LPA Receptor 4 deficiency attenuates experimental atherosclerosis

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Abstract

The widely expressed lysophosphatidic acid (LPA) selective receptor 4 (LPAR4) contributes to vascular development in mice and zebrafish. LPAR4 regulates endothelial permeability, lymphocyte migration, and hematopoiesis, which could contribute to atherosclerosis. We investigated the role of LPAR4 in experimental atherosclerosis elicited by adeno-associated virus expressing PCSK9 to lower LDL receptor levels. After 20 weeks on Western diet, cholesterol levels and lipoprotein distribution were similar in wild-type male and Lpar4/Y−/− mice (P = 0.94). Atherosclerotic lesion area in proximal aorta and arch was ~25% smaller in Lpar4/Y−/− mice (P = 0.009), and less atherosclerosis was detected in Lpar4/Y−/− mice at any given plasma cholesterol. Neutral lipid accumulation in aortic root sections occupied ~40% less area in Lpar4/Y−/− mice (P = 0.001), and CD68 ~ 25% lower (P = 0.045). No difference in smooth muscle α-actin staining was observed. Bone marrow derived macrophages isolated from Lpar4/Y−/− mice displayed significantly increased upregulation of the M2 marker Arg1 in response to LPA compared to wild-type cells. In aortic root sections from Lpar4/Y−/− mice, heightened M2 “repair” macrophage marker expression was detected by CD206 staining (P = 0.03). These results suggest that LPAR4 may regulate recruitment of specific sets of macrophages or their phenotypic switching in a manner that could influence the development of atherosclerosis.
Introduction

Lysophosphatidic acid (LPA) and several other related molecules constitute a family of bioactive lipid phosphoric acids that function as receptor-active mediators with roles in cell growth, differentiation, apoptosis and development (1-5). Vascular smooth muscle (6, 7), vascular endothelial cells (8), and platelets (9, 10) are notably responsive to LPA (11, 12). LPA, a potent trigger for Rho activation, promotes endothelial cell migration, disrupts endothelial barrier function, and induces phenotypic modulation of vascular smooth muscle cells. In addition to effects on endothelial (13, 14) and smooth muscle cells (6, 7), LPA is a weak activator of platelets (9, 10) and a potent stimulus for fibronectin-matrix assembly (15). LPA promotes inflammatory responses on endothelial cells and leukocytes (reviewed in (16)), and triggers neutral lipid accumulation in monocytes (17). The effects of LPA are largely mediated by members of a family of G-protein coupled receptors with 6 putative members (LPAR1-6) (1-4). Ongoing efforts seek to define the role of specific LPA receptors in blood and vascular cell functions in development and disease.

LPA is present in the lipid rich core of human atheroma and levels increase in lesions in mice during progression of atherosclerosis (18, 19). Advanced lesions in Ldlr-/− mice, generated by a combination of diet and collar placement around the carotid artery, contain ~20 fold higher levels of LPA than uninjured vessels, especially highly unsaturated long-chain acyl-LPA species (20) and may be influenced by cholesterol feeding (21). Multiple LPA species can be detected by mass spectrometry based analysis of lipid extraction imaging of human and murine atheroma. Sampling of blood from coronary arteries at the time of acute myocardial infarction (MI) reveals higher local levels of LPA (22). Hyperlipidemia may also increase steady state levels of LPA in plasma and/or enhance the capacity for LPA synthesis. Studies in rabbits suggest that systemic
LPA levels may be influenced by cholesterol feeding which elevates plasma levels of the LPA precursor lysophosphatidylcholine (LPC) and heightens the generation of LPA in serum (21). Recent work supports a link between levels of LPA in small intestine and experimental atherosclerosis, and feeding mice a diet supplemented with unsaturated LPA mimics the inflammatory effects of Western diet (23).

LPA may promote experimental atherosclerosis in a receptor dependent-manner. Treatment of Apoe-/- mice with LPA promotes monocyte adhesion to the endothelium, stimulates perivascular macrophage accumulation, and heightens atherosclerotic plaque burden in an LPAR1- and LPAR3-dependent manner (8). Similarly, pharmacological antagonism of LPAR1/LPAR3 alters inflammatory cell profiles in Ldlr-/- mice and retards progression of atherosclerosis (24). Whether other LPARs also contribute to atherosclerosis is not known, due in part to lack of selective pharmacologic tools to target specific receptors. Reports in both mice and zebrafish have clearly established a role for a particular LPA receptor subtype, LPAR4 in vascular development (25, 26) and vascular network formation by promoting cell-cell contacts (27, 28). LPAR4 may also regulate lymphocyte transmigration (29) and influence hematopoiesis (30). These effects could contribute to atherosclerosis development. Therefore, we sought to understand the role of LPAR4 in the development of experimental atherosclerosis.
Methods

Mice

All procedures conformed to the recommendations of “Guide for the Care and Use of Laboratory Animals” (Department of Health, Education, and Welfare publication number NIH 78-23, 1996) and were approved by the Institutional Animal Care and Use Committee. Mice lacking Lpar4 have previously been described (31) and were backcrossed to the C57BL/6J mice (The Jackson Laboratory, Stock # 000664), as the phenotype in the animals is dependent on the genetic background with approximately one-third of the animals dying before birth or weaning (25). All study mice were maintained in individually vented cages (maximally 5 mice/cage) on a 14-hour light and 10-hour dark cycle and euthanized 2 – 4 hours after the end of the dark cycle. Mice were fed a normal rodent laboratory diet (Diet # 2918, Envigo Teklad) and provided with drinking water from a reverse osmosis system ad libitum. Immediately after adeno-associated virus (AAV) injections, mice were fed a “Western” diet containing saturated fat (milk fat 21% wt/wt; Diet # TD.88137, Harlan Teklad) for 20 weeks unless it was otherwise stated.

Hyperlipidemia

AAV vectors (serotype 8) were produced by the Viral Vector Core at the University of Pennsylvania. These AAV vectors contained inserts expressing mouse PCSK9D377Y mutation (equivalent to human PCSK9D374Y gain-of-function mutation). AAV vectors were diluted in sterile PBS (200 µl per mouse) and injected intraperitoneally (2x10^{11} genomic copies) as previously reported (30). Total plasma cholesterol was measured using the Wako Diagnostics Cholesterol E Assay (Wako Diagnostics, Mountain View, CA; Cat#439-175001) according to the manufactures protocol. Plasma lipoprotein cholesterol distributions were determined by fast-performance liquid chromatography (FPLC). 50µL of plasma was separated using a Superose 6
size exclusion FPLC column. Cholesterol concentrations were determined by enzymatic colorimetric assay in fractions collected from the column eluate. Mice with cholesterol levels of <500 mg/dl were excluded from atherosclerosis analysis.

**Atherosclerosis analysis**

For *en face* atherosclerosis analysis, aortas were cleaned of the adventitia, dissected from the aortic root to the iliac bifurcation and stored in 10% formalin for 24 to 48 hours. Aortas were then transferred to a 0.9% saline solution and stored at 4°C for at least one day. Aortas were cut open longitudinally, exposing the intimal surface, and secured with pins to be photographed. Atherosclerosis was quantified on the intimal surface of the ascending aorta, aortic arch, and from the aortic orifice of left subclavian artery to 3 mm below by *en face* technique described previously (32).

For microscopic analysis of atherosclerosis at the aortic root, serial sections were taken at 10 µm intervals. Slides were fixed by immersion in chilled acetone at -20°C for 10 minutes. CD68 was detected with primary antibody (Abcam, Cambridge, MA; Cat# ab53444; 1:100) and secondary biotin conjugated antibody, amplified by ABC detector kit (Vector® ABC kit) and visualized by enzymatic precipitation of a chromogen substrate (VECTOR®NovaRED substrate kit sk-4800). For SMC α-actin, antibody staining (Sigma, St Louis, MO; A-5691; 1:100) was detected with VECTORS RED substrate kit (Vector, sk-5100). The areas were quantified using Metamorph software (Molecular Devices Inc). For detection of neutral lipid content in the atherosclerosis plaque, aortic root sections were stained with Oil Red-O and counter stained with hematoxylin. Atherosclerotic lesions were measured by manually tracing lesion areas on each section, as described previously (32). Measurements were made from 6 serial sections taken at 80 µm intervals and the average reported for each animal. Antibody controls are included in
Supplemental Data II. In situ hybridization and proximity ligation assays were performed as previously described (33). Imaging was performed using a Nikon (Melville, NY) A1R confocal microscope with a spectral detector and analysis performed using NIKON NIS software.

**Isolation and culture of cells**

Bone marrow-derived macrophages (BMDM) were isolated from mice by flushing the femur and tibia with DMEM. The bone marrow cells were resuspended in DMEM supplemented with 10% FBS, 1% Pen/Strep, and 10% L929 conditioned media containing M-CSF and plated at a density of 1x10^6/well in a 6-well plate. Cells were incubated for 7 days at 37 °C and 5% CO2 with medium change every 2 – 3 days. BMDMs were serum starved for 12 hours in 0.1% FBS, 1% Pen/Strep and then stimulated with LPA (5 µM; Avanti Polar, Alabaster, AL) or oxLDL (50 µg/ml; Kalen Biomedical, Germantown, MD) or vehicle for 3 hours.

Tissue, including aorta, were stored in RNAlater (Qiagen, Frederick, MD). Tissues were homogenized by mortar and pestle, and total RNA was extracted using Trizol (Invitrogen, ThermoFisher Scientific, Waltham, MA) following manufacturer’s instructions. cDNA was prepared with Multiscribe reverse-transcriptase enzyme as per manufacturer’s directions (High Capacity cDNA Archive Kit; Applied Biosystems, ThermoFisher Scientific, Waltham, MA). All the probes used in the study spanned an exon junction and thus would not detect genomic DNA. An RNA sample without reverse transcription was used as a negative control. Samples were assayed using a Quantstudio 7 flex (Applied Biosystems ThermoFisher Scientific). Threshold cycles (CT) were determined by an in-program algorithm assigning a fluorescence baseline based on readings prior to exponential amplification. Fold change in expression was calculated using the 2-ΔΔCT method using 18s RNA as an endogenous control. The TaqMan® gene expression (ThermoFisher Scientific) primers used are listed in Supplemental Table 1.
Statistics

All results are expressed as means ± SD. Statistical significance within strains was determined using a Student's t-test or two-way ANOVA with multiple pairwise comparisons as appropriate. In t-tests, if a sample failed the normality test, a rank t-test was used. In some cases of two-way ANOVA, data were log-transformed to be normally distributed. Statistical analysis was performed using Sigma-plot 13 software. A value of P < 0.05 was considered significant.
Results

LPA levels increase during experimental atherosclerosis in mice (Supplemental Data 1). To investigate a role for LPAR4 signaling in the development of experimental atherosclerosis, hyperlipidemia was elicited by infecting mice with adeno-associated virus (AAV) expressing a gain of function allele of PCSK9 (proprotein convertase subtilisin/kexin type 9) D377Y mutation (PCSK9D377Y.AAV) to lower LDL receptor levels and concurrently feeding mice Western diet to increase of plasma cholesterol. LPAR4 deficiency had no effect on the development of hypercholesterolemia (Figure 1A). Cholesterol levels in wild-type (WT) male mice (N = 20) were 839 ± 176 mg/dl (mean ± SD) and 844 ± 158 mg/dl in Lpar4Y/− mice (n = 10) (P = 0.94; two tailed t-test). No difference in the cholesterol lipoprotein distribution by size exclusion chromatography was observed between the genotypes (Figure 1B). Complete blood counts were also similar in the two genotypes (Supplemental Table 2).

Atherosclerotic lesion area was measured on the intimal surfaces of the proximal aorta and arch by en face analyses (Figure 1C). Lesion area to total aortic area in male WT mice (0.318 ± 0.06; mean ± SD) was significantly greater than in Lpar4Y/− mice (0.235 ± 0.08; P = 0.009 two tailed t-test; Figure 1D). To exclude the possibility that variability in plasma cholesterol levels after PCSK9D377Y.AAV treatment was affecting the development of atherosclerosis independent of genotype, we examined the relationship between total cholesterol and extent of atherosclerosis measured by en face analysis (Figure 1E). In both WT and Lpar4Y/− mice, a linear correlation between plasma cholesterol and extent of atherosclerosis was observed. However, even with normalization to plasma cholesterol levels, relatively less atherosclerosis was detected in Lpar4Y/− mice as compared to WT controls.
To gain insight into potential mechanisms underlying these effects on atherosclerosis we examined the cellular composition of lesions. Consistent with the *en face* analysis, neutral lipid accumulation, as detected by Oil Red O (ORO) staining, was significantly lower in serial sections taken at the aortic root of *Lpar4<sup>V<sup>-</sup></sup>* mice (Figure 2A). The area (mm<sup>2</sup>) of ORO staining was 0.547 ± 0.08 (mean ± SD) in WT (n = 10) mice versus 0.348 ± 0.122 in the knock out mice (n = 11) (P = 0.001; two tailed t-test). Macrophage accumulation and/or transdifferentiation of cells to a macrophage-like phenotype, as detected by CD68 expression, was also lower in the receptor-deficient mice (Figure 2B), with a mean (± SD) area of 2669 ± 699 units in WT (n = 10) and 2024 ± 380 in *Lpar4<sup>V<sup>-</sup></sup>* mice (n = 7) (P = 0.045 two tailed t-test). No differences in smooth muscle cell (SMC) area, as detected by SM α-actin staining, was observed between WT (470 ± 208 units; n = 10) and knock-out mice (591 ± 229; n = 11) (P = 0.27 two tailed t-test) (Figure 2C).

The LPAR4 receptor is carried on the X chromosome in mice. We therefore investigated sex differences in the role of the receptor in the development of atherosclerosis. No difference in cholesterol levels after PCSK9D377Y.AAV injection was observed between the genotypes in female mice, although total cholesterol levels in female mice were lower than those achieved in male mice. Cholesterol levels in WT female mice (N = 27) were 438 mg/dl (median) and 477 mg/dl in *Lpar4<sup>V<sup>-</sup></sup>* mice (n = 29) (Supplemental Data Figure IIIA). Lesion area to total aortic area in female WT mice (0.05 median with 25 – 75% CI of 0.05 – 0.288) was not significantly different than in *Lpar4<sup>V<sup>-</sup></sup>* females (0.09 median with 25 – 75% CI of 0.05 – 0.317; P = 0.9 by rank sum test) (Supplemental Data Figure IIIB). Supplemental Data Figure IIIC shows the relationship between plasma cholesterol and *en face* atherosclerosis development in female mice. While these observations may point to a sex-difference in the role of LPA signaling, it is
important to note that in our hands PCSK9D377Y.AAV is less effective at elevating cholesterol levels in female mice. The resulting lower lesion development likely limited our ability to detect a difference between genotypes.

To understand the role of LPAR4 in specific cellular signaling systems of relevance to atherosclerosis, we explored lipid-mediated responses in cells isolated from mice lacking LPAR4. Environmental lipid-induced transformation elicited by treatment of cells with oxidized LDL converts macrophages into lipid-laden foam cells and transdifferentiates SMC to assume a macrophage-like phenotype. LPA signaling has been implicated in responses that affect foam cell formation (17, 34-37) and SMC phenotypic modulation (38-40). The class B scavenger receptor CD36 and the class A scavenger receptor SRA are the major receptors responsible for the binding and uptake of ox-LDL. LPA signaling has been reported to increase SRA (35) and affects CD36 expression in certain circumstances (33). Thus, we examined the role of LPAR4 in these effects and responses to LPA in BMDM and primary cultures of aortic SMC from the mice.

Following exposure to LPA (Figure 3A), BMDM express higher levels of F3 (tissue factor) and Ctgf (Figure 3A) as has been reported in other model systems (41-43). Following exposure to ox-LDL, BMDM expression of Cd36 increases and expression of Il-10 declines. Neither LPA or ox-LDL significantly altered Sra1 expression under the experimental conditions examined. In comparison to WT cells, LPAR4-deficient cells expressed significantly higher levels of Arg1 mRNA in response to LPA (Figure 3A; P<0.05 by two-way ANOVA). We explored potential mechanisms by examining expression of Krüppel-like factors (KLF4 and KLF6), which are transcription factor that regulate phenotype modulation of cells and in the case of KLF4, reported to be influenced by LPA signaling in other cells. Interestingly, LPA exposure
increased KLF4 expression in LPAR4-deficient but not WT cells (Figure 3B), whereas KLF6 was increased in both genotypes of cells in response to ox-LDL. Together, these results suggest that LPAR4 may normally regulate phenotypic modulation. ARG1 is a marker of M2 “repair” macrophages, which have been associated with lesion regression (44). To determine if LPAR4 likewise increased M2 subsets of macrophages within atherosclerotic lesions, staining for the M2 marker CD206 was measured in sections from mice (Figure 3C). In comparison to CD206 in WT (n = 10) aortic root sections, the CD206+ area was significantly higher in Lpar4Y/- tissue (707 ± 142 versus 825 ± 102 µm²; P = 0.03 by one-tailed t-test). In both genotypes, CD206+ cells were abundant in the adventitial area external to the medial layer; however, LPAR4-deficient mice displayed more CD206+ cells within the atherosclerotic lesions (Figure 3D and E).

We also investigated the role of LPAR4 in SMC responses. Aortic SMC isolated from Lpar4-deficient mice have virtually no LPAR4 gene expression (P < 0.001 versus WT by 2-way ANOVA) (Figure 4A). Following exposure to ox-LDL, SMCs upregulate gene expression for the inflammatory markers Il-6 and Cd68 (P = 0.049 versus vehicle control by 2-way ANOVA) with no difference between WT and null genotypes. Interestingly, expression of Acta2, encoding α-SMC actin, was lower at baseline in the knock-out cells (P = 0.001 versus WT by 2-way ANOVA). Acta2 expression in SMC declined further after ox-LDL exposure (P = 0.001 versus vehicle by 2-way ANOVA), and was lowest in Lpar4-deficient cells treated with ox-LDL. In contrast, the lack of LPAR4 did not appear to influence SMC α-actin area of expression by immunohistochemistry in sections of atherosclerosis (Figure 2C). To determine if there was a difference in lineage committed SMC in the atherosclerotic plaques, we employed in situ hybridization and proximity ligation assays (ISH-PLA) to visualize the H3K4dime marker of the
MYH11 locus that is restricted to the SMC lineage (33). Quantification of the number of PLA+ cells area revealed a higher density of positive cells in atherosclerotic lesions at the aortic root of Lpar4<sup>−/−</sup> mice (4.1 ± 1.3 PLA+ cells per µm<sup>2</sup> area; n = 8) than WT mice (2.7 ± 1.1 PLA+ cells per µm<sup>2</sup> area; n = 9; P = 0.033, two-tailed t-test) (Figure 4B, 4C and Supplemental Data IV).

**Conclusions**

We provide evidence that LPA receptor signaling contributes to the development of experimental atherosclerosis. Specifically, the lack of LPAR4, a receptor previously demonstrated to regulate vascular development in mice, attenuated atherosclerosis in male mice. The reduction in atherosclerosis was accompanied by less accumulation of CD68+ cells, indicating a change in the inflammatory composition of the plaque. BMDM isolated from LPAR4-deficient animals displayed a paradoxical upregulation of gene expression for the M2 macrophage marker ARG1 and the transcriptional regulator KLF4 when exposed to LPA. Immunohistochemical analysis confirmed heightened M2 markers in atherosclerotic lesions of LPAR4-deficient mice. Previous results from *in vitro* assays indicated that LPA increases neutral lipid accumulation in macrophages and prevents their reverse migration across an endothelial layer (17). It is possible that LPAR4 has a role in these events during the course of atherosclerosis as well as effects on inflammatory profiles.

Alternative M2 macrophages are induced in response to Th2-type cytokines IL-4 and IL-13 and secrete anti-inflammatory factors. In general, M2 macrophages tend to resolve plaque inflammation and promote tissue repair, in part by taking up and oxidizing fatty acids and secreting high levels of collagen. KLF4 transcription factor promotes M2 and inhibits M1 macrophage polarization, and its absence accelerates atherosclerosis (45). LPA is known to regulate KLF4 (46) to affect cellular phenotypes. In cultured BMDM, we provide evidence that LPA4 appears to suppress LPA-mediated increases in KLF4 expression and, in the absence of
the receptor, higher KLF4 expression could promote the M2 phenotype. Additional pathways that may be involved include CD14, which has been reported to be a key mediator of both LPA and lipopolysaccharide-induced foam cell formation in vitro (36). In addition, LPA promotes ox-LDL uptake through class A scavenger receptor-dependent in macrophages (47). LPA has been proposed as a key molecule in serum responsible for SMC dedifferentiation (40) and through LPAR1 regulates SMC contraction (48).

Intriguingly, aortic SMCs isolated from mice lacking LPAR4 displayed lower gene expression for SMC α-actin basally and after ox-LDL exposure. However, we were unable to detect differences in SMC in atherosclerotic lesions from the LPAR4-deficient mice. It is possible that differences in developmental origins of the SMC in the two models could account for our findings. SMC in the aortic root arise from secondary heart filed from lateral plate mesoderm whereas the SMC isolated from descending thoracic and abdominal aorta are from somites. In other contexts, we have observed differences in LPA signaling in SMC from these developmentally distinct regions.

The reduction in atherosclerosis was independent of an effect on plasma lipoprotein content. This finding is different from observations with the LPAR1/3 antagonist Ki16425 that lacks activity at LPAR4, which also reduced experimental atherosclerosis in Ldlr<sup>−/−</sup> mice (8). Ki16425 mildly reduced total cholesterol in Ldlr<sup>−/−</sup> mice with a trend towards lowering LDL (24). LPAR1/3 antagonism also changed the inflammatory composition of the lesions by reducing monocyte and neutrophil accumulation and increasing regulatory T-cells. Together with our findings, these results support a role for LPAR signaling in promoting inflammation during atherosclerosis.
In cellular models, considerable overlap between LPA receptors and their downstream signaling pathways have been reported (49). However, selective targeting of LPAR subtypes in mice and zebrafish yields distinct phenotypes, pointing to non-redundant roles for the receptors pathophysiologically (25, 26). Given the broad ranging effects of LPA on cells and the observations with LPA receptor inhibitors, it seems that multiple LPA receptors contribute to experimental atherosclerosis. Receptor subtype expression in blood and vascular cells varies between humans and mice, for example, LPAR5 and LPAR6 are upregulated in human atheroma (50); and therefore it is probably not possible to extrapolate the findings in mouse models directly to humans.

The downstream signaling pathways that mediate effects of LPAR also demonstrates considerable overlap (49). Based on the similarities between the phenotypes in the LPAR4-deficient and the Go13-deficient mice (51), LPAR4 signaling regulating endothelial function has been suggested to through this pathway (25). LPAR1 and LPAR6 have also been linked to Ga12/13 signaling (52). In mouse embryonic fibroblasts, LPAR4 appears to negatively regulate migration stimulated by a LPAR1-G<sub>αi</sub>-PI3 Kinase–dependent pathway, in that LPA4-deficient cells display enhanced migratory responses to LPA. The negative regulation of LPA signaling by LPAR4 in this model is similar to our observations of enhanced LPA responses in BMDM lacking LPAR4. The downstream pathways mediating these effects are not known, although in addition to Ga12/13, Gαq and Gαs have also been proposed as mediating LPAR4 responses in other cells (53).

The role of LPA receptor signaling in experimental atherosclerosis certainly positions the pathway to contribute to disease pathology in humans. Additionally, a role for LPA signaling in the development of atherosclerosis could explain the observations that a genetic variant PLPP3,
encoding the LPA-inactivating enzyme LPP3, are strongly predictive of coronary artery disease and myocardial infarction in humans (54, 55). Furthermore, our results support the hypothesis that therapeutics targeting LPA pathways could be a novel anti-inflammatory approach to preventing complications of atherosclerotic disease.
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Figures

Figure 1

**Figure 1.** LPAR4-deficiency in male mice reduces atherosclerosis but has no effect on plasma cholesterol profiles. **A.** Total cholesterol in plasma from individual WT (dark circles) or Lpar4<sup>y/-</sup> (open circles) mice infected with PCSK9D377Y.AAV and fed Western diet for 20 weeks. P = 0.94; two tailed t-test. **B.** Plasma lipoprotein particles were resolved by size exclusion chromatography and total cholesterol in the indicated fractions (mean +/- SD, n = 3 mice/genotype determined. **C.** Images of representative aortas from WT and Lpar4<sup>y/-</sup> mice. **D.** *en face* analysis of atherosclerotic lesions from the ascending aorta to 3 mm below the subclavian artery. The atherosclerotic lesion area / total aortic area for individual WT (dark circles) or Lpar4<sup>y/-</sup> (open circles) mice is presented. P = 0.009 by two tailed t-test. **E.** Atherosclerosis lesion area plotted as a function of plasma cholesterol in WT (dark circles) or Lpar4<sup>y/-</sup> (open circles) mice.
Figure 2. LPAR4-deficiency in male mice reduces neutral lipid and CD68+ cell content but not SMC α-actin in aortic root atherosclerotic lesions

A. Quantification of lipid content (µm²) from oil red O staining of aortas from individual WT (dark circles) or Lpar4ymi (open circles) mice. (P = 0.001; two tailed t-test). Representative oil red O staining of neutral lipid in aortic root sections (bottom).

B. Quantification of CD68+ staining from individual WT (dark circles) or Lpar4ymi (open circles). P = 0.045 two tailed t-test. Representative CD68+ staining (bottom).

C. Quantification of SMC α-actin+ staining from individual WT (dark circles) or Lpar4ymi (open circles) mice (P = 0.045 two tailed t-test, P = 0.27 two tailed t-test). Representative SMC α-actin+ staining (bottom). In all cases, the area values for each animal are the average of 6 measurements taken 80µM apart.
Figure 3. LPAR4 deficiency influences oxidized LDL-elicited gene expression in bone marrow derived macrophages. 

A. Gene expression analysis in cells isolated from WT (open bars) or Lpar4y/- (hatched bars) mice. Cells were harvested from four mice, cultured for 7 days, and then exposed to LPA 18:1 (5 µM; yellow bars), ox-LDL (50 µg/ml; green bars), or vehicle control for 3 hours. Results are presented relative to expression with vehicle exposure and are summarized data from three separate experiments analyzed by 2-WAY ANOVA with Mann Whitney test. E = significant differences in gene expression between environmental exposures (P <0.05). GE = significant differences in gene expression based on genotype and environmental exposure (P <0.05). 

B. Klf4 and Klf6 gene expression analysis in BMDM isolated from WT (gray hatched bars; n = 4) or Lpar4y/- (open bars; n = 4) exposed to vehicle control, LPA 18:1 (2 µM; yellow bars), or LPS (100 ng/ml; blues bars) for 3 hours. Results are presented relative to expression with vehicle exposure and are summarized data from three separate experiments analyzed by 2-WAY ANOVA with Mann Whitney test. GE = significant differences in gene expression based on genotype and environmental exposure (P <0.05). 

C. CD206+ staining in aortic root sections from WT (dark circles; n = 10) or Lpar4y/- (open circles; n = 8) mice.
Individual values represent the average of 6 measurements taken 80µM apart. $P = 0.03$; one tailed t-test. **D.** Representative images of sections of WT aortic root stained for CD205 at 4x (left) and 20x (right). **E.** Representative images of sections of $Lpar4^{−/−}$ aortic root stained for CD205 at 4x (left) and 20x (right).
Figure 4. LPAR4 deficiency influences Ox-LDL-induced gene expression in aortic SMC. A. Gene expression analysis from SMC cells isolated from WT (gray hatched bars) or Lpar4<sup>y/-</sup> (open bars) aortas. Cells were exposed to ox-LDL (50 µg/ml; green bars) or vehicle control for 3 hours. Results are presented relative to expression in WT vehicle treated cells and are summarized data from three separate experiments analyzed by 2-WAY ANOVA with Mann Whitney test. E = significant difference in gene expression between environmental exposures (P <0.05). G = significant difference in gene expression between genotypes (P <0.05). GE = significant difference in gene expression based on genotype and environmental exposure (P <0.05). B. Quantification of MYH11 H3K4dime PLA+ cells from individual WT (dark circles) or Lpar4<sup>y/-</sup> (open circles) mice. Individual values represent the average of 6 measurements taken 80µM apart. P = 0.045; two tailed t-test. C. Representative immunostaining of aortic root
sections from WT (top) and \( Lpar4^{\text{+/–}} \) (bottom) mice with DAPI (blue) and ACTA2 (green) and MYH11 H3K4dime PLA signal (red). Scale bar = 50 μm.