SUPPLEMENTAL MATERIAL

Macrophage derived 25-hydroxycholesterol promotes vascular inflammation, atherogenesis and lesion remodeling.

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SUPPLEMENTAL METHODS

Animal experiments

C57BL6/J *(*WT*)* (stock number 000664), *Ldlr*$^{-/-}$ (stock number 002207) and *Ch25h*$^{-/-}$ (stock number 016263) mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). *Ldlr*$^{-/-}$ mice were bred to *Ch25h*$^{-/-}$ mice to generate the double knock out, *Ch25h*$^{-/-}$*;Ldlr*$^{-/-}$. To generate *Myh11ERT2CRE;mT/mG;Ldlr*$^{-/-}$ we crossed *Myh11ERT2CRE;mT/mG* mice, donated by Dr. Tellides (Yale University), with *Ldlr*$^{-/-}$ mice. To induce Cre-mediated excision, mice were injected intraperitoneally with tamoxifen (1 mg/mouse/day) at six weeks of age for five consecutive days. Tamoxifen (Sigma–Aldrich) was prepared by solubilizing 20 mg in 1 ml peanut oil (Sigma-Aldrich).

Bone marrow transplants were performed as previously described[51,95,96]. Briefly, eight-week-old male *Ldlr*$^{-/-}$ or *Myh11ERT2CRE;mT/mG;Ldlr-/-* mice were lethally irradiated twice with a dose of 550 rads (5.5 Gy) using a cesium source 4 h before transplantation. Bone marrow was collected from femurs of WT or *Ch25h*$^{-/-}$ mice by flushing with sterile Opti-MEM. Each recipient mouse was injected with $3x10^6$ bone marrow cells through retro-orbital injection. Four weeks after bone marrow transplantation (BMT), peripheral blood was collected by retro-orbital venous plexus puncture for PCR analysis of bone marrow reconstitution.

Atherosclerosis was induced by feeding mice for 12 weeks with a Western diet (WD) containing 40% fat and 1.25% cholesterol (D12108; Research Diets, Incorporated, New Brunswick, NJ, USA). Animal sample size for each study was chosen based on literature documentation of similar well-characterized experiments[24,51,95,96]. All experimental mice were housed in a barrier animal facility with constant temperature and humidity on a 12 h dark–light cycle with free access to water and food. All the experiments were approved by the Institutional Animal Care Use Committee of Yale University School of Medicine.

Human samples

Human left main coronary arteries were obtained from the explanted hearts of transplant recipients or cadaver organ donors as described previously [47]. Research protocols were approved by the Institutional Review Boards of Yale University and the New England Organ Bank. A waiver for consent was approved for surgical patients and written informed consent was obtained from a

member of the family for deceased organ donors. These samples were used for LC/MS-MS and immunohistochemistry analysis. Human samples utilized for the analysis of *CH25H* expression were described and analyzed from previously published single cell RNA-seq data of human plaques[48].

Lipoprotein profile and lipids measurements

Plasma lipids were measured as we previously described [51,96,97]. Briefly, mice were fasted for 12 h before blood samples were collected by retro-orbital venous plexus puncture for the "before diet" time point and by cardiac puncture for the "after diet" time point. Then, plasma was separated by centrifugation at 10,000xg for 10 min at 4ºC on a tabletop centrifuge. Total plasma cholesterol, high density lipoprotein (HDL)-cholesterol and triglycerides were enzymatically measured according to the manufacturer's instructions (Wako Pure ChemicalsTokyo, Japan). The lipid distributions in plasma lipoprotein fractions were assessed by fast-performance liquid chromatography gel filtration with 2 Superose 6 HR 10/30 columns (Pharmacia Biotech, Uppsala, Sweden). Total cholesterol was then measured in the different fractions.

Histology, immunohistochemistry, and morphometric analyses

After 12 months on WD, mice were euthanized and perfused with 10 ml PBS. Heart and aorta were collected and put in 10 ml 4% paraformaldehyde overnight. After incubation in paraformaldehyde, hearts were washed with PBS and left with PBS for 1 h. Next, hearts were put in 30% sucrose until the next day. Finally, hearts were embedded in OCT, frozen, and processed as described[96]. Serial sections were cut at 6 μm thickness using a cryostat. Three consecutive sections were collected on the same slide for a total of 20-25 slides, containing the whole aortic root. Every fifth slide from the library was stained with hematoxylin and eosin (H&E), and each consecutive slide was stained with Oil Red O (ORO) for quantification of the lesion area. Aortic lesion size of each animal was obtained by averaging the lesion areas in 9 to 12 sections from the same mouse. Necrotic core area was measured as a percentage of the total plaque area using the H&E slides[98]. Fibrous cap thickness was quantified by choosing the largest necrotic core from triplicate sections and taking a measurement from the thinnest part of the cap, determined by measuring the area between the outer edge of the cap and the necrotic core boundary[98]. Collagen content was assessed by Picrosirius red staining of 2 to 3 slides from the library. CD68 (Bio-Rad; #MCA1957; 1:200) and smooth muscle α-actin (Sigma; C6198; 1:500) immunodetection was used as a macrophage marker or SMC marker, respectively, in 2 to 3 slides from the library. Results were expressed as percentage of CD68 or SMA positive area with respect the total plaque area. VCAM (BD Biosciences; 550547; 1:100) immunodetection was performed in 2 slides form the library, and the measurement was expressed as percentage of VCAM positive area in the endothelium. Apoptotic cells in lesions were detected by TUNEL after proteinase K treatment, using the in-situ cell death detection kit, TMR red (Roche, Basel, Switzerland) according to the manufacturer's instructions. The data were expressed as the number of TUNEL-positive cells per square millimeter of cellular lesion area. In sections from *Myh11ERT2CRE;mT/mG;Ldlr*$^{-/-}$ animals, signal from SMCs was amplified using a GFP antibody (Abcam; ab13970; 1:200) for SMC lineage tracing. GFP positive area was represented as percentage of total plaque area or fibrous cap area. Fibrous cap area was delimited as previously described[94]. For all immunofluorescent preparations, the nuclei were counterstained with DAPI for 10 min. NIH ImageJ software (National Institutes of Health, Bethesda, MD, USA) was used for all the quantifications.

Fixed aortas were used to perform *en face* ORO staining as we previously described[51,96,97]. Briefly, aortas opened longitudinally were rinsed with 78% methanol, followed by staining with 0.16% ORO solution for 50 minutes, and then destained in 78% methanol for 5 min. The lesion area was quantified as a percentage of the ORO–stained area in the total aorta area[51,96,97].

Flow cytometry analysis of blood and aorta leukocytes

Blood was collected by retro-orbital puncture in heparinized microhematocrit capillary tubes and processed as previously described[96]. White blood cells were resuspended in 1% fetal bovine serum and 1% bovine serum albumin in PBS, blocked with 2 mg/ml FcgRII/III, then stained with a cocktail of antibodies. Cells were analyzed on a LSRII flow cytometer. Monocytes were identified as CD115^{high} and subsets as Ly6-C^{high} and Ly6-C^{low}. Neutrophils were identified as CD115^{low}, Ly5-C^{high}, and Ly6-G^{high}. B cells were identified as CD19^{+} CD45R/B220^{+}. T cells were divided into CD4^{+} and CD8^{+} cells. All antibodies used for flow cytometry experiments were purchased from BioLegend. Data was acquired on a BDTM LSR II, and FlowJoTM v10 was used to analyze the data.

Cytokines and chemokines analysis by Multiplex.

Plasma from *Ldlr*$^{-/-}$ transplanted with WT or Ch25h$^{-/-}$ BMfed on a WD for 12 weeks was collected by heart puncture and cytokines and chemokines were measured using the Cytokine 20-Plex Mouse Panel (Thermo Fisher Scientific, LMC0006M) according to the manufacturer's protocol.

Thioglycollate-elicited peritoneal lipid-laden macrophage isolation

20 week old *Ldlr*$^{-/-}$ and *Ch25h*$^{-/-}$*;Ldlr*$^{-/-}$ mice were intraperitoneal injected with 3 ml of 3% (w/v) of thioglycollate (BD; 211716). After 4 days, mice were euthanized, and peritoneal lavage was collected by washing the peritoneal cavity 3 times with 10 ml of PBS. After filtration by a 70μm cell strainer, the cells were pelleted by centrifugation (400xg for 5 min at 4°C). Cell pellet was resuspended in RPMI 1640 (Corning) with 10% FBS, 1% glutamine (Gibco), and 1% penicillin/streptomycin (HyClone), at a final concentration of 1x10^{6} cells/ml. For macrophage positive selection, cells were seeded on fibronectin coated tissue culture plates for 2 h, non-adherent cells were washed out, and macrophages were incubated in fresh medium.

Oil-red O and BODIPY cell staining

Hypercholesterolemic (*Ldlr*$^{-/-}$ background) lipid-laden thioglycollate-elicited peritoneal macrophages (TG-EPM) from WT*, Ch25h*$^{-/-}$*, Ldlr*$^{-/-}$ and *Ch25h*$^{-/-}$*;Ldlr*$^{-/-}$ mice were seeded on glass coverslips and fixed with 4% PFA in PBS for 1 h and stained for 30 min with 0.36% ORO solution in 60% isopropanol. Alternatively, peritoneal macrophages isolated from *Ldlr*$^{-/-}$ and *Ch25h*$^{-/-}$*;Ldlr*$^{-/-}$ mice were directly stained with 2 μM BODIPY (Thermo Fisher Scientific; D3922) for 15 min at 37°C. After 3 washes with PBS, cells were analyzed on a LSRII flow cytometer.

RNA isolation and quantitative real-time PCR

Total RNA from mouse aortas was isolated using the Bullet Blender Homogenizer (Next Advance, Averill Park, NY, USA) in TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Total RNA from bone marrow derived macrophages (BMDMs) or TG-EPM was isolated, extracted, and purified using the RNeasy isolation Kit (Qiagen) according to the manufacturer's instructions. One microgram of total RNA was reverse transcribed using the iScript RT Supermix (Bio-Rad, Hercules, CA), following the manufacturer's protocol. Quantitative real-time PCR was performed in triplicate using iQ SYBR green Supermix (Bio- Rad) on a Real-Time Detection System (Bio-Rad). The mRNA level was normalized to ribosomal RNA 18S as a housekeeping

gene. The following mouse primer sequences were used: CD68, 5′-CCAATTCAGGGTGGAAGAAA-3′ and 5′-CTCGGGCTCTGATGTAGGTC-3′; IL-6, 5′-AGTTGCCTTCTTGGGACTGA-3′ and 5′-TCCACGATTTCCCAGAGAAC-3′; TNF-*α*, 5′-CCCTCACACTCAGATCATCTTCT-3′ and 5′-GCTACGACGTGGGCTACAG-3′; IL-1β, 5′-CCAAAATACCTGTGGCCTTGG-3′ and 5′-GCTTGTGCTCTGCTTGTGAG-3′; MerTK, 5′-TGCGTTTAATCACACCATTGGA-3′ and 5′-TGCCCCGAGCAATTCTTTC-3′; 18S, 5'-TTCCGATAACGAACGAGACTCT-3' and 5'-TGGCTGAACGCCACTTGTC-3'.

Bulk RNA sequencing and analysis

Lipid-laden TG-EPM were treated with 100 ng/ml LPS (Sigma; L8274) for 4 or 12 h or mock treated with vehicle (PBS). RNA was extracted and purified using the RNeasy isolation Kit (Qiagen) and treated with DNAse to remove genomic contamination (RNA MinElute Cleanup, Qiagen). The purity and integrity of total RNA was verified using the Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA). rRNA was depleted from the RNA samples using Ribo-Zero rRNA Removal Kit (Illumina). RNA libraries were made using TrueSeq Small RNA Library preparation (Illumina) and were sequenced for 45 cycles on Illumina HiSeq 2000 platform (1 × 100bp read length). Using the PartekFlow® software, version 8.0.19.0405 (Partek, Inc., St. Louis, MO) the reads were aligned with the STAR algorithm to the mouse genome mmu10. The quantify to annotation model (Partek E/M) algorithm was used to estimate the transcript expression abundance. Counts were normalized using the recommended methods (CPM, Add 0.0001, Log2). To identify differential expression patterns the Differential Gene Expression-GSA algorithm was implemented. A default P-value ≤ 0.05 was considered statistically significant with a fold-change ≥ 1.5 for up-regulated transcripts or ≤ -1.5 for down-regulated transcripts. Multiple test correction was not applied for this data set. Using the significance criteria described before, we created a list of differentially expressed genes that we used to generate heat maps and KEGG pathway analysis using PartekFlow® software, version 8.0.19.0405 (Partek, Inc., St. Louis, MO). Additionally, we analyzed the list of differentially expressed genes with IPA® for upstream regulator analysis. Raw and normalized data are accessible in NCBI Gene Expression Omnibus and through GEO Series accession number GSE189079.

Bulk RNA-seq analysis of BODIPY^{hi},SSC^{hi} (foamy macrophages) and BODIPY^{lo},SSC^{lo} (non-foamy macrophages) isolated from the aortic tissues of *Apolipoprotein E* (*Apoe*) deficient mice[10], was done from extracted data accessible at NCBI GEO database [10] accession GSE116239. Analysis was performed using the PartekFlow® software, version 8.0.19.0405 (Partek, Inc., St. Louis, MO) as described above. For this data set we used multiple test correction and a default adjusted P value < ≤ 0.05 was considered statistically significant with a fold-change ≥ 2 for up-regulated transcripts or ≤ -2 for down-regulated transcripts.

To analyze LXR target genes in our RNA-seq data, we used a list of 363 genes published by Ramón-Vázquez A. et al. [60] as a true LXR target genes in macrophages. The authors created this list by comparing Chip-seq data with mRNA expression data of macrophages after LXR activation. We applied this list to our RNA-seq data, and we considered statistically significant those genes with a p-value ≤ 0.05 and with a fold-change ≥ 1.5 for up-regulated transcripts or ≤ -1.5 for down-regulated transcripts. Multiple test correction was not applied for this data set.

For SREBP2 target genes, we analyzed the genes that are directly involved in *de novo* biosynthesis of cholesterol [61]. This list includes a total of 27 gene. We considered statistically significant those genes with a p-value ≤ 0.05 and with a fold-change ≥ 1.5 for up-regulated

transcripts or ≤ -1.5 for down-regulated transcripts. Multiple test correction was not applied for this data set.

Single Cell RNA-sequencing analysis

Analysis of scRNA-seq to determine the expression of Ch25h in different macrophage population obtained from human plaques was performed from previous published data and as described[48].

Sequencing data was extracted from two moused data sets (*Ldlr*$^{-/-}$ 26 weeks WD [GSM4705598] from GSE15513[49] and total leukocytes from *Ldlr*$^{-/-}$ aorta [GSM3215435] from GSE116240)[10] subjected to 12 weeks of WD. These datasets were pre-processed using cellranger with default parameters and mm10 reference genome and annotation. Low-quality cells, doublets, and potentially dead cells were filtered based on the percentage of mitochondrial genes, number of genes and UMIs expressed in each cell. After filtering, we identified 6041 cells in dataset GSM4705598 and 3781 cells in dataset GSM3215435 for downstream analysis. Data clustering was performed using Seurat R package (version 3.0) with filtered genes by barcode expression matrices as inputs. Highly variable genes (HVGs) were calculated using Seurat function *FindVariableGenes* and used for downstream clustering analysis. Principal component analysis (PCA) was performed with *RunPCA* function (Seurat) using HVGs for dimensionality reduction, and the number of significant principal components was calculated using *JackStraw* function. We applied the *RunUMAP* function to significant principal components (PCs) identified by *JackStraw* analysis and presented data in two-dimensional coordinates through t-distributed stochastic neighbor embedding generated by R package ggplot2. Different number of clusters were identified in dataset GSM4705598 and in dataset GSM3215435 (Resolution = 0.25). From dataset GSM4705598, we subsetted on the CSF1R and ADGRE1 expressing macrophage population to identify a total of 4 macrophage populations, upon which downstream analysis was performed. Similar approach was also performed in dataset GSM4705598. Significantly differentially expressed genes in a cluster were analyzed using Seurat function *FindAllMarkers,* which were expressed in more than 25% of cells with at least 0.25-fold difference and reached statistical significance of an adjusted $P < 0.05$ as determined by the Wilcox test. Cell types were annotated based on marker identification genes and analyzed for expression of genes of interest. dataset GSM4705598

Western blotting

Lipid-laden TG-EPM were lysed in ice-cold Ripa buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, and 0.5% sodium deoxycholate) supplemented with 1 mM orthovanadate, and 1 mg/ml cOmplete™, Mini Protease Inhibitor Cocktail (Roche; 11836153001), 0.25 mg/ml AEBSF (Sigma; SBR00015-1ML) and 1 mg/ml PhosSTOP™ (Roche; 4906845001). Cell lysates were rotated at 4°C for 1 h before the insoluble material was removed by centrifugation at 12,000xg for 20 min. After normalizing for equal protein concentration, cell lysates were resuspended in Laemmli buffer (Bioworld; 50-196-785) before separation by SDS-PAGE.

Western blots were performed using the following antibodies: rabbit polyclonal antibodies from Cell Signaling against phospho-p65 (3033S), total p65 (8242S), phospho-p38 MAPK (4511S), total p38 MAPK (8690S), phospho-IRF3 (29047S), total IRF3 (4302S), and caspase-1 (24232S). Mouse monoclonal anti-ABCA1 antibody was purchased from Abcam (ab18180) and rabbit polyclonal anti-ABCG1 antibody from Novus Biologicals (NB400-132). From Gen Tex IL1β

(GTX74034). From Gen Tex IL1β (GTX74034). Mouse monoclonal antibody against HSP-90 was from BD Biosciences (610419). Secondary antibodies Alexa Fluor 700 (ThermoFisher Scientific; A21036) and Alexa Fluor 800 (ThermoFisher Scientific; A32730). Protein bands were visualized using the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA). Densitometry analysis of the gels was carried out using NIH ImageJ software (http://rsbweb.nih.gov/ij/).

NLRP3 inflammasome activation.

To study inflammasome activation, lipid-laden TG-EPM were plated $1x10^6$ cells/ml in RPMI 1640 containing 10% FBS. The following day, media was removed, cells were washed two times with PBS and RPMI 1640 without FBS was added. Then, cells were primed with LPS (100 ng/ml, 8 h) followed by treatment with ATP (5mM, Sigma-Aldrich, A7699) for the last 30 minutes. The cell lysates were collected after ATP treatment and analyzed for caspase-1 (Adipogene, AG-20B-0042-C100) and IL-1β (Genetex, GTX74034) by western blot. Media was also collected for IL-1β detection by western blot. The cell supernatants were collected and concentrated using Amicon Ultra 10K filtration units (Millipore) and IL-1β was detected by western blot. Mouse monoclonal antibody against b-actin was from BD Biosciences (610419).

Efferocytosis assay

$5x10^5$ lipid-laden TG-EPM were plated with RPMI 1640 (Invitrogen), with 20% FBS and 20% L-cell-conditioned medium for 2 days. Then, cells were washed twice with PBS and fresh RPMI 1640 10% FBS was added. Macrophages were treated with 100ng/ml of LPS for 12 h. After treatment, apoptotic cells (AC) were incorporated into the media in a 1:1 ratio (ACs: macrophages) and cultured at 37°C for 60 min to allow efferocytosis. After incubation with ACs, macrophages were washed several times with cold PBS with EDTA (0.6 μM) to remove free AC. Macrophages were harvested and stained with F4/80-FITC (BioLegend; 123107). Data was acquired by Amnis Imagestream-X MarkII Imaging Flow Cytometer and analysis was performed using IDEAS® Software. In average, 500 cells per experiment were analyzed. Efferocytosis was expressed as phagocytic index (PI): number of cells ingested per total number of macrophages × 100.

To generate AC, Jurkat T cells were labeled with CellTrackerTM Deep Red Dye (ThermoFisher, C34565) according to the manufacturer's instructions. Labeled cells were U.V. irradiated for 3 min 3 times, with gentle mixing before each time, using an 8W bulb and incubated at 37°C for 2 h to induce apoptosis. Fluorescent ACs were added to peritoneal macrophages in RPMI 1640 supplemented with 10% FBS.

Efferocytosis was determined *in situ* following stablished procedures described before [69-72]. Briefly, individual aortic root sections stained with TUNEL and CD68 (as explained at the Histology, immunohistochemistry, and morphometric analyses section) were used to count the number of macrophage-associated versus free apoptotic cells. We considered free apoptotic cells those TUNEL-positive nuclei that do not overlap with CD68, whereas TUNEL-positive nuclei that do overlap with CD68 were considered phagocytosed apoptotic cells. Pictures of three consecutive sections of the aortic root, were used for quantification. The results were represented as the ratio between phagocytosed and free apoptotic cells.

LC-MS/MS analysis

Peritoneal macrophages isolated from 20 week-old *Ldlr*$^{-/-}$ and *Ch25h*$^{-/-}$;*Ldlr*$^{-/-}$ mice were plated in RPMI 1640 with 10% FBS and treated with 100ng/ml LPS for 4 or 12 h. After treatment,

media was collected, cells were washed twice with cold PBS, and harvested. Lipids were extracted from the samples by a modified Bligh-Dyer extraction and sterols were analyzed by LC-MS/MS as described previously [99-101]. Briefly, lipid extracts from samples were dried under nitrogen and reconstituted in methanol. Sterols were analyzed using a Shimadzu LC20A HPLC (Kyoto, Japan) equipped with an Agilent Poroshell 120 EC-C18 column (2.1 × 150 mm, 2.7 micron beads, Agilent Technologies, Wilmington, DE). The elution was operated using a solvent gradient that transitioned linearly from 93% methanol/7% H2O to 100% methanol in 7 min. The column was washed for 5 min in 100% methanol and then returned to the initial solvent. Sterols were detected using an ABSciex (Framingham, MA) 4000 Qtrap MS/MS equipped with a Turbo V APCI source in positive mode with atmospheric pressure chemical ionization at a temperature of 350°C. The MS/MS detected mass to charge ratios (m/z) of 365–370, 393–400, 404, and 409–414, which spans the ion m/z plus 3 mass units for each sterol, along with the internal standards. The internal standards were commercially available for all but four of the sterols (dihydro-ff-MAS, dihydro-t-MAS, dehydrolathosterol, and dehydrodesmosterol) in the cholesterol biosynthetic pathway that were identified by their unique m/z values and retention times as described [99-101].

Mitosox and Mitotracker analysis

Lipid-laden TG-EPM isolated from *Ldlr*$^{-/-}$ and *Ch25h*$^{-/-}$;*Ldlr*$^{-/-}$ mice were plated in RPMI 1640 with 10% FBS and treated with 100ng/ml LPS for 4 or 12 h. After treatment, cells were washed twice with cold PBS, harvested, and stained with MitoSOX™ Red Mitochondrial Superoxide Indicator (ThermoFisher; M36008) and MitoTracker™ Green FM (ThermoFisher; M7514) according to the manufacturer's instructions. Data was acquired on a BDTM LSR II and FlowJoTM v10 was used to analyze the data.

VSMC migration assay

SMC were isolated from *Myh11ERT2CRE;mT/mG* mice. 6-week-old mice were injected intraperitoneally with tamoxifen (1 mg/mouse/day) for 5 consecutive days to induce eGFP expression in SMCs. 2 weeks later, the thoracic aorta of 3 mice was collected, digested as described above, and pooled together. Then, eGFP^{+} SMCs were sorted using a BD FACSAriaTM II and resuspended in DMEM. 5x10^{4} cells (per well) were plated on gelatin (0.1% w/v; Sigma; G1890) coated trans-well inserts (Sigma; CLS3464). Platelet-derived growth factor (PDGF-BB) at 10 ng/mL dissolved in DMEM medium containing 0.1% FBS was added in the bottom chamber. Ethanol (0.44% v/v) or 25-HC (5μM) dissolved in ethanol was added into the bottom chamber media. After 6 h of incubation, cells on both sides of the membrane were fixed and stained with the Differential Quik III Stain Kit (Polysciences; NC1796273). Cells on the upper side of the membrane were removed with a cotton swab. Images of 5 randomly selected fields from the lower side of each transwell were used for quantification.

PDGF signaling and dorsal ruffle assay

SMC were planted on 6 well plates at final density of 1 × 10^{6} cells. After 24 hours in growth media, cells were serum starved for 12h. After starvation SMC were pre-treated with 25-HC (5μM) or ethanol (0.44% v/v) for 2 hours. After that time, SMC were stimulated with PDGF-BB at 10 ng/mL for 2, 5, 10, 20 minutes. Then, media was removed, and cells were washed once with cold PBS and processed for western blot analysis. Antibodies against phospho-AKT (CST; 13038), AKT (CST; 4691), phospho-ERK1/2 (CST; 4370) and ERK1/2 (CST; 4695) were used.

SMC were planted on Nunc Lab Tek chamber slides (Thermo Fisher Scientific;154534) at a concentration of 3 × 10^{4} cells. After 24 hours in growth media, cells were serum starvated for

12h. After starvation SMC were pre-treated with 25-HC (5μM) or ethanol (0.44% v/v) for 2 hours. After that time, SMC were stimulated with PDGF-BB at 10 ng/mL for 5, 10, 20 minutes. Then, cells were fixed on 4% paraformaldehyde for 10 minutes at room temperature. After 3 washes with PBS, we permeabilized the cells with 0.1% Triton in PBS (v/v) for 15 minutes. Then, we washed 3 times with PBS to later incubate the cells with 1% BSA in PBS (w/v) for 30 minutes. Finally, cells were stained with 1X solution of fluorescent phalloidin (Thermo Fisher Scientific; A12381) and a of DAPI solution (5 ng/ml), to stain the nuclei for, 30 minutes. After staining, cells were washed 3 times with PBS and visualized with an EVOS M5000. Two random picture per chamber were taken using the EVOS M5000.

ALOD4 purification and labeling

ALOD4 expression vector (Addgene; 111026) was purified and labeled as described elsewhere[102]. Briefly, bacterial cultures overexpressing pALOD4 were collected in lysis buffer (50 mM Tris-HCl, pH 7.5, 1 mM Tris(2-carboxyethyl) phosphine, 150 mM NaCl, 1 mg/mL lysozyme, 400 µg/mL PMSF, and EDTA-free protease inhibitor) and homogenized using a Dounce homogenizer. Lysates were incubated for 3 h at 4°C for lysozyme disruption. After the incubation, the lysozyme-disrupted lysate was sonicated using a tip sonicator. Lysate was centrifuged for 1 hour at 220,000xg and the supernatant was filtered using a 0.22 μm filter. ALOD4 protein was purified using a pre-packed 5-mL HisTrap-HP Ni column on a FPLC system. Eluted fractions containing ALOD4 were pooled together and purified again using a pre-packed 1-mL HiTrap Q HP anion exchange column connected to the FPLC. Finally, a Tricorn 10/300 Superdex 200 gel filtration column was connected to the FPLC and the eluted ALOD4 was loaded and eluted. Protein rich fractions were pooled and concentrated using an Amicon Ultra-4 10 kDa cutoff centrifugal filter to a concentration of 1–2 mg/ml and stored at 4°C for use over the next 4 weeks.

For ALOD4 labeling, 20 nanomoles (320xg) of purified ALOD4 was mixed with 200 nanomoles of Alexa Fluor 680. Mix was placed on a rotator at 4°C for 16 h. The reaction was quenched using 10mM DTT. Unbound fluorescent label and DTT were removed by dialysis (Millipore Sigma; 71507).

ALOD4 binding to plasma membrane cholesterol assay

Freshly isolated TG-EPM were isolated and cultured as described above. Sphingomyelinase (Sigma; S9396) was added into the media (200 mU/ml), and cells were incubated for 30 min at 37°C before treatment with 100ng/ml of LPS. After the treatment, cells were washed twice with cold PBS and 3μM ALOD4 was added to the cells. ALOD4 was incubated for 1 h at 4°C. After incubation, cells were washed twice with cold PBS and directly lysed with lysing buffer for western blot. ALOD4 was detected using an Anti-His tag antibody (Abcam, ab18184).

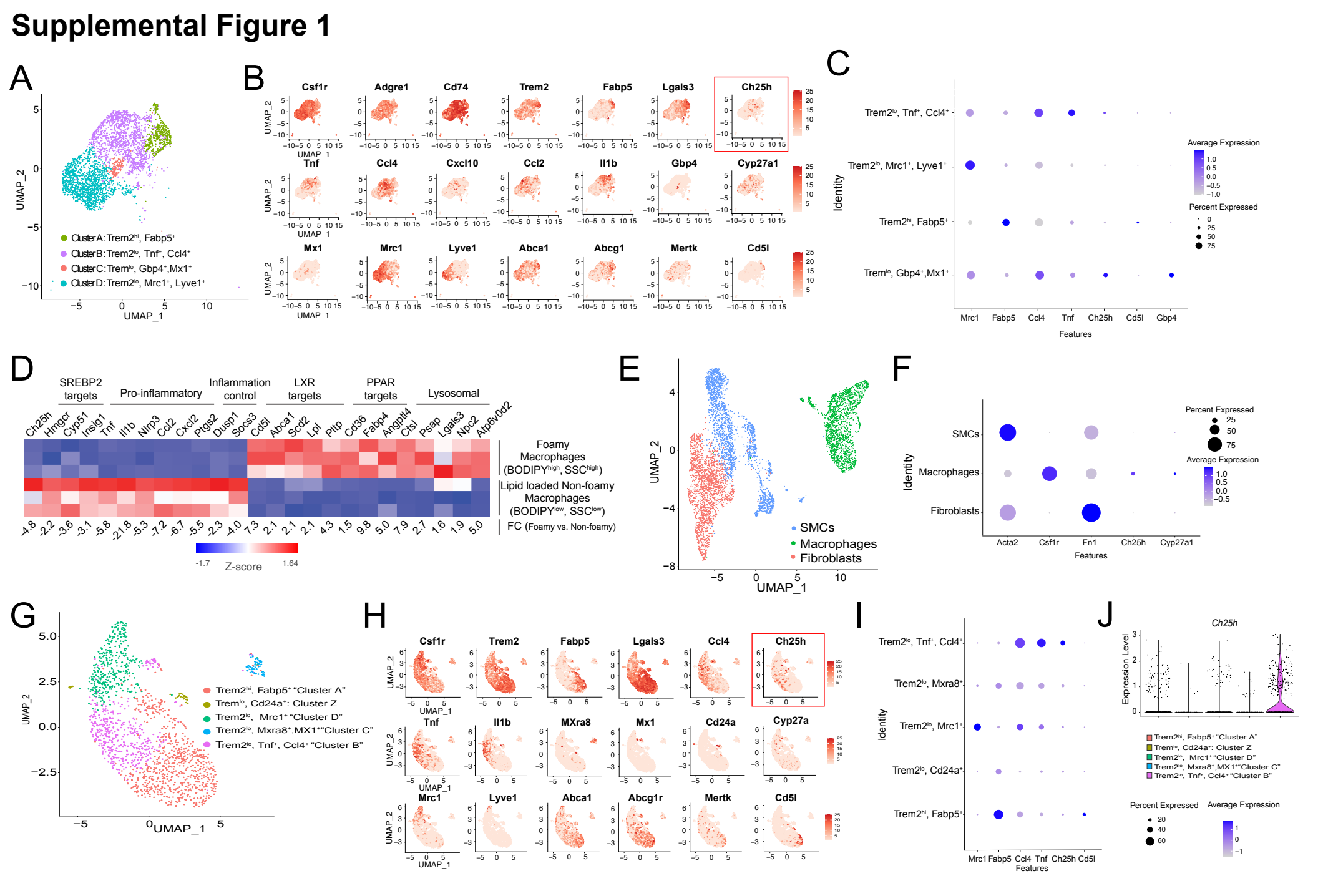
In other experiments, freshly isolated lipid-laden TG-EPM were directly stained with Alexa Fluor 680-ALOD4 at 4°C for 1 h and then macrophages were stained with F4/80 as described above. The level of ALOD4 bound to F4/80+ cells was quantified using a BDTM LSR II, and FlowJoTM v10 was used to analyze the data.

Statistical analysis

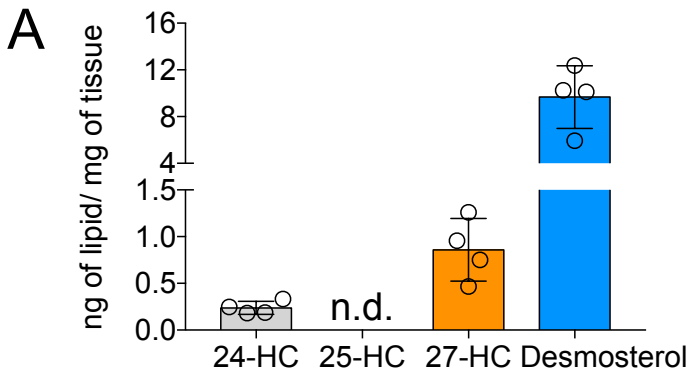
Animal sample size for each study was chosen based on literature documentation of similar well-characterized experiments. The number of animals used in each study is listed in the figure legends. *In vitro* experiments were routinely repeated at least three times unless otherwise noted. No inclusion or exclusion criteria were used, and studies were not blinded to investigators or

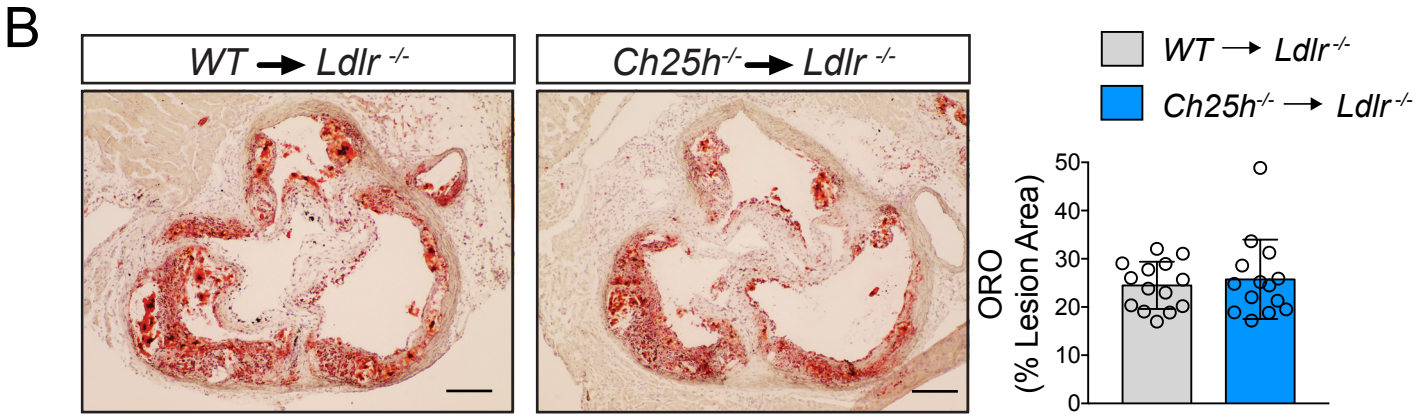
formally randomized. Data are expressed as average ± SD or ± SEM. Statistical differences were measured using an unpaired two-sided Student's *t*-test, one-way ANOVA with Bonferroni correction for multiple comparisons. Normality was checked using the Kolmogorov-Smirnov test. A nonparametric test (Mann-Whitney) was used when data did not pass the normality test or for experiments with small sample size. A value of $P \leq 0.05$ was considered statistically significant. Data analysis was performed using GraphPad Prism Software Version 7 (GraphPad, San Diego, CA).

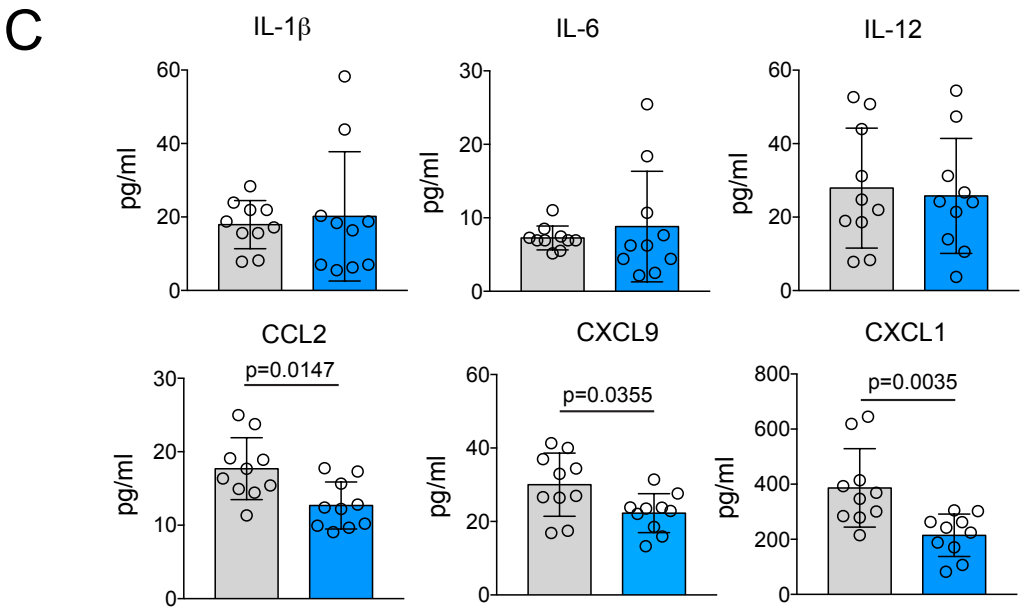
Supplemental Figure 1



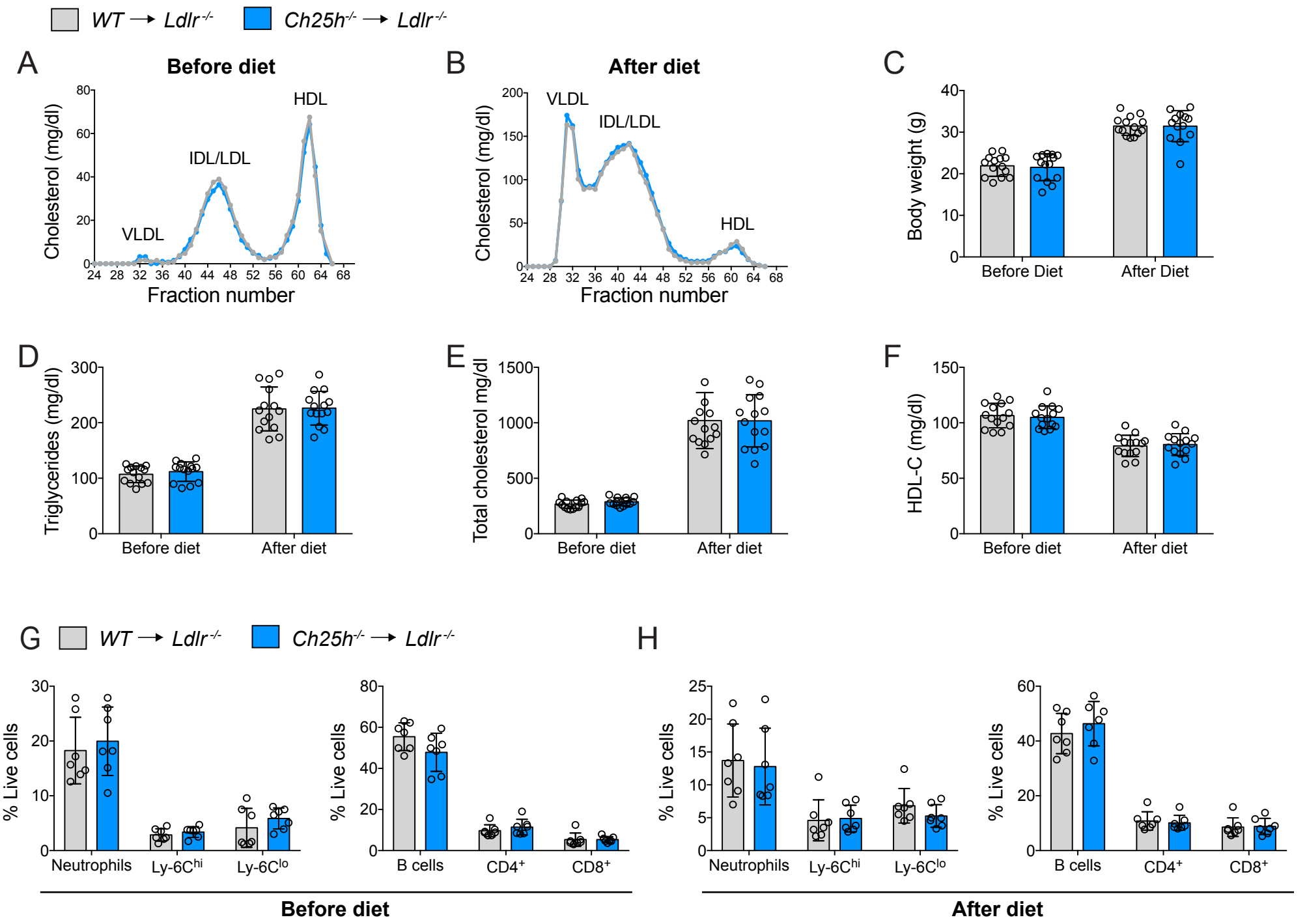
Supplemental Figure 2



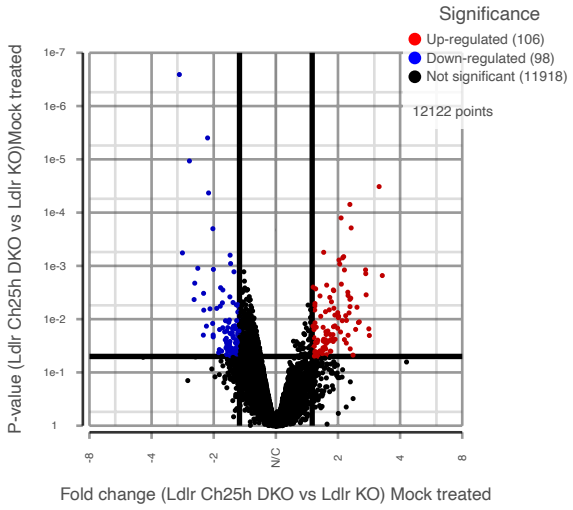


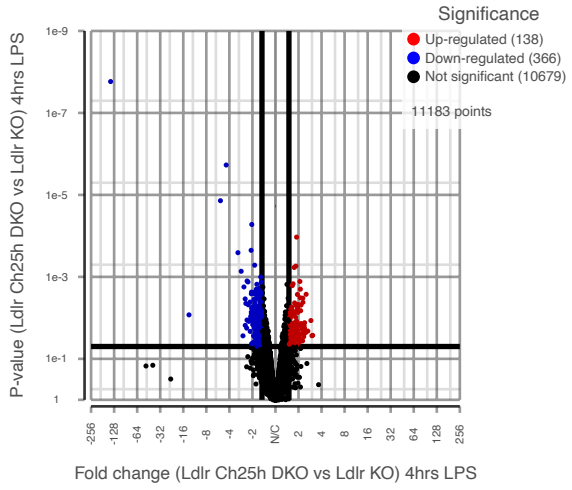


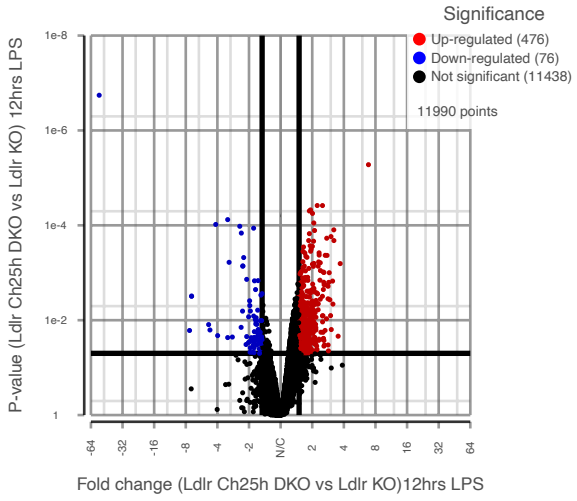
Supplemental Figure 3



Supplemental Figure 4

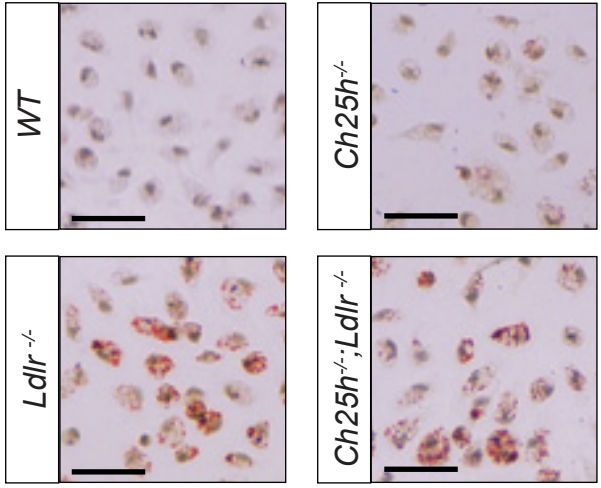






Supplemental Figure 5

A



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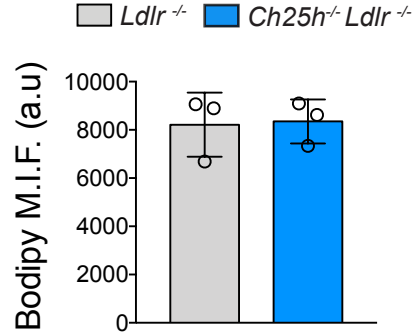
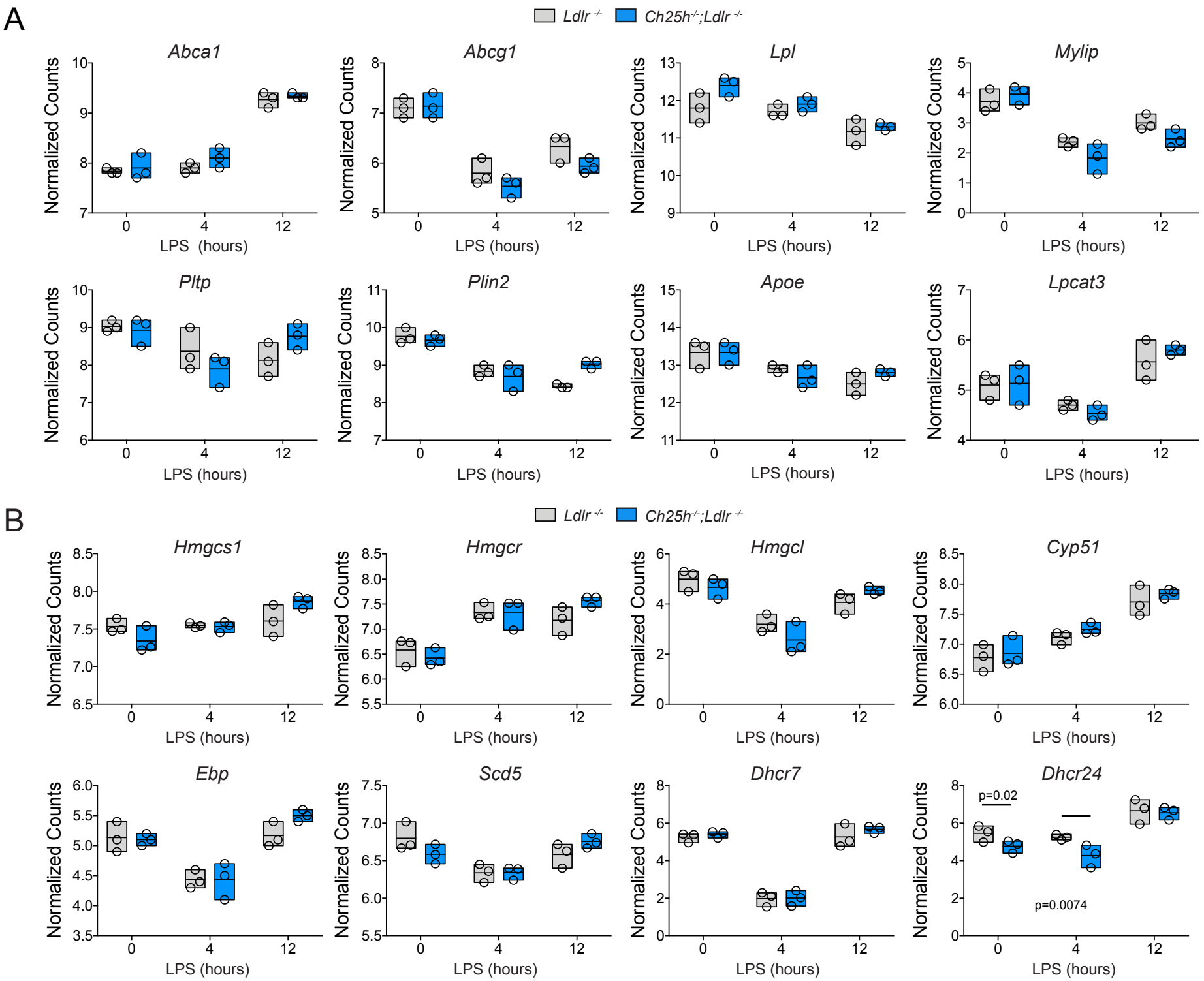
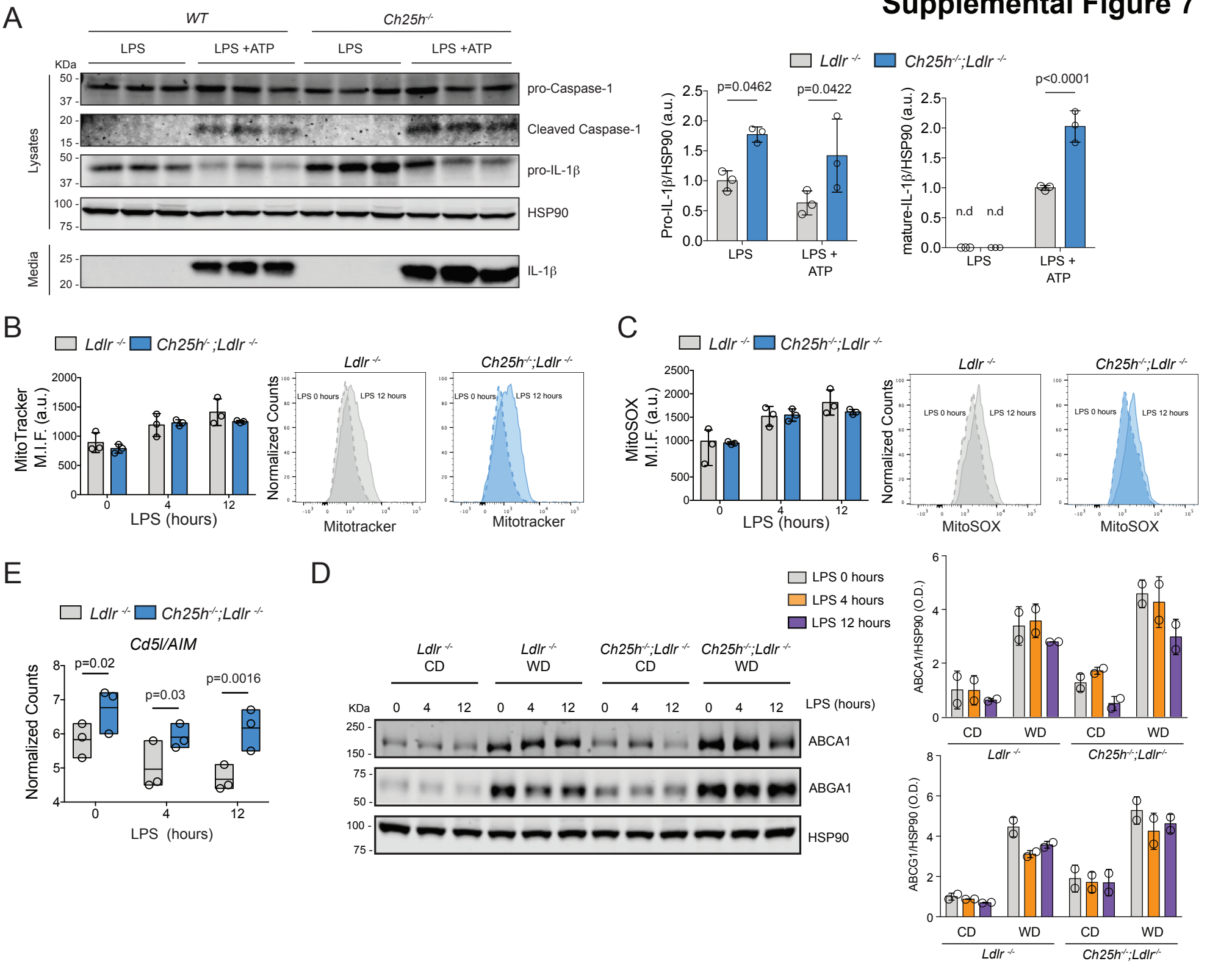


Supplemental Figure 6

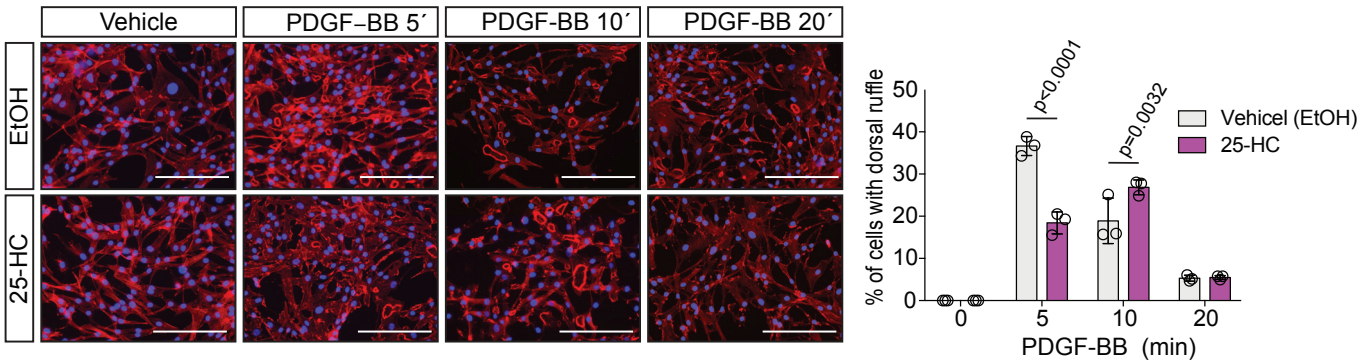


Supplemental Figure 7



Supplemental Figure 8

A



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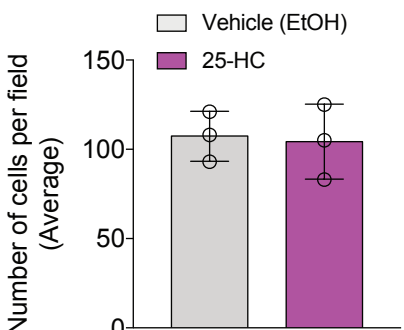

SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure 1. Single Cell RNA Sequencing and bulk RNA sequencing analyses of data extracted from datasets of cells isolated from mouse aortic tissues. A-C, scRNA-seq data set of total leukocytes isolated from *Ldlr-/-* aorta of mice fed on a WD for 12 weeks [GSM3215435] from GSE116240[10]. **A,** UMAP plot representation and **B**, Feature plots, on a UMAP plots, showing the expression of top marker genes of each cluster of macrophage population that was sub-set from leukocyte population based on of Csf1r and Adgre1 expression (See **B**, when referring to specific gene markers). Other genes of interests are also shown (*Ch25h, Cyp27a, Cd5L, Abca1, Abcg1* and *Mertk)*. **A**, Four different clusters were further detected based on gene markers of existing literature[103,104] and data extracted from GSE116239[10]. *Trem2* (increased by lipid levels[103,105] expressed in all different macrophage clusters with different expression levels[103]. Different macrophage clusters categorized as *Trem2*^{hi} and *Trem2*^{lo} when expression was higher or below the 20-threshold level, respectively. *Trem2*^{hi} macrophages were restricted to only one cluster of macrophages that presented almost exclusive expression of *Fabp5* (fatty acid binding protein 5, which regulates lipid uptake and has been linked to reduced inflammatory response of macrophages[106-109]), overlapping expression of *Lgals3* (Galectin-3 *aka* Mac 2, linked to anti-inflammatory macrophages)[110] and excluded expression of inflammatory markers (such as *Tnf, Ccl4, Cxcl10, Ccl2* and *Il1b*). This cluster was designated as Cluster A of lipid loaded foamy non-inflammatory macrophages (*Trem2*^{hi} and *Fabp5*^{hi}) with *Ch25h* almost undetectable expression of *Ch25h* (See **B**). The remaining three clusters were different subtypes of macrophages, based on different gene expression signatures (see **B**), but all lipid loaded macrophages toa certain extend, based on *Trem2* detection (*Trem2*^{lo}). Cluster B (inflammatory macrophages) was defined as *Trem2*^{lo}, *Tnf*$^{+}$, *Ccl4*$^{+}$. This Cluster also express additional inflammatory genes, such as *Il1b*, *Ccl2* and *Cxcl10*. Cluster C (lipid loaded, INF-responsive inflammatory macrophages) defined as *Trem2*^{lo}, *Gpb4*$^{+}$, *Mx1*$^{+}$, with exclusive expression of IFN-stimulated genes *Mx1*[111] and *Gbp4*[112] which is also induced by lipid loading[113]. This Cluster also express inflammatory genes that overlapped with Cluster B such as *Cxcl10*, *Ccl2*, *Tn*f and *Ccl4*, except for Il1b that was not present in Cluster C. *Ch25h* expression is also detected in these two clusters of inflammatory macrophages, Cluster C more unequivocally linked with lipid loaded macrophages (i.e., *Trem2*^{lo}, *Gbp4*$^{+}$). Cluster D of resident-like adventitial anti-inflammatory "M2-like" macrophages[104] 3.defined as *Trem2*^{lo}, *Mrc1*$^{+}$, *Lyve1*$^{+}$. **C**, Dot plot summary of expression of marker genes across clusters and for *Ch25h* and *Cd5L*. Circle size is proportional to the percentage of cells in each cluster expressing the marker and circle color represents average marker gene expression in the cluster. **D**, Heatmap of representative genes involve in atherogenesis differentially expressed in of BODIPY^{hi},SSC^{hi} (foamy macrophages) and BODIPY^{lo},SSC^{lo} (non-foamy macrophages) from the aortic tissues from *Apoe*$^{-/-}$ mice fed on a WD for 12 weeks (n= 6) GSE116239[10]. A default adjusted *P*-value ≤ 0.05 was considered statistically significant with a fold-change ≥ 2 for up-regulated transcripts or ≤ 2 for down-regulated transcripts. Fold-change (FC) for each gene is shown in the heatmap. **E-J**, Data set of total cells isolated from aortic tissue from *Ldlr-/-* 26 weeks in WD [GSM4705598] from GSE15513[49]. **E**, UMAP plot of the single cell RNA-seq clustering and **F**, Dot plot for marker genes of the defined clusters (SMC, Macrophages and Fibroblast) and for *Ch25h* and *Cyp27a* expression. **G**, UMAP plot of macrophage population from **E** that was sub-setted from total cell population based on of Csf1r and Adgre1. Five clusters are detected as indicated. Similar macrophages cluster identified as in **A** are denoted with same "cluster" naming. **H,** Feature plots show the expression of top marker genes of each cluster on a UMAP plot. Feature plot of genes of interests are also shown (*Ch25h, Cyp27a, Cd5L, Abca1, Abcg1 and Mertk*). **I**, Dot plot summary of expression of marker genes

across clusters. Gene of interest *Ch25h* and *Cd5L* are represented for comparison. **J**, Violin plot for *Ch25h* expression in all identified clusters in **G-I**.

Supplemental Figure 2. Absences of *Ch25h* in the hematopoietic compartment does not alter lipid accumulation and monocyte/macrophages percentage. A, LC/MS quantification of several lipids presents in the aorta of *Ldlr*$^{-/-}$ transplanted with Ch25h$^{-/-}$ BM (n= 4 aortas). **B**, Representative histological analysis of cross sections of the aortic sinus stained with ORO of *Ldlr*$^{-/-}$ mice transplanted with bone marrow from WT or *Ch25h*$^{-/-}$ donor mice and fed for 12 weeks a WD. Right panel, quantification of the ORO positive area is showing in the right panel and represents the mean ±SD (n=14 mice per group). Data were analyzed by Mann-Whitney non-parametric test. Scale bar: 100 µm. **C**, Cytokines and chemokines analysis in plasma of *Ldlr*$^{-/-}$ mice transplanted with WT or *Ch25h*$^{-/-}$ bone marrow and fed for 12 weeks on a WD (n=10 mice per group). Data were analyzed by Mann-Whitney non-parametric test.

Supplemental Figure 3. Absences of *Ch25h* hematopoietic cells does not affect, body weight, plasma lipids levels and circulating leukocytes. A-B, Lipoprotein profile in pooled plasma, **C**, body weight, **D**, triglyceride plasma levels, **E**, total cholesterol plasma levels, **F**, HDL-cholesterol plasma levels of *Ldlr*$^{-/-}$ mice transplanted with bone marrow from WT or *Ch25h*$^{-/-}$ donor mice and fed for 12 weeks a WD. **A-F** (n=14). **G-H**, percentage of circulating leukocytes analyzed by flow cytometry, measured in *Ldlr*$^{-/-}$ transplanted with bone marrow from WT or *Ch25h*$^{-/-}$ donors (n=7, analyzed per group out of 14 randomly selected). Data were analyzed by two-way ANOVA, post-hoc Bonferroni´s multiple comparison test.

Supplemental Figure 4. Volcano plots showing differentially expressed genes in macrophages isolated from *Ldlr*$^{-/-}$ and *Ch25h*$^{-/-}$*;Ldlr*$^{-/-}$. Macrophages were treated with LPS (100ng/ml) for 4 or 12 hours. (n=3 per genotype) Genes with a p-value < 0.05 and a fold change > 1.5 are represented by red dots. Genes with a p-value < 0.05 and a fold change < -1.5 are represented by blue dots.

Supplemental Figure 5. Absence of *Ch25h* does not affect their ability to form foam cells. A, Representative images of lipid-laden TG-EPM from 20 weeks old WT, *Ch25h*$^{-/-}$, *Ldlr*$^{-/-}$ or *Ch25h*$^{-/-}$*;Ldlr*$^{-/-}$ mice, stained with Oil Red O (ORO). **B**, Flow cytometry analysis of TG-EPM peritoneal macrophages freshly isolated and stained with BODIPY^{TM} 493/503. Quantification corresponds to the BODIPY fluorescence intensity (M.F.I.) in arbitrary units (a.u.). ±SD (n=3 per group). Data were analyzed by Mann-Whitney non-parametric test.

Supplemental Figure 6. Deficiency of *Ch25h* or lack of synthesis of 25HC in activated lipid-laden macrophages does not influences LXR-mediated or SREBP-mediated transcriptional gene expression. A, Comparison of normalize counts of well stablished LXR target genes[60] of lipid-laden TG-EPM from *Ldlr*$^{-/-}$ or *Ch25h*$^{-/-}$ *Ldlr*$^{-/-}$ treated with LPS (100 ng/ml) for 4 or 12 hours (n=3 per group). **B**, Comparison of normalize counts of well stablished SREBP target genes[61] of lipid-laden TG-EPM from *Ldlr*$^{-/-}$ or *Ch25h*$^{-/-}$*;Ldlr*$^{-/-}$ treated with LPS (100 ng/ml) for 4 or 12 hours

(n=3 per group). Data were analyzed by two-way ANOVA, post-hoc Bonferroni´s multiple comparison test.

Supplemental Figure 7. Lack of *Ch25h* in BMDMs increases Caspase 1 activation and IL-1B secretion and lipid-laden macrophages does not influence ABCA1 or ABCG1 protein levels. A, Representative Western blot analysis of pro-Caspase 1, cleaved caspase-1, Pro-IL-1B and Secreted IL-1B as a read out of inflammasome activation in WT or *Ch25h*$^{-/-}$ BMDMs treated with LPS (100 ng/ml) for 8 hours. ATP (5 mM) was added the last 30 minutes prior to samples collection. HSP90 was used as a loading control. Relative protein quantification by band densitometry is shown in the right panels. Data were analyzed by two-way ANOVA, post-hoc Bonferroni´s multiple comparison test (n=3). n.d. not detectable. **B**, Mitochondria mass quantification by Mitotracker and **C**, superoxide production by the mitochondria using MitoSOX in lipid-laden TG-EPM from *Ldlr*$^{-/-}$ or *Ch25h*$^{-/-}$*;Ldlr*$^{-/-}$ treated with LPS (100 ng/ml) for 4 or 12 hours. Data are average of the mean of Median Intensity Fluorescence (M.I.F.) in arbitrary units (a.u.) and analyzed by two-way ANOVA, post-hoc Bonferroni´s multiple comparison test (n=3 per group). **D**, Representative Total ABCA1, ABCG1 and TLR4 protein levels analyzed by western blot of lipid-laden TG-EPM from *Ldlr*$^{-/-}$ or *Ldlr*$^{-/-}$*;Ch25h*$^{-/-}$ mice fed a CD or a WD. After isolation macrophages were treated with LPS (100 ng/ml) for 4 or 12 hours as indicated. HSP90 was used as a loading control. Quantification of ABCA1 and ABCG1 by band densitometry is showing in the right panel and represent the mean ±SD. Data were analyzed by two-way ANOVA, post-hoc Bonferroni´s multiple comparison test. **E**, *Cd5l* mRNA normalized counts in lipid-laden TG-EPM from *Ch25h*$^{-/-}$*;Ldlr*$^{-/-}$ *vs Ldlr*$^{-/-}$ treated with LPS (100 ng/ml) for 4 or 12 hours. Data were analyzed by two-way ANOVA, post-hoc Bonferroni´s multiple comparison test (n=3).

Supplemental Figure 8. Treatment with 25-HC do not affect the number of SMC but reduces dorsal ruffle formation. A, Representative images of dorsal ruffle formation in SMC pre-treated with ethanol (EtOH) or 25-HC (5μM) for 2 hours, and stimulated with PDGF-BB at 10 ng/mL for 5, 10, 20 minutes. Two random picture per chamber were taken using the EVOS M5000. Data was represented as the percentage of SMC that present dorsal ruffle. Quantification of 3 independent experiments is represented in the right panel. Data were analyzed by two-way ANOVA, post-hoc Bonferroni´s multiple comparison test. Scale bar 100 μm **B**, Quantification of the number of SMC per field after 2 hours pre-treatment with EtOH or 25-HC (5μM) in 3 independent experiments. Data were analyzed by Mann-Whitney non-parametric test.