SUPPLEMENTAL MATERIAL

MATERIALS & METHODS

AAV8-PCSK9 viral vector preparation – DNA for pAAV/D377Y-mPCSK9 (Addgene plasmid #58376), a gift from Jacob Bentzon\(^1\) was packaged into adeno-associated virus serotype 8 (AAV8) using helper and capsid plasmids from the University of Pennsylvania.\(^2,3\) Viral stocks were sterilized via Millipore Millex-GV syringe filter (Billerica, MA), tittered by dot blot assay, aliquoted, and stored frozen until use. Final product will be referred to as AAV8-PCSK9.

Animals and tissue harvest – Animal protocols were approved by the LSU Health Sciences Center- Shreveport institutional animal care and use committee, and all animals were cared for according to the National Institute of Health guidelines for the care and use of laboratory animals. 6- to 8-week old, C57BL/6J mice were purchased from Jackson Laboratory (Bar Harbor, ME) and were used for the generation of bone marrow-derived macrophages (BMDMs) as described in the section titled “Generation of bone marrow-derived macrophages”. Mice harboring a Lpin1 allele with exons 3 and 4 of the Lpin1 gene flanked by LoxP sites (genetic background: C57BL/6J and SV129) were generously provided by Brian Finck and Roman Chrast.\(^4,5\) To generate mice with the Lpin1 gene selectively inactivated in myeloid-derived cells, the Lpin1 floxed (lipin-1\(^{\text{lox}/\text{lox}}\)) mice were mated with LysM-Cre transgenic mice purchased from Jackson Laboratory (Bar Harbor, ME). The resulting offspring were deficient in lipin-1 enzymatic activity within myeloid-derived cells (lipin-1\(^{\text{mEnzy}}\)KO). Experimental lipin-1\(^{\text{mEnzy}}\)KO mice were compared with lipin-1\(^{\text{lox/lox}}\) littermate control mice; 8- to 10-week old mice were used for all studies. Mice were given retro-orbital injections of 3x10\(^{10}\) vector genomes of AAV8-PCSK9. Immediately following the AAV8-PCSK9 injection the mice were switched to a high fat, Western diet (TD 88137; Harlan-Teklad, Madison, WI) that contained 21% fat by weight (0.15% cholesterol and 19.5% casein without sodium cholate) for 8 or 12 weeks before euthanasia. Mice were weighed once a week after starting high fat diet. After 8 or 12 weeks on high fat diet, mice were euthanized by exsanguination and pneumothorax under isoflurane anesthesia. Blood was collected by vena cava puncture into heparinized blood collection tubes, centrifuged at 5,000 rpm for 5 minutes, and plasma was isolated and frozen at -80°C until analysis. Following blood collection, mice were perfused with 1X phosphate-buffered saline (PBS), the heart and aorta were fixed in 4% neutral buffered formaldehyde for analysis, and additional tissue (heart and lung) was collected for Western blot or flow cytometric analysis.

Plaque & Blood Analysis – Aortas were harvested, cleaned of adventitia, cut open longitudinally, stained using 0.5% Oil Red O (Sigma Aldrich) prepared in 60% isopropanol, and pinned open. Vessels were visualized on a DS-Fil camera (Nikon) attached to a multizoom AZ100 microscope (Nikon), and plaque burden was analyzed using Nikon Elements software and expressed as a percentage of total aortic area. Total and HDL cholesterol (Wako) and triglyceride levels (Pointe Scientific) were measured using commercially available kits. LDL was calculated using the Friedewald equation.

Histology and Image Quantification – Aortic roots were fixed in 4% neutral buffered formaldehyde, embedded in paraffin, and cut into 5 \(\mu\)m sections. All sections were taken from the same site at equal distance from anatomical landmarks (initiation of valve leaflets). Russell-Movat Pentachrome staining was performed to determine plaque area. Images were collected using an Olympus BX40 microscope and quantification of the lesion area inside the internal elastic lamina was determined using Nikon Elements imaging software.

Immunofluorescence – Aortic roots were fixed in 4% neutral buffered formaldehyde for 24 hours and then processed for paraffin embedding. Paraffin blocks were sectioned into 5 \(\mu\)m sections. All sections within each regimen were taken from the same site at equal distance from anatomical landmark (initiation of value leaflets). Tissue sections were rehydrated and
antigen retrieval was performed in 10 mM citrate buffer (0.1M citric acid and 0.1M sodium citrate) using a microwave oven. Sections were blocked in blocking buffer (1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS)) for at least one hour at room temperature. Sections were rinsed with Tris-buffered saline with 0.1% Tween 20 (TBST) three times. Primary antibodies were added and kept at 4°C overnight. Sections were again rinsed with three washes of TBST. Secondary antibodies, all diluted 1:200, were added and allowed to incubate for 2 hours at room temperature. Sections were washed in PBS then 3-3'-diaminobenzidine (DAPI, Molecular Probes, D-3571) was added at a 1:50,000 dilution for 10 minutes at room temperature. Slides were imaged using a Nikon Eclipse Ti inverted epifluorescence microscope equipped with a Photometrics CoolSNAP120 ES2 camera and images were prepared using Nikon Elements software. Primary antibodies used include: 1:10,000 anti-mouse Mac2 (Accurate Chem., CL8942AP), 1:200 anti-rabbit smooth muscle myosin heavy chain 11 (Abcam, ab53219), and 1:400 anti-mouse α-smooth muscle actin-Cy3 (Sigma Aldrich, C6198). Secondary antibodies used include: Alexa Fluor® 647 donkey anti-rabbit IgG (A31573) and Alexa Fluor® 488 donkey anti-rat IgG (A21208) purchased from Life Technologies.

**Immunocytochemistry** – 1x10^6 C57BL/6 BMDMs were seeded on glass coverslips for 2 hours in a 6-well plate. BMDMs were treated with 25 μg/mL oxLDL for 4, 24, and 48 hours. Following treatments, cells were fixed in 4% PFA, washed twice with 1X phosphate buffered saline (PBS), and blocked in 30 minutes in 10% BSA in PBS with 0.1% saponin (BSP). Primary antibodies were diluted and added in BSP for 2 hours at room temperature. After washing three times with PBS, secondary antibody diluted 1:200 was added in BSP for 2 hours at room temperature. Coverslips were washed again three times with PBS and then mounted with DAPI slowfade (Invitrogen, S36938). Representative images were taken on a Leica TCS SP5 Confocal Microscope at 40X magnification with oil immersion.

Representative images are shown as equally enhanced using Image J software. Cells were scored for nuclear cJun or nuclear p65, and at least 100 cells were counted from at least 3 fields per condition for each experiment, performed in triplicate. Primary antibodies used included NF-kB p65 (1:70) (Cell Signaling, 8242S) and total cJun (1:50) (Thermo Scientific, MA5-15172). Secondary antibody used was Alexa Fluor® 594 AffiniPure F(ab’2) Fragment Donkey Anti-Rabbit IgG (Jackson Immunoresearch, 711-586-152).

**LDL oxidation** – Human LDL (Kalen Biomedical) was oxidized by incubation with copper (II) sulfate (CuSO₄) as previously described. In short, human LDL was dialyzed in 1X phosphate buffered saline (PBS) pH 7.6 for 24 hours at room temperature using a 7000 MWCO Slide-A-Lyzer® Dialysis cassette (Thermo Scientific) to remove EDTA. The buffer was changed to fresh 1X PBS containing 13.8 μM CuSO₄ for an additional 72 hours at room temperature. The cassette was transferred to fresh 1X PBS containing 50 μM EDTA for 4 hours at 4°C to remove excess copper. Finally, the cassette was transferred to fresh 1X PBS containing 50 μM EDTA for another 24 hours. Oxidized LDL (oxLDL) was then collected and stored at 4°C. All steps were performed under sterile conditions. Acetylated LDL (acLDL) was purchased from Alfa Aesar (J65029 BT-906). This methodology results in oxLDL that consistently displays a relative electrophoretic mobility between 2 and 3. Relative electrophoretic mobility of the oxLDL was 3.1 and the acLDL was 3.0.

**Limulus Amebocyte Lysate (LAL) Assay** – Oxidized LDL (oxLDL) prepared in the lab and acetylated LDL (acLDL) purchased from Alfa Aesar (J65029 BT-906) were tested for endotoxin. The Pierce LAL Chromogenic Endotoxin Quantitation Kit (ThermoFisher) was used as per manufacturers instructions. OxLDL contained 0.2 endotoxin units (EU) per mg and acLDL contained 0.5 EU per mg.

**Generation of bone marrow-derived macrophages** – Murine, bone marrow-derived macrophages (BMDMs) were generated by flushing the bone marrow from the femurs of male 6- to 8-week-old...
C57BL/6, lipin-1mEnzKO, or lipin-1floxflox mice with BMDM differentiation medium (KnockOut™ Dulbecco’s modified Eagle’s medium (DMEM; Gibco, 10829) supplemented with 30% L-cell conditioned medium, 20% fetal bovine serum (Atlas Biologicals), 2mM L-glutamine (HyClone), 100 U/ml penicillin-streptomycin (ATCC), 1mM sodium pyruvate (HyClone), and 0.2% sodium bicarbonate). Red blood cells were removed by ammonium chloride-potassium carbonate lysis. Isolated cells were incubated for 7 days in BMDM differentiation medium at 37°C and 5% CO₂. BMDMs were collected by removing medium, washing cells with 1X sterile phosphate buffered saline without Ca²⁺ or Mg²⁺ (PBS; HyClone) then incubated with 10 mM EDTA, pH 7.6, in 1X PBS to lift cells from the plate. BMDMs were then placed into complete DMEM (DMEM (Lonza, 12-741) 10% fetal bovine serum (Atlas Biologicals), 2mM L-glutamine (HyClone), 100 U/ml penicillin-streptomycin (ATCC), and 1mM sodium pyruvate (HyClone)).

**L-cell conditioned medium** – The murine fibroblast cell line L929 (ATCC CCL-1) was grown in RPMI 1640 (Hyclone, SH30027.01) supplemented with 10% FBS (Atlas Biologicals), 2 mM L-glutamine (HyClone), 1 mM sodium pyruvate (HyClone), and 100 U/mL penicillin/streptomycin (ATCC). In short, 3.75x10⁵ cells were seeded in a T225 tissue culture flask with 75 mL medium. The flask was incubated for 12 days at 37°C. Medium was collected, cleared of cell debris by centrifugation, filtered (0.22 µm), and stored at -80°C until use.

**siRNA treatment** – Bone marrow-derived macrophages (BMDMs) were collected in complete DMEM medium and counted. 5x10⁶ BMDMs were transfected using Amaxa™ P3 Primary Cell 4D-Nucleofector™ X Kit L (Lonza) with 300 pmols of appropriate siRNA. cJun Silencer® Select Pre-designed siRNA (Ambion® IDs: s68563 and s201552) and Accel™ Control siRNA eGFP siRNA #1 (Thermo Scientific, D-001940-01-20) were used. Cells were seeded in complete DMEM medium (DMEM (Lonza, 12-741) 10% fetal bovine serum (Atlas Biologicals), 2mM L-glutamine (HyClone), 100 U/ml penicillin-streptomycin (ATCC), and 1mM sodium pyruvate (HyClone)) at a concentration of 8x10⁵ cells/well in a 12-well plate. Cells were incubated at 37°C for 4 hours prior to treatment.

**Nile Red Staining** – 1.1x10⁶ BMDMs in complete DMEM (DMEM (Lonza, 12-741) 10% fetal bovine serum (Atlas Biologicals), 2mM L-glutamine (HyClone), 100 U/ml penicillin-streptomycin (ATCC), and 1mM sodium pyruvate (HyClone)) media were placed into a polypropylene flow cytometry tubes (VWR, 60818-500). 50 µg/mL of oxLDL (Alfa Aesar, J65261) was added to the tubes. BMDMs were incubated with oxLDL for 24 hours. Tubes were spun at 350Xg for 10 minutes. Complete DMEM medium was removed and BMDMs were fixed in 10% formalin for 20 minutes. BMDMs were then washed in FACS wash (1X phosphate buffered saline (PBS) with 1% bovine serum albumin and 0.1% sodium azide). Each sample was split equally in two new polypropylene flow tubes. Unstained tubes were left untreated and the remaining tubes were spun at 350Xg for 10 minutes and brought back up in 100 ng/mL of Nile Red (Thermo Scientific, N1142) in 1X PBS. After 5 minutes, BMDMs were washed with 1X PBS and reconstituted in 0.5 mL of 1X PBS. Flow cytometry was performed on an LSR II Flow Cytometer (BD Biosciences).

**Inhibitors** – Inhibitors used include 1 µM Gö6976 (EMD Millipore, 365253) and 10 µM U0126 (Sigma Aldrich, 662005). Inhibitors were added 24 hours after the initial stimulation (with either 25 µg/mL oxLDL or 10 ng/mL LPS) and cells or supernatants were collected at 48 hours after initial stimulation.

**Quantitative Real-Time PCR (qRT-PCR)** – mRNA was extracted from BMDMs with RNA-STAT 60 reagent (Ambio) per manufacturer’s instructions and converted to cDNA using qScript cDNA SuperMix (Quantabio). qRT-PCR was performed in a Biorad iCycler with SsoAdvanced Universal SYBER Green SuperMix (BioRad). Primers (Supplemental Table 1) were designed using online Beacon Designer software. PCR products were verified by the presence of a single peak in melt curve analysis. Results were normalized to the housekeeping gene, Rpl13a, and expressed as a fold change using the 2ΔΔCt method.
**Tissue processing for Western blot analysis** – Tissues were flash frozen in liquid nitrogen and stored at -80°C until use. Tissue was lysed in RIPA buffer (150 mM sodium chloride, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8, and 1 mM PMSF). Homogenization was performed using the Biojector® 2000 (iHealthNet). Homogenized tissue was placed on a rotator for 30 minutes at 4°C. Samples were then centrifuged for 5 minutes at 15,000Xg. Resulting pellets were resuspended in denaturing lysis buffer (1X NuPage LDS sample buffer containing 100 mM dithiothreitol (DTT; Life Technologies), 1X protease inhibitor cocktail (Thermo Scientific), 1X phosphatase inhibitor cocktail 1 (Sigma Aldrich), and 1X phosphatase inhibitor cocktail 2 (Sigma Aldrich)). Protein concentration of BMDM or tissue samples (described above) was determined by Peirce® 660 nm Protein Assay (Thermo Scientific). 20 µg of total protein for each sample was loaded onto a 4 to 12% polyacrylamide NuPAGE Novex gel (Invitrogen). MOPS (50 mM 4-morpholinepropanesulfonic acid, 50 mM Tris base, 0.1% SDS, 1 mM EDTA, pH 7.7) running buffer was used to run the gel. Proteins were separated at 200V for 55 minutes and semidry transfer (Novex, SD1000) was performed for 45 minutes at 20 V onto a polyvinylidene difluoride (Immobilion-FL) membrane (EMD Millipore). The membranes were blocked for 1 hour at room temperature in 10% Li-Cor blocking buffer (Li-Cor Biosciences) in 1X phosphate buffered saline (PBS). Primary antibodies were diluted 1:1,000 in 1% BSA in PBS with 0.01% sodium azide, excluding β-actin which was diluted 1:80,000, and were incubated on the membranes overnight at 4°C on a rocker. Secondary antibodies were diluted 1:2,000 in 5% milk with Tris-buffered saline with 0.1% Tween 20 (TBST) plus 0.01% SDS and incubated with membranes for 2 hours at room temperature. The membranes were washed three times, for 15 minutes each, with TBST while rocking after incubation with both primary and secondary antibodies. ImmunoCruz Western blotting luminol reagent (Santa Cruz, sc-2048) was mixed and added to blots for 1 minute. Exposures were taken with UltraCruz autoradiography film (Santa Cruz, sc-201697). Films were scanned and densitometry was performed using Image J analysis software. Bands of interest were normalized to β-actin or PCNA for statistical analysis. Primary antibodies were as follows: p38 MAPK (9212S), p-p38 MAPK (4631), SAPK/JNK (9252), p-SAPK/JNK (4668), p44/42 MAPK (9102), p-p44/42 MAPK (4370), p-c-Jun (3270), c-Fos (4384), p-c-Fos (5348), NF-κB p65 (8242), p-PKCα/β II (9375), p-PKCδ (9374), p-PKCδ (9377), PKD/PKCµ (2052), p-PKD/PKCµ (2054), lipin-1 (14906) (all purchased from Cell Signaling), c-Jun (Thermo Scientific, MA5-15172), PKCδ (sc-8402) and PCNA (sc-7907) (purchased from Santa Cruz), β-actin (Sigma Aldrich, A2228), and PKCβII (Abcam, ab38279). Secondary antibodies were as follows: goat anti-rabbit IgG-HRP (sc-2004) and goat anti-mouse IgG-HRP (sc-2005) (purchased from Santa Cruz).

**Cholesterol ester & Glycerolipid Analysis** – Extraction procedure and LC-MS/MS method adapted with modifications from Hutchins et al.®. Cell pellets were thawed on ice, resuspended in 1 mL PBS and sonicated for 1 minute. 500 µL of the cell homogenate was transferred to a borosilicate culture tube and spiked with 300 pmol of internal standards: 1,3(3d3)-dionadecanoloyl-2-dodecanoyl-glycerol, 1,3(3d3)-dionadecanoloyl-glycerol, 17:0 cholest-5-en-3β-yl heptadecanoate (Avanti® Polar Lipids, Inc). Samples were extracted with 2 mL of 75:25 (v/v) isooctane-ethyl acetate. The phases were separated by centrifugation (7 min, 1000 x g), and the organic layer was transferred to a clean borosilicate tube. The sample was then extracted further with another 2 mL of 75:25 (v/v) isooctane-ethyl acetate. Combined organic fractions were evaporated on a vacuum centrifuge. The residue was reconstituted in 100 µL of 4.5% methyl tert-butyl ether (MTBE) in hexane and transferred to a new vial for analysis. Vials were immediately placed in cooled (4°C) autosampler. Separation by normal phase high performance liquid chromatography (HPLC) was performed by loading 10 µL of each sample onto a 150 x 2 mm, 3 micron, Phenomenex Luna® silica column fitted with a 2.1 mm 2 µm assay frit. Lipid classes were separated by a 0.3 mL/min gradient of MTBE in hexane over 11 minutes. 4.5% MTBE was isocratic from 0 to 3
minutes; from 3 to 7.5 minutes MTBE was ramped to 45% where it was held until 9.5 minutes. MTBE was returned to 4.5% from 9.5 to 10 minutes where it remained until the end of the run at 11 minutes.

Before entry into the mass spectrometer, the eluent was modified via a mixing tee by the addition of 0.2 mL/min of an electrospray solvent containing 10 mM ammonium acetate in 45:45:5:5 (v/v/v/v), isopropanol-acetonitrile-water-dichloromethane delivered by a third HPLC pump (shimadzu LC-10AD).

Online LC-ESI MS/MS was performed on a QTRAP 6500 hybrid quadrupole/linear ion-trap mass spectrometer (AB Sciex). The relative abundance of glycerolipid species was monitored by neutral loss mass spectrometry. The instrument was operated in positive ion mode with an ion spray voltage of 5500 V. Neutral losses ([M+NH₄]⁺) of m/z 245 (14:0), 271 (16:1), 273 (16:0), 295 (18:3), 297 (18:2), 299 (18:1), 301 (18:0), 319 (20:5), 321 (20:4), were monitored using full scans over m/z 550 to m/z 1,000 collected every 2.4 seconds from 1.5 minutes to the end of the run at 11 minutes. Detection of cholesterol ester species was accomplished by using precursor ion scans in positive ion mode every 620 ms from 0-1.5 minutes with a product mass of m/z 369, [M+NH₄]⁺. Qualitative abundance of lipid species was determined using MultiQuant 2.1 software (Sciex). LC-MS/MS was conducted in the LIPID MAPS mass spectrometry facility in the Department of Pharmacology at the University of California San Diego, La Jolla, CA.

**ELISA Immunoassay Analysis** – TNF-α was measured using a commercially available Mouse TNF-alpha ELISA Ready-SET-Go® kit (Affymetrix eBioscience) according to the manufacturer’s instructions.

**Isolation and Staining of Splenocytes** – Spleens were homogenized in FACS wash buffer (1% bovine serum albumin, 1 mM EDTA, and 0.1% sodium azide in phosphate buffered saline). The spleens were strained with a 40 µm cell strainer (Falcon, 352340) followed by centrifugation at 300×g for 5 minutes. The supernatant was decanted and cells were dislodged in 3 mLs of ACK lysis buffer (0.15 M NH₄Cl, 10 mM KHCO₃, 0.1 mM Na₂EDTA, adjusted to pH 7.2 and filter sterilized in 0.22 µm filter). Cells were incubated on ice for 5 minutes. Cells were washed in FACS wash buffer with centrifugation. The pellet was re-suspended in 10 mLs RPMI 1640 (HyClone, SH30027.01), strained (Falcon, 352340), and counted. Cells were adjusted to 5×10⁶ cells/mL in RPMI. 100 µL of cells were mixed with 100 µL of blocking buffer (CD16/32 diluted in FACS wash buffer) and incubated at 4°C for 20 minutes. Cells were centrifuged at 1,500 rpm for 5 minutes and the supernatant was discarded. Primary labeled antibodies (50 µL per well) were added, light protected, and incubated at 4°C for 30 minutes. Cells were washed 3 times in FACS wash buffer with centrifugation. Cells were re-suspended in cold FACS/fix solution (FACS wash buffer with 0.1% formaldehyde), light protected, and incubated for 30 minutes at 4°C. Cells were washed in FACS wash buffer with centrifugation then cells were transferred to tubes in 500 µL FACS wash buffer. Flow cytometric analysis was performed on a BD LSRII (San Jose, CA). Antibodies used include: 1:800 CD11cBV786 (563735), 1:50 CD45.2 BV605 (56305), 1:1,000 CD3 PerCP (561089), and 1:800 Ly6G FItc (551460) purchased from BD Biosciences, and 1:4,000 CD4 e450 (48-0041-80), 1:2,000 CD8 APCe780 (47-0081-80), 1:400 NK1.1 APCe (17-5941-63), 1:4,000 CD11b PECyc7 (15-0112-81), 1:4,000 CD19 Pee610 (61-0193-80), 1:4,000 Ly6C PE (12-5932-80) and 1:200 CD16/CD32 (16-0161-81) purchased from eBioscience.

**Statistical analysis** – GraphPad Prism 5.0 (La Jolla, CA) was used for statistical analyses. Student T-test analysis was used for comparison between two data sets (FIG 1D). All other statistical significance was determined using a one-way ANOVA analysis of variance with a Dunnett’s post-test, P ≤ 0.05.


