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Myeloid-specific deficiency of *long-chain acyl CoA synthetase 4* (*Acsl4*) reduces inflammation *in vitro* and *in vivo* by remodeling phospholipids and reducing production of arachidonic acid-derived proinflammatory lipid mediators

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Abstract

In response to infection or tissue damage, resident peritoneal macrophages (rpMACs) produce inflammatory lipid mediators from the polyunsaturated fatty acid (PUFA), arachidonic acid (AA). Long-chain acyl-CoA synthetase 4 (ACSL4) catalyzes the covalent addition of a CoA moiety to fatty acids, with a strong preference for AA and other PUFA containing three or more double bonds. PUFA-CoA can be incorporated into phospholipids, which is the source of PUFA for lipid mediator synthesis. In this study, we demonstrated that deficiency of *Acsl4* in mouse rpMACs resulted in a significant reduction of AA incorporated into all phospholipid classes and a reciprocal increase in incorporation of oleic acid and linoleic acid. After stimulation with opsonized zymosan (opZym) a diverse array of AA-derived lipid mediators, including leukotrienes, prostaglandins, HETEs, and lipoxins, were produced and were significantly reduced in *Acsl4*-deficient rpMACs. The *Acsl4*-deficient rpMACs stimulated with opZym also demonstrated an acute reduction in mRNA expression of the inflammatory cytokines, *Il6*, *Ccl2*, *Nos2* and *Ccl5*. When *Acsl4*-deficient rpMACS were incubated *in vitro* with the TLR4 agonist, LPS, the levels of leukotriene B_4 (LTB_4) and prostaglandin E_2 (PGE_2) were also significantly decreased. In LPS-induced peritonitis, mice with myeloid-specific *Acsl4* deficiency had a significant reduction in LTB_4 and PGE_2 levels in peritoneal exudates, which was coupled with reduced infiltration of neutrophils in the peritoneal cavity as compared with WT mice. Our data demonstrate that chronic deficiency of *Acsl4* in rpMACs reduces the incorporation of AA into phospholipids, which reduces lipid mediator synthesis and inflammation.

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Introduction

Tissue-resident macrophages are sentinel cells that survey their environment to detect pathogens or tissue damage (1). Resident peritoneal macrophages (rpMACs) are long-lived cells derived from the yolk-sac during development and maintain their populations through self-renewal rather than recruitment from bone marrow-derived monocytes (2). Upon sensing infection or injury, resident macrophages become activated and produce inflammatory signaling molecules that lead to endothelial permeability, swelling, and the recruitment of immune cells (3, 4). Chronologically, neutrophils are typically the first myeloid cell-type recruited after tissue injury or infection, followed by monocytes and then macrophages that aid in the clearance of pathogens and dead cells, and help to orchestrate the resolution of inflammation and the repair and remodeling of tissue (5).

An important aspect of inflammatory activation of macrophages is the release of lipid mediators generated by the enzymatic oxidation of polyunsaturated fatty acids (PUFA). Arachidonic acid (AA, 20:4n-6) is an ω−6 PUFA that is the precursor for the biosynthesis of many pro-inflammatory lipid mediators. AA can be enzymatically oxidized to generate pro-inflammatory lipid mediators, such as prostaglandins, thromboxanes, and leukotrienes, as well as several other classes of lipid mediators, all of which have a broad range of biological activities. Each of the lipid mediators can have various, but specific, roles during inflammation. For instance, prostaglandin E_2 (PGE_2) can cause pain, increased blood flow to the injured area, and ultimately lead to edema (6, 7). The actions of PGE_2 on macrophages during inflammation are multi-factorial and include both pro-inflammatory and anti-inflammatory/immunosuppressive functions. For example, PGE_2 enhances Il-1β production in response to lipopolysaccharide (LPS), but can also blunt toll-like receptor 4 expression, enhance suppressor of cytokine 3 signaling, and diminish macrophage phagocytosis (8–11). Leukotriene B_4 (LTB_4) is a potent chemoattractant that recruits new pro-inflammatory immune cells such as neutrophils and macrophages (12, 13). Another group of AA-derived lipid mediators, the hydroxyeicosatetraenoic acids (HETE), have diverse effects on inflammation based on the position and stereochemistry of the hydroxyl group. The murine 12/15-lipoxegenase (12/15-LOX), for example, can generate both 12- or 15-HETE from AA and are thus markers of this lipoxygenase activity (14). The 12-HETE product is pro-inflammatory and leads to neutrophil aggregation and an itching sensation demonstrating that the position of an individual hydroxyl group is critical for the activity of many of these lipid mediators (15, 16).

The resolution of inflammation is an active process that macrophages contribute to as well, which involves reduction of inflammatory signals as well as coordinated remodeling of the damaged tissues by clearance of apoptotic immune cell infiltrate and dead cell debris (17, 18). AA can have a crucial role in resolving inflammation when it is sequentially oxidized by murine 12/15-LOX and 5-LOX to generate the specialized pro-resolving lipid mediators (SPM) termed lipoxins (13). Similar to the pro-inflammatory lipid mediators, the biological actions of SPM are mediated by activation of specific receptors that can result in a broad range of effects, such as suppressing inflammatory signaling and neutrophil recruitment, increasing macrophage efferocytosis, and promoting tissue repair, among many of their various functions (19).

Long-chain acyl-CoA synthetases (ACSL) catalyze the thioesterification of long-chain fatty acids and coenzyme-A (CoA) to form a fatty acyl-CoA. There are five ACSL isoforms (1, 3, 4, 5, and 6) in mammals that localize to several different subcellular compartments and differ in their preferences for specific fatty acids (20). Mouse, rat, and human ACSL4 have strong preferences for AA, EPA, and adrenic acid (22:4n-6) (21–24). Human ACSL4 has a very strong preference for highly unsaturated fatty acids that contain greater than 3 double bonds, such as AA, EPA, and DHA, with negligible activity towards saturated fatty acids and mono-unsaturated fatty acids (22). Once the fatty acyl-CoA is generated it can be utilized in various metabolic pathways such as synthesis of phospholipids (PL), triglycerides, cholesterol esters, as well as oxidative phosphorylation to generate ATP (25). Our laboratory previously demonstrated that ACSL4-deficient adipocytes from mice fed a high fat diet had reduced AA incorporation by approximately 30 to 50% into all PL classes within adipocytes and a reciprocal 25% to 89% increase in the incorporation of linoleic acid (LA, 18:2) in differing PL species (26). However, deficiency of ACSL4 in adipocytes did not alter the incorporation of any fatty acid (FA) species into triglycerides. Furthermore, mouse ACSL4 has also been demonstrated to direct adrenic acid, as well as AA, into phospholipids, which is necessary for ferroptosis, a non-apoptotic form of programmed cell death (23).

Diet is the predominant source of all PUFA. AA can come from the diet directly or, alternatively, dietary LA can be converted into AA through several enzymatic steps within cells. Both exogenous and endogenous FA can be incorporated into PL through the consecutive actions of ACSLs and acyltransferases. The FA composition of cellular PL is constantly being remodeled through a process of de-acylation by phospholipases and re-esterification by ACSLs and acyltransferases, termed the Lands Cycle, which enriches PUFA at the sn-2 position of PL due to the specificity of the acyltransferases (27–29). ACSL4 has been demonstrated to be involved in the re-esterification of AA during constitutive and stimulated lipid remodeling (30, 31).

To elucidate the role of ACSL4 in phospholipid remodeling and inflammation within macrophages we isolated resident peritoneal macrophages (rpMACs) from mice with or without myeloid-specific deficiency of *Acsl4*. Macrophage *Acsl4* deficiency resulted in dramatic reduction in AA and other specific PUFA within PL, decreased production of pro-inflammatory AA-derived lipid mediators and cytokine expression. Furthermore, neutrophil migration and lipid mediator production was decreased *in vivo* during LPS-induced peritonitis in myeloid *Acsl4*-deficient mice, which demonstrated a physiologic role of ACSL4 in mediating acute inflammation.

Materials and Methods

Animals

All animal breeding and procedures were approved by the Institutional Animal Care and Use Committee at Tufts University (protocol number H2019–145). Homozygous mice containing two LoxP sites flanking exon 3 and 4 of *Acsl4* (floxed *Acsl4*) were generated at the North Carolina Animal Models Core as previously reported (26). The floxed *Acsl4* mice were mated with heterozygous LysM-Cre mice from the Jackson Laboratory (B6.129P2-*Lyz2*$^{tm1(cre)Ifo}$/J), and the male progeny, *Acsl4*$^{FL/Y}$ *Lyz2*$^{Cre/WT}$ (*Acsl4*^{mKO}) or *Acsl4*$^{FL/Y}$

Lyz2$^{WT/WT}$ (*Acsl4*^{Flox}), were used for our studies. The mice were maintained on Teklad 2016 chow diet and were euthanized by CO_2 asphyxiation and cervical dislocation at 12–16 months of age, at which point there were no significant differences in body weights.

Isolation and culture of resident peritoneal macrophages

After euthanasia, the peritoneal cavity was exposed and 3mL of PBS containing 0.5% bovine serum albumin and 2mM ethylenediaminetetraacetic acid (EDTA) (MACS buffer) was injected into the peritoneal cavity and massaged for 30 seconds. The lavage fluid was removed with a 20-gauge needle and centrifuged at 300 x g for 10 min. The pellet was resuspended in MACS buffer and the macrophage population was purified by negative selection through magnetic columns as per the manufactures protocol (Miltenyi Biotec, Mouse Macrophage Isolation Kit (Peritoneum)), which resulted in a 99.5% pure population of macrophages as demonstrated by FACS analysis of $CD11b^+$ $F4/80^+$ cells (Supplemental Fig. 1).

For the culture of rpMACs, cells were resuspended in DMEM, low glucose (ThermoFisher, 11885), at 1×10^6 cells/mL and allowed to adhere to tissue-culture plastic for 45 min at 37°C. For lipid mediator analysis, 1 mL of the cell suspension per well of a 12-well plate was used, and for gene expression analysis, 0.5 mL of the suspension in each well of a 24-well plate was used. After adhesion, cells were washed once with PBS and new DMEM medium containing either opsonized Zymosan or LPS was added, and cells were incubated for indicated times.

Opsonized zymosan treatment and phagocytosis assay

Zymosan A (Sigma Aldrich, Z4250) was resuspended at 20 mg/mL in 500 μL PBS by vigorous vortexing. The zymosan suspension was centrifuged at 1000 x g for 15 minutes and resuspended again at 20 mg/mL in 500 μL PBS. An equal volume of opsonizing reagent (ThermoFisher, Z2850) was added, vortexed, and incubated at 37°C for 60 minutes. The opsonized zymosan (opZym) was then washed 2x with PBS and resuspended at 10 mg/mL in PBS. Particles of opZym were counted with a hemocytometer and used at 10 particles per cell. To assay phagocytosis in rpMACs, opsonized zymosan-FITC (ThermoFisher, Z2841), was incubated with cells for one hour. Fluorescence of non-phagocytosed zymosan-FITC particles was quenched with trypan blue (1 mg/mL) for 1 minute at room temperature and cells were then imaged with a Zeiss Axiovert 200 fluorescent microscope (32).

Pulse-chase with radio-labeled AA

Isolated rpMACs were adhered to Corning 12-well plates for 45 min and were then given new DMEM medium containing 8.75 μM 1-^{14}C-AA (0.5 μCi/mL) (PerkinElmer, NEC661050UC) bound 3:1, mol:mol, with fatty acid free bovine serum albumin (BSA) and incubated at 37°C in 5% CO_2 for 18 hours (pulse). Cells were then washed with PBS containing 2% BSA 3 times to remove any free AA and fresh DMEM medium was added to the cells containing 0.5% BSA and incubated for 6 hours. Medium was then collected, and cells were lysed using Qiagen RLT buffer and radioactivity from both was measured with a liquid scintillation counter.

Lipidomics Analysis

Multidimensional mass spectrometry-based shotgun lipidomics analysis of lipids for macrophages samples was performed as described (33, 34). In brief, premixed internal standard was added to 10^6 isolated macrophages for quantitation of lipid species and then normalized with the protein content (per mg protein), which was performed following the instruction of Pierce™ BCA protein assay kit (Cat #23225, Thermo Scientific). The lipids were extracted using a modified Bligh and Dyer procedure (35), and each lipid extract was reconstituted in chloroform/methanol (1:1, v/v) at a volume of 50 μL.

For shotgun lipidomics, lipid extracts were diluted to a final concentration of ~500 fmol total lipids μL^{-1}. Mass spectrometric analysis was performed on a triple quadrupole mass spectrometer (TSQ Altis, Thermo Fisher Scientific, San Jose, CA) and a Q Exactive mass spectrometer (Thermo Scientific, San Jose, CA), both of which were equipped with an automated nanospray device (TriVersa NanoMate, Advion Bioscience Ltd., Ithaca, NY) as described (36). Identification and quantification of lipid species were performed using an automated software program (37, 38). Data processing (ion peak selection, baseline correction, data transfer, peak intensity comparison and quantitation) was performed as described (38).

Lipid mediator analysis by targeted LC-MS/MS

The rpMACs were isolated as described above and plated in 6-well tissue culture plates in phenol red-free Dulbeco's Modified Eagles Medium (DMEM) for 45 min. After adhering to the plate, macrophages were washed with PBS 2x and media was replaced with 2 mL PBS (with 0.9 mM $CaCl_2$ and 0.49 mM $MgCl_2$-$6H_20$) with or without opsonized zymosan (10 particles per cell) for 2 hrs at 37°C. The cells and medium were then combined with two volumes of ice-cold methanol containing commercially available deuterium-labeled internal standards (d_4-LTB_4, d_8-5-HETE, d_4-PGE_2 and d_5-LXA_4; Cayman Chemical) for calculation of extraction efficiency. Following centrifugation (13,000 r.p.m.), supernatants were carefully separated from the resulting pelleted material and subjected to solid phase extraction (SPE) (39). The pellets were stored at −80°C and later used for protein determination via Pierce BCA protein assay (Thermo). SPE and LC–MS/MS analysis was conducted as described in part previously (39). Briefly, the pH of the samples was reduced via the addition of acidified water (pH 3.5). Acidified samples were then added to conditioned C18 SPE columns (Biotage). Following a column wash with n-hexanes, lipid mediators were liberated from the column and collected via the addition of methyl formate. Samples were concentrated by evaporating the solvent using N_2 gas and then resuspended in a solution of equal parts methanol and water (50:50). To ensure the elimination of any precipitate, samples were then centrifuged and transferred to clean glass inserts and amber auto sampler vials for LC-MS/MS analysis. Using a high-performance liquid chromatograph (Shimadzu) coupled to a QTrap5500 mass spectrometer (AB Sciex) operating in negative ionization mode, lipid mediators were identified using scheduled multiple reaction monitoring (MRM) transitions and by matching their retention time with synthetic standards run in parallel. The MRM transitions used included: m/z 351/189 (PGE_2, PGD_2), 353/193 ($PGF_{2\alpha}$), 369/169 (TXB_2), 335/195 (LTB_4 and isomers), 351/115 (LXA_4, 15epi-LXA_4), 351/221 (LXB_4), 303/259 (AA), 335/115 (5, 15-diHETE), 319/219

(15-HETE), 319/179 (12-HETE), and 319/115 (5-HETE). Lipid mediators were quantified by accounting for the extraction recovery of internal deuterated standards and by calibration curves of external standards for each individual mediator.

ACSL activity assay

ACSL activity was measured as previously described (26, 40), briefly, rpMACs from three mice were pooled and the cells were sheared with 30 passes through a 26-gauge needle in sucrose buffer (10 mM Tris pH 7.4, 1 mM EDTA, 0.25 M sucrose). Lysates were centrifuged at 100,000 x g and the pellet containing the membrane fraction was resuspended in sucrose buffer. Protein was quantitated with Pierce™ BCA Protein Assay Kit (ThermoFisher). Membrane protein fractions (5 μg) were added to 250 μL assay buffer (10 mM ATP, 250 μM CoA, 5 mM DTT, 8 mM $MgCl_2$*$6H_2O$, 175 mM Tris, pH 7.4, and 0.03% triton X-100) containing 10 μM 1-^{14}C-AA (PerkinElmer), and incubated at room temperature for 20 minutes. The reaction was stopped with the addition of 2.25 mL modified Dole's reagent (80:20:1, isopropanol:heptane:1 M H_2SO_4), incubated at room temp for 10 minutes, followed by the addition of 2 mL Heptane and 0.5 mL H_2O. After vortexing, phases separated for 15 min and the aqueous phase was measured with a liquid scintillation counter.

LPS-induced peritonitis and FACS analysis

Mice were injected i.p. with 10 μg/kg LPS and euthanized 4 hours later by cardiac puncture to collect blood while anesthetized with 2.5% isofluorane, followed by cervical dislocation. The mouse peritoneum was exposed and injected with 3 mL PBS containing 0.5% BSA and 2mM EDTA. The peritoneal lavage fluid was collected and centrifuged for 10 min at 400 x g to pellet cells. Cells were then stained with fluorochrome conjugated REAfinity antibodies (2 μL per 10^6 cells) against mouse CD11b (VioGreen), F4/80 (PE), and Ly6G (APC) (Miltenyi Biotec) for 15 minutes at 4°C. Cells were washed and centrifuged before FACS analysis on a BD LSR II. Analysis of the FACS data was performed with FlowJo software.

Lipid mediator purification and measurement by ELISA

Peritoneal lavage fluid (PLF) from mice after 4 hr LPS-induced peritonitis was collected and centrifuged for 10 minutes at 400 x g to remove cells. The PLF was acidified by addition of an equal volume of 1 M acetate buffer (pH 4.0). Purification of the lipid mediators was accomplished using SPE (C18) columns (Cayman Chemical, 400020) and eluted with ethyl acetate containing 1% methanol. The ethyl acetate was evaporated under nitrogen gas and the concentrations of LTB_4 and PGE_2 were measured by ELISA according to manufactures protocols (Cayman Chemical; LTB_4, 520111; PGE_2, 514010). No purification of lipid mediators was used for measurement of LTB_4 and PGE_2 in culture medium from rpMACs treated with E. coli O55:B5-derived LPS (1ug/mL) (Sigma Aldrich, L6529) for 6 hours. The ELISAs were performed according to manufacturer's protocols as described above.

Quantitative real-time PCR

Isolated rpMACs were lysed in RLT buffer (Qiagen) containing 1% β-mercaptoethanol. RNA was isolated using the Qiagen RNeasy Mini Kit according to manufacturer's protocol. The RNA concentration was quantified using a Nanodrop spectrophotometer (Nanodrop 1000, Wilmington, DE) and 500 ng of RNA was used for the generation of cDNA with the ThermoFisher High-Capacity cDNA Reverse Transcription kit. cDNA was further diluted 10x in nuclease free water. Quantitative real-time PCR was performed using SybrGreen (ThermoFisher) with the Applied Biosystems 7300 Real-Time PCR System. All primers were used at a concentration of 1 μM (primer sequences are listed in Table I). Gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method and normalizing to *Gapdh*.

Results

Deficiency of Acsl4 in macrophages decreases cellular AA

We generated a mouse line with a myeloid-specific deficiency of *Acsl4* (*Acsl4*^{mKO}) by transferring the LysM-Cre transgene into our line of *Acsl4* floxed mice. We used the littermate floxed males without Cre as controls (*Acsl4*^{Flox}). *Acsl4* is located on the X chromosome and therefore males have only one copy of the gene. First, we analyzed *Acsl* mRNA expression in isolated rpMACs from male mice between the ages of 12–16 weeks. RT-PCR analysis revealed that the rpMACs from *Acsl4*^{mKO} mice had an 80–90% reduction of *Acsl4* mRNA as compared with the control *Acsl4*^{Flox} mice (Fig. 1A). Furthermore, we observed no significant differences in mRNA expression between groups for other *Acsl* isoforms (1, 3, 5, or 6) demonstrating that our approach to reduce *Acsl4* expression in rpMACS was specific and did not result in any compensatory increases in other ACSL isoforms.

We next measured total membrane ACSL activity towards AA in rpMACs. We observed that *Acsl4*^{mKO} rpMACs exhibited a 56.5% decrease in ACSL activity that catalyzes the addition of a CoA moiety to AA, confirming a role for ACSL4 in regulating arachidonyl-CoA formation (AA-CoA) (Fig. 1B). To determine whether expression of *Acsl4* regulated the rate of AA retention within the rpMACs we performed pulse-chase experiments to quantitate the turnover of radiolabeled AA within cells. For these studies, cells were incubated with 1-^{14}C-labeled AA bound to BSA for 18 hours. The cells were then washed and chased with medium containing 0.5% BSA without labeled AA for 6 hours. The rate of release of radiolabel from *Acsl4*^{mKO} rpMACs into the medium was 1.88-fold higher than that of *Acsl4*^{Flox} cells (Fig. 1C). There was also a concurrent 33% reduction in the radiolabel measured within the *Acsl4*^{mKO} rpMACs after the 6 hrs chase (Fig. 1D). The radiolabel released in the medium likely consists of both oxidized AA metabolites and free AA bound to albumin in the medium, but those individual molecular species are not distinguished with the pulse-chase assay. Consistent with our pulse-chase data, previous studies have also demonstrated that the generation of AA-CoA by ACSLs traps the fatty acid within cells resulting in increased uptake of AA into cells (41–43).

In separate studies, we harvested rpMACS from the two lines of mice and quantitated the level of free AA by LC-MS/MS. We found that levels of free AA were significantly reduced

in the *Acsl4*^{mKO} rpMACs as compared with the floxed controls (Fig. 1E). To determine whether the reduced levels of AA within *Acsl4*^{mKO} rpMACs was due to a reduction in the expression of enzymes involved in the synthesis of AA from LA, we quantitated mRNA expression of the relevant enzymes by RT-PCR analysis. Surprisingly, expression of the fatty acid Δ6-desaturase (*Fads2*), the initial and rate limiting enzyme in the synthesis of AA from LA, was increased 77-fold in *Acsl4*^{mKO} rpMACs as compared to the floxed controls (Fig. 1F). Also, mRNA expression of the elongation of very long-chain fatty acids 5, (*Elovl5*) enzyme was upregulated 2.7-fold, though the difference was not significant ($p = 0.056$). *Fads1*, the Δ5-desaturase, and last enzyme involved in the synthesis of AA, was slightly, but significantly, upregulated in *Acsl4*^{mKO} rpMACs (Fig 1F). In summary, our results demonstrate that *Acsl4* deficiency increases the rate that radiolabeled-AA is released from cells, which is likely due to the reduced ability of these cells to generate AA-CoA that cannot diffuse out of the cell. Furthermore, these data demonstrate that the reduced levels of AA within cells is not secondary to decreased synthesis of AA from LA. In fact, the deficiency of *Acsl4* led to increased expression of the enzymes involved in AA synthesis, which is likely an attempt to compensate for the reduction of AA within the cells.

Acsl4 deficiency in rpMACs decreases the incorporation of specific PUFA into all phospholipid classes

Our work and others have shown that deficiency of *Acsl4* results in decreased incorporation of AA within PL (23, 26). To evaluate the effects of *Acsl4* deficiency on PL remodeling, we utilized rpMACs and performed shotgun lipidomic analysis with tandem MS/MS to determine the profile of fatty acid content of PL species. Our studies demonstrated that deficiency of *Acsl4* in rpMACs potently reduced the levels of AA between 75–90% in all phospholipid classes (Fig. 2, Supplemental Fig. 2), with a 98% reduction in phosphatidic acid (PA-18:0–20:4) (Fig. 2C). DPA (22:5n-3 or n-6) and the ω−3 PUFA, DHA (22:6n-3), were also significantly reduced in all phospholipids, though ω−3 PUFA was only a minor fraction of the total PUFA in the cells. We did not detect EPA (20:5n-3) in any phospholipids from rpMACs of either line of mice. Conversely, LA (18:2) and oleic acid (OA, 18:1) were significantly increased in all phospholipid classes with the largest increases found in phosphatidylethanolamine (PE) (Fig. 2A, B, Supplemental Fig. 2). Deficiency of *Acsl4* also resulted in decreased diacylpalmitoyl phosphatidylcholine (PC-16:0–16:0) and, to a lesser extent, 16:0–16:0-phosphatidic acid, without significant changes to 16:0–16:0 in other PL classes. These studies demonstrate that *Acsl4* is the predominant *Acsl* isoform in macrophages that facilitates the incorporation of AA into PL.

Lipid Mediators are reduced in Acsl4-deficient macrophages stimulated with opZym

In our studies investigating the effects of *Acsl4* deficiency on the PL profile in rpMACs, our most robust observation was a 75–90% reduction in the incorporation of AA into phospholipids. Given that AA within PLs serve as the primary source for the generation of lipid mediators after an inflammatory stimulation, we assessed the production of eicosanoids. To determine how reductions in AA content in PL, caused by *Acsl4* deficiency, altered the generation of lipid mediators we incubated rpMACS in the absence and presence of opZym, as an inflammatory stimulus. Of relevance to our studies, others have demonstrated that zymosan increases production of lipid mediators generated through the

COX-1/2, 5-LOX, and 12/15-LOX pathways to a much greater extent as compared to LPS in rpMACs (44). Indeed, after a 2-hour incubation with opZym (10 particles/cell), several eicosanoids were increased, including the prostaglandins (PGE_2, PGD_2, $PGF_{2\alpha}$), leukotrienes (LTB_4 and *trans* isomers), lipoxins (LXA_4, 15epi-LXA_4, LXB_4) and HETEs (5-HETE, 12-HETE, 15-HETE, 5,15-diHETE) (Fig. 3). Remarkably, all eicosanoids in our targeted analysis were significantly reduced, in *Acsl4*^{mKO} rpMACS as compared to *Acsl4*^{Flox} rpMACS (Fig. 3). Importantly, there was no difference in the uptake of opZym between *Acsl4*^{Flox} or *Acsl4*^{mKO} rpMACs that would explain the reductions in eicosanoids (Supplemental Fig. 3).

We next investigated whether mRNA expression of various enzymes involved in the biosynthesis of eicosanoids was altered. RT-PCR analysis revealed no differences in 5-lipoxygenase activating protein (*Alox5ap*), Leukotriene A_4 hydrolase (*Lta4h*), *Alox5,* or *Ptgs2* mRNA (Fig. 4A). We did observe a significant, 2.5-fold increase, in *Alox15* (Fig. 4A), although this did not overcome the suppression of eicosanoid biosynthesis in *Acsl4*^{mKO} rpMACs. Collectively, these results suggest that deficiency of *Acsl4* in rpMACS impairs biosynthesis of eicosanoids by reducing the bioavailability of AA.

Acsl4 deficiency in macrophages reduces expression of Il6, Ccl5, Ccl2 and Nos2 in response to opZym.

Given the decreased production of both inflammatory eicosanoids (leukotrienes, prostaglandins, and HETEs) and some pro-resolving mediators (lipoxins) in *Acsl4*^{mKO} rpMACs after opZym treatment, we investigated whether deficiency of *Acsl4* alters cytokine expression *in vitro*. Expression of *Il6*, *Nos2*, *Ccl5* and *Ccl2* were significantly increased in rpMACs stimulated with zymosan at 6 hrs, while they all returned to baseline by 24 hrs post-stimulation (Fig. 4B). We observed significant reductions in *Il6*, *Nos2*, *Ccl5* and *Ccl2* mRNA expression at 6 hours in the *Acsl4*^{mKO} rpMACs as compared to the *Acsl4*^{Flox} controls. The increase in *Nos2* mRNA levels was completely blunted in the *Acsl4*^{mKO} rpMACS. Also, *Nos2* mRNA levels were significantly decreased at 24 hrs post-stimulation in the *Acsl4*^{mKO} as compared to *Acsl4*^{Flox} rpMACs, but no differences were observed in expression of any of the other genes at 24 hrs (Fig. 4B). In summary, these data demonstrate that *Acsl4* deficiency reduced inflammatory cytokine expression in response to acute incubation with opZym.

Acsl4^{mKO} rpMACs have reduced levels of LTB_4 and PGE_2 and reduced recruitment of neutrophils into the peritoneal cavity in response to LPS.

We next extended our studies to investigate the TLR4 signaling pathway through LPS activation to determine if we could observe similar results as demonstrated with opZym and because rpMACs are the primary cell type that contributes to PMN recruitment during LPS-induced peritonitis (4). *Ex vivo*, *Acsl4*^{mKO} rpMACs treated with LPS generated significantly lower levels of LTB_4 and PGE_2 (Fig. 5A), though LPS did not stimulate LTB_4 production to the same extent as opZym. Given the reduction of the potent neutrophil chemotactic signaling molecule, LTB_4, in *Acsl4*^{mKO} rpMACs treated with LPS or opZym, we investigated how neutrophil recruitment into the peritoneum after i.p. injection of LPS would be affected by *Acsl4* deficiency. For these studies, mice were injected i.p. with

LPS (10ug/kg) and 4 hrs later peritoneal fluid was harvested to measure levels of LTB_4 and PGE_2 by ELISA, and FACS analysis was performed to quantitate the number of neutrophils. Both LTB_4 and PGE_2 levels were significantly reduced in peritoneal fluid of *Acsl4*^{mKO} mice as compared with the *Acsl4*^{Flox} controls after i.p. LPS injections (Fig. 5B). Significantly, the total number of neutrophils ($CD11b^+$ $Ly6G^+$ $F4/80^-$) in peritoneal fluid after LPS injection was reduced by 80% in *Acsl4*^{mKO} mice (Fig. 5C). The total number of peritoneal macrophages (CD11b+ F4/80+ Ly6G-) was 55% higher in the *Acsl4*^{mKO} mice after LPS stimulation, though it did not reach statistical significance. In the absence of LPS, we observed negligible numbers of neutrophils in the *Acsl4*^{Flox} mice (average of 4.6 neutrophils per 10^5 cells). In summary, these studies demonstrated that, in response to LPS, *Acsl4*-deficient rpMACS produced reduced levels of LTB_4 and PGE_2 *in vitro* and *in vivo,* and reduced recruitment of neutrophils *in vivo.*

Discussion

Macrophages are immune cells that respond to infection and various other insults in part by generating proinflammatory lipid mediators and cytokines. Pathogen-associated molecular patterns, such as yeast and bacterial cell wall components, can bind toll-like receptors on macrophages and activate phospholipase A_2, which then cleaves PUFA, including AA, out of PL. The AA can be subsequently oxidized by cyclooxygenases or lipoxygenases into the prostaglandins, leukotrienes, thromboxanes, and HETEs as well as other proinflammatory lipid mediators. In order for AA and other FA to be incorporated into PL, they must first be activated by ACSL enzymes that catalyze the thioesterification of a CoA to a FA, which generates a fatty-acyl-CoA that is trapped within the cell and can be incorporated into PL by members of the lysophosphatidylcholine acyltransferase (Lpcat) family (29). ACSL4 has been established as having a strong preference for AA and other PUFA with three or more double bonds to generate PUFA-CoA, and in particular AA-CoA. Our current findings involving both *in vitro* and *in vivo* studies demonstrate that *Acsl4* deficiency reduces the rate of AA-CoA formation and dramatically reduces AA incorporation into PL in rpMACs.

We previously generated floxed *Acsl4* mice with adipocyte-specific deficiency of *Acsl4* to elucidate the *in vivo* role of ACSL4. Those studies revealed that adipocytes from mice fed a high fat diet had a 30 to 50% reduction in the levels of AA incorporated into PL (26). *Acsl4* deficiency did not completely reduce the incorporation of AA into adipocyte PL, suggesting that other members of the ACSL family, in addition to ACSL4, may participate in the generation of AA-CoA necessary for incorporation into PL. In contrast to those observations in adipocytes, we now show that *Acsl4* deficiency in rpMACS results in a robust 75 to 90% reduction in the levels of AA incorporated into all classes of PL. Furthermore, we observed a 98% reduction of AA within PA, which is the precursor for all other PLs (45), indicating that *Acsl4* is necessary for the *de novo* generation of PL containing AA through the Kennedy pathway. Thus, our studies demonstrated that ACSL4 is the predominant ACSL isoform regulating the incorporation of AA into macrophage PL under homeostatic conditions. Consistent with this, we demonstrated with our pulse-chase experiment that the total retention of radiolabeled AA in cellular extracts was reduced, whereas the radiolabel present in the cells during the pulse was released into the chase medium from *Acsl4*-deficient rpMACs at nearly twice the rate of the floxed controls. We

note however that the radiolabel measurements cannot solely be attributed to native AA and could include further oxidized products of AA.

In addition to the reduced levels of AA incorporated into PL, we also found reduced free AA in the basal state of the rpMACs. One possible explanation for a reduction of total AA in the cells could have been that there was decreased expression of the enzymes involved in the generation of AA from LA. However, interestingly, our studies revealed a robust increase in the mRNA expression of the enzymes involved in the conversion of LA into AA, perhaps indicating a physiologic attempt by the cells to increase cellular AA levels. We therefore conclude that the most likely explanation for the reduced AA content in *Acsl4*-deficient rpMACS is that *Acsl4* deficiency results in a decreased ability to form AA-CoA and therefore free AA is not activated or trapped within the cell.

Along with the decrease of AA, we observed a reciprocal increase of OA and LA in the PL of *Acsl4*-deficient rpMACS. The increase in those unsaturated FA may be of relevance because previous studies have shown that reduced incorporation of both AA and LA in PL in mice with *Lpcat3* deficiency resulted in decreased membrane fluidity and diminished diffusion of FA into enterocytes (46). It is likely that the increase in OA and LA in *Acsl4*-deficient rpMACS may act to maintain membrane fluidity, though the mechanism for how this occurs is not currently understood and is a focus of investigation.

In our studies, *Acsl4* deficiency in isolated rpMACs resulted in dramatic reductions in nearly all AA-derived lipid mediators produced in response to inflammatory stimulation by opZym, which is consistent with the reductions of AA in PL. The decreased levels of eicosanoids included both pro-inflammatory (prostaglandins, leukotrienes) and pro-resolving lipid mediators (lipoxins). Of significance, in studies from the laboratories of Peters-Golden and Olefsky, LTB_4 has been previously demonstrated to regulate the expression of *Il6*, *Ccl5*, *Nos2*, and *Ccl2* in response to various TLR ligands in part by maintaining MyD88 expression and NF-κB activation in macrophages (47, 48). The reduction of LTB_4 that we observed in *Acsl4*-deficient rpMACS after opZym stimulation thus may be one factor that contributes to the observed reductions in mRNA expression of *Il6*, *Ccl5*, *Nos2*, and *Ccl2*. Nonetheless, it is also possible that the resulting alterations in membrane phospholipid remodeling with *Acsl4*-deficient rpMACS may dampen intracellular signaling pathways in response to TLR ligands and/or eicosanoid G-protein coupled receptors. The reduction in 12-HETE may also contribute to the reduced mRNA levels of *Ccl2* (49). Additionally, LTB_4 is a potent neutrophil chemoattractant and the reduction of LTB_4 in $Acsl4^{mKO}$ mice after LPS stimulation likely underlies the reduced neutrophil recruitment *in vivo*.

The lipoxins are SPM derived from AA that can act to resolve inflammation and stimulate tissue regeneration (50). We found that the AA-derived lipoxins, including LXA_4, 15*epi*-LXA_4, and LXB_4 were significantly reduced in $Acsl4^{mKO}$ rpMACs stimulated with zymosan. These mediators are potent regulators of inflammation-resolution and counter regulate pro-inflammatory actions of LTB_4 and PGE_2 (17, 51). The results demonstrate that, by acting to regulate substrate availability in membrane PL, ACSL4 regulates downstream production of both pro-inflammatory and pro-resolving lipid mediators derived from AA.

Our research focus was to investigate the effects of ACSL4 on rpMACs freshly isolated from the animal and *in vivo*. In contrast, a previous study by Kuwata *et al.* investigating the function of ACSL4 used bone marrow-derived macrophages that were cultured and differentiated *in vitro* from mice with whole-body knockout of *Acsl4* (24). In those studies, the *Acsl4* knockout BMDM were cultured in the presence of serum that could contain AA and other fatty acids, which contrasts with our studies done in serum-free media. Lipidomic analysis of these *Acsl4*-deficient BMDM demonstrated reductions in incorporation of AA and other PUFA into PL that were similar to our results, but to a lesser extent. They also noted a small reciprocal increase in OA and LA that were not as robust as what we observed. In direct opposition to our studies, when the *Acsl4* knockout BMDM were stimulated with LPS they had significant increases in prostaglandins, thromboxane, and HETEs, which occurred in the presence of serum. Importantly, our *in vivo* studies demonstrated a reduction of PGE_2 and LTB_4 in the peritoneal lavage fluid from $Acsl4^{mKO}$ mice after LPS injection that corroborated our *ex vivo* results that found similar decreases in PGE_2 and LTB_4 in the in LPS treated $Acsl4^{mKO}$ rpMACs. We hypothesize that the observed increase in AA-derived lipid mediators measured in the study by Kuwata *et al.* in BMDM could be due to the utilization of unesterified AA in serum. Conversely, long-term knockdown of *Acsl4* in smooth muscle cells, cultured in the presence of serum, was shown to reduce PGE_2 to a similar extent as we observed after acute stimulation of rpMACs with opZym in serum-free medium (30), indicating that the effects of *Acsl4* deficiency are time- and cell-type dependent.

In the BMDM study by Kuwata et al. they did not find differences in expression of *Nos2* or *Ccl2* after LPS treatment, whereas we saw significantly decreased expression of these pro-inflammatory genes in the *Acsl4*-deficient rpMACs after opZym treatment. BMDM, as compared with rpMACs, are known to have diminished 5-LOX activity necessary to produce LTB_4, especially with LPS as an inflammatory stimulus (44). As discussed, LTB_4 can increase expression of *Nos2* and *Ccl2*, and we measured high levels of LTB_4 after opZym stimulation in the control $Acsl4^{Flox}$ rpMACs compared with the $Acsl4^{mKO}$ rpMACs, which might explain why we saw differences in expression of those cytokines and the other researchers did not.

In summary, our studies have shown that ACSL4 is critical for maintaining normal PUFA content in PL within peritoneal macrophages through incorporation of AA into PA, a precursor for generation of diverse PL as well as esterification of PUFA containing more than three double bonds during homeostatic PL remodeling and generation of AA metabolites in response to TLR activation. Future directions aimed at investigating membrane fluidity due to the drastic alterations in PUFA content within PL of *Acsl4*-deficient macrophages will be important to explore, as well as the effects of these alterations on mobility, energy metabolism, and various signaling pathways. Lastly, the reductions in AA-derived lipid mediators and cytokines in *Acsl4*-deficient rpMACs after an inflammatory stimulus suggests that ACSL4 may be a good target for drug therapies aimed at treating diseases associated with chronic inflammation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Key points:

Deficiency of *Acsl4* in rpMACs reduces arachidonic acid levels in phospholipids.

Arachidonic acid-derived mediators are reduced in stimulated *Acsl4*-deficient rpMACS.

In response to stimulation, myeloid *Acsl4* deficiency reduces inflammation.

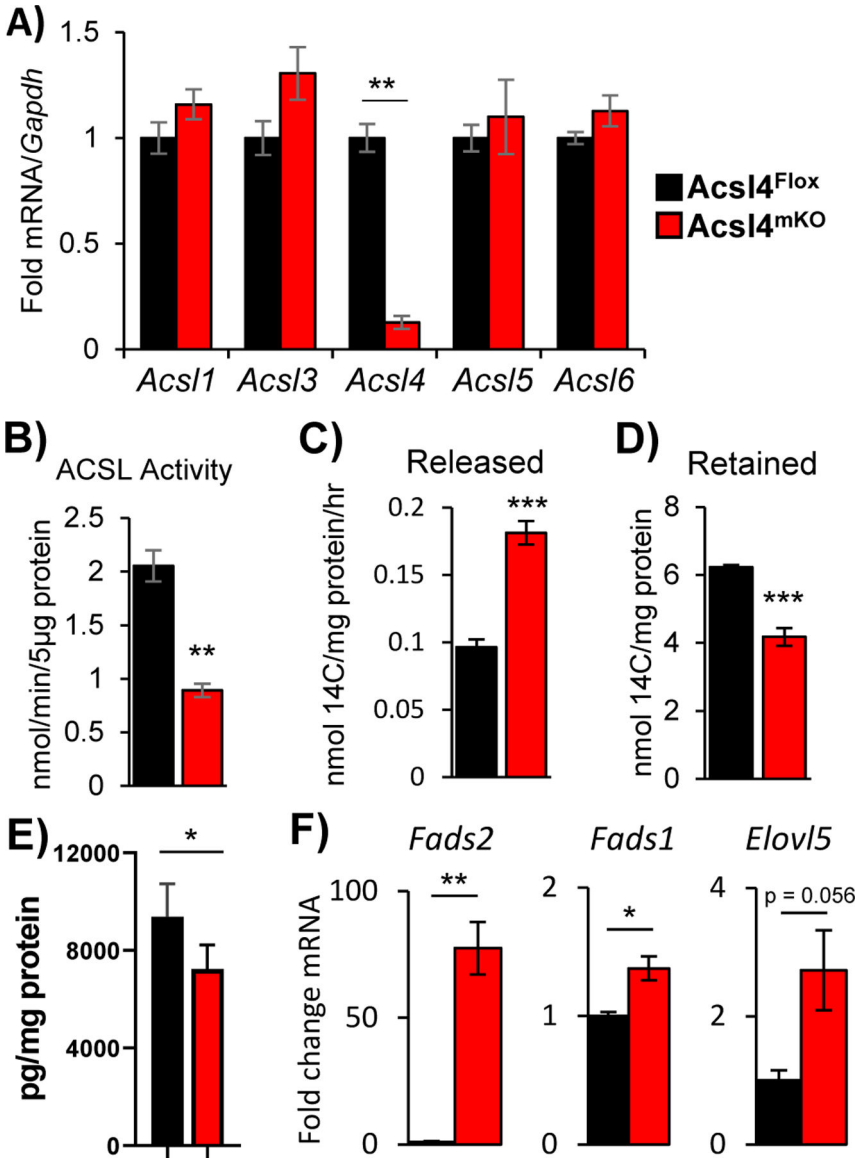

Figure 1.

Myeloid-specific knockout of Acsl4 reduces ACSL activity toward AA and alters AA content during homeostatic lipid remodeling in rpMACs. (A) mRNA expression of Acsl1–5. (B) Total membrane ACSL activity with 1–14C-AA. Protein pooled from 3 mice and assay performed in triplicate. (C-E) Incubation of rpMacs with 1–14C-AA for 18 hrs (pulse), washed with 2% BSA in PBS, then incubated for an additional 6 hours in 0.5% BSA in DMEM (chase). (C) Radiolabel released into media after a 6 hr chase, and (D) radiolabel retained in cellular lysate. (E) Free arachidonic acid measured by LC MS/MS in rpMACS. (F) Expression of fatty acid desaturase 1 and 2 (Fads1 & 2) and elongation of very long-chain fatty acid protein 5 (Elovl5) in rpMACs. Results are expressed as mean ± SEM; n = 4 unless otherwise stated. *p < 0.05, **p < 0.01, and ***p < 0.001 by Student's t-test.

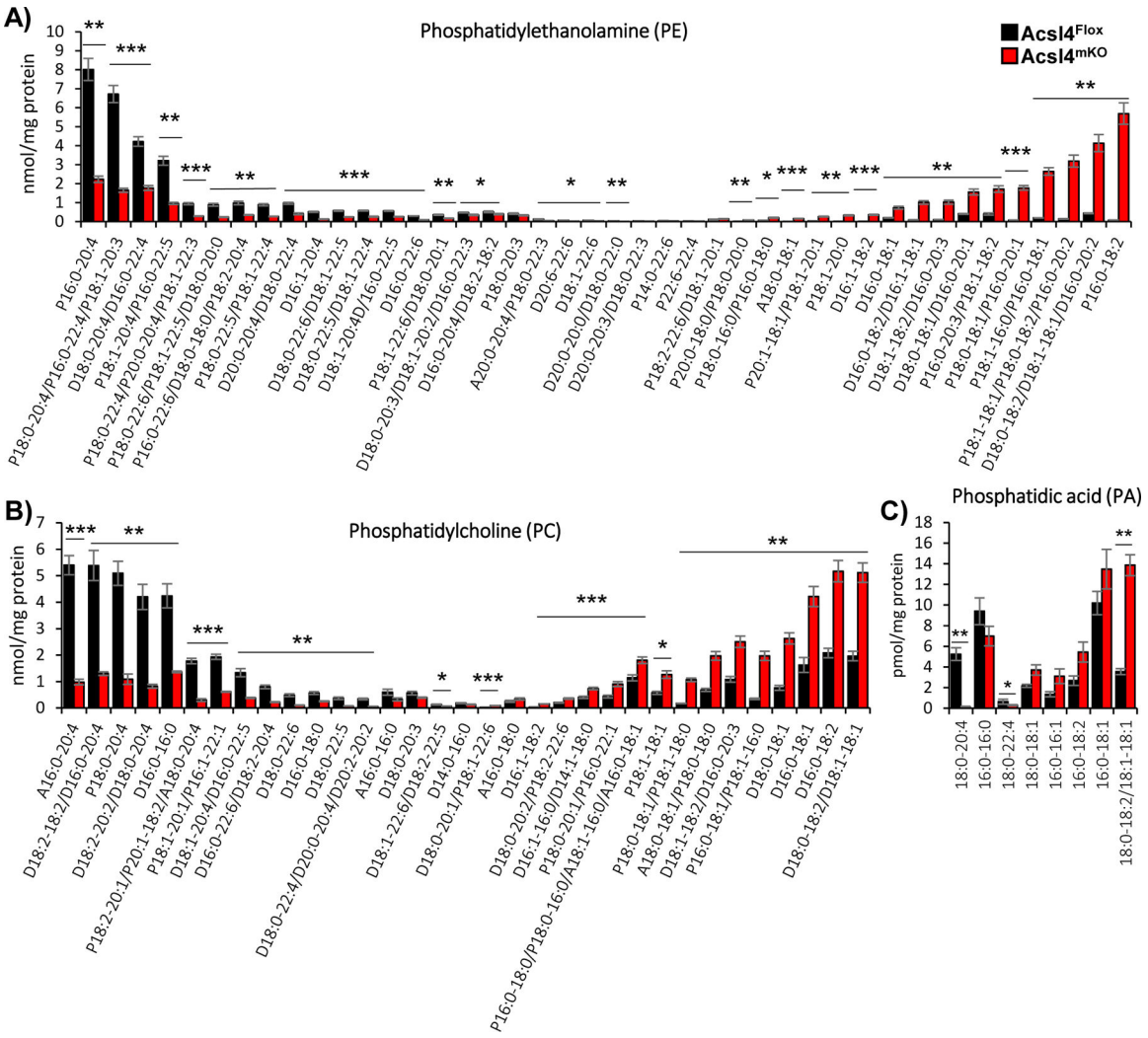

Figure 2.

Loss of Acsl4 reduces 20:4, 22:5, 22:6 fatty acids from phospholipids in rpMACs. Phospholipids were extracted from isolated rpMACs and analyzed by LC-MS/MS. Fatty-acyl species of (A) phosphatidylethanolamine (PE), (B) Phosphatidylcholine (PC), or (C) Phosphatidic acid (PA). D = diacyl phospholipids, P = plasmalogens (alkenyl acyl phospholipids), A = alkyl acyl phospholipids. Results are expressed as mean ± SEM; n = 4, *p < 0.05, **p < 0.01, and ***p < 0.001 by Student's t-test. * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001 by student's t-test.

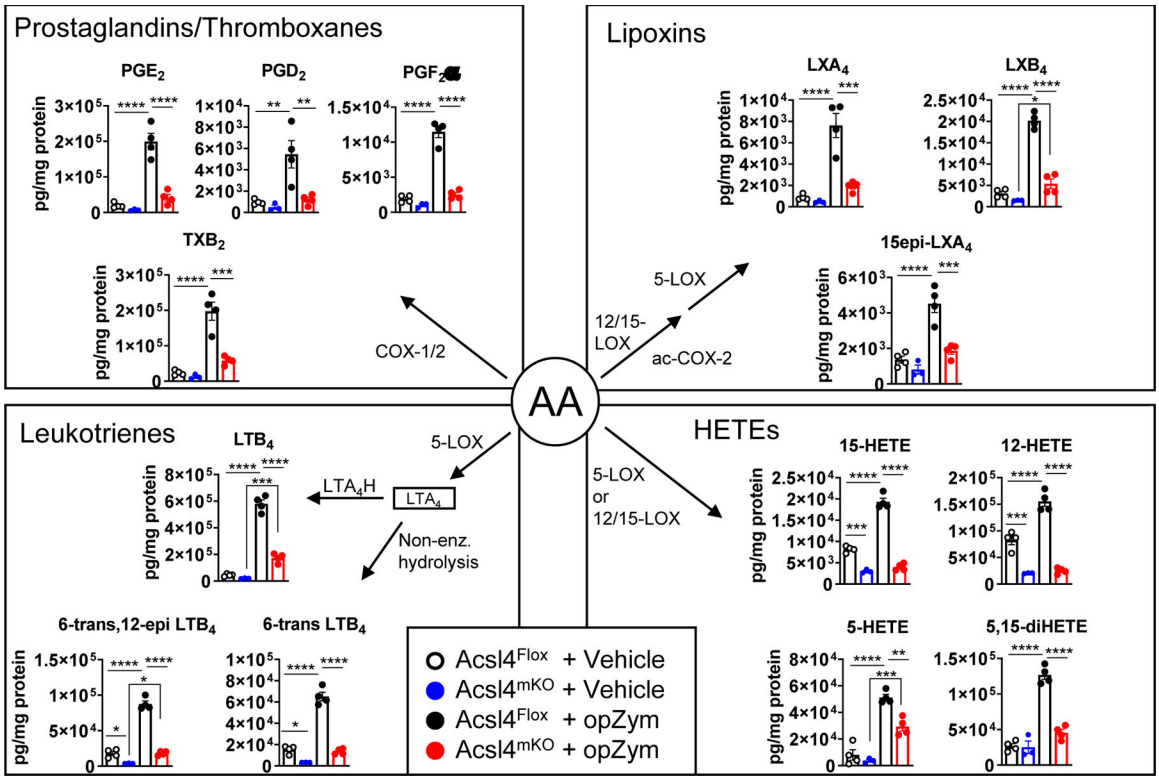

Figure 3.

Loss of Acsl4 reduces AA-derived lipid mediator production in rpMACs. rpMacs were isolated and treated with opsonized zymosan (opZym) (10 particles/cell) for 2 hours, reaction was quenched with 2 volumes cold methanol and lipid mediators were measured by LC-MS/MS. Results are expressed as mean ± SEM; n = 3–4, *p < 0.05, **p < 0.01, and ***p < 0.001 ****p < 0.0001 by two-way ANOVA with Tukey's multiple comparisons. Arachidonic acid (AA), prostaglandin (PG), thromboxane (TX), lipoxin (LX), leukotriene (LT), hydroxyeicosatetraenoic acid (HETE), cyclooxygenase (COX), acetylated (ac), lipoxygenase (LOX), LTA4 hydrolase (LTA4H), non-enzymatic hydrolysis (non-enz. hydrolysis).

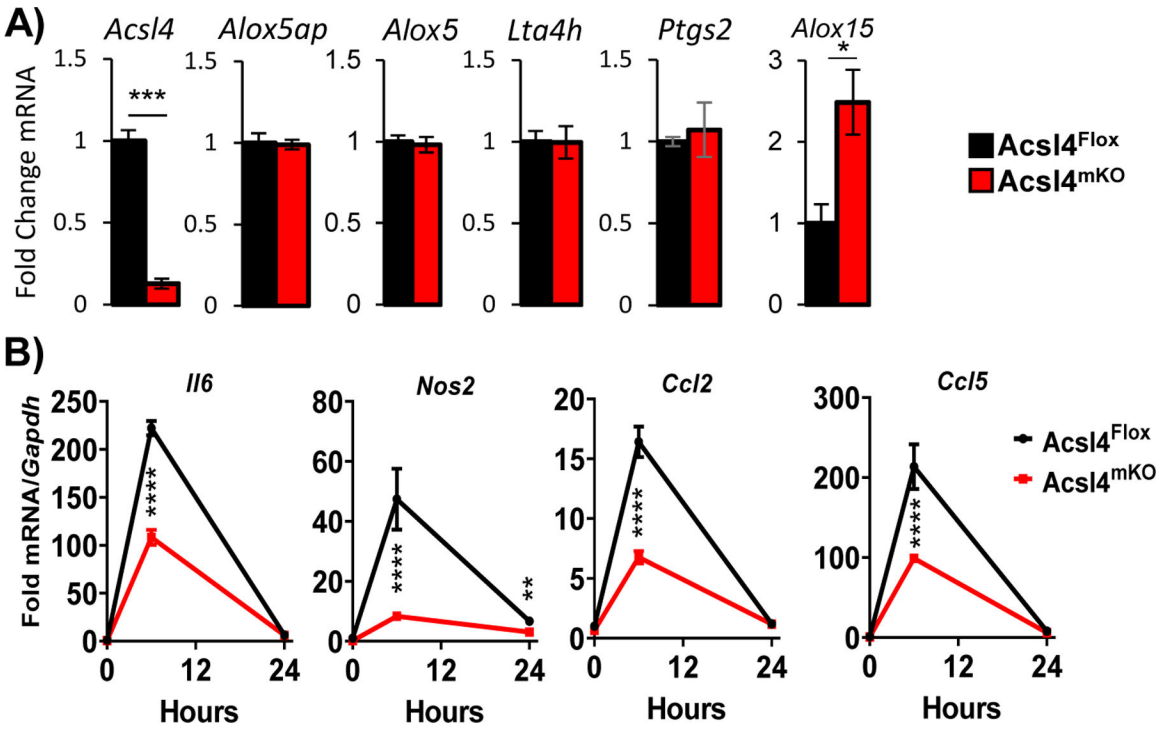

Figure 4.

Loss of Acsl4 blunts inflammatory gene expression in rpMACs. (A) Gene expression of key enzymes in the synthesis of LTB4 (5-lipoxegenase activating protein, Alox5ap; 5-lipoxegenase, Alox5; LTA4 hydrolase, Lta4h; prostaglandin-endoperoxide synthase, Ptgs2). The mRNA was normalized to GAPDH. (B) Gene expression of inflammatory markers throughout the time course of opsonized zymosan (10 particles/cell) stimulation at 0, 6, and 24 hrs. Results are expressed as mean ± SEM; n = 3–4. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ ****$p < 0.0001$ by Student's t-test or repeated measures two-way ANOVA with Sidak's multiple comparisons.

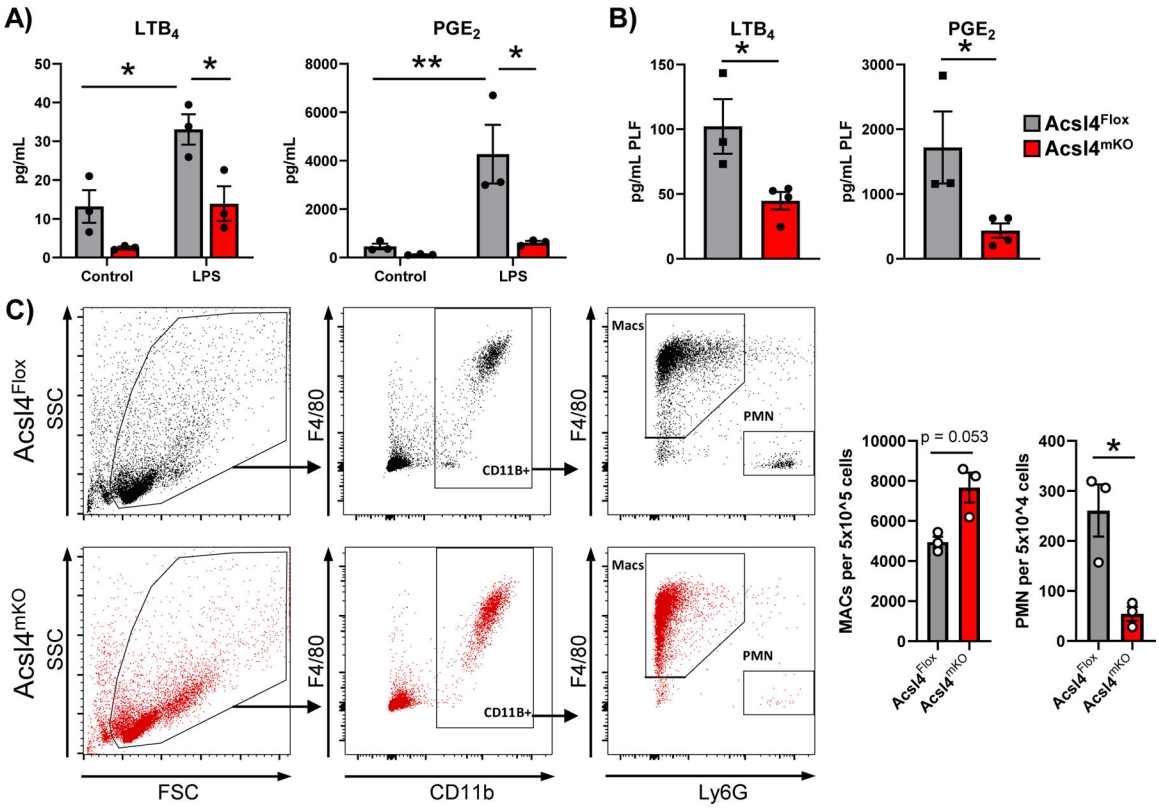

Figure 5.

LPS-induced neutrophil infiltration is reduced in the peritoneum with the loss of Acsl4. (A) rpMACs were isolated and treated with LPS (1μg/mL) for 6 hrs and medium was assayed by ELISA. Two-way ANOVA with Tukey's multiple comparisons, *p ≤ 0.05, n = 3–4/group. (B) Mice were i.p. injected with LPS (10 mg/kg) and peritoneal lavage fluid was assayed for LTB4 and PGE2 after 4 hrs. (C) FACS analysis of peritoneal neutrophils (PMN) in the mice injected with LPS. Results are expressed as mean ± SEM; n = 3–4, *p < 0.05, by Student's t-test unless otherwise noted. MACs, macrophages; PMN, polymorphonuclear cells.

Table I.

Primer sequences.

	Forward (5’ - 3’)	**Reverse (5’ - 3’)**
GAPDH	AATGTGTCCGTCGTGGATCT	CATCGAAGGTGGAAGAGTGG
ACSL1	CAGAACATGTGGGTGTCCAG	GTTACCAACATGGGCTGCTT
ACSL3	GGGACTACAATACCGGCAGA	CCGCTGTCCATTTTCATCTT
ACSL4	TGGAAGTCCATATCGCTCTGT	TTGGCTACAGCATGGTCAAA
ACSL5	AATGTGTTCAAAGGCTACCTAAAGGACCC	GCGACCAATGTCCCCAGTGTGA
ACSL6	TGAATGCACAGCTGGGTGTA	ATGTGGTTGCAGGGCAGAG
LTA4H	GGGGCACATAAAGCGAATGC	GCAGAGCCGTAACCATCTGA
Alox5	GGAACTGCAGGACTTCGTGA	GGGGAAACCTGAGGCCTTTT
FLAP	CGTTCTTTGCCCACAAGGTG	TCTACGCAGTTCTGGTTGGC
Alox15	TAAAACCAAGGACGCGACGA	CCCAGGGGCACCATAACAG
COX-2	CACAGCCTACCAAAACAGCCA	GCTCAGTTGAACGCCTTTTGA
TNFa	ATGGGCTTTCCGAATTCAC	GAGGCAACCTGACCACTCTC
MCP-1	AGGTCCCTGTCATGCTTCTG	TCTGGACCCATTCCTTCTTG
IL-1b	CTGGTGTGTGACGTTCCCATT	CCGACAGCACGAGGCTTT
IL-6	CCAGTTGCCTTCTGGGACT	GGTCTATTGGGAGTGGTATCC
NOS2	CAGAGGACCCAGAGACAAGC	TGCTGAAACATTTCCTGTGC
CCL5	GCTGCTTTGCCTACCTCTCC	TCGAGTGACAAACACGACTGC
FADS1	CAGTGCAACTCCTTCGAACA	AAAAACAGCTTCTCCCAGCA
FADS2	ACAGCCTTTGTCCTCGCTAC	GTTGGCTGAGGCACCCTTTA
ELOVL5	CCTTCCTCTTGCATCGCGG	TTGACTCTTGTATCTCGGGGG