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Lipin-1 Contributes to Modified Low-Density Lipoprotein-Elicited Macrophage Pro-Inflammatory Responses

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Abstract

Atherosclerosis is a chronic inflammatory disease of large and medium-sized arteries and the underlying cause of cardiovascular disease, a major cause of mortality worldwide. The over-accumulation of modified cholesterol-containing low-density lipoproteins (e.g. oxLDL) in the artery wall and the subsequent recruitment and activation of macrophages contributes to the development of atherosclerosis. The excessive uptake of modified-LDL by macrophages leads to a lipid-laden “foamy” phenotype and pro-inflammatory cytokine production. Modified-LDLs promote foam cell formation in part by stimulating *de novo* lipid biosynthesis. However, it is unknown if lipid biosynthesis directly regulates foam cell pro-inflammatory mediator production. Lipin-1, a phosphatidate phosphohydrolase required for the generation of diacylglycerol during glycerolipid synthesis has recently been demonstrated to contribute to bacterial-induced pro-inflammatory responses by macrophages. In this study we present evidence demonstrating the presence of lipin-1 within macrophages in human atherosclerotic plaques. Additionally, reducing lipin-1 levels in macrophages significantly inhibits both modified-LDL-induced foam cell formation *in vitro*, as observed by smaller/fewer intracellular lipid inclusions, and ablates modified-LDL-elicited production of the pro-atherogenic mediators tumor necrosis factor- α , interleukin-6, and prostaglandin E2. These findings demonstrate a critical role for lipin-1 in the

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regulation of macrophage inflammatory responses to modified-LDL. These data begin to link the processes of foam cell formation and pro-inflammatory cytokine production within macrophages.

Keywords

lipin-1; macrophage; foam cell; lipid droplet; atherosclerosis; inflammation

INTRODUCTION

Atherosclerosis, a chronic inflammatory condition of large and medium-sized arteries mediated by macrophages, is the most common cause of CVD¹. Hypercholesterolemia can stimulate the deposition of cholesterol carrying low-density lipoproteins (LDL) in susceptible areas of the artery wall, initiating the development of atherosclerotic lesions². The deposition of LDL in the arterial intima stimulates the recruitment of macrophages to remove cholesterol, thereby preventing cholesterol-induced necrosis³. If LDLs are not promptly removed from the intima, local reactive oxygen species can modify LDLs to oxidized-LDL (oxLDL)⁴. The oxidation of LDL causes it to be taken up by macrophages via scavenger receptors⁵, resulting in a positive feedback response and enhanced oxLDL uptake. Excessive oxLDL uptake initiates *de novo* lipid synthesis which is necessary to store incoming cholesterol and lipids. Free cholesterol-induced foam cell formation requires the activity of acyl-CoA synthetase, the enzyme responsible for the generation of fatty acid acyl-CoA precursors that are needed for glycerolipid synthesis and the esterification of cholesterol⁶. Additionally, fatty acid-induced intracellular lipid accumulation in the form of lipid droplets requires the generation of phosphatidylcholine⁷. Thus, the *de novo* production of lipids (both neutral and glycerophospho- lipids) is required for the intracellular storage and trafficking of lipids (i.e. endosomes and/or lipid droplets)⁸. In addition to enhanced uptake, macrophages loaded with oxLDL display a reduced ability to efflux cholesterol⁹. The net result of excessive LDL uptake and the inability to efflux cholesterol leads to lipid-laden macrophages called foam cells¹⁰. Eventually, foam cells begin to secrete pro-inflammatory mediators such as TNF- α , IL-6 and PGE₂. The combination of excessive uptake, reduced cholesterol efflux, and pro-inflammatory mediator production promotes atheroma formation, plaque progression, and ultimately plaque instability that culminate in the catastrophic events associated with CVD. It is currently unclear if lipid synthetic events elicited by oxLDL contribute to the foam cell pro-inflammatory state.

Lipin-1 belongs to the evolutionarily conserved three-member lipin family in mammals (reviewed in ¹¹). Lipin-1 converts phosphatidate into DAG through phosphohydrolase enzymatic activity and exhibits the highest phosphatidate-specific phosphohydrolase activity of the identified lipins¹². Lipin-1 contributes to triglyceride and likely glycerophospholipid synthesis. The contribution of lipin-1 to glycerophospholipid synthesis is suggested to be necessary for new membrane generation during B cell differentiation¹³. In human and mouse macrophages, lipin-1 contributes to lipid droplet formation following fatty acid loading¹⁴. Additionally, we and others have demonstrated that lipin-1 regulates macrophage inflammatory activity in response to both the intracellular pathogen *Francisella tularensis*¹⁵ and to lipopolysaccharide (LPS)¹⁶. These studies demonstrate that lipin-1 is involved in two

cellular events that are known to exist in macrophage foam cells; storage of engulfed cholesterol/lipids and pro-inflammatory activity, suggesting to us that lipin-1 may link lipid synthesis pathways that contribute to foam cell formation to foam cell inflammatory mediator production. Therefore, we sought to determine if lipin-1 is present within atherosclerotic plaques and if it contributes to modified LDL-elicited foam cell formation and pro-inflammatory responses by macrophages.

RESULTS

Macrophage-associated lipin-1 is present in atherosclerotic lesions

If lipin-1 contributes to atherosclerotic plaque progression, it should be present in atherosclerotic plaques. Staining of human atherosclerotic plaques revealed a strong localization of lipin-1 within the macrophage-rich region (Fig 1A–B). Furthermore, lipin-1 in atherosclerotic plaques is located within CD68⁺ cells, a surface marker for macrophages (Fig 1C). Within CD68⁺ cells lipin-1 was observed in both the cytosol and the nucleus (5 cells observed by confocal microscopy in various stages of plaque development). Furthermore, lipin-1 staining appears at a perinuclear position that could be the endoplasmic reticulum (ER). Due to the poor specificity of commercially available anti-mouse lipin-1 antibodies, a similar experiment demonstrating the presence of lipin-1 within murine plaques could not be performed. Therefore, we examined lipin-1 mRNA levels in a murine model of atherosclerosis. *ApoE*^{−/−} mice were fed normal chow or a high-fat chow for 8 weeks, and lipin-1 mRNA expression in the aortic arch was measured by qRT-PCR. *ApoE*^{−/−} mice fed a high-fat diet displayed a significant increase in lipin-1 expression compared to mice fed a normal diet (Fig 1D). Taken together, these data suggest that macrophage-associated lipin-1 may contribute to atherosclerosis.

Lipin-1 contributes to modified-LDL induced foam cell formation

Lipin-1 regulates lipid droplet generation in hepatocytes and adipocytes, and recently it has been demonstrated that lipin-1 regulates saturated fatty acid-induced macrophage foam cell formation¹⁴. However, it is unknown if lipin-1 regulates modified LDL-elicited foam cell formation. Others have established that the treatment of macrophages *in vitro* with modified-LDL (oxidized or acetylated) generates lipid-laden macrophages that are equivalent to plaque macrophage foam cells¹⁷. OxLDL is inefficiently processed following uptake and a portion of the lipoprotein becomes trapped in the endocytic pathway^{18–19} while the remaining can be broken down and the cholesterol transferred to lipid droplets. Conversely, acLDL is readily processed and leads to the accumulation of cholesterol ester-rich lipid droplets²⁰. To investigate the contribution of lipin-1 to foam cell formation, we used the RAW264.7 macrophage cell line, in which we generated stable lipin-1 knockdown using a lentivirally delivered shRNA (80% knockdown). We have previously demonstrated both the successful and specific knockdown of lipin-1 in RAW264.7 macrophages¹⁵. Wild-type (WT), non-target (NT) or lipin-1 depleted RAW264.7 macrophages were mock treated or treated with oxLDL (10 µg/mL) or acLDL (50 µg/mL) for 24 hours. To visualize intracellular neutral-lipid content we stained the macrophages with Nile Red, a stain that fluoresces intensely in neutral lipid-rich environments²¹, which includes both lipid droplets and internalized lipoproteins²². We will refer to Nile Red positive structures as “lipid

inclusions.” To differentiate between internalized lipoproteins and lipid droplets we stained macrophages with anti-ADRP (adipose-related differentiation protein) antibody. ADRP is a protein known to be present on the outer surface of lipid droplets²³. We acquired images of macrophages and quantified lipid inclusions and lipid droplets using the Cellomics (Thermo Scientific) high-content imaging system. Depletion of lipin-1 reduced both oxLDL-elicited lipid inclusions and acLDL-elicited lipid droplets in macrophages as observed by a reduction their number and intensity of staining when compared to WT macrophages (Fig 2A–B). No differences were observed between WT and NT RAW264.7 macrophages in any analysis performed in this study, thus only WT data is shown in this manuscript. Together, these data suggest that lipin-1 contributes to foam cell formation regardless of the LDL species being metabolized.

Depletion of lipin-1 does not affect uptake of modified-LDL

The failure of lipin-1 depleted macrophages to become foam cells may be due to an inability to endocytose modified LDLs. The scavenger receptor CD36 is known to be the predominant oxLDL receptor present on the surface of macrophages²⁴. Depletion of lipin-1 did not alter the amount of CD36 on the cellular membrane of macrophages either prior to, or after oxLDL treatment when compared to WT macrophages (Supp. Fig 1). We next decided to measure uptake of labeled modified-LDLs. WT or lipin-1 deficient macrophages were treated with either with fluorescently labeled oxLDL (DiI-oxLDL), [³H]-cholesterol loaded oxLDL, or [³H]-cholesterol loaded acLDL. At 4 and 24 hours after treatment, macrophages were collected and mod-LDL uptake was quantified by flow cytometry (DiI-oxLDL) or scintillation count ([³H]-cholesterol mod-LDLs). Depletion of lipin-1 resulted in a partial reduction in the uptake of labeled cholesterol at both 4 and 24 hours after treatment (Figure 3A–C). However, this reduction is not sufficient to account for the nearly complete loss of mod-LDL-elicited lipid inclusions observed in lipin-1 depleted macrophages.

To address modified-LDL uptake by macrophages in a manner distinct from tracking LDL/cholesterol via fluorescence or labeling techniques (Nile Red, DiI-oxLDL, [³H]-cholesterol), we quantified the amount of cellular cholesterol, a portion of which will be derived from incoming LDL. WT and lipin-1 depleted macrophages were mock treated or treated with oxLDL or acLDL for 4 or 24 hours. Total and free cholesterol was quantified by mass spectrometry. In contrast to wild type macrophages, lipin-1 depleted macrophages failed to significantly accumulate intracellular total or free cholesterol in response to either oxLDL or acLDL (Fig 3D–E). However, there was a detectable increase in the esterified cholesterol to total cholesterol ratio (EC/TC), suggesting that lipin-1 depleted macrophages are processing cholesterol in response to mod-LDL treatment (Fig 3F–G). The data obtained using labeled mod-LDLs demonstrate that lipin-1 depleted macrophages are capable of internalizing mod-LDLs, while measurement of total intracellular cholesterol by mass spectrometry suggests they are able to quickly processes and remove internalized cholesterol.

Depletion of lipin-1 reduces modified-LDL-elicited macrophage proinflammatory cytokine production

Chronic exposure to modified-LDLs causes macrophages to progress toward the lipid-laden “foamy macrophage” phenotype. A hallmark of lipid-laden foamy macrophages is their

secretion of pro-inflammatory factors. We hypothesized that the inflammatory responses of modified-LDL-treated macrophages is due to intracellular hyperlipidemia mediated by lipin-1's contribution to glycerolipid biosynthesis, specifically the production of diacylglycerol. The direct product of lipin-1 is DAG, which is a key substrate for both neutral- and phospho-lipid synthesis. Mod-LDL treatment of macrophages results in DAG formation²⁵, and we hypothesize that lipin-1 is required for this generated DAG. To test this, WT or lipin-1 depleted macrophages were left untreated or treated with oxLDL for 24 hours. Total lipids were extracted and DAG was visualized and quantified by thin-layer chromatography. DAG levels increased in WT macrophages following 24 hour oxLDL treatment while lipin-1 depleted macrophages do not show this increase (Fig 4A). These data suggest that lipin-1 is participating the generation of DAG in oxLDL treated macrophages.

To begin to establish a connection between lipin-1 and foam cell inflammation, we quantified the production of inflammatory mediators following modified-LDL treatment of WT or lipin-1 depleted macrophages. Supernatant concentrations of the pro-atherogenic mediators TNF- α , IL-6 and prostaglandin E2 (PGE₂) were determined by ELISA 24 and 48 hours following treatment. Only elevated PGE₂ production was detected by oxLDL-treated macrophages 24 hours after treatment, with no difference between WT and lipin-1 depleted macrophages (Fig 4B). However, after 48 hours of oxLDL treatment, increased TNF- α , PGE₂ and IL-6 production was detected and depletion of lipin-1 resulted in a complete loss of oxLDL-elicited production of TNF- α and IL-6, and reduced PGE₂ synthesis (Fig 4B). AcLDL treatment of macrophages resulted in a similar increase in TNF- α and PGE₂ production that was also dependent upon lipin-1 (Fig. 4C). We did not observe an increase in IL-6 production following exposure to acLDL (data not shown). Because oxLDL and acLDL induce different lipid synthesis events, it is possible that the accumulation of oxLDL in endocytic compartments and/or the lipid pathways required to support these inclusions is critical to the induction of IL-6. Future experiments will mechanistically define how lipin-1 contributes to both foam cell formation and foam cell pro-inflammatory response.

Due to the known role for elevated intracellular cholesterol-induced toxicity in macrophages, we tested whether oxLDL treatment enhanced cell death of lipin-1 deficient macrophages compared to WT macrophages. Propidium iodide exclusion assays demonstrated no increase in cell death in WT, NT or lipin-1 deficient macrophages following 24 or 48 hour oxLDL treatment (Supp. Fig. 2). To confirm that lipin-1 depletion does not result in an unresponsive macrophage, WT, NT shRNA or lipin-1 deficient macrophages were treated with LPS and the phosphorylation of NF- κ B p65 and p38-MAPK (correlates of activity) were quantified by western blot (Supp. Fig 3A & B). There were no observed differences in the phosphorylation of p65 and p38 following LPS stimulation in both WT and lipin-1 deficient macrophages, suggesting that lipin-1 may be more critical to modLDL-induced inflammation than bacterial-induced inflammation.

Alternative splicing of lipin-1 results in two isoforms: lipin-1 α and lipin-1 β which localize to unique subcellular locations in adipocytes when over-expressed. Yet, it remains unknown whether or not these isoforms have non-overlapping functions. To gain insight into the possible differential function of lipin-1 splice variants during oxLDL-elicited responses by

macrophages we examined mRNA levels of lipin-1 α and lipin-1 β in macrophages. WT macrophages were treated with 10 μ g/ml oxLDL for 4, 24, or 48 hours. RNA was isolated and end-point PCR was conducted to differentiate between lipin-1 α and lipin-1 β . We did not observe a significant alteration in mRNA levels nor a change in the ratio of lipin-1 α and lipin-1 β (Fig 4D). Thus, at this time we cannot state which lipin-1 isoform contributes more critically to foam cell biology.

DISCUSSION

Macrophages are critical to the initiation and progression of atherosclerosis. Two key functional characteristics observed in the plaque-associated macrophage population are foam cell formation and pro-inflammatory cytokine production^{26–27}. Macrophage foam cell formation is partially due to enhanced uptake of modified-LDLs and *de novo* lipid synthesis. Pro-inflammatory cytokine responses are suggested to be due to both TLR recognition of oxLDL²⁸ and inflammasome activation by cholesterol crystals²⁹. It is currently unknown whether lipid synthetic events that contribute to foam cell formation and pro-inflammatory cytokine production are separate functional consequences of macrophage exposure to modified-LDL or if these two events are linked. Our studies provide evidence that lipid synthesis and pro-inflammatory responses in foam cells may be connected. Specifically, we demonstrate here that lipin-1 deficiency renders macrophages refractory to modified-LDL-induced lipid inclusion accumulation and pro-inflammatory cytokine production.

Lipin-1 was observed in plaque-rich regions of both human and mouse vasculature. Lipin-1 mRNA was enriched in the aorta of atherosclerotic-prone mice fed a high-fat diet and lipin-1 protein was detected in the macrophage-rich region of human atherosclerotic plaques, which was observed at the stage of fatty-streak formation, and persisted throughout plaque progression (data not shown). At the cellular level, lipin-1 was observed in both the nucleus and in the cytosol of plaque macrophages (observed in all cells examined). Cytoplasmic lipin-1 seems to be enriched in a region juxtaposed to the nucleus that is likely the ER. Lipin-1 is known to perform its enzymatic activity at the ER³⁰ and its transactivator function in the nucleus³¹. Detection of lipin-1 at these locations in the same cell suggests that both activities could simultaneously contribute to macrophage function within human atherosclerotic plaques. Further studies are needed to provide more detail on the physiological location and cell type expression pattern of lipin-1 in human atherosclerotic lesions, as well as how each lipin-1 activity may contribute to foam cell biology.

Both oxLDL and acLDL treatment resulted in a significant increase in intracellular lipid inclusions that was not observed in RAW264.7 macrophages depleted of lipin-1. Foam cell formation in response to oxLDL requires uptake of oxLDL via receptor-mediated endocytosis, typically following its binding to the scavenger receptor CD36²⁴. We did not detect a difference in the amount of CD36 present at the cell surface. The use of labeled mod-LDLs demonstrated that the failure of lipin-1 depleted macrophages to become foam cells was not due to an overt defect in mod-LDL uptake. Interestingly, the use of mass spectrometry demonstrated that there was no significant increase in total cholesterol levels in lipin-1 depleted macrophages over the same time period. The detection of labeled mod-LDL uptake and no corresponding change in total cholesterol are not mutually exclusive

observations. The use of labeled mod-LDLs allows us to measure internalization, while mass spectrometry allows us to determine intracellular cholesterol levels but not the source of cholesterol. Taking these observations together, we suggest that lipin-1 depleted macrophages more efficiently process and export cholesterol than wild type macrophages. The observation of an increase in the ratio of esterified to total cholesterol in lipin-1 depleted macrophages provides some evidence these cells are processing incoming cholesterol. Improved processing of cholesterol may be due to enhanced autophagy, as lipin-1 depleted cells have been observed to have a higher rate of autophagy³², and autophagy is a critical pathway for removal of cholesterol from macrophages⁹. The fate of mod-LDL and its lipid components following uptake by lipin-1 depleted macrophages will be the subject of future investigation. Regardless of mechanism, the loss of lipin-1 reduced the accumulation of lipid inclusions without an increase in cellular death.

Macrophages require lipin-1 for PGE₂ and pro-inflammatory cytokine production in response to LPS and the intracellular bacteria *Francisella tularensis*^{15–16}. We demonstrate here that short-term oxLDL treatment (24 hours) fails to elicit pro-inflammatory cytokine responses, in agreement with work by Spann *et.al*, who demonstrated that cholesterol uptake by macrophages results in an anti-inflammatory macrophage phenotype³³. However, prolonged treatment of macrophages with oxLDL (48 hours) elicits the production of PGE₂, IL-6 and TNF- α by RAW264.7 by macrophages that require lipin-1; similar responses were observed with acLDL treatment. In contrast to LPS and *F. tularensis* in which lipin-1 only partially contributes to TNF- α production, loss of lipin-1 essentially eliminates oxLDL and acLDL-elicited TNF- α and oxLDL IL-6 production. One possible explanation for the severe defect in modified-LDL-induced pro-inflammatory cytokine production by lipin-1-deficient macrophages could involve lipin-1's central role in glycerolipid synthesis. Lipin-1 generates DAG as part of the glycerolipid synthesis pathway³⁴ resulting in the synthesis of triglyceride (TG) and the glycerophospholipids, phosphatidylcholine (PC) and phosphatidylethanolamine (PE)³⁵. These lipids (TG, PC and PE) are known components of lipid droplets and endosomes^{36–37}. Lipin-1 contributes to the production of diacylglycerol during LPS stimulation³⁸, and we have demonstrated that lipin-1 regulates DAG formation in foam cells as well. DAG is known to contribute to pro-inflammatory responses in macrophages through the activation of protein kinase Cs (PKCs)³⁹. PKCs have been suggested to contribute to oxLDL-elicited inflammatory responses⁴⁰. Thus, we suggest that the mechanism by which lipin-1 regulates oxLDL elicited pro-inflammatory responses is due to the sustained generation of lipids that can activate signaling cascades, such as diacylglycerol mediated activation of PKCs. Future work will focus on defining how the enzymatic activity of lipin-1 and potentially the glycerophospholipid synthetic pathway contributes to both foam cell formation and to foam cell inflammatory responses.

CONCLUSION

We demonstrated that lipin-1 was necessary for both modified LDL-elicited foam cell formation and pro-inflammatory cytokine production. Furthermore, lipin-1 was detected in human atherosclerotic plaques. This work suggests that the process of lipid synthesis, when allowed to proceed unrestricted in foam cells, can directly contribute to pro-inflammatory responses via lipin-1 activity. We propose during modLDL uptake, new lipids are required

to be synthesized for either endosome or lipid droplet generation. We propose that the sustained production of lipids (such as lipin-1 generated diacylglycerol) within foam cells leads to the inadvertent activation of signaling cascades that result in macrophage pro-inflammatory cytokine responses. Furthermore, the observation that lipin-1 seems to more significantly contribute to oxLDL-elicited pro-inflammatory responses than that observed for LPS stimulation¹⁶ suggests that targeting lipin-1 enzymatic activity may reduce atherosclerotic inflammation without rendering individuals susceptible to bacterial infection. Whether the modulation of lipin-1 activity can provide therapeutic benefits for the treatment of atherosclerosis and CVD will be the focus of future research.

MATERIALS AND METHODS

Cell lines

The murine macrophage cell line RAW264.7 (ATCC #TIB-71) was cultured at 37°C, 5% CO₂ in DMEM supplemented with 10% FBS (ATLAS), 2 mM L-glutamine, 1 mM sodium pyruvate (HyClone), 100 U/mL penicillin, 100 µg/mL streptomycin (ATCC). Lipin-1 in the RAW264.7 macrophage cell line was depleted as previously described.¹⁵

Preparation of oxidized LDLs

Human LDLs (Intracel or Kalen Biomedical) were oxidized by incubation with copper (II) sulfate (CuSO₄) as previously described⁴¹. Briefly, human LDL was dialyzed against phosphate buffered saline (PBS) pH 7.6 for 24 hours at room temperature using a 7,000 MWCO Slide-A-Lyzer® Dialysis cassette (Thermo Scientific) to remove EDTA. The buffer was then changed to fresh PBS containing 13.8 µM CuSO₄. The LDL was incubated with CuSO₄ for 72 hours at room temperature. The buffer was then changed to fresh PBS containing 50 µM EDTA for 24 hours at 4°C to remove excess copper. The buffer was then changed to fresh PBS containing 50 µM EDTA and allowed to incubate an additional 24 hours. Oxidized LDL (oxLDL) was then collected and stored under argon gas at 4°C. It has been described previously that this methodology results in oxLDL that consistently displays a relative electrophoretic mobility between 2 and 3⁴¹. All steps performed under sterile conditions. Acetylated human LDL and DiI-labeled oxidized human LDL were purchased from Kalen Biomedical.

Enzyme immunoassays

PGE₂ (Assay Designs), TNF-α and IL-6 (eBioscience) were measured using commercially available immunoassay kits according to manufacturer's instructions.

Macrophage imaging and quantification of intracellular lipid content

5,000–10,000 Wild-type (WT), non-target shRNA (NT) or lipin-1 shRNA RAW264.7 macrophages were seeded into a sterile, black-walled 96-well half-area high content imaging plate with a 0.2 mm glass bottom (Corning 4580) and incubated overnight at 37°C to allow cells to adhere. The macrophages were then mock treated (PBS), treated with 10 µg/mL oxLDL or 50 µg/mL acLDL for 24 hours at 37°C. The media was then aspirated and the cells were washed once with PBS. The macrophages were then fixed with 4% formaldehyde in PBS for 20 minutes at room temperature. Following fixation, the macrophages were

washed once with PBS and then incubated with 100 nM Nile Red (ACROS Organics) in PBS for 5 minutes at room temperature protected from light. Following Nile Red incubation, the macrophages were washed once with PBS and incubated with Hoechst solution (500 nM in PBS) for 30 minutes at room temperature protected from light. The Hoechst solution was then removed and PBS added to each well. For ADRP immunostaining, following fixation, the cells were permeabilized with 0.2% TritonX100 in PBS for 30 minutes at room temperature. Cells were then blocked with anti-mouse CD16/32 (ebioscience) at a concentration of 1:250 in 1% BSA in PBS for 30 minutes at room temperature. Cells were then stained with rabbit anti-ADRP (abcam, ab52356) at 1:250 in 0.25% BSA in PBS for 1 hour at 37°C. Cells were washed with PBS and then stained with goat anti-rabbit secondary AlexaFluor488 (Life Technologies) at 1:2000 in 0.25% BSA in PBS for 30 minutes at 37°C. Cells were washed and incubated with Hoechst as above. The plates were scanned using the Cellomics imaging system (Thermo Scientific). Lipid inclusion images presented as an inverted grayscale image with densely stained areas in black. Approximately 3,000 cells were analyzed per treatment group per experiment for quantitation of macrophage images.

Cytokine assays and western blot analysis

100,000 WT, NT or lipin-1 shRNA RAW264.7 macrophages were seeded in a sterile 12-well tissue culture treated plate and incubated for 24 hours at 37°C. The macrophages were then mock treated (PBS) or treated with oxLDL (10 µg/mL) acLDL (50 µg/mL) or LPS (100 ng/mL).

Flow cytometry

Cell viability: Cell death following treatment with oxLDL was determined using propidium iodide exclusion. 100,000 macrophages in 1 mL media added to a sterile 5 mL Falcon tube. Macrophages were then mock treated (PBS) or treated with oxLDL (10 µg/mL) for 24 or 48 hours at 37°C. Propidium iodide at 2 µg/mL in PBS was added to each tube immediately prior to analysis.

CD36 surface expression: The macrophages were mock treated (PBS) or treated with oxLDL (10 µg/mL) for 24 hours at 37°C. Macrophages were washed once with FACS wash (1% BSA, 0.1% sodium azide in PBS) and re-suspended in 400 µL FACS wash. Macrophages were treated with anti-mouse CD16/32 (1:400) in FACS wash for 30 minutes on ice to block Fc Receptors and then incubated with anti-mouse CD36 antibody (APC) (1:400) for 30 minutes on ice. The macrophages were fixed (4% formaldehyde in PBS) for 20 minutes on ice. Cells were stored at 4°C protected from light until analysis.

Modified LDL uptake

For uptake of mod-LDL, macrophages were treated with 1:10 DiI-oxLDL/unlabeled oxLDL at 37°C, radiolabeled cholesterol loaded oxLDL or acLDL. OxLDL and acLDLs were loaded with [1,2-³H(N)]-cholesterol (Perkin Elmer) at 30 µCi/mg LDL as previously described⁴². At 4 and 24 hours after treatment, DiI content was determined by flow cytometry while [³H]-cholesterol was determined by scintillation counting using a Beckman LS 6500 Scintillation System.

Quantification of cellular lipid content

Measurement of Diacylglycerol: Following treatment, macrophages were collected and washed twice with phosphate buffered saline. Total lipids were extracted from 10^7 macrophages by the method of Bligh & Dyer⁴³. 5 μ g of DAG standard (Avanti Polar Lipids) and extracted lipids solubilized in chloroform were spotted onto silica gel-coated plastic-backed thin-layer chromatography plates (Sigma) and separated using 75:25 petroleum ether:diethyl ether. DAG was visualized by exposure of plates to Iodine vapor. Images were captured and band intensity quantified using ImageJ software.

Total and Free Cholesterol Determination: Following treatment, cells were collected and washed extensively with PBS. 10^6 macrophages per sample were pelleted in a 1.5mL microcentrifuge tube and stored at -80°C until analysis. Samples were spiked with the internal standard, cholesterol-3,4- $^{13}\text{C}_2$ and total lipids were extracted by the method of Bligh & Dyer as previously described⁴⁴. Measurement of total and free cholesterol was measured using a Finnigan TSQ Quantum XLS tandem mass spectrometer interfaced to a trace gas chromatograph (GC/MS/MS) as previously described⁴⁵. Cholesteryl esters were calculated as (total cholesterol – free cholesterol).

Immunoblot analysis

Cell lysis, protein determination, and immunoblotting was performed as previously described¹⁵. Bands of interest were normalized to β -actin for statistical analysis. Antibodies were as follows: antibodies from Cell Signaling included rabbit anti-NF- κ B p65, rabbit anti-phospho-NF- κ B p65 (Ser536), rabbit anti phospho-p38 MAPK, β -actin was from Sigma; and IRDye 680LT donkey (polyclonal) anti-rabbit IgG secondary and blocking buffer from Li-Cor Biosciences. All primary antibodies were diluted 1:1,000 in 1% BSA in PBS with sodium azide. β -actin and IRDye secondary antibody were diluted 1:20,000 in 5% milk in Tris-buffered saline with 0.1% Tween 20 (TBST) plus 0.01% SDS.

Analysis of lipin-1 in human and mouse plaques

All animal protocols were approved by the LSU Health Sciences Center-Shreveport Animal Care and Use Committee and followed the National Institute of Health guidelines for the care and use of laboratory animals. Eight week old, male ApoE knockout mice were purchased from Jackson Laboratories and fed either standard chow or a high fat Western diet (Teklad TD88137; containing 21% fat by weight with 0.15% cholesterol and 19.5% casein) for 8 weeks. Mice were euthanized, perfused with saline by intracardiac injection, and the aorta was excised. The aorta was cleaned of perivascular adipose tissue and lysed in TRIzol reagent for mRNA analysis. mRNA was converted to cDNA using the BioRad iScript cDNA synthesis kit. Lipin-1 primer sequence: FW '5-CATGCTTCGGAAAGTCCTTCA-3' RV '5-GGTTATTCTTTGGCGTCAACCT-3' and rpl13a (F 5'-TCCACCCTATGACAAGAA-3', R 5'-GTAAGCAAACCTTCTGGTAG-3'). qRT-PCR was performed using Sybr Green 2X master mix (BioRad) on a BioRad CFX96 real-time PCR machine. Gene expression was normalized to the housekeeping genes peptidylprolyl isomerase A (PPIA) and ribosomal protein L13A (Rpl13a) using the $2^{-\text{Ct}}$ method. Experiments involving the use of human tissue were deemed non-human research by the local IRB due to the exclusive use of post-mortem samples. Patient data from this

collection of human tissue has been previously published⁴⁶. Human atherosclerotic plaque tissue, collected postmortem during routine autopsy, was fixed in 4% formaldehyde, embedded in paraffin, and cut into 5 mm sections. Slides were stained by standard immunohistochemistry, visualized using Alexa dye-conjugated secondary antibodies, and imaged using a Leica TCS SP5 Confocal Microscope. Antibodies used included mouse anti-CD68 (1:200, Dako) and rabbit anti-lipin-1 (1:50, Abcam ab92316).

RNA extraction and mRNA expression analysis for lipin-1 α and lipin-1 β

RNA was collected in STAT60 (TEL-TEST, INC). cDNA was synthesized from 1 μ g of total RNA using the iScript cDNA Synthesis Kit (Bio-Rad). Thereafter, end-point PCR was performed to identify lipin-1 α , lipin-1 β and rpl13a with Phusion Polymerase (Thermo Scientific) according to the manufacturer's instructions. Primer sequences used: lipin-1 α /lipin-1 β (F 5'-CCCTCGATTTCACGTACCC-3', R 5'-GCAGCCTGTGGCAATTCA-3')⁴⁷ and rpl13a (F 5'-TCCACCCTATGACAAGAA-3', R 5'-GTAAGCAAACCTTCTGGTAG-3'). All reactions were performed on the GeneAmp PCR System 9700 Thermocycler (Applied Biosystems). Samples were combined and loaded on a 2% agarose gel containing GelRed (Phenix Research) and visualized on the Gel Doc XR+ (BioRad). Band intensities were quantified using ImageJ. Lipin-1 α and lipin-1 β were normalized to rpl13a.

Statistical analysis

GraphPad Prism 5.0 was used for statistical analyses. Statistical significance was determined using a one-way ANOVA analysis of variance with a Dunnett's post-test, $P < 0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

- The phosphatidate phosphohydrolase lipin-1 was detected in human atherosclerotic plaques.
- Lipin-1 was required for modified LDL-elicited foam cell formation and production of pro-inflammatory mediators TNF- α , IL-6 and prostaglandin E2
- The loss of lipin-1 did not inhibit modified LDL uptake or enhance apoptosis of macrophages.
- These data begin to link the process of foam cell formation and foam cell inflammatory responses.

Figure 1. Lipin-1 is found within human atherosclerotic plaques

(A) Representative immunofluorescent staining of a stage III human atherosclerotic plaque showing lipin-1 (red) near the macrophage-rich region (CD68; green) $n=9$ of plaques ranging from stage I to stage V. (B) H&E stain of human plaque. (C) A single cell from the plaque displayed in A that is positive for the macrophage marker CD68 (1 of Five closely examined). Lipin-1 is present within the nucleus and cytosol. All closely examined plaque macrophages contained cytosolic and nuclear lipin-1. (D) qRT-PCR of lipin-1 mRNA from the aortic arch of ApoE^{-/-} mice fed normal or high-fat chow. The data presented is representative (1 experiment $n=3$) of two independent experiments. * indicates statistical significance compared to normal chow group ($P < 0.05$).

Figure 2. Lipin-1 is required for full lipid inclusion formation by macrophages in response to mod-LDL

Fluorescent microscopy of wild type, or lipin-1 depleted macrophages treated with oxLDL or acLDL for 24 hours. Macrophages were then stained with Nile Red or anti-ADRP. Representative grayscale images are shown on the left, displaying densely stained, neutral lipid-rich areas (Nile Red) or lipid droplets (ADRP) in black. Quantification of spots from 3–7 independent experiments (a minimum of 1,000 cells per group per experiment were examined). # indicates statistical significance compared to WT oxLDL or acLDL group (P 0.05).

Figure 3. Lipin-1 is not required for oxLDL uptake by macrophages

Wild type, or lipin-1 depleted RAW264.7 macrophages were mock treated or stimulated with labeled oxLDL or acLDL. (A) Wild type or lipin-1 depleted RAW264.7 macrophages were incubated with diI-oxLDL (10 μ g/mL) for 4 or 24 hours. DiI-oxLDL uptake was quantified by flow cytometry and the data is reported as mean fluorescence intensity (MFI). (B–C) RAW264.7 macrophages were treated with [³H]-cholesterol loaded oxLDL (10 μ g/mL) or acLDL (50 μ g/mL) for 4 or 24 hours. Radioactivity in the cellular fraction was determined by scintillation counting. (D–E) Total (TC) and free cholesterol (FC) content of RAW264.7 macrophages was determined by mass spectrometry at baseline or following stimulation with oxLDL (10 μ g/mL) or acLDL (50 μ g/mL) for 4 or 24 hours. Esterified cholesterol (EC) was determined as TC – FC (n=4–6). # statistical significance compared to corresponding WT treatment group, * statistical significance compared to WT baseline

cholesterol value, ∇ statistical significance from baseline lipin-1 shRNA EC/TC value (P 0.05).

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Figure 4. Depletion of lipin-1 reduces macrophage inflammatory responses to mod-LDL
 (A) Total lipids were extracted from WT or lipin-1 shRNA macrophages following mock or 24 treatment with oxLDL. Lipids were separated by thin-layer chromatography. Graph represents quantification of three independent experiments. (B–C) Wild type or lipin-1 depleted RAW264.7 macrophages were left untreated or stimulated with oxLDL (10 μ g/mL) or acLDL (50 μ g/mL) for 24 or 48 hours. Concentrations of TNF α , IL-6 and PGE₂ were quantified by ELISA. Data represents the mean $\pm$ SEM of 3–6 independent experiments. (D) Wild-type RAW264.7 macrophages were left untreated or stimulated with 10 μ g/mL of oxLDL for 4, 24, or 48 hours. RNA was isolated, converted to cDNA and then amplified by end-point PCR. Lipin-1 α and lipin-1 β were normalized to the rpl13a loading control. Graphical data represents the mean $\pm$ SEM of three independent experiments (n=3). * indicates statistical significance compared to WT oxLDL (Fig. A–C) (P < 0.05)