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Macrophage-associated lipin-1 enzymatic activity contributes to modified LDL-induced pro-inflammatory signaling and atherosclerosis

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Abstract

Objective—Macrophage pro-inflammatory responses induced by modified low-density lipoproteins (modLDL) contribute to atherosclerotic progression. How modLDL causes macrophages to become pro-inflammatory is still enigmatic. Macrophage foam cell formation induced by modLDL requires glycerolipid synthesis. Lipin-1, a key enzyme in the glycerolipid synthesis pathway, contributes to modLDL-elicited macrophage pro-inflammatory responses *in vitro*. The objective of this study was to determine if macrophage-associated lipin-1 contributes to atherogenesis and to assess its role in modLDL-mediated signaling in macrophages.

Approach and Results—We developed mice lacking lipin-1 in myeloid-derived cells and used adeno-associated viral vector 8 expressing the gain-of-function mutation of mouse proprotein

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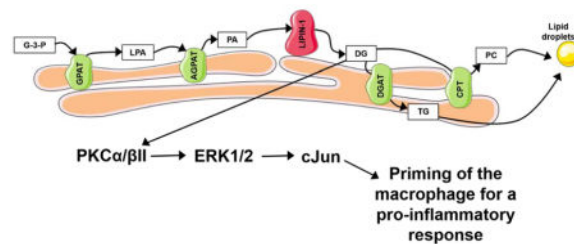
DISCLOSURES

None.

convertase subtilisin/kexin type 9 (AAV8-PCSK9) to induce hypercholesterolemia and plaque formation. Mice lacking myeloid-associated lipin-1 had reduced atherosclerotic burden compared to control mice despite similar plasma lipid levels. Stimulation of bone marrow-derived macrophages with modLDL activated a persistent PKC α / β II-ERK1/2-cJun signaling cascade that contributed to macrophage pro-inflammatory responses that was dependent on lipin-1 enzymatic activity.

Conclusions—Our data demonstrate that macrophage-associated lipin-1 is atherogenic, likely through persistent activation of a PKC α / β II-ERK1/2-cJun signaling cascade that contributes to foam cell pro-inflammatory responses. Taken together these results suggest modLDL-induced foam cell formation and modLDL-induced macrophage pro-inflammatory responses are not independent consequences of modLDL stimulation, but rather are both directly influenced by enhanced lipid synthesis.

Graphical abstract



Keywords

Lipin-1; macrophage; atherosclerosis; inflammation

Subject code

Animal models of human disease; inflammation; lipids and cholesterol; metabolism; cardiovascular disease; atherosclerosis

INTRODUCTION

Macrophage responses to modified low-density lipoproteins (e.g. oxidized LDL) contribute to atherosclerosis progression. Internalization of modified LDL (modLDL) by macrophages results in the generation of a lipid-laden phenotype (foam cell) and generation of pro-inflammatory mediators. The foam cell phenotype results from both enhanced modLDL uptake via scavenger receptor recognition and reduced ability to process cholesterol and deliver it to high-density lipoproteins.¹ Uptake of modLDLs is also known to induce production of pro-inflammatory cytokines, such as TNF- α , IL-6, IL-1, PGE2, CCL2, etc., that promote atherosclerosis.² However, the mechanism through which foam cells become pro-inflammatory has been elusive. ModLDLs can be recognized by macrophages *in vitro* as “pathogens” via pattern recognition receptors (PRRs) such as TLR2, TLR4, and NLRP3, resulting in acute signaling responses that elicit pro-inflammatory mediator production.^{3–6} However, *in vivo* the induction of macrophage pro-inflammatory responses is clearly more

complex as hematopoietic deletion of TLR2, TLR4, or NLRP3 does not reduce atherosclerosis burden.^{7, 8} We and others have demonstrated that glycerolipid synthesis can not only contribute to foam cell formation but can also mediate macrophage pro-inflammatory responses.^{9–11} Together, these data suggest glycerolipid synthesis may provide an additional mechanism by which modLDLs influence macrophage pro-inflammatory responses.

ModLDLs are engulfed by macrophages and broken down by lysosomal acid lipases within endosomes, resulting in the generation of free cholesterol and free fatty acids.¹² Excess free cholesterol is esterified at the endoplasmic reticulum (ER) and then stored in lipid droplets, which give foam cells their phenotypic appearance.¹ Although the composition of foam cell lipid droplets is dominated by cholesterol esters, they also contain triacylglycerol (TAG) and phosphatidylcholine (PC).^{12, 13} One lipid synthetic pathway that contributes to lipid droplet generation in macrophages is the glycerolipid synthetic pathway, which produces TAG and PC.¹⁴ Glycerolipid synthesis consists of four enzymatic steps in which glycerol-3-phosphate (G3P) is converted into TAG or PC at the ER (Graphical abstract). Sequential esterification of G3P with acyl-CoA charged long-chain-fatty-acids by glycerol phosphate acyl-transferase and lysophosphate-acyl transferase enzymes produces phosphatidic acid (PA). PA is dephosphorylated by the phosphatidic acid phosphatase (PAP), lipin-1, to generate diacylglycerol (DAG). DAG can then be converted into TAG by diglyceride acyltransferase (DGAT) enzymes or PC via the activity of CDP-DAG:cholinephosphotransferase.¹⁵ The inhibition of long-chain-fatty-acid-CoA ligase (with triacin C), the genetic deletion of DGAT1, or genetic reduction of lipin-1 all result in reduced foam cell formation in response to modLDLs.^{10, 11, 16} Thus, these studies provide evidence that glycerolipid synthesis contributes to foam cell formation.

Glycerolipid synthesis has been shown to influence immune cell function and enhance inflammation.^{17–19} Our lab and others have demonstrated that lipin-1, a key regulatory enzyme in the glycerolipid synthetic pathway, mediates DAG production and contributes to pro-inflammatory responses in LPS-, oxLDL-, and acetylated LDL- (acLDL) stimulated macrophages.^{9, 10} Lipin-1 is a bi-functional enzyme with both enzymatic PAP activity and transcriptional cofactor activity.^{14, 20} However, only lipin-1 enzymatic activity is required for glycerolipid synthesis.²¹ The contribution of lipin-1 to macrophage pro-inflammatory responses led us to hypothesize that macrophage-associated lipin-1 enzymatic activity influences atherosclerosis development. To evaluate the role of macrophage-associated lipin-1 enzymatic activity in atherosclerosis, we developed a mouse model with a myeloid-specific deletion of lipin-1 enzymatic activity (lipin-1^{mEnzy}KO) and induced atherosclerosis using the adeno-associated viral vector 8 expressing a gain-of-function mutation (D377Y) of mouse proprotein convertase subtilisin/kexin type 9 (referred to as AAV8-PCSK9) and high-fat diet.²² Additionally, our *in vitro* studies provide evidence as to the molecular mechanisms by which macrophage-associated lipin-1 enzymatic activity promotes atherosclerosis.

MATERIALS AND METHODS

Materials and Methods are available in the online-only Data Supplement.

RESULTS

Myeloid-associated lipin-1 is atherogenic

To investigate the potential contribution of macrophage-associated lipin-1 on atherosclerosis, we generated mice lacking lipin-1 enzymatic activity in myeloid-derived cells. *Lpin1* floxed (lipin-1^{flox/flox}) mice were crossed with *LysM-Cre* mice to generate myeloid-specific lipin-1 enzymatic knockout (lipin-1^{mEnzyKO}) mice. Due to the location of the flox sites in these lipin-1^{flox/flox} mice, the original translational start site is lost, but an alternative start site is enforced and the resulting truncated lipin-1 protein lacks enzymatic activity while transcriptional coregulatory function is retained.²³

Equal amounts of lipin-1 were present in non-myeloid-derived tissue from the heart and lung in lipin-1^{mEnzyKO} and lipin-1^{flox/flox} littermate control mice (Figure 1A). BMDMs derived from lipin-1^{mEnzyKO} mice have a smaller lipin-1 band compared to the lipin-1 band detected in the lipin-1^{flox/flox} BMDMs, the smaller band represents the truncated form of lipin-1²³ (Figure 1A). We first wanted to verify that BMDMs from lipin-1^{mEnzyKO} mice responded to oxLDL in a manner similar to the lipin-1-depleted RAW264.7 macrophage cell line.¹⁰ RAW264.7 macrophages lacking lipin-1 have reduced foam cell formation and DAG production, while cholesterol uptake and scavenger receptor expression remains unaltered in response to oxLDL as compared to control RAW264.7 macrophages.¹⁰ Consistent with this, LC-MS/MS analysis showed lipin-1^{mEnzyKO} BMDMs had reduced DAG production 24 and 48 hours after oxLDL stimulation as compared to lipin-1^{flox/flox} control BMDMs (Figure 1B). Furthermore, analysis of cholesterol esters showed no difference in cholesterol content between the lipin-1^{mEnzyKO} and lipin-1^{flox/flox} control BMDMs (Figure 1C). Nile red analysis also demonstrated a reduction in lipid content of lipin-1^{mEnzyKO} BMDMs compared to lipin-1^{flox/flox} control BMDMs (Figure 1D). Similar cholesterol content suggests oxLDL engulfment is unaltered in the lipin-1^{mEnzyKO} BMDMs, which was supported by similar levels of scavenger receptor gene expression as measured by qRT-PCR analysis (Supplemental Figure 1A). Together these data demonstrate that lipin-1^{mEnzyKO} BMDMs have reduced foam cell formation and DAG production but continue to engulf oxLDLs.

To elicit atherosclerosis, lipin-1^{flox/flox} and lipin-1^{mEnzyKO} mice were retro-orbitally injected with 3×10^{10} vector genomes of AAV8-PCSK9 and placed on high fat diet for 8 or 12 weeks. Both lipin-1^{flox/flox} and lipin-1^{mEnzyKO} mice gained weight in response to high fat diet at similar rates (Supplemental Figure IIA&B). Serum lipid analysis was performed at the time of euthanasia to determine the state of hypercholesterolemia. Comparable levels of total cholesterol (TC), triglycerides (TG), high-density lipoprotein (HDL) cholesterol, and low-density lipoprotein (LDL) cholesterol were observed between the lipin-1^{mEnzyKO} and lipin-1^{flox/flox} mice (Supplemental Figure IIC&D). To ensure the lipin-1^{mEnzyKO} did not have alterations in immune composition, we analyzed splenocytes in lipin-1^{mEnzyKO} and lipin-1^{flox/flox} mice at time of euthanasia and demonstrated equivalent percentages and numbers of granulocytes, macrophages, dendritic cells, T cells, B cells, and NK cells between the mouse strains (Table 1). We also determined atherosclerotic burden in lipin-1^{flox/flox} and lipin-1^{mEnzyKO} mice. Oil Red O staining of *en face* aortas revealed less

plaque development in the aortas of lipin-1^{mEnzyKO} mice compared to the corresponding lipin-1^{flox/flox} controls (Figure 1E). Additionally, MOVAT staining and subsequent analysis of the aortic root demonstrated reduced plaque area in lipin-1^{mEnzyKO} mice compared to the corresponding lipin-1^{flox/flox} mice (Figure 1F). Immunofluorescent analysis of the aortic root showed no significant difference in plaque composition (Supplemental Figure IIE&F), suggesting that monocyte recruitment is similar between lipin-1^{mEnzyKO} and lipin-1^{flox/flox} mice.

Our data suggests lipin-1 enzymatic activity is responsible for the decreased severity of atherosclerosis seen in the lipin-1^{mEnzyKO} mice. However, as truncated proteins are known to undergo enhanced degradation *in vivo*, it is possible that the loss of lipin-1 transcriptional coregulatory activity contributes to changes in atherosclerosis severity in our mouse model. However, this is unlikely, as this lipin-1^{flox/flox} mouse has been used in other studies to investigate lipin-1 transcriptional coregulatory activity in hepatocytes.²⁵ Furthermore, lipin-1 has been demonstrated to enhance the activity of transcription factors such as PPAR γ , PPAR α , and PGC-1 α , which are known to induce an atheroprotective response.^{26–30} The known interactions of lipin-1 transcriptional coregulatory activity suggest the degradation of the truncated lipin-1 protein in our mice would enhance atherosclerosis severity, which is in contrast to our data. We also evaluated PPAR γ -regulated gene expression by qRT-PCR to determine if lipin-1 transcriptional co-regulator activity was altered in our lipin-1^{mEnzyKO} BMDMs and observed no differences between lipin-1^{mEnzyKO} and lipin-1^{flox/flox} BMDMs after oxLDL stimulation (Supplemental Figure IB). These data suggest lipin-1 transcriptional coregulatory activity is either not active or is unaltered in lipin-1^{mEnzyKO} BMDMs in response to oxLDL.

These data demonstrate that lipin-1^{mEnzyKO} mice have less severe atherosclerosis, even when plasma cholesterol levels are similar to control mice, suggesting that myeloid-associated lipin-1 enzymatic activity positively contributes to the development of atherosclerosis *in vivo*.

ModLDL stimulation of BMDMs results in activation of a PKC α / β II-ERK1/2-cJun signaling cascade

Having demonstrated that myeloid-associated lipin-1 enzymatic activity is pro-atherogenic *in vivo* (Figure 1), we sought to determine the molecular mechanism by which macrophage-associated lipin-1 enzymatic activity may contribute to atherosclerosis. Lipin-1 mediates oxLDL- and LPS-induced DAG production and LPS-induced MAPK and AP-1 activity in macrophages.^{9, 10} ModLDL stimulation of macrophages induces the activation of DAG responsive proteins (PKC α / β II and PKC δ), MAPKs (JNK1/2, ERK1/2 and p38), and AP-1.³¹ Furthermore, the engulfment, breakdown, and storage of cholesterol and lipids from modLDLs is an ongoing process in the macrophage as long as modLDLs are present. These observations led us to hypothesize that lipin-1 enzymatic activity is responsible for persistent activation of a PKC-MAPK-AP-1 signal transduction pathway in macrophages in response to modLDL-stimulation. We first set out to determine if there is persistent activation of a PKC-MAPK-AP-1 signal transduction pathway in oxLDL-stimulated macrophages.

The phosphorylation status of PKCs, MAPKs, and the AP-1 subunits were monitored via Western blot analysis as a correlate of protein activity at 4, 24, and 48 hours after oxLDL-stimulation. Phosphorylation of PKC α / β II, PKC δ , PKC θ , and PKC μ were evaluated in wildtype (WT) BMDMs stimulated with oxLDL. We failed to detect total or phosphorylated PKC θ and PKC μ in WT BMDMs. Although phosphorylation of PKC δ was detected, the level of phosphorylation remained unchanged over the 48 hour period (Figure 2A). PKC α / β II phosphorylation was elevated at 4, 24, and 48 hours after oxLDL stimulation (Figure 2A&B). Analysis of MAPK phosphorylation demonstrated increased phosphorylation of ERK1/2 at 4, 24, and 48 hours after oxLDL stimulation (Figure 2C&D). Sustained phosphorylation of JNK1/2 and p38 was not observed at these time points (Figure 2C). Finally, phosphorylation of the AP-1 subunits, cJun and cFos, was evaluated. The phosphorylation of both cJun and cFos was elevated after 4 hours of oxLDL-stimulation (Figure 2E). However, only cJun remained phosphorylated at 24 and 48 hours after treatment, whereas cFos returned to basal levels (Figure 2E&F). The phosphorylation of cJun initiates its translocation to the nucleus where it can either bind to cFos or homodimerize to form AP-1.^{32, 33} To determine if cJun was localizing to the nucleus of oxLDL-stimulated BMDMs, we performed immunocytochemistry and observed nuclear cJun in WT BMDMs at 4, 24, and 48 hours after oxLDL stimulation (Figure 2G&H). To determine if these three proteins were linked in a signaling cascade, we used the small molecule inhibitors Gö6376 (PKC α / β II) and U0126 (inhibits MEK1/2 directly upstream of ERK1/2) to determine if PKC α / β II and ERK1/2 were upstream of cJun. Due to the fact that PKCs and MAPKs directly contribute to oxLDL internalization by macrophages, inhibitors were added 24 hours after oxLDL treatment as to not confound the data interpretation.³⁴ Both small molecules inhibited the phosphorylation of cJun (Supplemental Figure IIIA&B), suggesting that we identified a linked PKC α / β II-ERK1/2-cJun signaling pathway. To ensure the signaling responses observed in oxLDL-stimulated macrophages were not due to LPS contamination, WT macrophages were stimulated with 1.2 pg/mL LPS (maximum calculated endotoxin present in the modLDL preparations), 60 pg/mL (50 times the maximum endotoxin present in the modLDL preparations), or 10 ng/mL of LPS (amount of LPS used in Figure 5). Persistent phosphorylation of the PKC α / β II-ERK1/2-cJun pathway was not observed in any of the LPS treatments (Supplemental Figure IV).

To ensure that persistent activation of the PKC α / β II-ERK1/2-cJun pathway was not due to a single type of LDL modification, WT macrophages were stimulated with 25 μ g/mL acLDL or 25 μ g/mL oxLDL. Stimulation with either oxLDL or acLDL resulted in sustained phosphorylation of PKC α / β II, ERK1/2, and cJun at 24 and 48 hours (Figure 3A&B). Together these results suggest that modLDL stimulation of macrophages induces persistent activation of a PKC α / β II-ERK1/2-cJun signaling axis.

OxLDL stimulation of BMDMs activates the lipin-1-dependent signaling cascade PKC α / β II-ERK1/2-cJun

To determine if the PKC α / β II-ERK1/2-cJun pathway is lipin-1 dependent, the phosphorylation status of PKC α / β II, ERK1/2, and cJun was evaluated in BMDMs generated from lipin-1^{mEnzyKO} and lipin-1^{fllox/fllox} mice. BMDMs were stimulated with 25 μ g/mL oxLDL and protein was collected at 4, 24, and 48 hours for Western blot analysis.

Lipin-1^{mEnzyKO} BMDMs had reduced phosphorylation of PKC α / β II, ERK1/2, and cJun at the 24 and 48 hour time points compared to the lipin-1^{flox/flox} BMDMs (Figure 4A&B), suggesting the persistent phosphorylation events of these proteins are dependent upon lipin-1 enzymatic activity. These data demonstrate PKC α / β II-ERK1/2-cJun is a lipin-1-dependent signaling cascade activated in response to oxLDL-stimulation.

Lipin-1 contributes to oxLDL-elicited TNF- α production in BMDMs

We previously demonstrated oxLDL elicits TNF- α production by RAW264.7 macrophages in a lipin-1-dependent manner.¹⁰ To determine if lipin-1 enzymatic activity regulates TNF- α production in BMDMs in response to oxLDL, lipin-1^{mEnzyKO} and lipin-1^{flox/flox} BMDMs were stimulated with 25 μ g/mL of oxLDL and TNF- α production in supernatants was analyzed by ELISA 48 hours after stimulation. This late time point (48 hours) was chosen as it corresponds to the time of maximal TNF- α production by oxLDL-stimulated RAW264.7 macrophages.¹⁰ However, stimulation of BMDMs with oxLDL alone failed to elicit TNF- α from either the lipin-1^{mEnzyKO} or lipin-1^{flox/flox} BMDMs (Figure 5A). Based upon previous findings in RAW264.7 macrophages, we reasoned the lack of TNF- α production by BMDMs might be a result of very low basal NF- κ B activity.¹⁰ In contrast to untreated RAW264.7 macrophages, there was no NF- κ B (p65) phosphorylation in untreated WT BMDMs as detected by Western blot analysis (Figure 5D). Additionally, immunocytochemistry confirmed that NF- κ B (p65) was not present in the nucleus of untreated or oxLDL-stimulated WT BMDMs (Figure 5E).

The presence of pre-existing NF- κ B activity in the RAW264.7 macrophage cell line is noteworthy because oxLDL alone does not induce NF- κ B activity in BMDMs, which is required for TNF- α production.³⁵ Additionally, AP-1 transcriptional activity is not sufficient for TNF- α production, but can enhance the activity of other transcription factors in macrophages, including NF- κ B, to augment TNF- α production.³⁶ Thus, we hypothesized that lipin-1-mediated activation of cJun (AP-1) triggered by oxLDL treatment may prime macrophages to be more responsive to a subsequent NF- κ B activating stimulus, such as LPS. To examine the effect of an NF- κ B stimulus on oxLDL-stimulated macrophages, we treated WT (lipin-1^{flox/flox}) BMDMs with either (1) 25 μ g/mL oxLDL, (2) 10 ng/mL LPS, or (3) 25 μ g/mL oxLDL for 24 hours, then 10 ng/mL LPS for an additional 24 hours. Supernatants were collected 48 hours after oxLDL addition and evaluated by ELISA for TNF- α production. LPS stimulation resulted in TNF- α production in WT BMDMs and stimulation of WT BMDMs with oxLDL and LPS resulted in augmented TNF- α production (Figure 5A).

We next wanted to determine if the identified lipin-1-dependent PKC α / β II-ERK1/2-cJun signaling axis was responsible for augmented TNF- α production in BMDMs stimulated with both oxLDL and LPS. Lipin-1^{mEnzyKO} and lipin-1^{flox/flox} BMDMs were treated with either (1) 25 μ g/mL oxLDL, (2) 10 ng/mL LPS or (3) 25 μ g/mL oxLDL for 24 hours followed by addition of 10 ng/mL LPS for an additional 24 hours. LPS alone induced TNF- α production in both the lipin-1^{mEnzyKO} and lipin-1^{flox/flox} BMDMs (Figure 5A). However, pre-loading lipin-1^{flox/flox} BMDMs with oxLDL augmented LPS-elicited TNF- α production, which was not observed in the lipin-1^{mEnzyKO} BMDMs (Figure 5A). Next, WT BMDMs were

stimulated with either (1) oxLDL, (2) LPS, or (3) oxLDL and LPS with inhibitors of PKC α / β II (Gö6376) and ERK1/2 (U0126). Inhibitors were added at 24 hours along with LPS stimulation. Inhibition of PKC α / β II and ERK1/2 ablated oxLDL-augmented LPS-induced TNF- α production (Figure 5B). Finally, to determine if the augmented TNF- α production by lipin-1^{flox/flox} BMDMs stimulated with (3) oxLDL and LPS was due to elevated cJun activity, cJun was depleted using siRNA and TNF- α production was examined following treatment. WT BMDMs were transfected with either non-target siRNA or cJun-targeted siRNA (Figure 5C) and left untreated or stimulated with 25 μ g/mL oxLDL for 24 hours prior to 10 ng/mL LPS treatment for an additional 24 hours. Depletion of cJun prevented the augmentation of LPS-elicited TNF- α production, similar to what was seen in the lipin-1^{mEnzyKO} BMDMs in Figure 5A (Figure 5C).

Inhibition of the macrophages ability to produce TNF- α *in vivo* results in reduced serum TNF- α levels in atherosclerotic mice.³⁷ Therefore, we wanted to determine if there was a reduction in serum TNF- α in the lipin-1^{mEnzyKO} mice compared to lipin-1^{flox/flox} mice. Serum from lipin-1^{mEnzyKO} and lipin-1^{flox/flox} mice was collected after 8 weeks on high fat diet and analyzed by ELISA for TNF- α production. The lipin-1^{mEnzyKO} mice had a significant reduction in serum TNF- α compared to lipin-1^{flox/flox} mice (Figure 5F). Although this data is not definitive, this begins to provide evidence that lipin-1 enzymatic activity is contributing to the pro-inflammatory responses *in vivo* that promote atherosclerosis. These data suggest that oxLDL induces activation of cJun (AP-1) via lipin-1 enzymatic activity, which effectively primes the macrophage for enhanced responses to a pro-inflammatory stimulus above what would be exhibited by the pro-inflammatory stimulus alone.

DISCUSSION

Using the lipin-1^{mEnzyKO} mouse model with the viral model of atherosclerosis, we demonstrated the loss of myeloid-associated lipin-1 enzymatic activity results in reduced atherosclerosis in mice fed high fat diet for 8 and 12 weeks. Furthermore, we demonstrated that macrophage-associated lipin-1 enzymatic activity contributes to foam cell formation and regulates a DAG-dependent signaling cascade that results in persistent AP-1 activation in response to modLDL. Persistent activation of AP-1 primes the macrophage to be hyper-responsive to LPS-induced TNF- α production. Although the glycerolipid synthetic pathway is likely not the only lipid synthesis pathway contributing to foam cell formation, it is a critical pathway that influences modLDL-elicited pro-inflammatory responses. Taken together, our data highlights the dynamic interaction between modLDL-elicited lipid synthetic events and their contribution to macrophage pro-inflammatory mediator production that promotes atherosclerosis.

The combination of our *in vitro* and *in vivo* data suggest that the reduced plaque development observed in the lipin-1^{mEnzyKO} mice is due primarily to monocytes and macrophages. However, *LysM*-Cre was used to knockout lipin-1 enzymatic activity in these mice and *LysM* expression is not restricted to monocytes and macrophages. Analysis of *LysM*-Cre-mediated gene deletion demonstrates gene excision in dendritic cells and granulocytes, as well as monocytes and macrophages.^{38, 39} The contribution of dendritic

cells to atherosclerosis is unclear because tools to specifically deplete this population long term are currently lacking. However, the few studies using CD11cDTR mice, in which dendritic cells are transiently depleted, demonstrate no alteration in plaque size or composition.⁴⁰ The contribution of lipin-1 to dendritic cell function is completely unknown, and will need to be looked at in the future. Neutrophils clearly contribute to atherosclerosis progression as they can be identified in both early and advanced murine and human plaques, and antibody depletion of neutrophils has been shown to contribute to plaque burden in mice.^{41–43} However, lipin-1 is not readily detected in neutrophils, suggesting lipin-1 has little contribution to neutrophil function.⁹ Thus, we propose that the loss of lipin-1 enzymatic activity in monocytes and macrophages is most likely responsible for the reduction in atherosclerosis observed in the lipin-1^{mEnzy}KO mice.

Our studies identified a novel mechanism through which lipin-1, a regulatory enzyme in the glycerolipid synthesis pathway, initiates a DAG-mediated PKC α / β II-ERK1/2-cJun signaling cascade that contributes to macrophage pro-inflammatory responses to modLDL. Glycerolipid synthesis is a universal pathway required for the storage of incoming lipids. We demonstrate that stimulation of macrophages with both oxLDL and acLDL results in sustained phosphorylation of the PKC α / β II-ERK1/2-cJun pathway up to 48 hours. The initiation of glycerolipid synthesis by any modLDL would then be capable of activating the DAG-dependent PKC α / β II-ERK1/2-cJun signaling pathway leading to persistent macrophage pro-inflammatory responses. Acute macrophage pro-inflammatory responses have been investigated as a result of modLDLs being recognized by toll-like receptors or the inflammasome.^{3–6} However, our data highlights another molecular mechanism by which modLDLs can elicit signaling that contribute to macrophage pro-inflammatory responses that may be independent of receptor-mediated recognition of modLDLs. Our data investigating PKC α / β II supports this proposed alternative mechanism, as PKC β is required for modLDL uptake *in vitro*.⁴⁴ However, the lipin-1^{mEnzy}KO macrophages do not have an oxLDL uptake deficiency even with a loss of PKC β phosphorylation, suggesting that late activity of PKC α / β II (24 and 48 hours after oxLDL stimulation) is dependent on lipin-1, while early activation (up to 4 hours) is not. This supports the idea that there are multiple signals that contribute to modLDL-induced pro-inflammatory responses in a temporal manner. Our studies identified ERK1/2 and cJun as downstream proteins of PKC α / β II. Although JNK1/2 is the classical kinase associated with phosphorylation of cJun, ERK1/2 can also phosphorylate cJun specifically at Ser63 and Ser72.^{45, 46} The ability of ERK1/2 to phosphorylate cJun may also be temporally regulated. Studies using total knockout of JNK1/2 in mice show prevention of the early steps of atherosclerosis and thus reduction in plaque burden.⁴⁷ However, inhibition of JNK1/2 during late stage atherosclerosis in humans showed minimal effects on levels of pro-inflammatory mediators in serum.⁴⁸ We hypothesize that targeting JNK1/2 in the human study was ineffective because ERK1/2 may be responsible for chronic activation of cJun in macrophages during later stages of atherosclerosis. Overall, our data supports the idea that there is a temporal difference in signaling responses that is determined by macrophage interactions with modLDLs. Early signaling seems to be dependent on receptor interactions and later signaling events are dependent on prolonged lipid synthetic events.

The persistent activation of the PKC α / β II-ERK1/2-cJun signaling axis primes macrophages to be hyper-responsive to secondary pro-inflammatory stimuli, such as LPS. Many of the comorbidities that contribute to atherosclerosis progression are associated with increased endotoxin levels in the plasma, called endotoxemia. As expected, chronic bacterial infections, such as periodontitis and chlamydial infections, increase plasma endotoxin levels and non-infectious comorbidities such as obesity, consumption of a high-fat diet (both in mice and humans), type 2 diabetes mellitus, and alcoholism also result in increased serum endotoxin levels.^{49–55} Enhanced macrophage pro-inflammatory responses induced via cooperation of AP-1 and NF- κ B have previously been demonstrated to enhance vascular inflammation when compared to NF- κ B (via LPS stimulation) alone.³⁶ Our data provide a molecular mechanism by which hypercholesterolemia and endotoxemia promote pro-inflammatory responses that mediate atherosclerosis. We have provided evidence that oxLDL elicits persistent DAG generation that leads to AP-1 activation via a PKC α / β II-ERK1/2-cJun signaling axis in macrophages, and interruption of this pathway reduces enhanced pro-inflammatory responses (TNF- α) (Figure 5). *In vivo*, the loss of lipin-1 from myeloid-derived cells reduced circulating TNF- α levels in response to high-fat diet. Serum TNF- α levels often correspond to degree of plaque inflammation and growth.⁵⁶ This supports the hypothesis that macrophage-associated lipin-1 contributes to modLDL-elicited pro-inflammatory responses. Combining our *in vitro* observations of the contribution of lipin-1 to oxLDL-elicited pro-inflammatory responses with our *in vivo* data demonstrating that myeloid-associated lipin-1 contributes to atherosclerotic progression, suggests that lipid synthetic events required to store incoming lipids from modLDLs also mediate pro-inflammatory responses in macrophages that contribute to atheroma progression. We suggest hypercholesterolemia *in vivo* results in prolonged enhancement of glycerolipid synthesis within macrophages, priming the macrophage with active AP-1, resulting in macrophage foam cells that are hyper-responsive to additional pro-inflammatory stimuli (such as LPS).

A better understanding of the fundamental mechanisms of intrinsic plaque inflammation may provide additional therapeutic targets to reduce atherosclerosis better than statin therapy alone. Interestingly, the statins most effective at reducing cardiovascular disease risk are those that also exhibit anti-inflammatory properties.⁵⁷ We currently lack mechanisms to target plaque intrinsic inflammation without causing global immune-suppression. Macrophage foam cell formation is largely unique to atherosclerosis, and our results suggest that modLDL-induced foam cell formation and modLDL-induced macrophage pro-inflammatory responses are linked via enhanced lipid synthesis. Thus, understanding of modLDL-elicited lipid synthetic events that contribute to macrophage pro-inflammatory responses may lead to an ideal therapeutic target to reduce plaque intrinsic inflammation without causing global immune-suppression to reduce cardiovascular disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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ABBREVIATIONS

AAV8	adeno-associated viral vector 8
ABCA1	ATP binding cassette subfamily A member 1
ABCG1	ATP binding cassette subfamily G member 1
AcLDL	acetylated low-density lipoprotein
AP-1	activator protein-1
BMDM	bone marrow-derived macrophage
CCL2	C-C motif chemokine ligand 2
cFos	fos proto-oncogene, AP-1 transcription factor subunit
cJun	jun proto-oncogene, AP-1 transcription factor subunit
DAG	diacylglycerol
DGAT	diglyceride acyltransferase
ER	endoplasmic reticulum
ERK	extracellular receptor kinase
G3P	glycerol-3-phosphate
IL-1	interleukin 1
IL-6	interleukin 6
IL-10	interleukin 10
LXRα	liver x receptor alpha
JNK	c-Jun NH ₂ -terminal kinase
HDL	high-density lipoprotein
LC-MS/MS	liquid chromatography tandem mass spectrometry
LDL	low-density lipoprotein
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells

oxLDL	oxidized low-density lipoprotein
PA	phosphatidic acid
PAP	phosphatidic acid phosphohydrolase
PC	phosphatidylcholine
PGC-1α	PPAR-gamma coactivator-1 alpha
PCSK9	proprotein convertase subtilisin/kexin type 9
PGE₂	prostaglandin E2
PKC	protein kinase C
PPARα	peroxisome proliferator-activated receptor alpha
PPAR	peroxisome proliferator-activated receptor gamma
p38	protein 38
TAG	triacylglycerol
TC	total cholesterol
TG	triglyceride
TLR4	toll-like receptor 4
TNF-α	tumor necrosis factor alpha
WT	wildtype

References

1. Mori M, Itabe H, Higashi Y, Fujimoto Y, Shiomi M, Yoshizumi M, Ouchi Y, Takano T. Foam cell formation containing lipid droplets enriched with free cholesterol by hyperlipidemic serum. *J Lipid Res.* 2001; 42:1771–1781. [PubMed: 11714846]
2. Kleemann R, Zadelaar S, Kooistra T. Cytokines and atherosclerosis: A comprehensive review of studies in mice. *Cardiovasc Res.* 2008; 79:360–376. [PubMed: 18487233]
3. Mullick AE, Tobias PS, Curtiss LK. Modulation of atherosclerosis in mice by toll-like receptor 2. *J Clin Invest.* 2005; 115:3149–3156. [PubMed: 16211093]
4. Michelsen KS, Wong MH, Shah PK, Zhang W, Yano J, Doherty TM, Akira S, Rajavashisth TB, Arditi M. Lack of toll-like receptor 4 or myeloid differentiation factor 88 reduces atherosclerosis and alters plaque phenotype in mice deficient in apolipoprotein e. *Proc Natl Acad Sci U S A.* 2004; 101:10679–10684. [PubMed: 15249654]
5. Stewart CR, Stuart LM, Wilkinson K, van Gils JM, Deng J, Halle A, Rayner KJ, Boyer L, Zhong R, Frazier WA, Lacy-Hulbert A, El Khoury J, Golenbock DT, Moore KJ. Cd36 ligands promote sterile inflammation through assembly of a toll-like receptor 4 and 6 heterodimer. *Nat Immunol.* 2010; 11:155–161. [PubMed: 20037584]
6. Duewell P, Kono H, Rayner KJ, Sirois CM, Vladimer G, Bauernfeind FG, Abela GS, Franchi L, Nunez G, Schnurr M, Espevik T, Lien E, Fitzgerald KA, Rock KL, Moore KJ, Wright SD, Hornung V, Latz E. Nlrp3 inflammasomes are required for atherogenesis and activated by cholesterol crystals. *Nature.* 2010; 464:1357–1361. [PubMed: 20428172]

7. Curtiss LK, Black AS, Bonnet DJ, Tobias PS. Atherosclerosis induced by endogenous and exogenous toll-like receptor (tlr)1 or tlr6 agonists. *J Lipid Res.* 2012; 53:2126–2132. [PubMed: 22822027]
8. Menu P, Pellegrin M, Aubert JF, Bouzourene K, Tardivel A, Mazzolai L, Tschopp J. Atherosclerosis in apoe-deficient mice progresses independently of the nlrp3 inflammasome. *Cell Death Dis.* 2011; 2:e137. [PubMed: 21451572]
9. Meana C, Pena L, Lorden G, Esquinas E, Guijas C, Valdearcos M, Balsinde J, Balboa MA. Lipin-1 integrates lipid synthesis with proinflammatory responses during tlr activation in macrophages. *J Immunol.* 2014; 193:4614–4622. [PubMed: 25252959]
10. Navratil AR, Vozenilek AE, Cardelli JA, Green JM, Thomas MJ, Sorci-Thomas MG, Orr AW, Woolard MD. Lipin-1 contributes to modified low-density lipoprotein-elicited macrophage pro-inflammatory responses. *Atherosclerosis.* 2015; 242:424–432. [PubMed: 26288136]
11. Namatame I, Tomoda H, Arai H, Inoue K, Omura S. Complete inhibition of mouse macrophage-derived foam cell formation by triacsin c. *J Biochem.* 1999; 125:319–327. [PubMed: 9990129]
12. McGookey DJ, Anderson RG. Morphological characterization of the cholesteryl ester cycle in cultured mouse macrophage foam cells. *J Cell Biol.* 1983; 97:1156–1168. [PubMed: 6684660]
13. Bozza PT, Melo RC, Bandeira-Melo C. Leukocyte lipid bodies regulation and function: Contribution to allergy and host defense. *Pharmacol Ther.* 2007; 113:30–49. [PubMed: 16945418]
14. Reue K, Brindley DN. Thematic review series: Glycerolipids. Multiple roles for lipins/ phosphatidate phosphatase enzymes in lipid metabolism. *J Lipid Res.* 2008; 49:2493–2503. [PubMed: 18791037]
15. Gibellini F, Smith TK. The Kennedy pathway--de novo synthesis of phosphatidylethanolamine and phosphatidylcholine. *IUBMB Life.* 2010; 62:414–428. [PubMed: 20503434]
16. Harris CA, Haas JT, Streeper RS, Stone SJ, Kumari M, Yang K, Han X, Brownell N, Gross RW, Zechner R, Farese RV Jr. Dgat enzymes are required for triacylglycerol synthesis and lipid droplets in adipocytes. *J Lipid Res.* 2011; 52:657–667. [PubMed: 21317108]
17. Huang YL, Morales-Rosado J, Ray J, Myers TG, Kho T, Lu M, Munford RS. Toll-like receptor agonists promote prolonged triglyceride storage in macrophages. *J Biol Chem.* 2014; 289:3001–3012. [PubMed: 24337578]
18. Fagone P, Sriburi R, Ward-Chapman C, Frank M, Wang J, Gunter C, Brewer JW, Jackowski S. Phospholipid biosynthesis program underlying membrane expansion during B-lymphocyte differentiation. *J Biol Chem.* 2007; 282:7591–7605. [PubMed: 17213195]
19. Ruiz M, Jove M, Schluter A, Casanovas C, Villarroya F, Guilera C, Ortega FJ, Naudi A, Pamplona R, Gimeno R, Fourcade S, Portero-Otin M, Pujol A. Altered glycolipid and glycerophospholipid signaling drive inflammatory cascades in adrenomyeloneuropathy. *Hum Mol Genet.* 2015; 24:6861–6876. [PubMed: 26370417]
20. Reue K, Zhang P. The lipin protein family: Dual roles in lipid biosynthesis and gene expression. *FEBS Lett.* 2008; 582:90–96. [PubMed: 18023282]
21. Han GS, Carman GM. Characterization of the human lipin1-encoded phosphatidate phosphatase isoforms. *J Biol Chem.* 2010; 285:14628–14638. [PubMed: 20231281]
22. Bjorklund MM, Hollensen AK, Hagensen MK, Dagnaes-Hansen F, Christoffersen C, Mikkelsen JG, Bentzon JF. Induction of atherosclerosis in mice and hamsters without germline genetic engineering. *Circ Res.* 2014; 114:1684–1689. [PubMed: 24677271]
23. Mitra MS, Chen Z, Ren H, Harris TE, Chambers KT, Hall AM, Nadra K, Klein S, Chrast R, Su X, Morris AJ, Finck BN. Mice with an adipocyte-specific lipin 1 separation-of-function allele reveal unexpected roles for phosphatidic acid in metabolic regulation. *Proc Natl Acad Sci U S A.* 2013; 110:642–647. [PubMed: 23267081]
24. Kent C. Eukaryotic phospholipid biosynthesis. *Annu Rev Biochem.* 1995; 64:315–343. [PubMed: 7574485]
25. Schweitzer GG, Chen Z, Gan C, McCommis KS, Soufi N, Chrast R, Mitra MS, Yang K, Gross RW, Finck BN. Liver-specific loss of lipin-1-mediated phosphatidic acid phosphatase activity does not mitigate intrahepatic tg accumulation in mice. *J Lipid Res.* 2015; 56:848–858. [PubMed: 25722343]

26. McCarthy C, Lieggi NT, Barry D, Mooney D, de Gaetano M, James WG, McClelland S, Barry MC, Escoubet-Lozach L, Li AC, Glass CK, Fitzgerald DJ, Belton O. Macrophage ppar gamma co-activator-1 alpha participates in repressing foam cell formation and atherosclerosis in response to conjugated linoleic acid. *EMBO Mol Med*. 2013; 5:1443–1457. [PubMed: 23964012]
27. Kim HE, Bae E, Jeong DY, Kim MJ, Jin WJ, Park SW, Han GS, Carman GM, Koh E, Kim KS. Lipin1 regulates ppargamma transcriptional activity. *Biochem J*. 2013; 453:49–60. [PubMed: 23627357]
28. Finck BN, Gropler MC, Chen Z, Leone TC, Croce MA, Harris TE, Lawrence JC Jr, Kelly DP. Lipin 1 is an inducible amplifier of the hepatic pgc-1alpha/pparalpha regulatory pathway. *Cell Metab*. 2006; 4:199–210. [PubMed: 16950137]
29. Ricote M, Huang J, Fajas L, Li A, Welch J, Najib J, Witztum JL, Auwerx J, Palinski W, Glass CK. Expression of the peroxisome proliferator-activated receptor gamma (ppargamma) in human atherosclerosis and regulation in macrophages by colony stimulating factors and oxidized low density lipoprotein. *Proc Natl Acad Sci U S A*. 1998; 95:7614–7619. [PubMed: 9636198]
30. Soskic SS, Dobutovic BD, Sudar EM, Obradovic MM, Nikolic DM, Zaric BL, Stojanovic SD, Stokic EJ, Mikhailidis DP, Isenovic ER. Peroxisome proliferator-activated receptors and atherosclerosis. *Angiology*. 2011; 62:523–534. [PubMed: 21467121]
31. Lin CS, Lin FY, Ho LJ, Tsai CS, Cheng SM, Wu WL, Huang CY, Lian CH, Yang SP, Lai JH. Pkcdelta signalling regulates sr-a and cd36 expression and foam cell formation. *Cardiovasc Res*. 2012; 95:346–355. [PubMed: 22687273]
32. Grondin B, Lefrancois M, Tremblay M, Saint-Denis M, Haman A, Waga K, Bedard A, Tenen DG, Hoang T. C-jun homodimers can function as a context-specific coactivator. *Mol Cell Biol*. 2007; 27:2919–2933. [PubMed: 17283046]
33. Halazonetis TD, Georgopoulos K, Greenberg ME, Leder P. C-jun dimerizes with itself and with c-fos, forming complexes of different dna binding affinities. *Cell*. 1988; 55:917–924. [PubMed: 3142692]
34. Feng J, Han J, Pearce SF, Silverstein RL, Gotto AM Jr, Hajjar DP, Nicholson AC. Induction of cd36 expression by oxidized ldl and il-4 by a common signaling pathway dependent on protein kinase c and ppar-gamma. *J Lipid Res*. 2000; 41:688–696. [PubMed: 10787429]
35. Ohlsson BG, Englund MC, Karlsson AL, Knutsen E, Erixon C, Skribeck H, Liu Y, Bondjers G, Wiklund O. Oxidized low density lipoprotein inhibits lipopolysaccharide-induced binding of nuclear factor-kappa b to dna and the subsequent expression of tumor necrosis factor-alpha and interleukin-1beta in macrophages. *J Clin Invest*. 1996; 98:78–89. [PubMed: 8690807]
36. Wiesner P, Choi SH, Almazan F, Benner C, Huang W, Diehl CJ, Gonen A, Butler S, Witztum JL, Glass CK, Miller YI. Low doses of lipopolysaccharide and minimally oxidized low-density lipoprotein cooperatively activate macrophages via nuclear factor kappa b and activator protein-1: Possible mechanism for acceleration of atherosclerosis by subclinical endotoxemia. *Circ Res*. 2010; 107:56–65. [PubMed: 20489162]
37. Yu M, Zhou H, Zhao J, Xiao N, Roychowdhury S, Schmitt D, Hu B, Ransohoff RM, Harding CV, Hise AG, Hazen SL, DeFranco AL, Fox PL, Morton RE, Dicorleto PE, Febbraio M, Nagy LE, Smith JD, Wang J, Li X. Myd88-dependent interplay between myeloid and endothelial cells in the initiation and progression of obesity-associated inflammatory diseases. *J Exp Med*. 2014; 211:887–907. [PubMed: 24752299]
38. Clausen BE, Burkhardt C, Reith W, Renkawitz R, Forster I. Conditional gene targeting in macrophages and granulocytes using lysmcrc mice. *Transgenic Res*. 1999; 8:265–277. [PubMed: 10621974]
39. Abram CL, Roberge GL, Hu Y, Lowell CA. Comparative analysis of the efficiency and specificity of myeloid-cre deleting strains using rosa-eyfp reporter mice. *J Immunol Methods*. 2014; 408:89–100. [PubMed: 24857755]
40. Rombouts M, Cools N, Grootaert MO, de Bakker F, Van Brussel I, Wouters A, De Meyer GR, De Winter BY, Schrijvers DM. Long-term depletion of conventional dendritic cells cannot be maintained in an atherosclerotic zbtb46-dtr mouse model. *PLoS One*. 2017; 12:e0169608. [PubMed: 28060909]

41. Drechsler M, Megens RT, van Zandvoort M, Weber C, Soehnlein O. Hyperlipidemia-triggered neutrophilia promotes early atherosclerosis. *Circulation*. 2010; 122:1837–1845. [PubMed: 20956207]
42. Rotzius P, Thams S, Soehnlein O, Kenne E, Tseng CN, Bjorkstrom NK, Malmberg KJ, Lindbom L, Eriksson EE. Distinct infiltration of neutrophils in lesion shoulders in apoe^{-/-} mice. *Am J Pathol*. 2010; 177:493–500. [PubMed: 20472897]
43. Ionita MG, van den Borne P, Catanzariti LM, Moll FL, de Vries JP, Pasterkamp G, Vink A, de Kleijn DP. High neutrophil numbers in human carotid atherosclerotic plaques are associated with characteristics of rupture-prone lesions. *Arterioscler Thromb Vasc Biol*. 2010; 30:1842–1848. [PubMed: 20595650]
44. Harja E, Chang JS, Lu Y, Leitges M, Zou YS, Schmidt AM, Yan SF. Mice deficient in pckbeta and apolipoprotein e display decreased atherosclerosis. *Faseb j*. 2009; 23:1081–1091. [PubMed: 19036858]
45. Pulverer BJ, Kyriakis JM, Avruch J, Nikolakaki E, Woodgett JR. Phosphorylation of c-jun mediated by map kinases. *Nature*. 1991; 353:670–674. [PubMed: 1922387]
46. Morton S, Davis RJ, McLaren A, Cohen P. A reinvestigation of the multisite phosphorylation of the transcription factor c-jun. *Embo j*. 2003; 22:3876–3886. [PubMed: 12881422]
47. Osto E, Matter CM, Kouroedov A, Malinski T, Bachschmid M, Camici GG, Kilic U, Stallmach T, Boren J, Iliceto S, Luscher TF, Cosentino F. C-jun n-terminal kinase 2 deficiency protects against hypercholesterolemia-induced endothelial dysfunction and oxidative stress. *Circulation*. 2008; 118:2073–2080. [PubMed: 18955669]
48. Meijer CA, Le Haen PA, van Dijk RA, Hira M, Hamming JF, van Bockel JH, Lindeman JH. Activator protein-1 (ap-1) signalling in human atherosclerosis: Results of a systematic evaluation and intervention study. *Clin Sci (Lond)*. 2012; 122:421–428. [PubMed: 22092038]
49. Lehr HA, Sagban TA, Ihling C, Zahringer U, Hungerer KD, Blumrich M, Reifenberg K, Bhakdi S. Immunopathogenesis of atherosclerosis: Endotoxin accelerates atherosclerosis in rabbits on hypercholesterolemic diet. *Circulation*. 2001; 104:914–920. [PubMed: 11514379]
50. Kalayoglu MV, Libby P, Byrne GI. Chlamydia pneumoniae as an emerging risk factor in cardiovascular disease. *Jama*. 2002; 288:2724–2731. [PubMed: 12460096]
51. Pussinen PJ, Tuomisto K, Jousilahti P, Havulinna AS, Sundvall J, Salomaa V. Endotoxemia, immune response to periodontal pathogens, and systemic inflammation associate with incident cardiovascular disease events. *Arterioscler Thromb Vasc Biol*. 2007; 27:1433–1439. [PubMed: 17363692]
52. Yoshimatsu M, Terasaki Y, Sakashita N, Kiyota E, Sato H, van der Laan LJ, Takeya M. Induction of macrophage scavenger receptor marco in nonalcoholic steatohepatitis indicates possible involvement of endotoxin in its pathogenic process. *Int J Exp Pathol*. 2004; 85:335–343. [PubMed: 15566430]
53. Pendyala S, Walker JM, Holt PR. A high-fat diet is associated with endotoxemia that originates from the gut. *Gastroenterology*. 2012; 142:1100–1101. e1102. [PubMed: 22326433]
54. Jayashree B, Bibin YS, Prabhu D, Shanthirani CS, Gokulakrishnan K, Lakshmi BS, Mohan V, Balasubramanyam M. Increased circulatory levels of lipopolysaccharide (lps) and zonulin signify novel biomarkers of proinflammation in patients with type 2 diabetes. *Mol Cell Biochem*. 2014; 388:203–210. [PubMed: 24347174]
55. Rao R. Endotoxemia and gut barrier dysfunction in alcoholic liver disease. *Hepatology*. 2009; 50:638–644. [PubMed: 19575462]
56. Puz P, Lasek-Bal A, Ziaja D, Kazibutowska Z, Ziaja K. Inflammatory markers in patients with internal carotid artery stenosis. *Arch Med Sci*. 2013; 9:254–260. [PubMed: 23671435]
57. Antonopoulos AS, Margaritis M, Lee R, Channon K, Antoniadou C. Statins as anti-inflammatory agents in atherogenesis: Molecular mechanisms and lessons from the recent clinical trials. *Curr Pharm Des*. 2012; 18:1519–1530. [PubMed: 22364136]

HIGHLIGHTS

- Macrophage-associated lipin-1 is pro-atherogenic.
- ModLDLs elicit activation of a lipin-1-dependent PKC α / β II-ERK1/2-cJun signaling cascade in macrophages.
- Lipin-1 primes the macrophage to be hyper-responsive to additional pro-inflammatory stimuli.

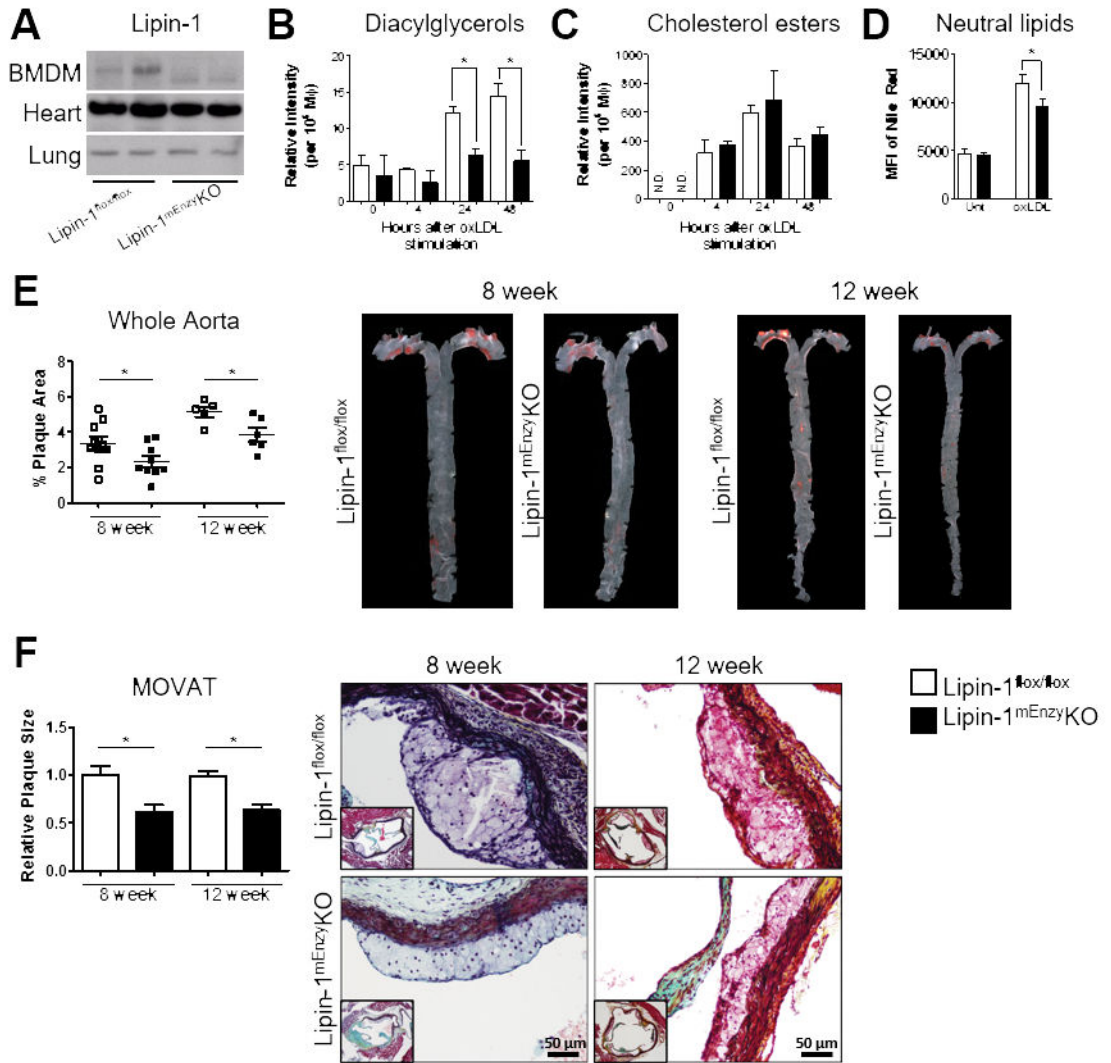


Figure 1.

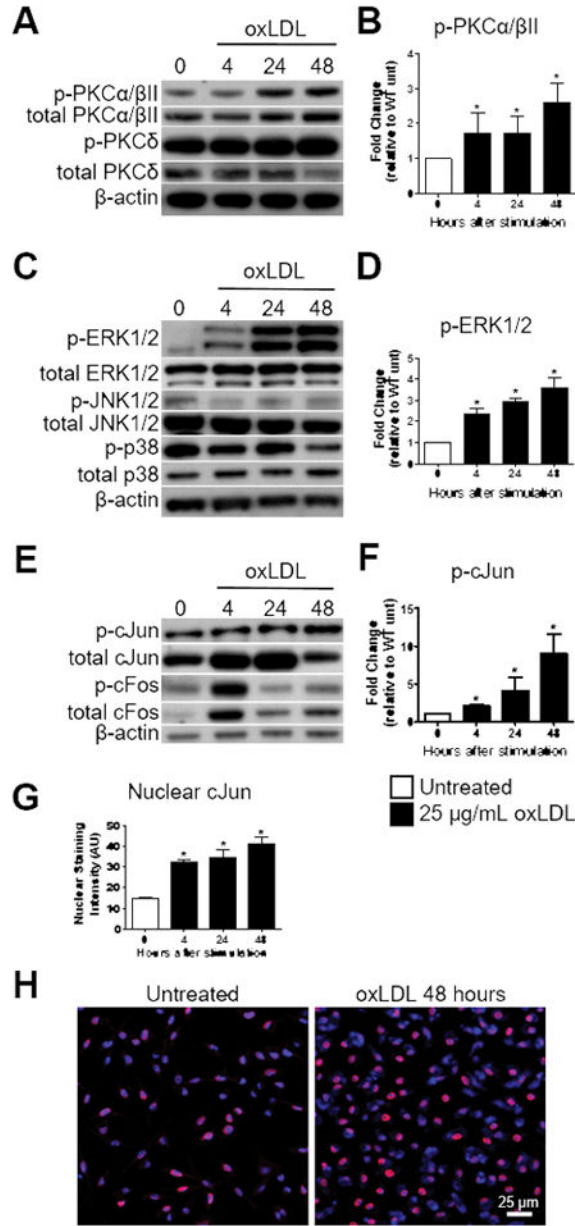


Figure 2.

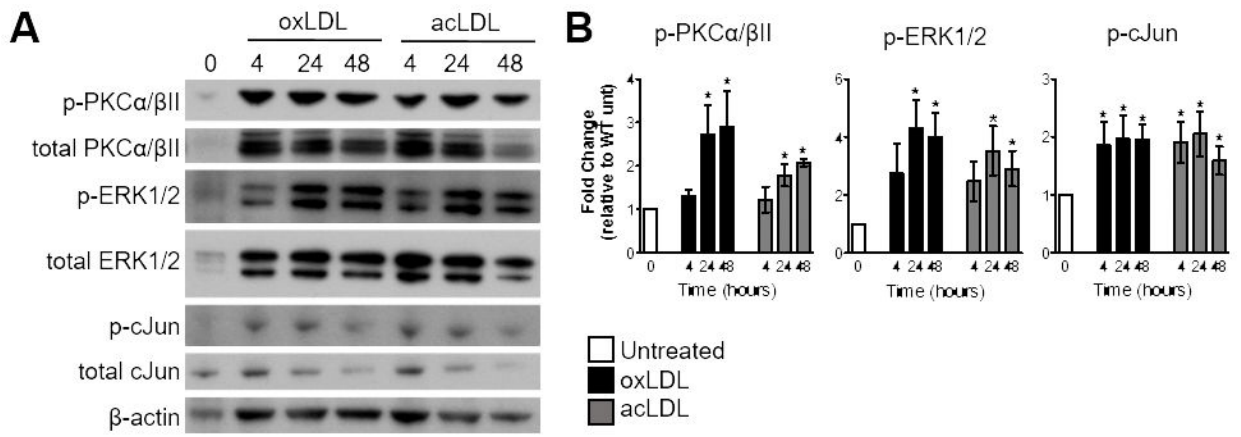
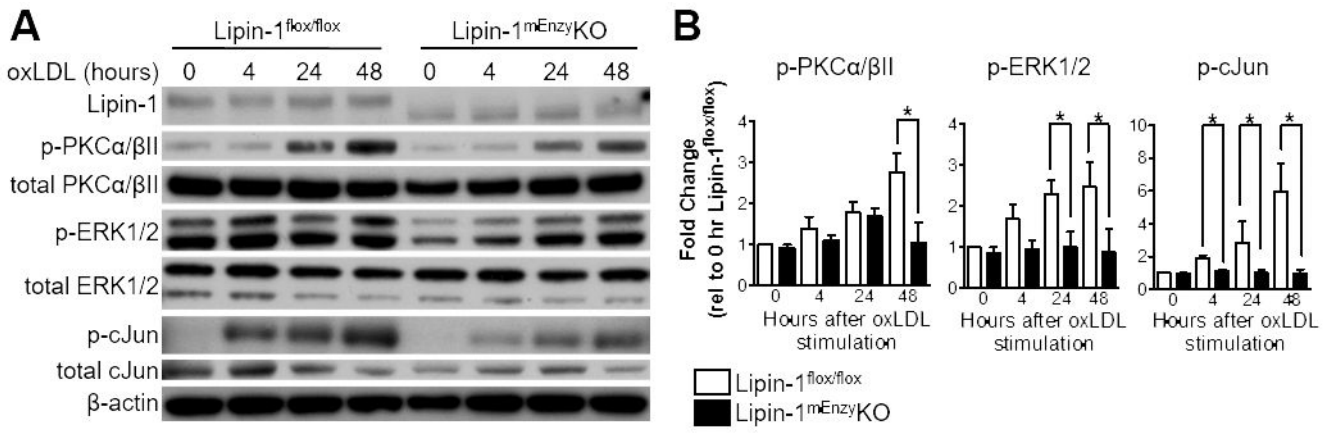


Figure 3.



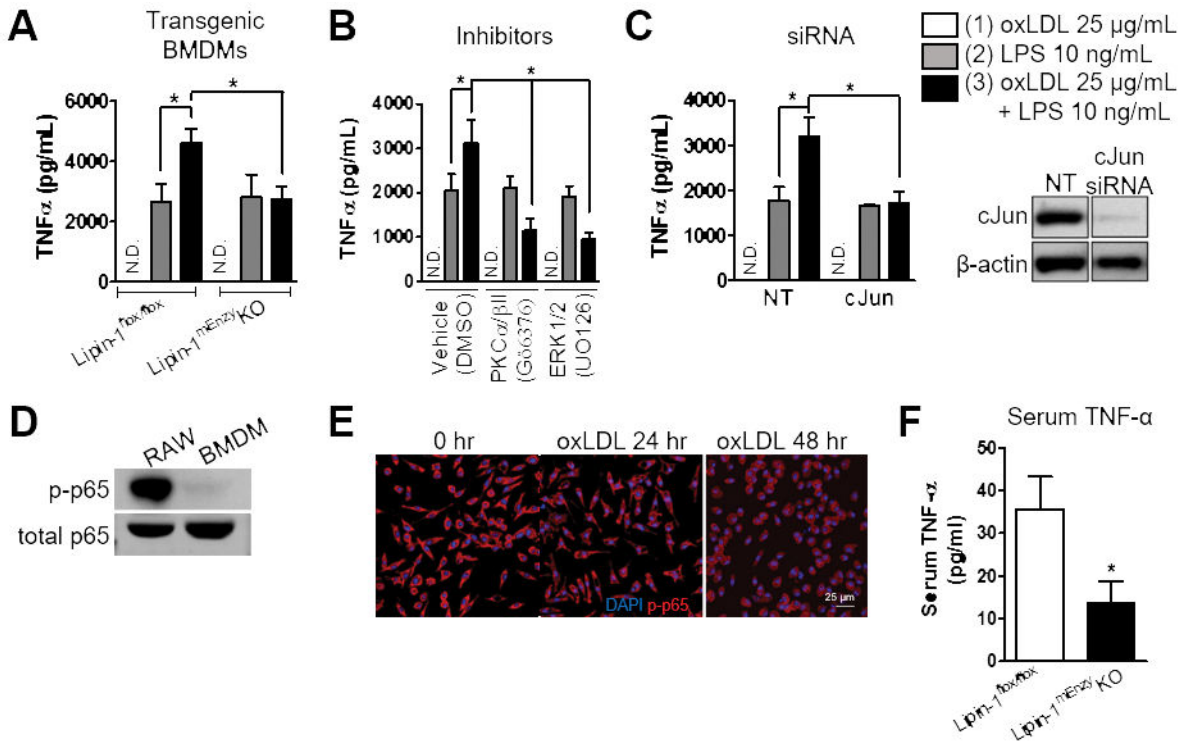


Figure 5.

TABLE 1

Analysis of Splenocyte Population in Mice*

Cell Type [†]	Marker [‡]	Mouse Strain			
		Lipin-1 ^{fllox/flox}		Lipin-1 ^{mEnzyKO}	
		Cell %	Cell #	Cell %	Cell #
Macrophages	CD11b ⁺	10.2 (2.4) [§]	12.5 (3.5) ^{//}	8.8 (1.8)	11.1 (2.3)
Dendritic Cells	CD11c ⁺	0.2 (0.04)	0.2 (0.04)	0.2 (0.05)	0.2 (0.07)
T Helper Cells	CD3+CD4 ⁺	8.8 (3.0)	10.5 (2.3)	10.0 (2.4)	12.7 (3.4)
Cytotoxic T Cells	CD3+CD8 ⁺	2.9 (1.8)	3.3 (1.9)	3.8 (2.0)	5.0 (2.6)
NK cells	CD161 (NK1.1)	0.8 (0.2)	1.0 (0.2)	0.8 (0.2)	1.0 (0.1)
B Cells	CD19 ⁺	51.1 (2.4)	62.5 (10.5)	51.9 (5.3)	66.8 (13.4)

* Mice were injected with 3×10^{10} AAV8-PCSK9 vector genomes then placed on high-fat diet for 8 weeks. Splenocytes were isolated and immune cells were quantified by flow cytometric analysis.

[†] Characterization of cell type

[‡] Cell differentiation (CD) marker used to define cellular population

[§] Cellular percentage (marker/total cells) +/- (SD) n=7

^{//} Cell number (splenocyte count x cell percentage) +/- (SD) n=7