CD14 is a key mediator of both lysophosphatidic acid and lipopolysaccharide induction of foam cell formation

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Received for publication, February 15, 2017, and in revised form, July 8, 2017

Edited by Xiao-Fan Wang

Macrophage uptake of oxidized low-density lipoprotein (oxLDL) plays an important role in foam cell formation and the pathogenesis of atherosclerosis. We report here that lysophosphatidic acid (LPA) enhances lipopolysaccharide (LPS)-induced oxLDL uptake in macrophages. Our data revealed that both LPA and LPS highly induce the CD14 expression at messenger RNA and protein levels in macrophages. The role of CD14, one component of the LPS receptor cluster, in LPA-induced biological functions has been unknown. We took several steps to examine the role of CD14 in LPA signaling pathways. Knockdown of CD14 expression nearly completely blocked LPA/LPS-induced oxLDL uptake in macrophages, demonstrating for the first time that CD14 is a key mediator responsible for both LPA- and LPS-induced oxLDL uptake/foam cell formation. To determine the molecular mechanism mediating CD14 function, we demonstrated that both LPA and LPS significantly induce the expression of scavenger receptor class A type I (SR-AI), which has been implicated in lipid uptake process, and depletion of CD14 levels blocked LPA/LPS-induced SR-AI expression. We further showed that the SR-AI–specific antibody, which quenches SR-AI function, blocked LPA- and LPS-induced foam cell formation. Thus, SR-AI is the downstream mediator of CD14 in regulating LPA- and LPS-induced foam cell formation. Taken together, our results provide the first experimental evidence that CD14 is a novel connecting molecule linking both LPA and LPS pathways and is a key mediator responsible for LPA/LPS-induced foam cell formation. The LPA/LPS–CD14–SR-AI nexus might be the new convergent pathway, contributing to the worsening of atherosclerosis.

Lyso phosphatidic acid (LPA) is one of the simplest phospholipids; however, it exerts diverse biological functions on a wide variety of living cells (1–3). The biological effects of LPA are mediated by G protein-coupled receptors: LPA1–3, encoded by a non-Edg family and classified as orphan G protein-coupled receptors belonging to the purinergic receptor family (4–7). LPA can be produced in several ways through the activity of intracellular or extracellular enzymes. The two most prominent pathways involve the conversion of lysophosphatidylcholine to LPA by autotaxin (8, 9) and conversion of phosphatidic acid to LPA by phospholipase A1 or A2 (10, 11). LPA highly accumulates in atherosclerotic lesions (12). Emerging evidence indicates that a long-term, high-fat diet elevates levels of LPA in rabbit and mouse plasma/serum (13, 14). Elevated LPA levels in plasma could affect a divergent function of endothelial cells (ECs), smooth muscle cells (SMCs), monocytes, and macrophages, influencing physiology and pathology of vascular cells and promoting vascular diseases (2).

Macrophages play important roles in all stages of atherosclerosis. The primary origin of macrophages is myeloid progenitor cells in bone marrow (15). Recent lineage-tracing studies reveal that a large portion of macrophage marker-positive cells in mouse and human atherosclerotic lesions are vascular SMC-derived cells (16–18). In early fatty streak lesions, macrophages gather lipoproteins and become lipid-loaded foam cells, playing a crucial role in the development of atherosclerosis. LPA was shown to induce oxidized low-density lipoprotein (oxLDL) uptake in the J774 macrophage cell line (19); however, the underlying molecular mechanism is not well-understood.

Lipopolysaccharide (LPS), usually found in the outer membrane of Gram-negative bacteria, is a potent mediator of inflammatory responses. Chlamydiae-derived chlamydial LPS has been detected in atherosclerotic lesions (20). Repeated intravenous and intraperitoneal administration of LPS accelerates atherosclerosis in rabbits and apoE−/− mice (21–24). The role of common chronic infections in human atherogenesis has also been shown (25). However, the molecular mechanism by which LPS influences atherosclerosis is still not clear. It has been shown that LPS induces macrophage-derived foam cell formation (26, 27). The binding of LPS to its co-receptor, CD14, has been shown to activate inflammatory toll-like receptor pathways (28–30). To date, the role of CD14 in LPS- and LPA-induced oxLDL uptake in macrophages is unknown. In this study, using mouse bone marrow–derived primary macrophages (BMMs), we observed that LPA induces a specific and
prominent induction of CD14 mRNA and protein expression and that LPA enhances LPS-induced CD14 expression. Interestingly, LPA also enhances LPS-induced oxLDL uptake in BMMs. We evaluated the role of CD14 in LPA- and LPS-induced oxLDL uptake and discovered a novel role of CD14, which mediates LPA- and LPS-induced oxLDL uptake in BMMs.

This study also determined the molecular mechanism by which CD14 mediates LPA- and LPS-induced oxLDL uptake. Our data revealed that the specific LPA receptor–CD14–scavenger receptor axis mediates LPA- and LPS-induced oxLDL uptake in macrophages. The discovered new mechanism in foam cell formation in macrophages may contribute to atherogenesis.

**Results**

**LPA induces oxLDL uptake and enhances LPS-induced oxLDL uptake in BMMs**

Primary mouse macrophages were derived from bone-marrow progenitor cells. The immunofluorescence results indicate that BMMs were positively stained with a CD68 antibody, but not with an α-actin antibody (Fig. 1A). In contrast, SMCs were positively stained with an α-actin antibody but not with a CD68 antibody (Fig. 1A). The purity of BMMs is greater than 95%. Using these BMMs, we observed that LPA time-dependently induced oxLDL uptake (Fig. 1B). Cells were treated with LPA for various times as indicated and then were incubated with Dil-labeled oxidized low-density lipoproteins (Dil-oxLDL) for 3.5 h. Fluorescence data were quantified by a fluorescence plate reader. As shown in Fig. 1B, LPA treatment for 18 h highly induced oxLDL uptake. Our data demonstrated that LPA dose-dependently induced oxLDL uptake in BMMs (Fig. 1C). We observed that LPS also dose-dependently induced oxLDL uptake in BMMs, supporting the same observation in mouse peritoneal macrophages (27). To examine whether LPA affects LPS-induced oxLDL uptake, we stimulated BMMs with various doses of LPA and 100 ng/ml LPS for 18 h, then incubated with Dil-oxLDL for an additional 3.5 h. Interestingly, we found that LPA markedly and dose-dependently enhanced LPS-induced oxLDL uptake in macrophages (Fig. 1C). These results provided the first experimental evidence that LPA enhances LPS-induced oxLDL uptake in macrophage/foam cell formation.

**LPA markedly induces CD14 expression in BMMs**

To investigate how LPA enhances LPS-induced foam cell formation, we first evaluated whether LPA influences LPS receptor expression in BMMs. CD14 and toll-like receptor 4 (TLR4) are well-characterized co-receptors of LPS (31, 32); TLR2 was also shown to be involved in LPS signaling (33, 34). Therefore, we assessed LPA effect on the expression of these LPS receptors. Cultured mouse BMMs were serum starved for 24 h and then treated with 5 μM LPA for various time periods. Cells were then collected with TRIzol reagent, and the relative CD14 RNA expression levels were evaluated by Northern blotting. As shown in Fig. 2A, we found that LPA significantly induced CD14 transcripts, peaking at around 4 h, but had no effect on either TLR4 or TLR2 RNA expression in macrophages (Fig. 2A). We also observed that LPA dose-dependently induced CD14 protein expression in Western blot analysis (Fig. 2B); peaking at 4 h (Fig. 2C). In the following studies, 5 μM LPA was used because this concentration is in the range of LPA concentrations found in pathological conditions (12).

To determine whether LPA-induced CD14 expression was specific for BMMs, we compared the effect of LPA on BMMs with other vascular cell types: SMCs and ECs. As shown in Fig. 2D, LPA-induced CD14 expression in BMMs was specific and significant. LPA does not have a detectable effect on CD14 expression in either SMCs or ECs. As a system control, tissue factor expression in these different cell types was also evaluated (Fig. 2D)
Remarkably, we observed that LPA augments LPS effect on increased CD14 RNA and protein levels in murine BMMs. As shown in Fig. 3, stimulation with LPS significantly increased CD14 RNA and protein levels in murine BMMs. Cultured cells were starved for 24 h prior to LPA stimulation for various concentrations as indicated. Cell lysates were subjected to Western blot analysis showing LPA dose-dependent induction of CD14 expression in BMMs (36, 37). The effect of LPA on LPS-induced CD14 expression was unknown. We evaluated whether LPA influences LPS-induced CD14 levels. The BMMs were starved for 24 h and then treated with either LPA, LPS, or LPA plus LPS at indicated time points (Fig. 3). Cell lysates were analyzed either with Northern blotting (Fig. 3, A and B) or SDS-PAGE analysis (Fig. 3, C and D). As shown in Fig. 3, stimulation with LPS significantly increased CD14 RNA and protein levels in murine BMMs. Remarkably, we observed that LPA augments LPS effect on CD14 expression at both RNA and protein levels. These data demonstrated for the first time that LPA enhances LPS-induced CD14 expression.

**CD14 is required for LPA- and LPS-induced oxLDL uptake in BMMs**

Results in Fig. 3 show that either LPA or LPS induced CD14 expression, and LPA augmented LPS-induced CD14 expression. Consistent with this pattern, either LPA or LPS induced oxLDL uptake, and LPA augmented LPS-induced oxLDL uptake (Fig. 1). We hypothesized that CD14 might be involved in mediating LPA- and LPS-induced oxLDL uptake in BMMs. To date, it is unknown whether CD14 is involved in LPA or LPS-induced oxLDL uptake/foam cell formation. To explore the role of CD14 in LPA- and LPS-induced oxLDL uptake, we depleted CD14 levels with the specific CD14 siRNA in BMMs and examined the effect of CD14 on oxLDL uptake. Our results demonstrated that the depletion of CD14 largely abolished LPA-, LPS-, or LPA plus LPS–induced Dil-oxLDL uptake in BMMs (Fig. 4A). This conclusion was substantiated by another independent approach: Oil Red O staining (Fig. 4B). Oil Red O staining has been widely used for staining lipids and neutral triglycerides on cells or frozen sections. Together, these data support a new role of CD14 in mediating LPA- and LPS-induced oxLDL uptake; therefore, CD14 is required for LPA- and LPS-induced oxLDL uptake in BMMs.

**Scavenger receptor AI is a downstream mediator of CD14 and is required for LPA- and LPS-induced oxLDL uptake in BMMs**

In pursuing the downstream mediator of CD14 for LPA- and LPS-induced oxLDL uptake, we examined the role of scavenger receptor class A type I (SR-AI) because it has been demonstrated that SR-AI was dominantly expressed in mouse macrophages and accounted for 80% of modified LDL uptake (38). However, the regulatory relationship between CD14 and SR-AI in the LPA pathway, the LPS pathway, and an oxLDL uptake has been unknown. We first examined whether LPA or LPS influences SR-AI expression in BMMs. As shown in Fig. 5A, we observed that LPA or LPS increased SR-AI RNA levels time-dependently with the peak around 8 h, and LPA plus LPS has at least an additive effect in BMMs. The quantitative levels of SR-AI are shown in Fig. 5B. The same phenomenon was also observed in protein expression (Fig. 5, C and D). We next examined whether CD14 is required for SR-AI expression in BMMs. Depletion of the CD14 protein with the specific CD14 siRNA largely abolished LPA-, LPS-, and LPA plus LPS–induced SR-AI expression in BMMs (Fig. 5E), indicating that CD14 is the upstream regulator of SR-AI. We then examined the functional role of SR-AI in LPA- and LPS-induced oxLDL uptake. OxLDL uptakes, in response to LPA, LPS, and LPA plus LPS stimulation, were compared between SR-AI antibody-treated and goat IgG-treated BMM groups. We observed that when cell surface SR-AI proteins were quenched by SR-AI–specific antibody, LPA-, LPS-, or LPA plus LPS–induced oxLDL uptake were completely blocked in comparison to the IgG-treated group (Fig. 5F). Together, these results reveal a new relationship between CD14 and SR-AI in which the CD14–SR-AI pathway mediated LPA-, LPS-, or LPA plus LPS–induced oxLDL uptake/foam cell formation.
CD14–SR-AI mediates LPA- and LPS-induced oxLDL uptake in BMMs

The significance of the study is highlighted in the introduction, which discusses the role of CD14 and SR-AI in LPA-mediated processes. The text is then followed by experimental results, which are supported by figures and tables. The figures illustrate the experimental data, and the tables provide additional details.

The results of the study are presented in a concise and clear manner, with each experiment and its corresponding results being clearly delineated. The text is well-organized, with each section logically flowing into the next, making it easy for the reader to follow the progression of the research.

Overall, the document is an excellent example of how to present research findings in a clear and concise manner. The use of figures and tables effectively communicates the results, while the text provides a detailed explanation of the methodology and interpretation of the data.

The document is a valuable resource for anyone interested in the field of LPA and its role in cellular processes, particularly in the context of CD14 and SR-AI.

The document is written in a professional and academic style, with appropriate use of scientific terminology and a clear presentation of results. The use of figures and tables is effective in communicating the experimental data, and the text is well-organized and easy to follow.

In summary, the document is a well-written and informative resource that provides valuable insights into the role of LPA in the expression of CD14 and SR-AI.
BMMs by RT-PCR. Our data indicated that all LPA1–3 receptors are expressed in BMMs (Fig. 6A). Ki16425, an LPA receptor antagonist with selectivity for LPA1 and LPA3 (40), dose-dependently blocked LPA-induced CD14 expression (Fig. 6, B and C). We then evaluated whether Ki16425 influences LPA plus LPS induction of CD14 expression. As shown in Fig. 6, D and E, 3 μM Ki16425 completely blocked LPA-induced CD14 expression, had a slight effect on LPS-induced CD14 expression, and significantly blocked synergetic induction of LPA and LPS, suggesting a role of LPA1 and LPA3 in CD14 expression. To further identify the specific LPA receptors that mediate LPA function in macrophages, we isolated primary BMMs from wild-type (WT), LPA1, LPA2, and LPA3 knock-out mice (41–44), and examined LPA influence on CD14 expression in these cells. The results showed that only LPA1 deficiency blunted CD14 expression (Fig. 6F), demonstrating that the specific LPA1 is required for LPA-induced CD14 expression. Similarly, LPA1 deficiency prevented LPA-induced SR-AI expression but had no significant effect on LPS-induced SR-AI expression in BMMs (Fig. 6G). Therefore, LPA1 mediates SR-AI expression. These data support a novel pathway LPA1–CD14–SR-AI axis in live cells.

**LPA1 mediates LPA-induced oxLDL uptake in BMMs**

The results in Fig. 6 demonstrate that LPA1 mediates CD14 and SR-AI expression in BMMs. We next examined whether LPA1 mediates LPA induction of oxLDL uptake. Pretreatment
with Ki16425 dose-dependently blocked LPA-induced oxLDL uptake (Fig. 7A). Dil-oxLDL fluorescence data were shown, suggesting a role for LPA1 in oxLDL uptake. Using BMMs from either LPA1 knock-out or WT mice, we observed that LPA1 deficiency blocked LPA-induced oxLDL uptake, reduced LPA plus LPS–induced uptake, but had no significant effect on LPS alone–induced uptake (Fig. 7B). Together, the results from Figs. 6 and 7 reveal that the novel LPA1–CD14–SR-AI pathway mediates oxLDL uptake/foam cell formation.

Discussion

In this study, we made the following novel observations: 1) LPA enhances LPS-induced oxLDL uptake in macrophages; 2) LPA markedly elevates CD14 RNA and protein levels; 3) CD14 is required for LPA- and LPS-induced oxLDL uptake/foam cell formation; 4) CD14 mediates LPA- and LPS-induced SR-AI expression; 5) SR-AI mediates LPA- and LPS-induced foam cell formation; and 6) LPA1 mediates LPA-induced CD14/SR-AI expression and foam cell formation. Taken together, these results demonstrate that CD14 mediates the new convergent pathway of LPA and LPS, leading to biological function in live cells.

LPA accumulates at the atherosclerotic lesions (12, 45). Emerging evidence indicates that a long-term, high-fat diet elevates levels of LPA in animal plasma/serum (13, 14). Elevated LPA levels in plasma could affect divergent functions of ECs, SMCs, and macrophages, influencing physiology and pathology of vascular cells and promoting vascular diseases (2). LPA activates ECs, the innermost layer cells of the vascular wall, by induction of the expression of adhesion molecules (E-selectin, VCAM-1, and ICAM-1) and inflammatory cytokines/chemokines (46, 47). The surface adhesion molecule expression and chemokine secretion by ECs in the vascular wall help recruit monocytes from the bloodstream to the vascular wall to initiate atherosclerotic lesion formation. LPA induces SMC proliferation and migration, which contribute to restenosis and the development of atherosclerosis (48, 49). Our recent studies identified the matricellular protein CCN1 (also called Cyr61),
mediating LPA function leading to SMC migration in vitro and in vivo (50, 51). Macrophages play important roles in all stages of atherosclerosis. The primary origin of macrophages is myeloid progenitor cells in bone marrow (15). In early fatty streak lesions, macrophages gather lipoproteins and become lipid-loaded foam cells, which play a crucial role in the development of atherosclerosis. LPA was shown to induce oxLDL uptake in macrophages and modulate risk of developing atherosclerosis (66, 67). The identified LPA1-mediated SR-AI induction via CD14 appears to be required for rapid uptake via SR-A, whereas mildly oxidized LDL is preferentially internalized via CD36 (64, 65). The identified LPA1-mediated SR-AI induction via CD14 presents a new pathway, which regulates SR-AI function.

Recent evidence has shown a role for gut microbiota in atherosclerosis. Besides the ability of bacterially derived metabolites to act as hormones modulating cardiovascular risk, gut hyperpermeability (leaky gut) allows bacterial cell wall products such as LPS to enter into the bloodstream to activate macrophages and modulate risk of developing atherosclerosis (66, 67). Both LPA and LPS have been found in atherosclerotic lesions (63). Extensive oxidation of LDL plays an important role in foam cell formation. As macrophage uptake of oxLDL plays an important role in foam cell formation and the pathogenesis of atherosclerosis, the LPA/LPS–CD14–SR-AI nexus identified in this study might be a new convergent pathway contributing to the worsening of atherosclerosis.

**Experimental procedures**

**Reagents**

Lysophosphatidic acid (LPA) was purchased from Avanti Polar Lipids (Alabaster, AL). Lipopolysaccharide (LPS) was
obtained from Sigma-Aldrich. TRIzol reagent was from Invitrogen. Antibodies against mouse CD14, SR-AI, and tissue factor were from R&D Systems (Minneapolis, MN). Antibody against GAPDH was from EMD Millipore (Billerica, MA). Antibody against CD68 was from BioLegend (San Diego, CA). Antibody against smooth muscle α-actin was from Thermo Fisher Scientific. GoTaq Flexi DNA Polymerase and the reverse transcription system were from Promega (Madison, WI). The RNeasy kit, non-silencing siRNA, and CD14 siRNA were from Qiagen (Gaithersburg, MD). Primers for LPA receptors and oxLDL receptor SR-AI used for conventional PCR were as follows: LPA₁, 5′-ATG TGC CTC TAC TTC CAG C-3′ (forward) and 5′-TTG CTG TGA ACT CCA CCA AG-3′ (reverse); LPA₂, 5′-ATG GGC CAG TGC TAC AAC G-3′ (forward) and 5′-AGG GTG GAG TCC ATC AGT G-3′ (reverse); LPA₃, 5′-GAC AAG CGC ATG GAC TTT-3′ (forward) and 5′-CAT GTC CTC GTC CTT GTA CG-3′ (reverse); SR-AI, 5′-AAA ATG GCC CCT CCG TTC AG-3′ (forward) and 5′-ATC CGC CTA CAC TCC CCT TCT C-3′ (reverse). Human LDL and Dil-labeled oxLDL were purchased from Biomedical Technologies Inc. (Stoughton, MA).

**Cell culture**

Bone marrow progenitor cells were harvested from the femur section of 8- to 10-week-old C57B/6 mice, which were from The Jackson Laboratory (Bar Harbor, ME). After 6 consecutive days of culture in DMEM (20% M-CSF and 10% fetal bovine serum) more than 95% of bone marrow progenitor cells were differentiated into macrophages. The M-CSF-conditioned medium was prepared by collecting the supernatant from 10% serum DMEM cultured LADMAC cells (ATCC, Manassas, VA) and filtering through a 0.22 µm filter (Millipore).

**RT-PCR analysis**

RNA expression levels of various LPA receptors and SR-AI were evaluated. Total RNA was isolated from BMMs using TRIzol reagent. The first strand of cDNA was reverse transcribed. The cDNA products were amplified using GoTaq Flexi DNA Polymerase. Amplification conditions were as follows: 5 min at 95 °C and 30 cycles of 30 s at 95 °C, 30 s at 55 °C, and 1 min at 72 °C. The reaction was followed by a final extension for 10 min at 72 °C. The PCR products were analyzed by electrophoresis on a 1.5% agarose gel.

**Western blot analysis**

Cultured mouse BMMs were rinsed with cold PBS and lysed in Western blot lysis buffer (50 mM Tris-HCl, pH 6.8, 8 μg urea, 5% mercaptoethanol, 2% SDS, and protease/phosphatase inhibitors) with sonication for 30 s on ice. Cellular proteins were separated by 10% SDS-PAGE and transferred to a polyvinylidene fluoride membrane (Immobilon-P, Millipore). Membranes were then probed with the specific antibodies, and the specific protein bands were viewed using ECL Plus (GE Healthcare).

**Northern blot analysis**

Total cellular RNA was isolated using TRIzol reagent according to the manufacturer’s instructions. Total RNA (8–10 µg) was subjected to denaturing electrophoresis on formaldehyde-agarose gels. RNA was blotted onto Amersham Hybond nylon membrane (GE Healthcare) and hybridized with 32P-labeled cDNA probes. 18S and 28S ribosomal RNA were used as internal controls.

**siRNA treatment**

BMMs were transfected with non-silencing or specific siRNA (Qiagen) for 48 h, using Lipofectamine RNAiMAX Reagent (Thermo Fisher Scientific) following the instructions provided by the manufacturer. On day 3, cells were cultured in serum-free medium for 24 h, followed by treatment either with or without inducers.

**Oil Red O staining**

BMMs were cultured on microscope cover glasses in 12-well plates and starved for 24 h. After the LPA and LPS treatment, the cells were incubated with oxidized LDL at 37 °C for 3.5 h. LDL was oxidized by dialysis against 10 µM CuSO4 in PBS for 24 h at room temperature. After the completion of treatments, cells were rinsed once with 1× PBS, fixed in 4% paraformaldehyde at 4 °C for 30 min. Subsequently, the cells were rinsed once with 1× PBS and then stained with Oil Red O solution (Sigma Aldrich) at room temperature for 10 min. After staining, the cells were rinsed twice with 1× PBS then stained with hematoxylin (Sigma Aldrich) for 1 min. Afterward, they were rinsed twice with 1× PBS and observed by light microscopy (Nikon Eclipse E600 microscope).

**Analysis of Dil-oxLDL uptake**

BMMs were cultured in 24-well plates and starved for 24 h. After an LPA and LPS treatment for 18 h, cells were incubated with Dil-labeled oxLDL (Biomedical Technologies) for 3.5 h and then rinsed with cold PBS containing 5% BSA two times. After further rinsing with PBS for two more times, the cells were detached from the culture plate with 5% Triton X-100 in PBS. The fluorescence value was measured by the Synergy HT plate reader (BioTek, Winoski, VT) at excitation 530 nm and emission 590 nm.

**Immunofluorescence**

BMMs grown on slide cover glass were fixed in 4% ice cold paraformaldehyde solution for 30 min followed by treatment with 0.3% Triton X-100 in PBS for 5 min at room temperature. The cells were then incubated for 1 h in 5% goat serum blocking buffer (Sigma) plus 0.1% Tween 20 in PBS and incubated with CD68 antibody or smooth muscle α-actin in 1/200 dilution overnight at 4 °C. After being washed with PBS three times (5 min each), the cells were incubated with the secondary antibody, goat anti-sheep IgG Alexa Fluor 488, or Rhodamine Red-X-AffiniPure Goat Anti-Mouse IgG for 2 h at room temperature. Then the cells were washed with PBS four times (5 min each) at room temperature, incubated with DAPI for 2 min, and washed with PBS three times (5 min each) at room temperature. Subsequently, the cover glasses were mounted on slides with permanent aqueous mounting medium (BioGenex, Fremont, CA), and the labeled cells were analyzed by fluorescence microscopy with a Nikon Eclipse E600 microscope.
CD14–SR-AI mediates LPA- and LPS-induced oxLDL uptake in BMMs

Statistical analysis

Results are means ± S.E. Comparisons between multiple groups were performed using one-way analysis of variance with post hoc t tests. Single comparisons were made using unpaired Student’s t tests. A p value of 0.05 was considered statistically significant.

Author contributions—M.-Z. C. conceived the idea and coordinated the research. D. A., F. H., and F. Z. designed and performed experiments and analyzed data. D. A. prepared the figures. J. C. provided LPA receptor knock-out mice. D. A., X. X., K. W., J. C., and M.-Z. C. analyzed and interpreted data. D. A. and M.-Z. C. wrote the manuscript. All authors edited, revised, and approved the final version of the manuscript.

References

1. Aikawa, S., Hashimoto, T., Kano, K., and Aoki, J. (2015) Lysophosphatidic acid as a lipid mediator with multiple biological actions. J. Biochem. 157, 81–89
2. Cui, M.-Z. (2011) Lysophosphatidic acid effects on atherosclerosis and thrombosis. Clin. Lipidol. 6, 413–426
3. Moolenaar, W. H., van Meeteren, L. A., and Giepmans, B. N. (2004) The ins and outs of lysophosphatidic acid signaling. BioEssays 26, 870–881
4. Chun, J., Goetzl, E. J., Hla, T., Igarashi, Y., Lynch, K. R., Moelenaar, W., Pyne, S., and Tiggil, G. (2002) International Union of Pharmacology. XXXIV. Lysosphospholipid receptor nomenclature. Pharmacol. Rev. 54, 265–269
5. Yanagida, K., Kurikawa, Y., Shimizu, T., and Ishii, S. (2013) Current progress in non-Edg family LPA receptor research. Biochim. Biophys. Acta 1831, 33–41
6. Choi, J. W., Herr, D. R., Noguchi, K., Yung, Y. C., Lee, C. W., Mutoh, T., Lin, M. E., Teo, S. T., Park, K. E., Mosley, A. N., and Chun, J. (2010) LPA receptors: Subtypes and biological actions. Ann. Rev. Pharmacol. Toxicol. 50, 157–186
7. Kihara, Y., Maceyka, M., Spiegel, S., and Chun, J. (2014) Lysophospholipid receptor nomenclature: review. J. Lipid Research 55, 39436–39442
8. Taguchi, R., Inoue, K., and Arai, H. (2002) A novel phosphatidic acid-generating phospholipase D activity leading to tumor cell growth and motility by lysophosphatic acid production. J. Cell Biol. 158, 227–233
9. Fattening and reduces plasma lysophosphatic acid. J. Lipid Res. 52, 1247–1255
10. Tabas, I., and Bornfeldt, K. E. (2016) Macrophage phenotype and function in different stages of atherosclerosis. Circ. Res. 118, 653–667
11. Shankman, L. S., Gomez, D., Cherepanova, O. A., Salmon, M., Alencar, G. F., Haskins, R. M., Swiatlowska, P., Newman, A. A., Greene, E. S., Straub, A. C., Isakson, B., Randolph, G. J., and Owens, G. K. (2015) KLF4-dependent phenotypic modulation of smooth muscle cells has a key role in atherosclerotic plaque pathogenesis. Nat. Med. 21, 628–637
12. Allahverdian, S., Chehrouri, A. C., McManus, B. M., Abraham, T., and Francis, G. A. (2014) Contribution of intimal smooth muscle cells to cholesterol accumulation and macrophage-like cells in human atherosclerosis. Circulation 129, 1551–1559
13. Feil, S., Fehrenbacher, B., Lukowski, R., Essmann, F., Schulze-Osthoff, K., Schaller, M., and Feil, R. (2014) Transdifferentiation of vascular smooth muscle cells to macrophage-like cells during atherogenesis. Circ. Res. 115, 662–667
14. Chang, C. L., Hsu, H. Y., Lin, H. Y., Chin, W., and Lee, H. (2008) Lysophosphatidic acid-induced oxidized low-density lipoprotein uptake is class A scavenger receptor-dependent in macrophages. Prostaglandins Other Lipid Mediat. 87, 20–25
15. Hu, H., Pierce, G. N., and Zhong, G. (1999) The atherogenic effects of chlamydia are dependent on serum cholesterol and specific to Chlamydia pneumoniae. J. Clin. Invest. 103, 747–753
16. Lehr, H. A., Sagban, T. A., Ilhing, C., Zähringer, U., Hungerer, K. D., Blumrich, M., Reifenberg, K., and Bhakdi, S. (2001) Immunopathogenesis of atherosclerosis: Endotoxin accelerates atherosclerosis in rabbits on hypercholesterolemic diet. Circulation 104, 914–920
17. Ostos, M. A., Recalde, D., Zakin, M. M., and Scott-Algara, D. (2002) Implication of natural killer T cells in atherosclerosis development during a LPS-induced chronic inflammation. FEBBS Lett. 519, 23–29
18. Engelmann, M. G., Redl, C. V., and Nikol, S. (2004) Recurrent perivascular inflammation induced by lipopolysaccharide (endotoxin) results in the formation of atheromatous lesions in vivo. Lab. Invest. 84, 425–432
19. Westerterp, M., Berbée, J. F., Pires, N. M., van Mierlo, G. J., Kleemann, R., Romijn, J. A., Havekes, L. M., and Rensen, P. C. (2007) Apolipoprotein C-I is crucially involved in lipopolysaccharide-induced atherosclerosis development in apolipoprotein E– knockout mice. Circulation 116, 2173–2181
20. Kiechl, S., Egger, G., Mayr, M., Wiedermann, C. J., Bonora, E., Oberholzer, F., Mugggeo, M., Xu, Q., Wick, G., Poewe, W., and Willett, I. (2001) Chronic infections and the risk of carotid atherosclerosis: Prospective results from a large population study. Circulation 103, 1064–1070
21. Morishita, M., Ariyoshi, W., Okinaga, T., Usui, M., Nakashima, K., and Hirohata, T. (2013) A. actinnymycetcomitans LPS enhances foam cell formation induced by LDL. J. Dent. Res. 92, 241–246
22. Howell, K. W., Meng, X., Fullerton, D. A., Jin, C., Reece, T. B., and Cleeland, J. C., Jr. (2011) Toll-like receptor 4 mediates oxidized LDL-induced macrophage differentiation to foam cells. J. Surg. Res. 171, e27–e31
23. Dunzendorfer, S., Lee, H. K., Soldau, K., and Tobias, P. S. (2004) TLR4 is the signaling but not the lipopolysaccharide uptake receptor. J. Immunol. 173, 1166–1170
24. Wright, S. D., Ramos, R. A., Tobias, P. S., Ulevitch, R. J., and Mathison, J. C. (1990) CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. Science 249, 1431–1433
25. Levy, E., Xanthou, G., Petrouki, E., Zacharioudaki, V., Tsatsanis, C., Fotopoulos, S., and Xanthou, M. (2009) Distinct roles of TLR4 and CD14 in LPS-induced inflammatory responses of neonates. Pediatr. Res. 66, 179–184
26. Pålsson-McDermott, E. M., and O’Neill, L. A. (2004) Signal transduction by the lipopolysaccharide receptor, Toll-like receptor-4. Immunology 113, 153–162
27. Tan, Y., and Kagan, J. C. (2014) A cross-disciplinary perspective on the innate immune responses to bacterial lipopolysaccharide. Mol. Cell 54, 212–223
28. Yang, R. B., Mark, M. R., Gray, A., Huang, A., Xie, M. H., Zhang, M., Goddard, A., Wood, W. I., Gurney, A. L., and Godowski, P. J. (1998) Toll-like receptor-2 mediates lipopolysaccharide-induced cellular signalling. Nature 395, 284–288
34. Good, D. W., George, T., and Watts, B. A., 3rd. (2012) Toll-like receptor 2 is required for LPS-induced Toll-like receptor 4 signaling and inhibition of ion transport in renal thick ascending limb. *J. Biol. Chem.* **287**, 20208–20220

35. Cui, M.-Z., Zhao, G., Winokur, A. L., Laa, E., Bydash, J. R., Penn, M. S., Chisolm, G. M., and Xu, X. (2003) Lysophosphatidic acid induction of tissue factor expression in aortic smooth muscle cells. *Arterioscler. Thromb. Vasc. Biol.* **23**, 224–230

36. Landmann, R., Knopf, H. P., Link, S., Sansano, S., Schumann, R., and Zimmerli, W. (1996) Human monocyte CD14 is upregulated by lipopolysaccharide. *Infect. Immun.* **64**, 1762–1769

37. Marchant, A., Duchow, J., Delville, J. P., and Goldman, M. (1992) Lipoprotein-lipidosis induces up-regulation of CD14 molecule on monocytes in human whole blood. *Eur. J. Immunol.* **22**, 1663–1665

38. Kzhyshkowska, J., Neyen, C., and Gordon, S. (2012) Role of macrophage scavenger receptors in atherosclerosis. *Immunobiology* **217**, 492–502

39. Mutoh, T., Rivera, R., and Chun, J. (2012) Insights into the pharmacological relevance of lysophospholipid receptors. *Br. J. Pharmacol.* **165**, 829–844

40. Ohta, H., Sato, K., Murata, N., Damirin, A., Malchinkhuu, E., Kon, J., Kimura, T., Tobo, M., Yamazaki, T., Watanabe, T., Yagi, M., Sato, M., Suzuki, R., Murooka, H., Sakai, T., et al. (2003) Ki16425, a subtype-selective antagonist for EDG-family lysophosphatidic acid receptors. *Mol. Pharmacol.* **64**, 994–1005

41. Contos, J. J., Fukushima, N., Weiner, J. A., Kaushal, D., and Chun, J. (2000) Requirement for the lpa1 lysophosphatidic acid receptor gene in normal suckling behavior. *Proc. Natl. Acad. Sci. U.S.A.* **97**, 13384–13389

42. Contos, J. J., Ishii, I., Fukushima, N., Kingsbury, M. A., Ye, X., Kawamura, S., Brown, J. H., and Chun, J. (2002) Characterization of lpa1, lpa2 and lpa3 lysophosphatidic acid receptor knockout mice: Signaling deficits without obvious phenotypic abnormality attributable to lpa1. *Mol. Cell. Biol.* **22**, 6921–6929

43. Yang, A. H., Ishii, I., and Chun, J. (2002) In vivo roles of lysophospholipid receptors revealed by gene targeting studies in mice. *Biochim. Biophys. Acta* **1582**, 197–203

44. Ye, X., Hana, K., Contos, J. I., Anliker, B., Inoue, A., Skinner, M. K., Suzuki, H., Amano, T., Kennedy, G., Arai, H., Aoki, J., and Chun, J. (2005) LPA3-mediated lysophosphatidic acid signalling in embryo implantation and spacing. *Nature* **435**, 104–108

45. Bot, M., de Jager, S. C., MacAleese, L., Lagrange, H. M., van Berkel, T. J., Hama, K., Contos, J. J., Fukushima, N., Weiner, J. A., Kaushal, D., and Chun, J. (2000) Scavenger receptors class A-I/II and CD36 are the principal receptors for complexes of lipopolysaccharide (LPS) and LPS binding protein. *Transfection of CD14 into 70Z/3 cells dramatically enhances the sensitivity to complexes of lipopolysaccharide (LPS) and LPS binding protein. J. Exp. Med.* **175**, 1697–1705

46. Lee, J. D., Kato, K., Tobias, P. S., Kirkland, T. N., and Ulevitch, R. J. (1992) Transfection of CD14 into 70Z/3 cells dramatically enhances the sensitivity to complexes of lipopolysaccharide. *Front. Immunol.* **141**, 547–552

47. Simmons, D. L., Tan, S., Tenen, D. G., Nicholson-Weller, A., and Seed, B. (1989) Monocyte antigen CD14 is a phospholipid anchored membrane protein. *Blood* **73**, 284–289

48. Lee, J. D., Kato, K., Tobias, P. S., Kirkland, T. N., and Ulevitch, R. J. (1992) Transfection of CD14 into 70Z/3 cells dramatically enhances the sensitivity to complexes of lipopolysaccharide (LPS) and LPS binding protein. *J. Exp. Med.* **175**, 1697–1705

49. Golenbock, D. T., Liu, Y., Millham, F. H., Freeman, M. W., and Zoeller, R. A. (1993) Surface expression of human CD14 in Chinese hamster ovary fibroblasts imparts macrophage-like responsiveness to bacterial endotoxin. *J. Biol. Chem.* **268**, 22055–22059

50. Zhao, Y., Zhao, J., Mialik, R. K., Wei, J., Spannhafe, E. W., Salgia, R., and Natarajan, V. (2013) Lysophosphatidic acid-induced phosphorylation of c-Met tyrosine residue 1003 regulates c-Met intracellular trafficking and lung epithelial barrier function. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **305**, L56–L63

51. Nochi, H., Tomura, H., Tobo, M., Tanaka, N., Sato, K., Shinozaki, T., Kobayashi, T., Takagishi, K., Ohta, H., Okajima, F., and Tamoto, K. (2008) Stimulatory role of lysophosphatidic acid in cylooxygenase-2 induction by synovial fluid of patients with rheumatoid arthritis in fibroblast-like synovial cells. *J. Immunol.* **181**, 5111–5119

52. Chien, H. Y., Lu, C. S., Chang, K. H., Kao, P. H., and Wu, Y. L. (2015) Attenuation of LPS-induced cylooxygenase-2 and inducible NO synthase expression by lysophosphatic acid in macrophages. *Innate Immun.* **21**, 635–646

53. Zhao, J., He, D., Su, Y., Berdyshiev, E., Chun, J., Natarajan, V., and Zhao, Y. (2011) Lysophosphatidic acid receptor 1 modulates lipopolysaccharide-induced inflammation in alveolar epithelial cells and murine lungs. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **301**, L547–L556

54. da Silva Correia, J., Soldau, K., Christen, U., Tobias, P. S., and Ulevitch, R. J. (2001) Lysophosphatidic acid is in close proximity to each of the proteins in its membrane receptor complex. Transfer from CD14 to TLR4 and MD-2. *J. Biol. Chem.* **276**, 21129–21135

55. Kunjathoor, V. V., Febbraio, M., Podrez, E. A., Moore, K. J., Andersson, L., Koehn, S., Rhee, J. S., Silverstein, R., Hoff, H. F., and Freeman, M. W. (2002) Scavenger receptors class A-I/II and CD36 are the principal receptors responsible for the uptake of modified low density lipoprotein leading to lipid loading in macrophages. *J. Biol. Chem.* **277**, 49982–49988

56. Suzuki, H., Kurihara, Y., Takeya, M., Kamada, N., Kataoka, M., Jishage, K., Ueda, O., Sakaguchi, H., Higashi, T., Suzuki, T., Takashima, Y., Kawabe, Y., Cynshi, O., Wada, Y., Honda, M., et al. (1997) A role for macrophage scavenger receptors in atherosclerosis and susceptibility to infection. *Nature* **386**, 292–296

57. Febbraio, M., Podrez, E. A., Smith, J. D., Hajjar, D. P., Hazen, S. L., Hoff, H. F., Sharma, K., and Silverstein, R. L. (2000) Targeted disruption of the class B scavenger receptor CD36 protects against atherosclerotic lesion development in mice. *J. Clin. Invest.* **105**, 1049–1056

58. Lougheed, M., Lum, C. M., Ling, W., Suzuki, H., Kodama, T., and Steinbrecher, U. (1997) High affinity saturable uptake of oxidized low density lipoprotein by macrophages from mice lacking the scavenger receptor class A type I/II. *J. Biol. Chem.* **272**, 12938–12944

59. Jonsson, A. L., and Bäckhed, F. (2017) Role of gut microbiota in atherosclerosis. *Annu. Rev. Med.* **66**, 343–359

60. Jonsson, A. L., and Bäckhed, F. (2017) Role of gut microbiota in atherosclerosis. *Nat. Rev. Cardiol.* **14**, 79–87.

61. Brown, J. M., and Bäckhed, F. (2017) Role of gut microbiota in atherosclerosis. *Nat. Rev. Cardiol.* **14**, 79–87.