

in buffer containing 20 mM Tris-HCl (pH 7.4), 300 mM sucrose, and 1 mM EDTA, and protein concentration was measured using the Bio-Rad Protein Assay (Bio-Rad) with BSA as a standard. LPCAT activity was measured according to Harayama et al. [2014]. Briefly, 0.01 μ g protein was added to equal volumes of reaction mixtures containing 200 mM Tris-HCl (pH 7.4), 4 mM CaCl₂, 2 mM EDTA, 0.03% Tween-20 (Wako Pure Chemical Industries, Osaka, Japan), 2 μ M each of 16:0-CoA, 18:1-CoA, 18:2-CoA, 20:4-CoA, and 22:6-CoA, and 50 μ M deuterium labeled 16:0 LPC in a total volume of 0.1 ml. After incubation at 37°C for 10 min, reactions were stopped by the addition of 0.3 ml of chloroform:methanol (1:2, vol/vol) containing dilaurylphosphatidylcholine as an internal standard. Total lipids were extracted by the method of Bligh and Dyer [Bligh and Dyer, 1959] and measured by an Acquity ultra performance liquid chromatography system (Waters, Milford, MA) and a TSQ Vantage triple stage quadrupole mass spectrometer (Thermo Fisher Scientific, Waltham, MD) (LC-MS).

SMALL INTERFERING RNA TRANSFECTION

Small interfering RNA (siRNA) targeting was used to knockdown LPCAT3 expression in U937 cells. siRNA against human LPCAT3 and siRNA control were purchased from Santa Cruz Biotechnology. LPCAT3 siRNA consisted of a pool of three target-specific 19–25 nt siRNAs designed to knock down gene expression. U937 cells were transfected using Lipofectamine[®] RNAiMAX (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. In brief, cells (5×10^5 cells/well) were seeded into 6-well plates in 2 ml of RPMI supplemented with 5% FBS and treated with PMA (100 ng/ml) for 12 h. After washing with PBS, the cells were resuspended in 2 ml of RPMI supplemented with 5% FBS. Lipofectamine[®] RNAiMAX was first diluted in Opti-MEM (150 μ l; Invitrogen) for 5 min before mixing with an equal volume of Opti-MEM containing the siRNA (12.5 nM). After 20 min of incubation, 250 μ l of the resulting RNAiMAX/siRNA was added directly onto the cells. After 24 h of incubation at 37°C (5% CO₂ atmosphere), the cells were rinsed with 2 ml of RPMI supplemented with 5% FBS and treated with IL-4 (20 ng/ml).

STATISTICAL ANALYSIS

Statistical analyses were performed using Student's *t*-test (two groups) or ANOVA (>two groups) followed by post hoc tests, the Tukey-Kramer test. A *P*-value of <0.05 was considered statistically significant. All statistical calculations were performed using EZR (Easy R, Saitama Medical Center, Saitama, Japan; <http://www.jichi.ac.jp/saitama-sct/SaitamaHP.files/statmedEN.html>), which is based on R and R commander [Kanda, 2013].

RESULTS

PMA PROMOTES THE DIFFERENTIATION OF HUMAN U937 MONOCYTIC CELLS INTO MACROPHAGE-LIKE CELLS

Human U937 monocytic cells are non-adherent cells. After treatment with PMA (100 ng/ml) for 12 h, U937 cells attached to the culture dish and developed elongated projections (Fig. 1A). PMA treatment significantly increased the mRNA expression of the macrophage marker CD68 (Fig. 1B) and the phagocytic activity compared with those of DMSO-treated U937 cells (Fig. 1C). These

results suggest that PMA promotes the differentiation of U937 cells into macrophage-like cells.

LPS PROMOTES THE DIFFERENTIATION OF PMA-TREATED U937 CELLS INTO M1-POLARIZED MACROPHAGES

LPS changed the shape of PMA-treated U937 cells from rounded to spindle-shaped (Fig. 2A, upper left), whereas IL-4 had no effect (Fig. 2A, upper right). LPS-activated U937 cells showed a significantly higher proportion of spindle-shaped cells than IL-4-activated U937 cells (Fig. 2A, lower). LPS promoted the phagocytic activity of PMA-treated U937 cells, whereas IL-4 had no effect (Fig. 2B). These results suggest that PMA-treated U937 cells themselves seem to be polarized into M2-polarized macrophages and that LPS may induce M1-polarized macrophages in PMA-treated U937 cells.

LPS upregulated the mRNA expression of the M1-markers CXCL10, TNF- α , and IL-1 β at 12–24 h in PMA-treated U937 cells (Fig. 3A–C, left), increased the secretion of CXCL10 and TNF- α (Fig. 3A and B, right), and upregulated the protein expression of IL-1 β at

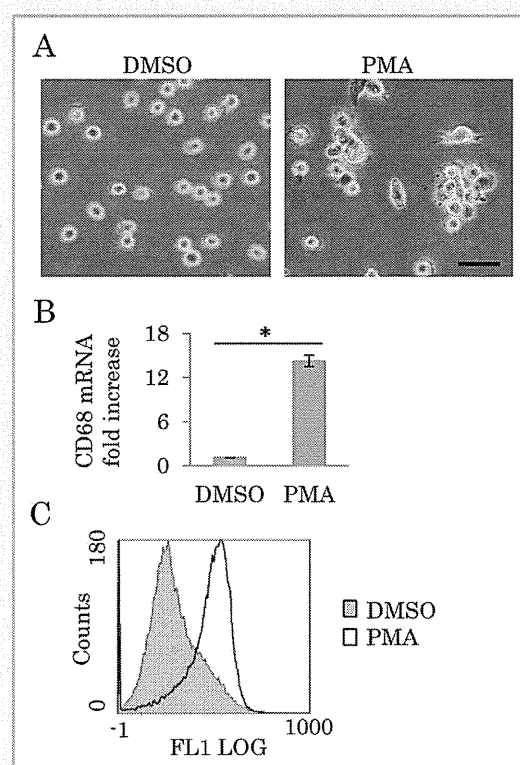


Fig. 1. PMA promotes the differentiation of human U937 monocytic cells into macrophage-like cells. U937 cells (5×10^5 cells/well) were seeded into 6-well plates and treated with DMSO or PMA (100 ng/ml) for 12 h. A: Representative images of DMSO-treated U937 cells (DMSO) and PMA-treated U937 cells (PMA). Scale bar = 50 μ m. B: The mRNA expression of the macrophage differentiation marker CD68 was analyzed by quantitative real-time PCR. Data are presented as the mean \pm SD of three independent experiments. **P* < 0.01 versus DMSO. C: Phagocytic activity was measured by flow cytometry. Filled and unfilled histograms represent DMSO-treated U937 cells (DMSO) and PMA-treated U937 cells (PMA), respectively. The experiment was performed three times. Similar results were obtained for each experiment and a representative histogram is shown.

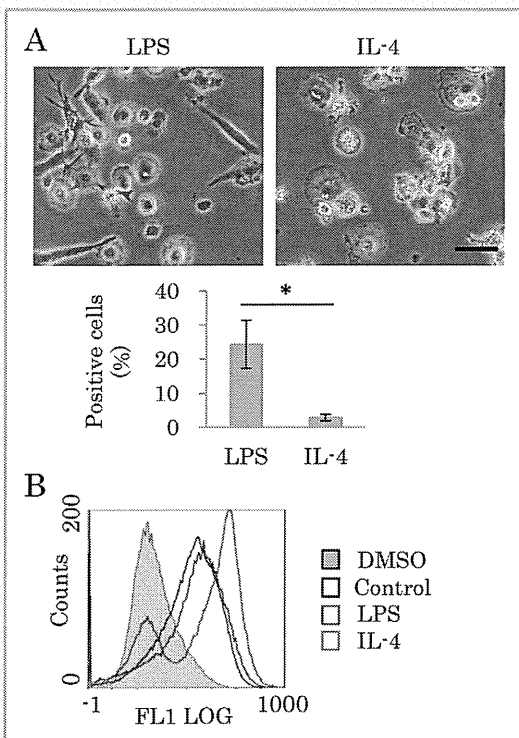


Fig. 2. LPS and IL-4 alter the morphology and function of PMA-treated U937 cells. U937 cells (5×10^5 cells/well) were seeded into 6-well plates and treated with PMA (100 ng/ml) for 12 h. After washing with PBS, the samples were stimulated with LPS (100 ng/ml) or IL-4 (20 ng/ml) for various periods of time (12, 24, 48, and 72 h). **A:** Representative images of M1- and M2-polarized macrophages (upper). Scale bar = 50 μ m. The percentage of spindle-shaped cells (positive cells) was quantified as described in the materials and methods section (lower). * $P < 0.01$ versus IL-4. **B:** Phagocytic activity was measured by flow cytometry. Filled, black, red, and blue histograms represent DMSO-treated U937 cells (DMSO), PMA-treated U937 cells (Control), LPS-activated U937 cells (LPS), and IL-4-activated U937 cells (IL-4), respectively. The experiment was performed three times. Similar results were obtained for each experiment and a representative histogram is shown. Data are presented as the mean \pm SD of three independent experiments.

12–72 h (Fig. 3C, right). These results indicate that LPS promoted M1-polarization of macrophages in PMA-treated U937 cells.

In PMA-treated U937 cells, which show certain properties of M2-polarized macrophages, IL-4 upregulated the mRNA expression of the M2-markers CD206, IL-1ra, and TGF- β at 24–72 h (Fig. 4A–C, left), and upregulated the protein expression of CD206 and IL-1ra (Fig. 4A, middle and B, right). Flow cytometric analysis showed that IL-4-activated U937 cells expressed the surface marker CD206 (Fig. 4A, right). However, IL-4 did not affect the secretion of TGF- β (Fig. 4C, right), indicating that IL-4 increased expression of M2-markers, not all, in PMA-treated U937 cells. These results suggest that IL-4 enhanced M2-polarization in PMA-treated U937 cells, which already had some properties of M2-polarized macrophages.

LPS DOWNREGULATES LPCAT3 IN PMA-TREATED U937 CELLS

The gene expression profiles of LPCATs in LPS or IL-4-activated U937 cells were examined by real-time PCR (qRT-PCR). LPS significantly downregulated the mRNA expression of LPCAT1, LPCAT2, and LPCAT3 at 12–72 h and that of LPCAT4 at 12 h in PMA-treated U937 cells (Fig. 5A). The LPS-induced downregulation of LPCAT2 in U937 cells differed from the previously reported upregulation of LPCAT2 by LPS in mouse peritoneal macrophages; this discrepancy could be due to differences between the U937 cell line and primary macrophages. LPCAT3 mRNA expression in LPS-activated U937 cells was reduced to one-tenth of that in untreated cells. IL-4 treatment caused a mild upregulation of LPCAT1 and LPCAT3 mRNA expression at 24–48 h and of LPCAT4 at 24 h. LPS significantly decreased LPCAT activity toward linoleoyl-CoA and arachidonoyl-CoA, whereas IL-4 did not (Fig. 5B). Neither LPS nor IL-4 changed LPCAT activity toward palmitoyl-CoA, oleoyl-CoA, and docosahexaenoyl-CoA.

KNOCKDOWN OF LPCAT3 IN PMA-TREATED U937 CELLS DECREASES LPCAT ACTIVITY TOWARD LINOLEOYL-COA AND ARACHIDONOYL-COA

PMA-treated U937 cells were transfected with control siRNA or LPCAT3 siRNA. Transfection of LPCAT3 siRNA into PMA-treated

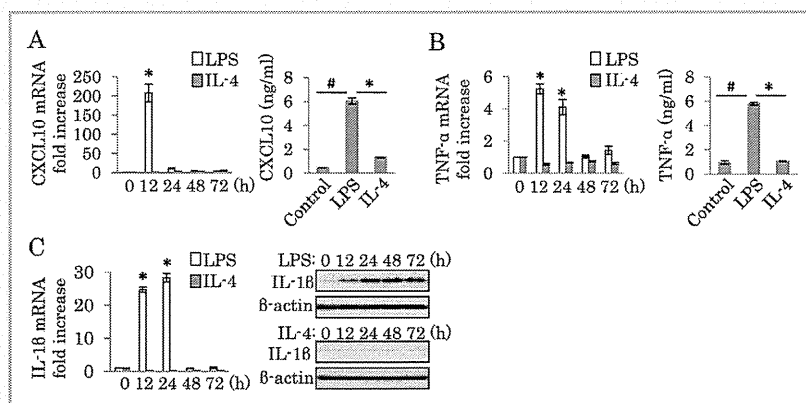


Fig. 3. LPS induces the expression of M1-markers. **A–C:** The mRNA expression of the M1 macrophage markers CXCL10, TNF- α , and IL-1 β was analyzed by quantitative real-time PCR. * $P < 0.01$ versus LPS at 0 h (left panels). The secretion of CXCL10 and TNF- α in culture after 72 h was analyzed by ELISA. # $P < 0.01$ versus control, * $P < 0.01$ versus IL-4 (A and B, right panels). The protein expression of IL-1 β was analyzed by western blotting. The experiment was performed three times. Similar results were obtained for each experiment and a representative immunoblot is shown (C, right).

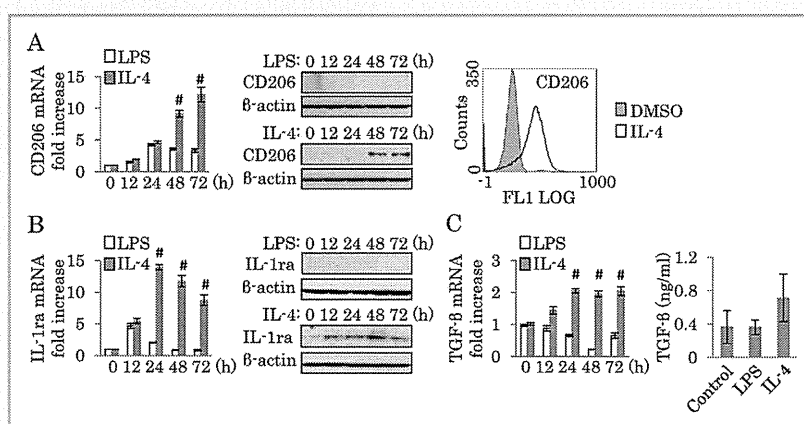


Fig. 4. IL-4 induces the expression of M2-markers. A–C: The expression of M2 macrophage markers CD206, IL-1ra, and TGF- β was analyzed by quantitative real-time PCR. $^{\#}P < 0.01$ versus IL-4 at 0 h (left panels). The protein expression of CD206 and IL-1ra was analyzed by western blotting. The experiment was performed three times. Similar results were obtained for each experiment and representative immunoblots are shown (A, middle and B, right panels). CD206 membrane expression was measured by flow cytometry. Filled and unfilled histograms represent DMSO-treated U937 cells (DMSO) and IL-4-activated U937 cells (IL-4), respectively. The experiment was performed three times. Similar results were obtained for each experiment and a representative histogram is shown (A, right). The secretion of TGF- β in culture after 72 h was analyzed by ELISA (C, right).

U937 cells efficiently downregulated the mRNA expression of LPCAT3 compared with the control siRNA (Fig. 6A). LPCAT activities toward linoleoyl-CoA and arachidonoyl-CoA were significantly decreased in LPCAT3 siRNA-transfected cells (Fig. 6B).

KNOCKDOWN OF LPCAT3 SHIFTS POLARIZATION TO M1 IN PMA-TREATED U937 CELLS

LPCAT3 siRNA-transfected cells showed a spindle-shaped morphology similar to that of M1-polarized macrophages, whereas control siRNA-transfected cells showed a rounded morphology typical of M2-polarized macrophages (Fig. 7A, left). LPCAT3 siRNA-transfected cultures had a significantly higher proportion of spindle-shaped cells than control siRNA-transfected cultures (Fig. 7A, right). Analysis of the mRNA and protein expression of the M1/M2-markers CXCL10 and CD206, which

showed the highest expression among the investigated markers, indicated that LPCAT3 knockdown significantly increased the secretion of CXCL10. IL-4 had no effect on CXCL10 production by the M1-polarized macrophages that were induced by LPCAT3 knockdown of PMA-treated U937 cells (Fig. 7B). Knockdown of LPCAT3 prevented upregulation of CD206 protein expression visualized by IL-4 (Fig. 7C). These results suggest that knockdown of LPCAT3 shifts PMA-treated U937 cells to M1-polarized macrophages.

DISCUSSION

M1- and M2-polarized macrophages are distinguished by their cellular morphology [Pelegrin and Surprenant, 2009; Zhang et al.,

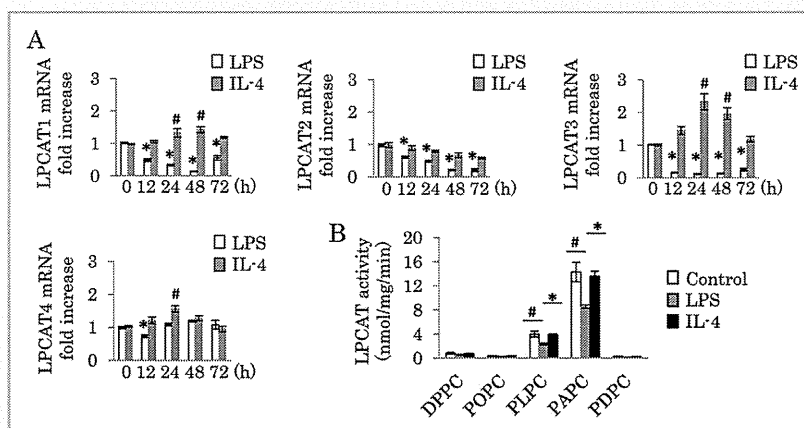


Fig. 5. LPS significantly decreases the mRNA expression of LPCATs and their enzymatic activities. A: The mRNA expression of LPCAT1, LPCAT2, LPCAT3, and LPCAT4 was analyzed by quantitative real-time PCR. $^*P < 0.01$ versus LPS at 0 h. $^{\#}P < 0.01$ versus IL-4 at 0 h. B: LPCAT activity was measured using LC-MS/MS. $^*P < 0.01$ versus control, $^{\#}P < 0.01$ versus IL-4. Data are presented as the mean \pm SD of three independent experiments. DPPC, dipalmitoyl phosphatidylcholine; POPC, palmitoyl oleoyl phosphatidylcholine; PLPC, palmitoyl linoleoyl phosphatidylcholine; PAPC, palmitoyl arachidonoyl phosphatidylcholine; PDPC, palmitoyl docosahexaenoyl phosphatidylcholine.

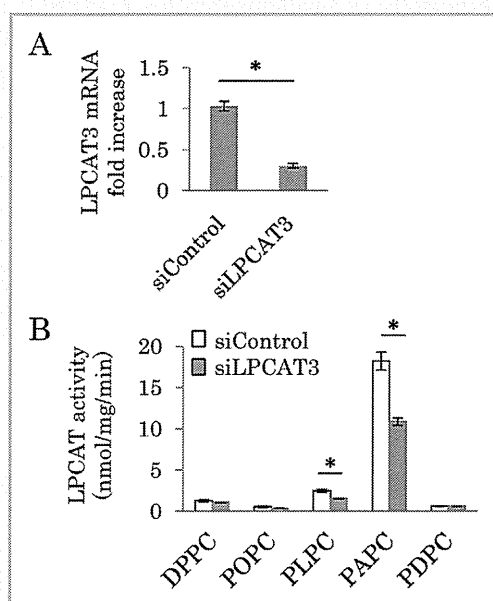


Fig. 6. Knockdown of LPCAT3 in PMA-treated U937 cells decreases LPCAT activity toward linoleoyl-CoA and arachidonoyl-CoA. U937 cells (5×10^5 cells/well) were seeded into 6-well plates and treated with PMA (100 ng/ml) for 12 h. After washing with PBS, the samples were transfected with control siRNA (siControl) and LPCAT3 siRNA (siLPCAT3) using Lipofectamine[®] RNAiMAX and incubated for 48 h. A: The mRNA expression of LPCAT3 was analyzed by quantitative real-time PCR. B: LPCAT activity was measured using LC-MS/MS. Data are presented as the mean \pm SD of three independent experiments. * $P < 0.01$ versus control siRNA-transfected cells.

2015], surface antigen presentation, production of chemokines and cytokines [Biswas et al., 2006; Umemura et al., 2008], and phagocytic activity [Vereyken et al., 2011]. CXCL10, TNF- α , and IL-1 β are known to be released by M1-polarized macrophages and are referred to as M1-markers [Martinez et al., 2006; Müller-Quernheim et al., 2012]. The macrophage mannose receptor CD206, IL-1ra, and TGF- β are anti-inflammatory and M2-markers. The mRNA and protein expression of CD206 is well known to be potently upregulated by IL-4 stimulation [Stein et al., 1992; Porcheray et al., 2005]. However, some studies described that CD206 was not always good marker for human M2-macrophages [Daigneault et al., 2010; Jaguin et al., 2013]. To confirm that CD206 is a proper marker of M2-polarized macrophages, we examined the surface expression of CD206 in IL-4-activated U937 cells by flow cytometry (Fig. 4A, right). Our results certainly showed that IL-4 increased the expression of CD206 in PMA-treated U937 cells.

A previous study suggested that the M1/M2-polarization pattern depends on the characteristics of the tissue microenvironment in U937 cells [Sanchez-Reyes et al., 2014]. In the present study, we showed that PMA-treated U937 cells stimulated with LPS and IL-4 are fully polarized to M1- and M2-macrophages, respectively, indicating that M1/M2-macrophage polarization in U937 cells may be a useful model for the investigation of macrophage function. Human monocytic THP-1 cells are reported to differentiate into M2-polarized macrophages in response to PMA treatment [Tju et al., 2009]. In the present study, PMA-treated U937 cells showed low levels of CXCL10, TNF- α , and IL-1 β and the typical cellular morphology and phagocytic activity of M2-polarized macrophages. These results suggest that

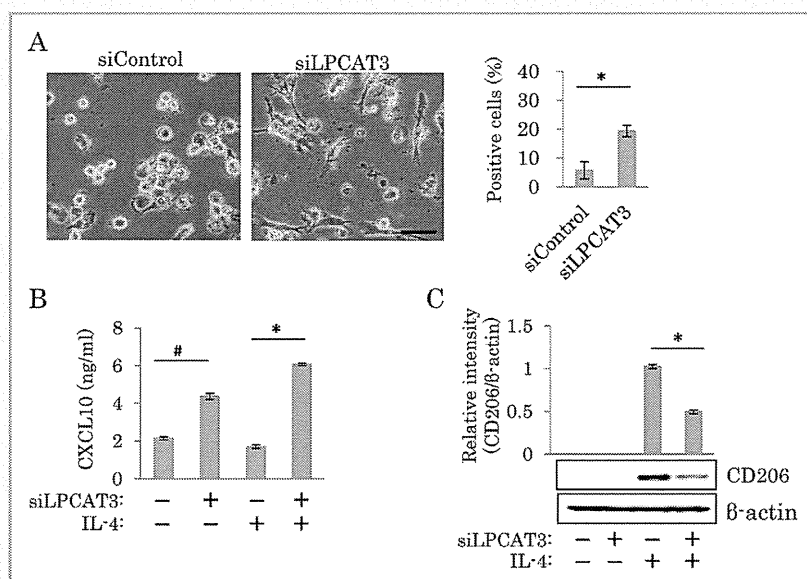


Fig. 7. Knockdown of LPCAT3 shifts PMA-treated U937 cells to M1-polarized macrophages. A: Representative images of control siRNA (siControl) and LPCAT3 siRNA (siLPCAT3) in PMA-treated U937 cells (left panels). Scale bar = 50 μ m. The percentage of spindle-shaped cells was quantified as described in the materials and methods section (right). * $P < 0.01$ versus control siRNA-transfected cells. B: U937 cells (5×10^5 cells/well) were seeded into 6-well plates and treated with PMA (100 ng/ml) for 12 h. After washing with PBS, the samples were transfected with control siRNA and LPCAT3 siRNA for 24 h. The cells were rinsed with 2 ml of RPMI supplemented with 5% FBS and then stimulated with or without IL-4 (20 ng/ml) and incubated for 48 h. The secretion of CXCL10 was analyzed by ELISA. Data are presented as the mean \pm SD of three independent experiments. # $P < 0.01$ versus control siRNA-transfected cells, * $P < 0.01$ versus control siRNA-transfected cells with IL-4. C: CD206 protein expression was analyzed by western blotting. The experiment was performed three times. Similar results were obtained for each experiment and a representative immunoblot is shown. Band intensity levels were normalized to β -actin. Data are presented as the mean \pm SD of three independent experiments. * $P < 0.01$ versus LPCAT3 siRNA-transfected cells with IL-4.

PMA-treated U937 cells, which already have some properties of M2-polarized macrophages, shift into cells with more properties of M2-polarized macrophages in response to IL-4.

Accumulation of saturated fatty acids, such as palmitic acid, causes lipotoxicity and induces inflammation, ER stress, and cell death [Ariyama et al., 2010; Prieur et al., 2011]. By contrast, polyunsaturated fatty acids, such as linoleic acid, arachidonic acid, and docosahexaenoic acid, reduce inflammation and ER stress induced by palmitic acid [Ishiyama et al., 2011; Rong et al., 2013]. Furthermore, saturated fatty acids induce the expression of M1-markers, whereas polyunsaturated fatty acids strongly induce the expression of M2-markers in adipose tissue macrophages [Prieur et al., 2011]. Sphingolipids, which are one of the essential lipid components of cellular membranes, regulate the differentiation of monocytes into macrophages in U937 cells [Yamamoto et al., 2011]. LPCAT3 is reported to be a major contributor to increase polyunsaturated fatty acids, including linoleic acid and arachidonic acid, and is associated with inflammatory responses in human primary macrophages [Ishibashi et al., 2013]. Therefore, among various LPCATs, LPCAT3 may particularly affect M1/M2-macrophage polarization associated with inflammation. In the present study, we showed that LPCAT3 mRNA expression was strongly downregulated and LPCAT activity toward linoleoyl-CoA and arachidonoyl-CoA was reduced in M1-polarized macrophages (Fig. 5).

Induction of LPCAT3 activity increases the abundance of polyunsaturated PCs and downregulates the expression of inflammatory mediators such as CXCL10, TNF- α , and IL-1 β in mouse primary macrophages. By contrast, knockdown of LPCAT3 decreases the abundance of polyunsaturated PCs and induces inflammatory responses [Rong et al., 2013]. We showed that knockdown of LPCAT3 significantly increased the secretion of the M1 macrophage marker CXCL10 in PMA-treated U937 cells and IL-4-activated U937 cells. In addition, knockdown of LPCAT3 suppressed the expression of the M2 macrophage marker CD206 induced by IL-4 treatment (Fig. 7). Our results suggest that knockdown of LPCAT3 induces a phenotypic shift to M1-polarized macrophages.

Polyunsaturated fatty acids increase membrane fluidity and the flexibility of cellular membranes [Holzer et al., 2011], whereas excessive contents of saturated fatty acids reduce membrane fluidity. The decrease in the content of PCs containing linoleic acid or arachidonic acid in biological membranes caused by the suppression of LPCAT3 may influence membrane fluidity, curvature and function. In fact, LPCAT3 knockdown in HEK293 cells was reported to have a remarkable effect on cell morphology [Jain et al., 2009]. Therefore, LPCAT3 may also cause morphological changes, such as those of M1-polarized macrophages, in LPCAT3 siRNA-transfected cells.

In summary, we showed that modulation of LPCAT3 expression regulates M1-macrophage polarization. This study supports the development of new anti-inflammatory drugs capable of altering macrophage phenotypes via the remodeling pathway of glycerophospholipids.

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