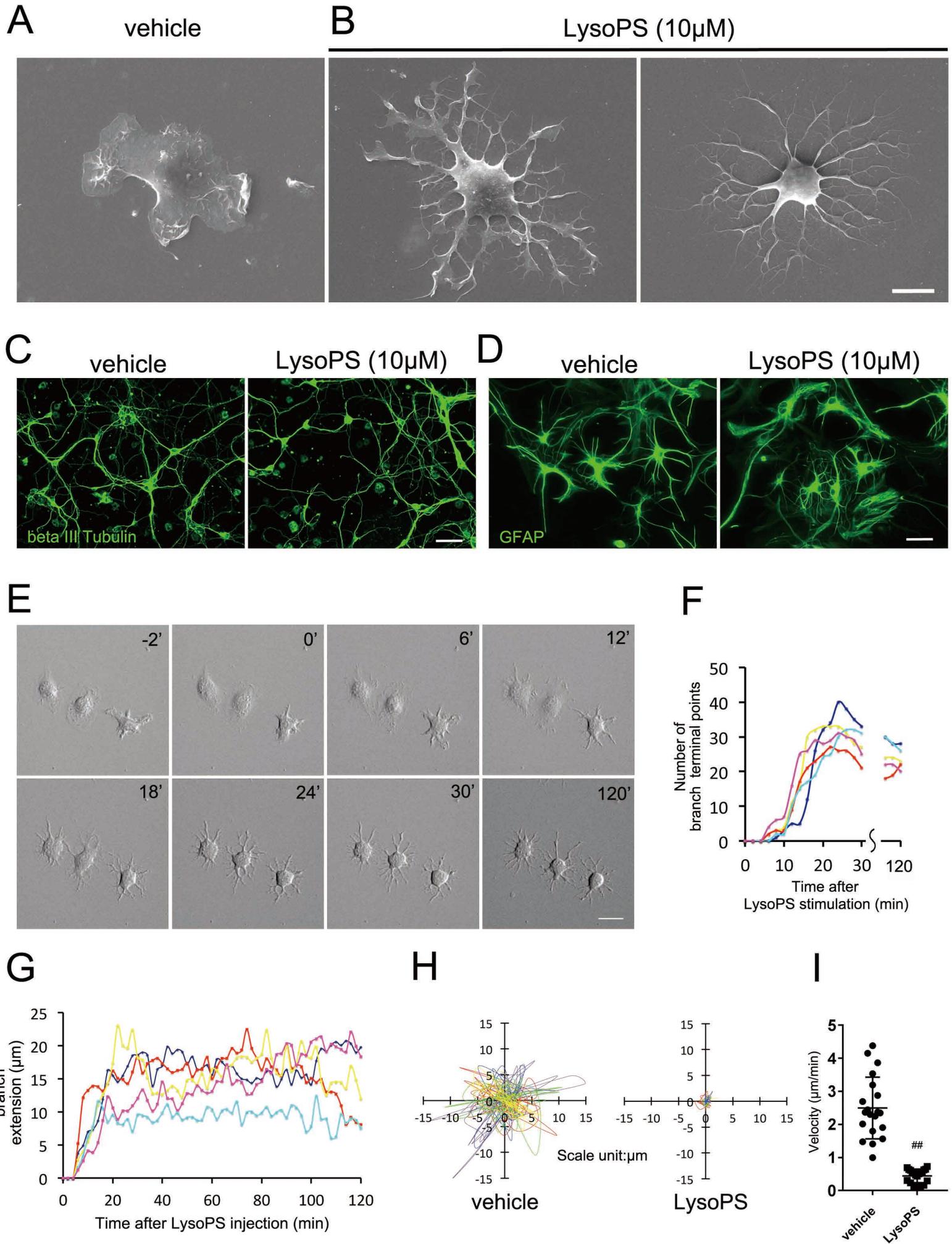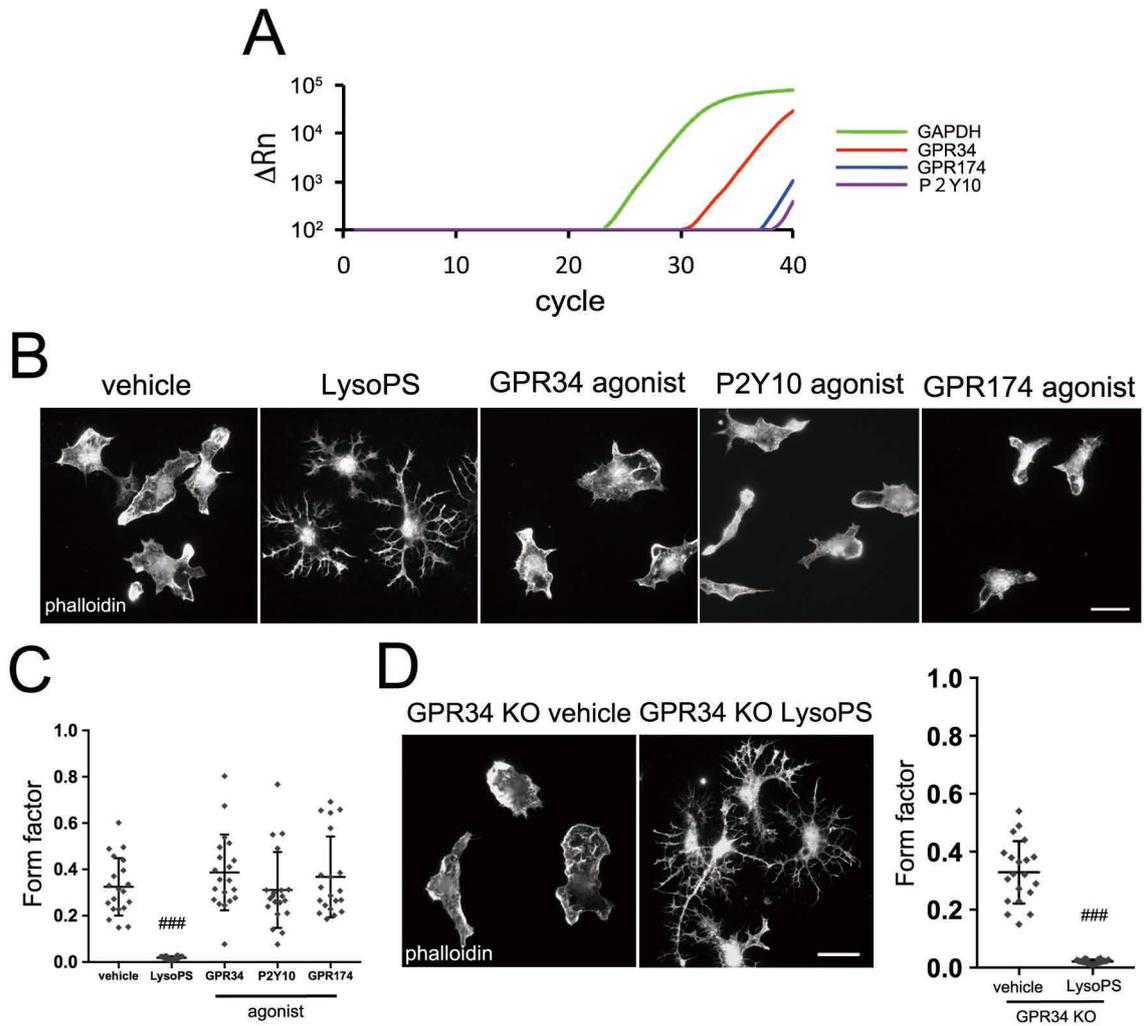**FIGURE 7: LysoPS suppresses the LPS-induced proinflammatory phenotype in primary microglia.**

Primary cultured microglia were obtained from WT (A) and GPR34-deficient (B)

mice (postnatal 1–2 days), and treated with bacterial LPS and either vehicle or LysoPS for 3 h. The mRNA for proinflammatory and anti-inflammatory markers were quantified by quantitative real-time RT-PCR. Results were normalized to and are shown as ratios to LPS-treated microglia cells. Values show mean ± S.D. of three independent experiments. $^{#}P < 0.05$, $^{##}P < 0.01$, $^{###}P < 0.001$, two-tailed unpaired Student's *t*-test. (C) Microglial cells were incubated with vehicle or 10 µM of LysoPS for 60 min in the presence or absence of LPS (100 ng/ml). The cells were simultaneously stained with p65 (green), DAPI (blue), and phalloidin (red). The column third from the left shows merged images, and the column fourth from the left shows the higher magnification in the merged images. (D) Line-scan graphs of representative cells to the right of each panel indicate fluorescent intensities of NF-κB p65 (green) and DAPI (blue) along the yellow lines. Scale bar, 20 µm. (E) Localization of NF-kB p65 are represented by cytoplasm localization (yellow), cytoplasm and nuclear localization (grey), and nuclear localization (black). Data are means from 100 cells pooled from four individual experiments.

Fig.1

A
vehicle

B
LysoPS (10μM)

C
vehicle   LysoPS (10μM)

beta III Tubulin

D
vehicle   LysoPS (10μM)

GFAP

E

-2'   0'   6'   12'

18'   24'   30'   120'

F

Number of branch terminal points vs. Time after LysoPS stimulation (min)

G

branch extension (μm) vs. Time after LysoPS injection (min)

H

Scale unit:μm

vehicle   LysoPS

I

Velocity (μm/min)

vehicle   LysoPS

##

Figure 2

A

B

C

D

Figure 3

Figure 4

Figure 5

A
vehicle | LysoPS | KT5720+LysoPS | LY294002+LysoPS

phalloidin

Y27632+LysoPS | U0126+LysoPS | NSC23766+LysoPS | ML141+LysoPS

B
pAAV-CAGGS-EGFP-Rac1-N17

vehicle

LysoPS

EGFP
Iba1

Form factor

Rac1 DN (-) | Rac1 DN (+) | Rac1 DN (-) | Rac1 DN (+)
|
vehicle | LysoPS

C
pAAV-CAGGS-EGFP-Cdc42-N17

vehicle

LysoPS

EGFP
Iba1

Form factor

Cdc42 DN (-) | Cdc42 DN (+) | Cdc42 DN (-) | Cdc42 DN (+)
###
vehicle | LysoPS

D
Rac1 GTP
Total Rac1

Rac1 GTP (% vehicle)
250
200
150
100
50
0
vehicle | LysoPS

E
Cdc42 GTP
Total Cdc42

Cdc42 GTP (% vehicle)
500
400
300
200
100
0
#
vehicle | LysoPS

Figure 6

Figure 7

A WT

mRNA relative expression (fold)

IL1-β   IL6   TNF-α   iNOS   IL10   TGF-β   IGF-1

□ LPS + vehicle
■ LPS + LysoPS

###

B GPR34 KO

mRNA relative expression (fold)

IL1-β   IL6   TNF-α   iNOS   IL10   TGF-β   IGF-1

□ LPS + vehicle
■ LPS + LysoPS

###

C

vehicle

LysoPS

LPS

LPS + LysoPS

NFκ-B p65   DAPI   phalloidin

D Intensity profile

vehicle

LysoPS

LPS

LPS + LysoPS

Fluorescence intensity
Line Distance

— NFκ-B p65
— DAPI

E

Percentage of cells

vehicle   LysoPS   LPS   LPS +LysoPS

NFκ-B p65
■ cytoplasm localization
■ cytoplasm and nuclear localization
■ nuclear localization