

SUPPLEMENTARY DATA

Monoglyceride lipase deficiency modulates endocannabinoid signaling and improves plaque stability in ApoE-knockout mice

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Detailed Methods

Animals and diets

ApoE^{-/-}-MGL^{-/-} (DKO) mice were generated by crossing ApoE^{-/-} (The Jackson Laboratory, Bar Harbor, ME) and MGL^{-/-} mice [1]. ApoE^{-/-} and DKO mice were kept with unlimited access to standard chow diet (4% fat and 19% protein; Altromin Spezialfutter GmbH & Co, Lage, Germany) and water in a regular light-dark cycle (12 h light, 12 h dark). At the age of 6 to 8 weeks, mice were fed a Western-type diet (WTD, 21% fat, 0.2% cholesterol; Ssniff Spezialdiaeten GmbH, Soest, Germany) for 9 weeks to induce atherosclerotic plaque formation. Body weights were measured weekly throughout the WTD feeding period. Food intake was measured in single-housed animals after 7 weeks of WTD feeding. All protocols were approved by the Austrian Federal Ministry of Science and Research, Division of Genetic Engineering and Animal Experiments, Vienna, Austria (BMWF-66.010/0057-II/3b/2011; BMWF-66.010/0159-II/3b/2012; BMWF-66.010/0085-II/3b/2013).

Plasma parameters

Mice were fasted for 12 h before 100 µl blood were drawn from *v. facialis*. Plasma was separated by centrifugation at 5,200 x *g* for 7 min at 4°C and triglyceride (TG), total cholesterol (TC), and free cholesterol (FC) concentrations were measured using enzymatic test kits (Triglycerides FS, Cholesterol FS, Free Cholesterol; DiaSys, Holzheim, Germany) according to manufacturers' instructions.

Plasma lipoprotein profiles

Two hundred μl of pooled plasma per genotype were subjected to fast protein liquid chromatography (FPLC) on a Pharmacia FPLC system (Pfizer Pharma, Karlsruhe, Germany) equipped with a Superose 6 column (Amersham Biosciences, Piscataway, NJ). Lipoproteins were eluted with 10 mM Tris-HCl, 1 mM EDTA, 0.9% NaCl, and 0.02% NaN_3 (pH 7.4). Fractions of 0.5 ml each were collected and TG and TC concentrations were assayed enzymatically using above mentioned kits. To enhance sensitivity, reaction buffers were supplemented by the addition of sodium 3,5-dichloro-2-hydroxy-benzenesulfonate.

Isolation and cultivation of peritoneal macrophages and neutrophils

Peritoneal macrophages were collected 72 h after an intraperitoneal injection of 2.5 ml 3% thioglycolate broth. The peritoneum was flushed with 10 ml PBS/EDTA. Cells were cultivated in DMEM (Gibco®, Life Technologies, Carlsbad, CA) containing 10% lipoprotein-deficient serum (LPDS) and 1% penicillin/streptomycin (P/S) for 2-3 h. Thereafter, cells were washed twice with pre-warmed PBS and adherent cells (macrophages) were cultured in DMEM containing 25 mM glucose, 4 mM glutamine, 1 mM pyruvate, 10% LPDS, and 1% P/S for 24 h. Cells were washed twice with pre-warmed PBS and cultured for additional 24 h either in the absence or presence of VLDL or acetylated (ac)LDL (100 μg protein/ml medium) \pm 1 μM SR144528 (Cayman chemicals, Ann Arbor, MI) in ethanol or vehicle (final concentration of ethanol 0.1%). Cells were washed twice with pre-warmed PBS and lipids were extracted with 2 ml hexane:isopropanol (3:2, v:v) for 2 h at 4°C. The organic phase was transferred to a clean tube. After the addition of 100 μl of 2% Triton X-100 in chloroform, samples were vortexed vigorously. The organic phase was completely dried under a stream of N_2 . Extracted lipids were redissolved in 100 μl dH_2O in an ultrasonic bath (15 min, 37°C). TG and cholesterol concentrations were determined in 40 and 20 μl extracts, respectively, using above-mentioned DiaSys kits. Results were normalized to protein concentrations after complete cell lysis with 2 ml 0.3 M NaOH/well for 2 h at RT.

Neutrophils were collected 16 h after thioglycolate injection. Five million neutrophils/ml of medium were cultivated for 24 h in DMEM containing 10% LPDS and 1% P/S. Thereafter, conditioned media were collected and used for zymography.

Quantification of 2-arachidonoyl glycerol (2-AG) in plasma, aorta, and macrophages

Aortae were homogenized in 800 μ l dH₂O. Two hundred μ l of plasma were mixed with 800 μ l dH₂O. Peritoneal macrophages were scraped from the 6-well plates with ice-cold PBS and lysed by sonication (2 x 15 sec with 30 sec intervals on ice). Thereafter 800 μ l of lysate was used for further analysis. Lipids were extracted twice with 4 ml CHCl₃/MeOH/H₂O (2:1:0.6, v:v:v), containing 2 μ g C17:0 monoglyceride (Avanti Lipids, Alabaster, AL) as internal standard. The lipid-containing organic phase was dried and monoglycerides were isolated by solid phase extraction using a self-packed silica gel column. Fractions were obtained by eluting lipids with 99:1 and 90:10 CHCl₃:MeOH (v:v) consecutively. 2-AG concentrations were quantitated in the latter fraction using an AQUITY-UPLC (Waters, Manchester, UK) equipped with a BEH-C18-column (2.1x150 mm, 1.7 μ m; Waters, Manchester, UK) coupled to a SYNAPT™ G1 qTOF HD mass spectrometer (Waters, Manchester, UK) equipped with an ESI source [2].

Complete blood cell count and immunophenotyping of bone marrow

One hundred μ l of blood was drawn from v. facialis into EDTA-coated tubes (Kabe Labortechnik, Nuembrecht-Eisenroth, Germany). Complete blood cell count was performed with the Cell Counter Analyzer MS9-5V (Melet Schloesing Laboratories GmbH, Maria Enzersdorf, Austria).

For bone marrow analysis, femurs and tibiae were collected and the marrow was flushed out of the bones with Hanks' balanced salt solution containing 1 mM EDTA. Washed cell pellets were resuspended in 200 μ l antibody cocktail and incubated at RT for 10 min. Finally, cells were washed in PBS (containing 0.5% BSA, 0.025% sodium azide) and subsequently analyzed. A forward-side scatter gate excluded cell debris and remaining red blood cells.

Dead cells were excluded by 7-aminoactinomycin D (7-AAD; BD Biosciences, San Jose, CA) uptake. LSK populations were defined as Lin⁻Sca-1⁺c-Kit⁺, myeloid progenitor LK cells as Lin⁻Sca-1⁻c-Kit⁺. Granulocyte/monocyte progenitors (GMP) were defined as Lin⁻c-Kit⁺CD34⁺FcγRII/III⁺ and monocyte/dendritic cell progenitors (MDP) as Lin⁻c-Kit^{+/Int}CD115⁺Flt3⁺. Common lymphoid progenitors (CLP) were identified as Lin⁻c-Kit^{lo}Sca-1^{lo}IL7Rα⁺. CD115-PE, CD117(c-Kit)-PE-Cy7, CD16/32(FcγRII/III)-eFluor450, and Ly-6A/E(Sca-1)-PE-Cy7 were purchased from eBioscience (San Diego, CA). IL7Rα(CD127)-brilliant violet and Flt3(CD135)-APC were purchased from BioLegend (San Diego, CA), and CD117(c-Kit)-APC as well as the lineage antibody cocktail encompassing anti-Gr1, anti-CD11b, anti-Ter119, anti-B220, and anti-CD3e from BD Biosciences (San Jose, CA). All antibodies were titrated prior use. Data were acquired on an LSR II flow cytometer with DIVA 6.1.2 software (BD Biosciences, San Jose, CA) and analyses were performed using Flowjo (Treestar Inc, San Carlos, CA). Frequencies of specific cell types were calculated as the percentage of living cells.

White blood cell (WBC) half-life

Mice were injected with 3 mg EZ-Link Sulfo-NHS-Biotin (Pierce Biotechnology, Rockford, IL) into the tail vein. Two and a half hours, 1, 3, 5, and 7 days post-injection, blood was drawn from *v. facialis*. Red blood cells were lysed with ACK lysis buffer. Remaining WBCs were washed with PBS/EDTA (400 x *g*, 5 min, 4°C). Cells were then fixed with 10% neutral buffered formalin (methanol-free) for 10 min at 4°C, washed once again with PBS/EDTA, and stained with Streptavidin-PE antibody (eBioscience, San Diego, CA) in PBS containing 3% FCS (20 min, 4°C). Data were acquired on a FACSCalibur (BD Biosciences, San Jose, CA) and analyses were performed using Flowjo (Treestar Inc, San Carlos, CA). Frequencies of specific cell types were calculated as the percentage of living cells.

MCP-1 and IL-6 ELISAs

Blood was drawn from WTD-fed ApoE^{-/-} and DKO mice. After 1 h at RT, serum was isolated by centrifugation at 660 x *g* for 15 min at RT. MCP-1 (R&D Systems Inc., Minneapolis, MN) and IL-6 (Enzo Life Sciences, Lausen, Switzerland) concentrations were determined by ELISA according to the manufacturer's protocol.

Gel zymography

Fifty μ l of neutrophil-conditioned media were separated by SDS-PAGE (8%, 0.2% gelatine) for 2 h at 90 V. SDS was removed from the gel by washing with 2.5% Triton X-100 in PBS for 40 min at RT. Thereafter, the gel was incubated in buffer (50 mM Tris-HCl, 0.15 M NaCl, 10 mM CaCl₂, pH 7.9) for 20 h at 37°C. Gels were then stained with 0.05% Coomassie brilliant blue solution and the gelatinolytic activity was measured by inverted densitometry of clear bands using ImageJ software.

Western blotting

Macrophages and aortae were lysed in RIPA buffer and protein concentrations from cell lysates were determined (Bio-Rad DC protein assay; Bio-Rad Laboratories, Hercules, CA). One hundred μ g of macrophage protein and 50 μ g of aortic protein/lane were separated by SDS-PAGE (15%). Proteins were transferred to nitrocellulose membranes (Hybond-C Extra; Amersham Biosciences, Piscataway, NJ). Non-specific binding sites were blocked by incubating the membrane with 5% non-fat dry milk in 1x TBS-T buffer (150 mM NaCl, 10 mM Tris, 0.1% Tween 20, pH 8) for 2 h at RT. Blots were incubated with rabbit polyclonal antibodies against CB2R (1:1,000) (Abcam, Cambridge, UK) or caspase 3 (1:1,000) (Cell Signaling Technology, Danvers, MA) and mouse monoclonal antibody against β -actin (1:20,000) (Santa Cruz, Heidelberg, Germany). HRP-conjugated goat anti-rabbit (1:2,500) and rabbit anti-mouse antibodies (1:500) (Dako, Glostrup, Denmark) were visualized by enhanced chemiluminescence detection (ClarityTM Western ECL substrate; Bio-Rad) using a ChemiDocTM MP imaging system (Bio-Rad, Hercules, CA).

MG hydrolase activity assay

MG hydrolase activity was determined as previously described [1]. Macrophages were lysed in MG hydrolase lysis buffer (250 mM sucrose, 1 mM EDTA, 0.1% Triton X-100, 20 μ M DTT, pH 7.0). Thereafter, 120 μ g protein were incubated with 100 μ l substrate (2 mM *rac*-1(3)-oleoyl glycerol bound to fatty acid-free BSA in equimolar ratio in 50 mM potassium phosphate buffer, pH 7.0) for 30 min at 37°C. The reaction was stopped by adding 200 μ l chloroform, samples were vortexed vigorously and centrifuged at 10,000 x *g* for 5 min at 4°C. Free glycerol was estimated in 50 μ l of the aqueous phase (Sigma-Aldrich, St. Louis, MO) according to manufacturer's instructions.

Cytosolic Ca²⁺ imaging using Fura-2/AM

Ca²⁺ imaging was performed as previously described [3]. Macrophages of ApoE^{-/-} (n=4) and DKO (n=6) mice were plated on 30 mm glass coverslips. Prior to experiments, cells were incubated for 40 min with 2 μ M Fura-2/AM (TEFLabs; Austin, TX) and maintained in HEPES buffer (138 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 1 mM HEPES, 2.6 mM NaHCO₃, 0.44 mM KH₂PO₄, 0.34 mM Na₂HPO₄, 1 mM glucose, 0.1% vitamins, 0.2% essential amino acids, 1% P/S, pH 7.4). All buffers were supplemented with 0.25 mM sulfinpyrazone (Sigma-Aldrich, St. Louis, MO) to prevent Fura-2/AM efflux from macrophages. During experiments, cells were perfused with HEPES buffer (145 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM glucose, 10 mM HEPES, pH 7.4), and stimulated with 20 μ M 2-AG followed by 200 μ M ATP (final concentrations). Single cell measurements were performed on a Zeiss AxioVert inverted microscope (Zeiss; Goettingen, Germany) equipped with a polychromator illumination system (VisiChrome, Visitron Systems; Puchheim, Germany) and a thermoelectric-cooled CCD camera (Photometrics CoolSNAP HQ, Visitron Systems). Macrophages were imaged with a 40X oil-immersion objective (Zeiss). Cells were alternately illuminated at 340 and 380 nm (340HT15, 380HT15, Omega Optical; Brattleborough, VT), while fluorescence emission was recorded at 510 nm (510WB40, Omega Optical). Data were

acquired by VisiView 2.0.3 (Visitron Systems) software. Results of Fura-2/AM measurements are shown as normalized ratios $(F_{380}/F_{340})/R_0$ to correct for photobleaching.

Cellular cholesterol efflux

Macrophages were incubated with 50 $\mu\text{g/ml}$ acLDL (pre-loaded with 0.5 $\mu\text{Ci/ml}$ [^3H]cholesterol; ARC Inc., St. Louis, MO) and 30 $\mu\text{g/ml}$ non-labeled cholesterol in DMEM/0.2% fatty acid-free BSA (Sigma-Aldrich, St. Louis, MO) for 32 h at 37°C. After washing cells twice with PBS, cells were cultivated for 16 h in equilibration medium (DMEM/0.2% fatty acid-free BSA). We determined cholesterol efflux after incubating cells in DMEM/0.2% fatty acid-free BSA in the absence or presence of 15 $\mu\text{g/ml}$ ApoA-I (Calbiochem, La Jolla, CA) or 100 $\mu\text{g/ml}$ HDL₃ as extracellular acceptors. Radioactivity in 80 μl medium and cells was measured by liquid scintillation counting after 1, 3, 6, 9 and 12 h of incubation. Cholesterol efflux is expressed as the percentage of total cell [^3H]cholesterol present in the medium. Basal efflux in the absence of ApoA-I and HDL₃ was subtracted from the data.

Nile red staining and fluorescence microscopy

Thioglycolate-elicited peritoneal macrophages were plated in 6-well plates for 24 h in DMEM/10% LPDS/1% P/S. Cells were washed twice with pre-warmed PBS and cultured for 24 h in the absence or presence of VLDL or acLDL (100 μg protein/ml medium). Thereafter, cells were washed twice with pre-warmed PBS and fixed with 10% methanol-free formalin for 10 min. Lipid droplets were visualized after Nile red staining (2.5 $\mu\text{g/ml}$) by confocal laser scanning microscopy using an LSM 510 META microscope system (Carl Zeiss GmbH, Vienna, Austria). Pictures (x63) were taken at excitation 543 nm and signals were recorded using a 560 nm long pass filter.

Apoptosis assay

Apoptosis of thioglycolate-elicited peritoneal macrophages was assayed by BD Pharmingen™ FITC Annexin V apoptosis detection kit I (BD Biosciences, San Jose, CA). Two million cells were incubated with DMEM + 10% LPDS + 1% P/S for 24 h. Thereafter, cells were either incubated with or without 100 µg protein/ml VLDL or acLDL for additional 24 h. Cells were then washed three times with ice-cold PBS. FITC Annexin V and PI staining was performed according to manufacturer's instructions. Data were acquired using Guava easyCyte™ 8 flow cytometer (Merck Millipore, Darmstadt, Germany).

RNA isolation and quantitative real-time PCR analysis

Total RNA from abdominal aortae was isolated using TriFast™ reagent according to the manufacturer's protocol (Peqlab, Erlangen, Germany). Macrophage RNA was isolated with PerfectPure RNA Cultured Cell Kit (5Prime GmbH, Hilden, Germany). One µg of total RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA). Quantitative real-time PCR was performed on a Roche LightCycler 480 (Roche Diagnostics, Palo Alto, CA) using the Quantifast™ SYBR® Green PCR kit (Qiagen, Hilden, Germany). Samples were analyzed in duplicate and normalized to the expression of cyclophilin A (aortae) or hypoxanthine-guanine phosphoribosyltransferase (Hprt) (macrophages) as reference genes. Expression profiles and associated statistical parameters were determined using the $2^{-\Delta\Delta CT}$ method. Following primer sequences were used:

Abca1-fwd: 5'- CTCTTCATGACTCTAGCCTGGA -3'

Abca1-rev: 5'- ACACAGACAGGAAGACGAACAC -3'

Abcg1-fwd: 5'-GACGCTGACTATAAGAGAGACC-3'

Abcg1-rev: 5'-GGAGTTGCTCAGGACCTTCTTG-3'

Abhd6-fwd: 5'- TATTGGTACTGGCGGAGGAC -3'

Abhd6-rev: 5'- AAGGATGGATGGCTTGTGTC -3'

Abhd12-fwd: 5'- GTCACCTTGGAGCATGAGC -3'

Abhd12-rev: 5'- GCAATGTAGAACCCCAGAACAC -3'
Arg1-fwd: 5'- TGGCTTGCGAGACGTAGAC-3'
Arg1-rev: 5'- GCTCAGGTGAATCGGCCTTTT-3'
Atgl-fwd: 5'- GCCACTCACATCTACGGAGC -3'
Atgl-rev: 5'- GACAGCCACGGATGGTGTTC -3'
Cb1r-fwd: 5'-AAGTCGATCTTAGACGGCCTT-3'
Cb1r-rev: 5'-TCCTAATTTGGATGCCATGTCTC-3'
Cb2r-fwd: 5'-ACGGTGGCTTGGAGTTCAAC-3'
Cb2r-rev: 5'-GCCGGGAGGACAGGATAAT-3'
Ccl5-fwd: 5'-GCTGCTTTGCCTACCTCTCC-3'
Ccl5-rev: 5'-TCGAGTGACAAACACGACTGC-3'
Cd36-fwd: 5'-GCAGGTCTATCTACGCTGTG-3'
Cd36-rev: 5'- GGTTGTCTGGATTCTGGAGG-3'
Chi3l3-fwd: 5'-CAGGTCTGGCAATTCTTCTGAA-3'
Chi3l3-rev: 5'-GTCTTGCTCATGTGTGTAAGTGA-3'
Cox-2-fwd: 5'-TTCAACACACTCTATCACTGGC-3'
Cox-2-rev: 5'-AGAAGCGTTTGCGGTACTIONCAT-3'
Cyclophilin A-fwd: 5'-CCATCCAGCCATTCAGTCTT-3'
Cyclophilin A-rev: 5'- TTCCAGGATTCATGTGCCAG-3'
Dagl α -fwd: 5'- GTCCTGCCAGCTATCTTCCTC -3'
Dagl α -rev: 5'- CGTGTGGGTTATAGACCAAGC -3'
Dagl β -fwd: 5'- AGCGACGACTTGGTGTTC -3'
Dagl β -rev: 5'- GCGTGAGATAACAACGTCAGACT -3'
E-selectin-fwd: 5'- TACTGTCAGCGGGACTACACA-3'
E-selectin-rev: 5'- CCACCCAGATCCATACGTTATTG-3'
Gro-1-fwd: 5'-CTGGGATTCACCTCAAGAACATC-3'
Gro-1-rev: 5'-CAGGGTCAAGGCAAGCCTC-3'
Hprt-fwd: 5'-TCAGTCAACGGGGGACATAAA-3'

Hprt-rev: 5'-GGGGCTGTAAGCTTAACCAG-3'
Hsl-fwd: 5'- GCTGGTGACACTCGCAGAAG -3'
Hsl-rev: 5'- TGGCTGGTGTCTCTGTGTCC -3'
Icam1-fwd: 5'-GTGATGCTCAGGTATCCATCCA-3'
Icam1-rev: 5'-CACAGTTCTCAAAGCACAGCG-3'
IL-1 β -fwd: 5'-CAGCAGCACATCAACAAGAG-3'
IL-1 β -rev: 5'-GTCACACACCAGCAGGTTAT-3'
IL-6-fwd: 5'- TAGTCCTTCCTACCCCAATTTCC-3'
IL-6-rev: 5'- TTGGTCCTTAGCCACTCCTTC-3'
IL-10-fwd: 5'-CTATGCTGCCTGCTCTTACT-3'
IL-10-rev: 5'-GCATTAAGGAGTCGGTTAGC-3'
Itgax-fwd: 5'-CTGGATAGCCTTTCTTCTGCTG-3'
Itgax-rev: 5'-GCACACTGTGTCCGAACTCA-3'
Lal-fwd: 5'- CGGCTTGCTGGCAGATTCTA -3'
Lal-rev: 5'- GTGCAGCCTTGAGAATGACC -3'
Lfa1-fwd: 5'-TGCACCAAGTACAAAGTCAGC-3'
Lfa1-rev: 5'-GCGCAAGGAGTCAGGTTCT-3'
L-selectin-fwd: 5'-TACATTGCCCAAAGCCCTTAT-3'
L-selectin-rev: 5'-CCTCCTTGGACTTCTTGTTGTT-3'
Mcp-1-fwd: 5'-ACTGAAGCCAGCTCTCTCTTCCTC-3'
Mcp-1-rev: 5'-TTCCTTCTTGGGGTCAGCAC-3'
Mcp-5-fwd: 5'-ATTTCCACACTTCTATGCCTCCT-3'
Mcp-5-rev: 5'-ATCCAGTATGGTCCTGAAGATCA-3'
Mgl-fwd: 5'- GCAGCCACTAGGATGGAGATG -3'
Mgl-rev: 5'- GCAATGTAGAACCCAGAACAC -3'
Mgl2-fwd: 5'-CAATGTGCTTAGCTGGATGGG-3'
Mgl2-rev: 5'-CCATGCCAGTTATCCGGCTG-3'
Mmp2-fwd: 5'-ACCTGAACACTTTCTATGGCTG-3'

Mmp2-rev: 5'-CTTCCGCATGGTCTCGATG-3'
Mmp9-fwd: 5'-GGACCCGAAGCGGACATTG-3'
Mmp9-rev: 5'-CGTCGTTCGAAATGGGCATCT-3'
Mrc1-fwd: 5'-GCTGAATCCCAGAAATTCCGC-3'
Mrc1-rev: 5'-ATCACAGGCATACAGGGTGAC-3'
Mrc2-fwd: 5'-TCTCCCGGAACCGACTCTTC-3'
Mrc2-rev: 5'-AACTGGTCCCCTAGTGTACGA-3'
Msr1-fwd: 5'-TGGAGGAGAGAATCGAAAGCA-3'
Msr1-rev: 5'-CTGGACTGACGAAATCAAGGAA-3'
P-selectin-fwd: 5'- CATCTGGTTCAGTGCTTTGATCT-3'
P-selectin-rev: 5'- ACCCGTGAGTTATTCCATGAGT-3'
Selplg-fwd: 5'-CCCTGGCAACAGCCTTCAG-3'
Selplg-rev: 5'-GGGTCCTCAAATCGTCATCC-3'
Sr-bl-fwd: 5'-TTTGGAGTGGTAGTAAAAGGGC-3'
Sr-bl-rev: 5'-TGACATCAGGGACTCAGAGTAG-3'
Timp1-fwd: 5'-TCCTCTTGTTGCTATCACTGATAGCTT-3'
Timp1-rev: 5'- CGGTGGTATAAGGTGGTCTCGTT-3'
Timp2-fwd: 5'- TCAGAGCCAAAGCAGTGAGC -3'
Timp2-rev: 5'- GCCGTGTAGATAAACTCGATGTC -3'
Tnf- α -fwd: 5'-CTTCCAGAACTCCAGGCGGT-3'
Tnf- α -rev: 5'-GGCTACAGGCTTGTCACCTCG-3'
Vcam1-fwd: 5'-TGCACAGTCCCTAATGTGTATCC-3'
Vcam1-rev: 5'-GACTTTATGCCCATTTCTCCA-3'
Vla4-fwd: 5'-AAAATGGCTCTATC-3'
Vla4-rev: 5'-CAGAAGGCATGACGTAGCAAAT-3'

***In vivo* macrophage phagocytosis assay**

Mice were intraperitoneally (i.p.) injected with 200 μ l of fluorescein-labeled *E. coli* BioParticles® (Molecular Probes®, Life Technologies, Carlsbad, CA) suspended in Hanks' balanced salt solution. After 2 h, macrophages were collected by flushing the peritoneal cavity with 10 ml of PBS/EDTA and incubated in DMEM containing 25 mM glucose, 10% LPDS, and 1% P/S for 150 min. Cells were washed three times with PBS and the fluorescence was determined after addition of trypan blue to quench the extracellular probe. After aspiration of trypan blue, the fluorescence was measured at 484 (excitation) and 535 nm (emission) on a Victor 1420 multilabel counter (PerkinElmer Life Sciences, Waltham, MA). Experimental readings were normalized to protein content.

***In vivo* treatment of mice with SR144528**

ApoE^{-/-} and DKO mice were treated with the peripheral CB2R inverse agonist SR144528 (Cayman Chemicals, Ann Arbor, MI) for 3 weeks (during the last 3 weeks of 8 week WTD feeding). SR144528 was dissolved in vehicle (0.2% ethanol in 1% Tween80/PBS) and administered daily in a dose of 0.75 mg/kg body weight via gastric gavage [4].

Preparation of histological sections for atheroassays

Mice were anesthetized by i.p. injections of sodium-pentobarbital (50 mg/kg body weight). Mice were perfused with PBS/EDTA for 10-15 min. The abdominal part of the aorta was removed and frozen in liquid N₂ for RNA isolation. Animals were then perfused with 10% neutral buffered formalin (methanol-free) for 15 min. Adventitial adipose tissue was carefully removed from the upper part of the aorta, which was then excised from the thoracic cavity, cut open in a Y-formation, and stored in formalin until staining. Upper two thirds of the heart were fixed with formalin for 24 h. Fixed tissues were stored in 30% sucrose. One day before sectioning, hearts were transferred into Neg-50™ frozen section medium (Richard-Allan Scientific, Kalamazoo, MI). Serial sections (7-8 μ m) of aortic root were cut at -20°C using a cryostat-microtome (HM 560 Cryo-Star; Microm International GmbH, Walldorf, Germany).

Oil red O staining of aortae and aortic valve sections

Oil red O (0.5 g) was mixed with 100 ml isopropanol under constant stirring for 24 h. The working solution (30 ml of filtered stock plus 20 ml dH₂O, mixed for 10 min, filtered) was freshly prepared before use.

Aortae were washed with PBS for 1 min and then placed in 70% isopropanol for 5 min. Thereafter, aortae were stained with oil red O working solution for 20 min and then dipped into 70% isopropanol to remove excessive oil red O. Aortas were pinned on black dissecting wax and images were taken using a stereo zoom microscope (Olympus SZX12). Aortic root sections were fixed in 10% methanol-free neutral buffered formalin for 10 min. Sections were incubated in 70% isopropanol for 5 min, oil red O for 15 min, dipped into 70% isopropanol, counterstained with hematoxylin for 5 min, and washed in 0.1% NaHCO₃. Sections were air dried and mounted with Kaiser's glycerol mounting medium (Merck, Darmstadt, Germany). Mean lesion area (mm²) was calculated from 14-15 consecutive oil red O-stained sections per mouse. Images were taken with ScanScope T3 whole slide scanner (Aperio Technologies, Bristol, UK). Plaque areas were quantitated with ImageJ software.

Monoclonal antibody to macrophages-2 (MoMa-2) immunostaining

Histological sections were fixed for 15 min in formalin and washed three times with PBS. Sections were blocked for 30 min with peroxidase blocking solution (Lab Vision, Fremont, CA) and washed two times with PBS. Thereafter, Ultra V block (Lab Vision, Fremont, CA) was added for 7 min. Sections were incubated with primary monoclonal antibody against macrophages-2 (MoMa-2, 1:600; Acris, Hiddenhausen, Germany) for 1 h at RT and then at 4°C overnight. Sections were washed two times with PBS, incubated for 3 h at RT with polyclonal rabbit anti-rat secondary antibody coupled to horseradish peroxidase (1:100, Dako Denmark A/S, Glostrup, Denmark), and washed once with PBS. AEC substrate (Vector Laboratories, Burlingame, CA) was added to the sections for 20 min and then sections were washed 3 times with dH₂O. Sections were stained with hematoxylin for 5 min, washed in

0.1% NaHCO₃, and fixed with Kaiser's glycerol mounting medium (Merck, Darmstadt, Germany). Images were taken with ScanScope T3 whole slide scanner (Aperio Technologies, Bristol, UK). Quantification of MoMa-2-stained sections was performed with ImageJ software.

Monoclonal antibody to alpha-smooth muscle actin (α -SMA) immunostaining

Antigen retrieval was performed with 10 mM sodium-citrate buffer (pH 6) in a pressure chamber (Pascal, Dako Denmark A/S, Glostrup, Denmark) at 120°C for 170 seconds. Thereafter, slides were blocked with 1% H₂O₂ in methanol for 15 min and washed three times with PBS. For detection of smooth muscle cells, histological sections were treated with the primary antibody against alpha smooth muscle actin (α -SMA, 1:50; Abcam, Cambridge, UK) in Dako REAL™ antibody diluent (Dako Denmark A/S, Glostrup, Denmark) for 1 h at RT. Slides were washed 5 times with PBS and treated with the secondary antibody Dako REAL™ EnVision™ HRP Rabbit/Mouse (Dako Denmark A/S, Glostrup, Denmark) for 30 min at RT. After washing with PBS, AEC substrate was added to the sections (DakoCytomation AEC Substrate Chromogen Ready-to-Use), incubated for 7 min and the reaction was stopped with PBS. Slides were counterstained with Mayer's hematoxylin, washed under running tap water, and fixed with Aquatex mounting medium (Merck, Darmstadt, Germany). Images were taken with ScanScope T3 whole slide scanner (Aperio Technologies, Bristol, UK). Quantification of α -SMA-stained sections was performed with ImageJ software.

Monoclonal antibody to active caspase 3 immunostaining

Antigen retrieval was performed with 10 mM sodium-citrate buffer (pH 6) in a microwave at 150 W for 40 min. Thereafter, slides were blocked with 3% H₂O₂ in methanol for 15 min and washed three times with PBS. For detection of active caspase 3, histological sections were treated with the primary antibody against active caspase 3 (1:25; R&D Systems, Minneapolis, MN) in Dako REAL™ antibody diluent (Dako Denmark A/S, Glostrup, Denmark) for 1 h at RT. Slides were washed 5 times with PBS and treated with EnVision™ Dual Link

System HRP (Dako Denmark A/S, Glostrup, Denmark) for 30 min at RT. After washing with PBS, AEC substrate was added to the sections (DakoCytomation AEC Substrate Chromogen Ready-to-Use), incubated for 5 min and the reaction was stopped with PBS. Slides were counterstained with Mayer's hematoxylin, washed under running tap water, and fixed with Aquatex mounting medium (Merck, Darmstadt, Germany). Images were taken with ScanScope T3 whole slide scanner (Aperio Technologies, Bristol, UK). Quantification of caspase 3-stained sections was performed with ImageJ software.

Monoclonal antibody to cluster of differentiation (CD) 31 immunostaining

Antigen retrieval was performed with Target Retrieval Solution pH 9 (DAKOCytomation A/S, Glostrup, Denmark) in a microwave at 150 W for 40 min. Thereafter, slides were blocked with 3% H₂O₂ in methanol for 15 min and washed three times with PBS. For detection of neovessels, histological sections were treated with the primary antibody against CD31 (1:50; Thermo Fischer Scientific, Waltham, MA) in Dako REAL™ antibody diluent (Dako Denmark A/S, Glostrup, Denmark) for 1 h at RT. Slides were washed 5 times with PBS and treated with Ultravision LP HRP Polymer (Thermo Scientific, Waltham, MA) for 30 min at RT. After washing with PBS, DAB substrate was added to the sections (Dako ChemMate™ detection kit, peroxidase/DAB), incubated for 10 min and the reaction was stopped with PBS. Slides were counterstained with Mayer's hematoxylin, washed under running tap water, dehydrated in ethanol gradient, and fixed with Entellan® mounting medium (Merck, Darmstadt, Germany). Images were taken with ScanScope T3 whole slide scanner (Aperio Technologies, Bristol, UK). Quantification of CD31-stained sections was performed with ImageJ software.

Masson's Trichrome staining

Slides were incubated in Bouin's solution (Sigma-Aldrich, St. Louis, MO) overnight at RT, washed under running tap water until histological sections were clear from yellow color, and then washed with dH₂O for 1 min. Working Accustain® Wigert's Iron Hematoxylin solution

(Sigma-Aldrich, St. Louis, MO) was freshly prepared and sections were stained for 5 min, washed with running tap water for 5 min and dH₂O for 1 min. Sections were then stained with Masson's Trichrome stain (Sigma-Aldrich, St. Louis, MO) according to manufacturer's instructions, washed with dH₂O, and dehydrated (90, 95, and 100% of ethanol and xylene). Sections were fixed with Cytoseal™-60 mounting medium (Thermo Scientific, Kalamazoo, MI). Images were taken with ScanScope T3 whole slide scanner (Aperio Technologies, Bristol, UK). Collagen in Trichrome-stained sections (blue) and necrotic core (acellular area within the plaque) were quantitated with ImageJ software. Fibrotic cap thickness was measured in the zone of minimal fibrosis, perpendicularly to the necrotic core at a magnification of 20x, using ImageJ software.

Statistics

Statistical analyses were performed using GraphPad Prism 5.0 software. Significances were determined by Student's unpaired *t*-test and Welch correction (in case of unequal variances) for two group comparison and ANOVA followed by Bonferroni correction for multiple group comparison. Data are presented as mean ± SD. Values of $p < 0.05$ were considered to be significantly different.

Supplementary Figures and Figure Legends

Supplementary Figure S1

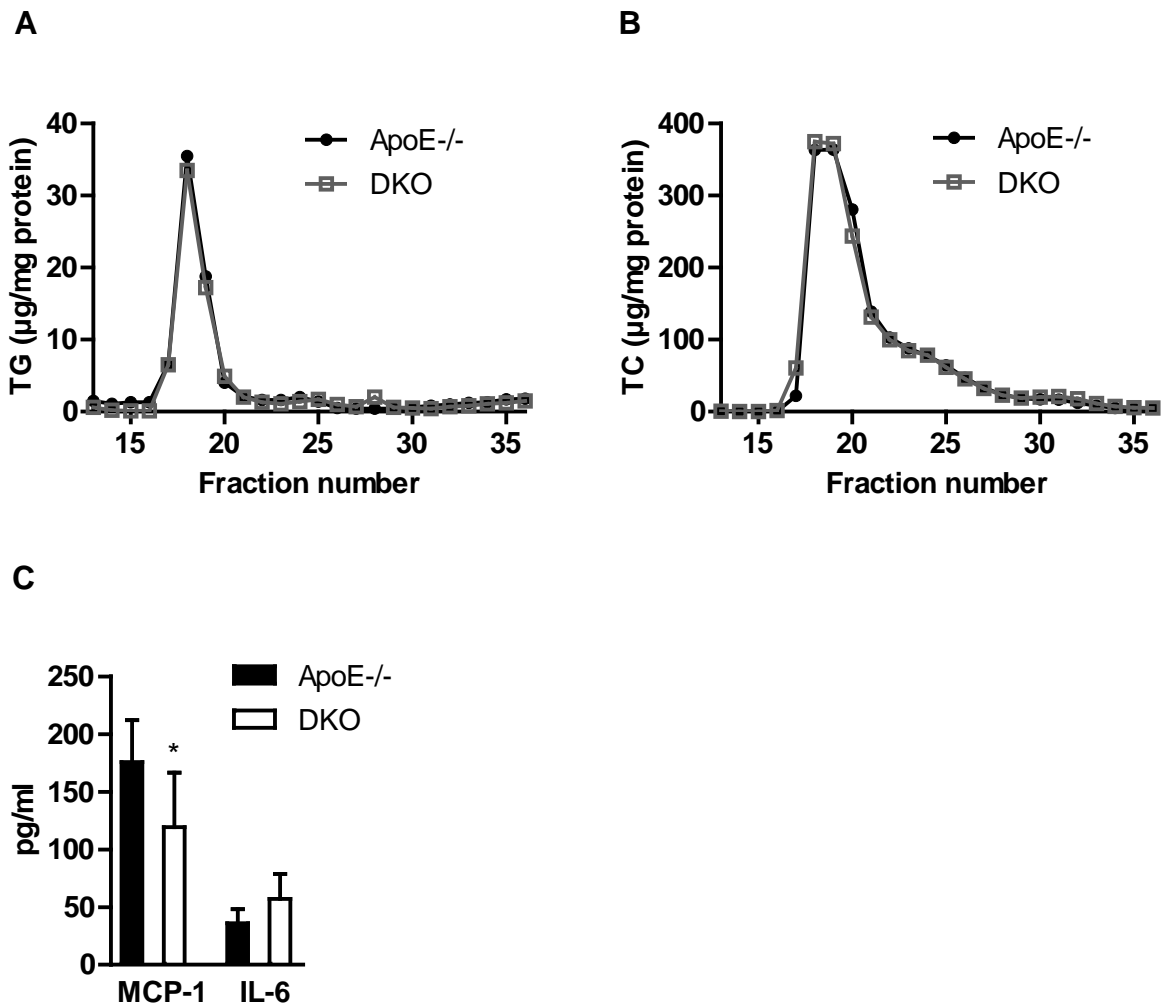
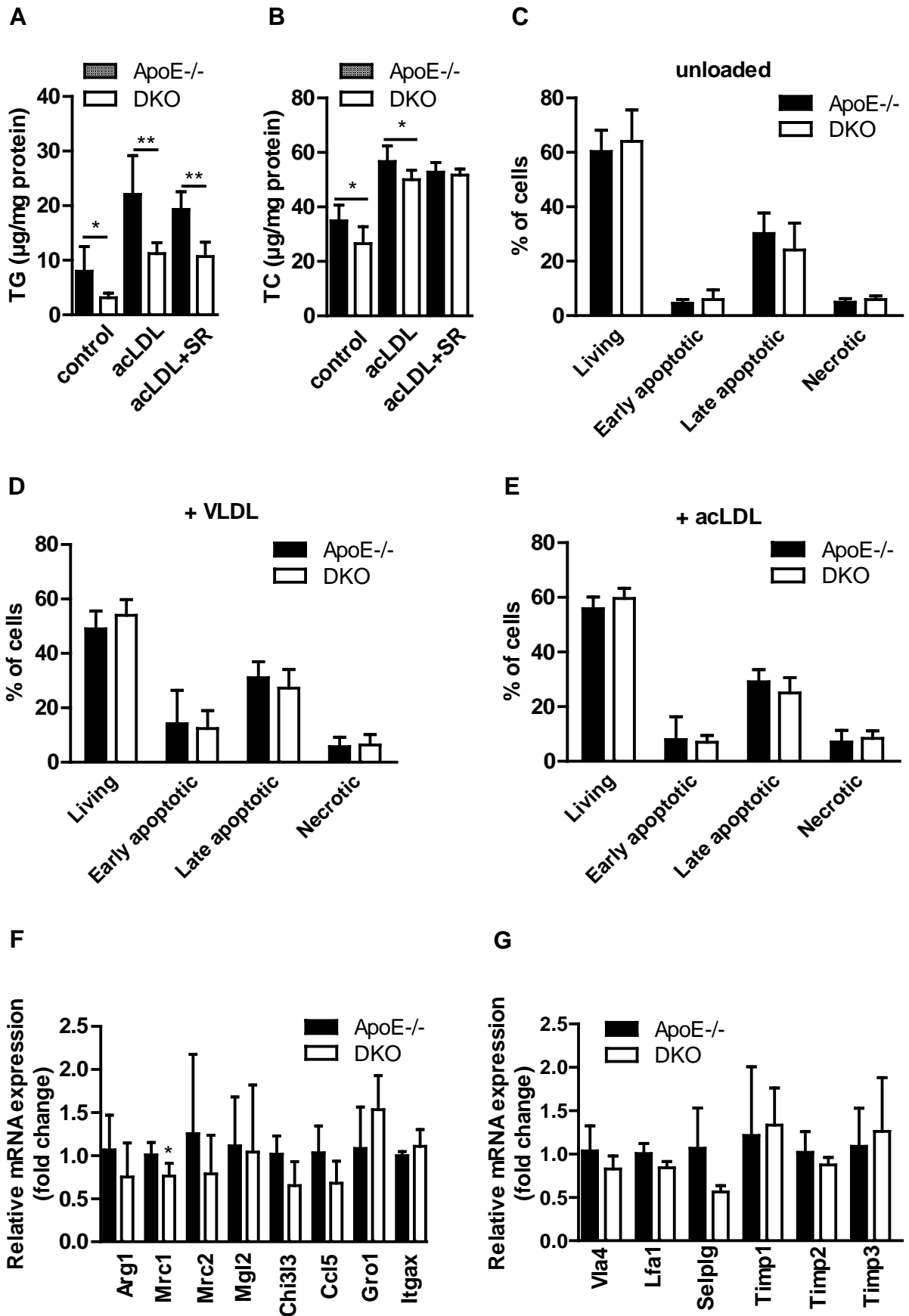


Figure S1. Comparable plasma lipoprotein profiles in ApoE^{-/-} and DKO mice. (A, B) Plasma lipoprotein profiles from ApoE^{-/-} and DKO mice fed WTD. Plasma samples from each genotype (n=5) were pooled and 200 µl were subjected to fast protein liquid chromatography using a Superose 6 column. (A) TG and (B) TC concentrations in each fraction were determined using enzymatic assays. (C) Serum MCP-1 and IL-6 concentrations were determined by ELISA. Data represent means (n=8) + SD. *, p < 0.05.

Supplementary Figure S2



H

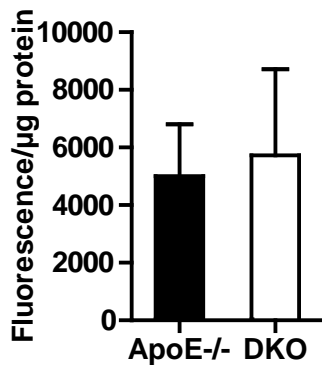


Figure S2. Comparable macrophage phenotype between ApoE^{-/-} and DKO mice. (A, B) Peritoneal macrophages were plated in 6-well plates for 24 h ± acetylated (ac)LDL (100 µg protein/ml medium) or acLDL + SR144528 (1 µM/ml medium). After lipid extraction, (A) TG and (B) TC concentrations were measured spectrophotometrically. Data represent means (n=5-7) + SD. (C-E) Apoptosis of macrophages was determined using Annexin V/PI staining. Peritoneal macrophages were cultured for 24 h, (C) in the absence or (D) presence of VLDL (100 µg protein/ml medium) or (E) acLDL (100 µg protein/ml medium) and analyzed by flow cytometry. Data represent means (n=6-7) + SD. (F, G) Total RNA was isolated from macrophages and subjected to real time PCR analysis. mRNA expression of target genes was analyzed in duplicate and normalized to the expression of Hprt as reference gene. mRNA expression and associated statistical parameters were determined using the $2^{-\Delta\Delta CT}$ method. Data represent means (n=4-5) + SD. (H) Fluorescein-labeled *E. coli* particles (200 µl) were injected into ApoE^{-/-} and DKO mice to measure phagocytosis ability. After 2 h, the peritoneum was flushed with 10 ml PBS/EDTA, macrophages were isolated and assayed for internalized fluorescence after quenching of extracellular fluorescence by trypan blue. Data represent means (n=6-7) + SD *, p < 0.05, **, p ≤ 0.01.

Figure S3

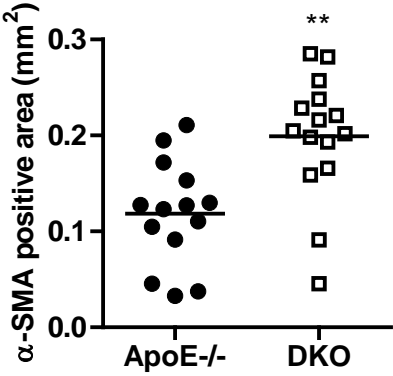


Figure S3. Increased abundance of α -smooth muscle actin (α -SMA)-positive lesion area in DKO mice. α -SMA-positive lesion size was determined by measuring immunohistochemically-stained surface size (Fig. 6F, red). Data represent mean values of 3 aortic valve sections in the area of maximal plaque size for α -SMA for each mouse. Bars represent means (n=14-15). **, p ≤ 0.01.

Supplementary Figure S4

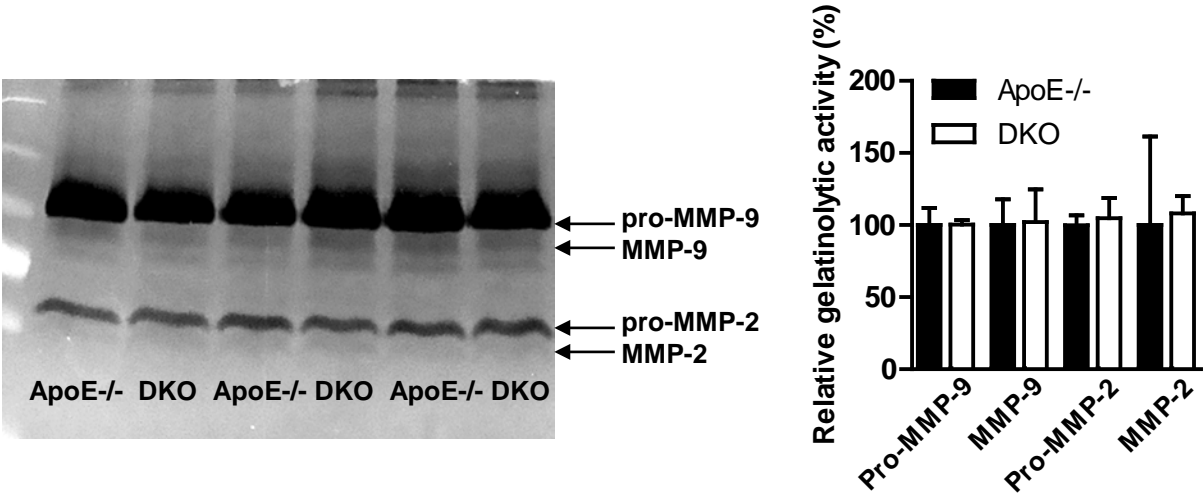
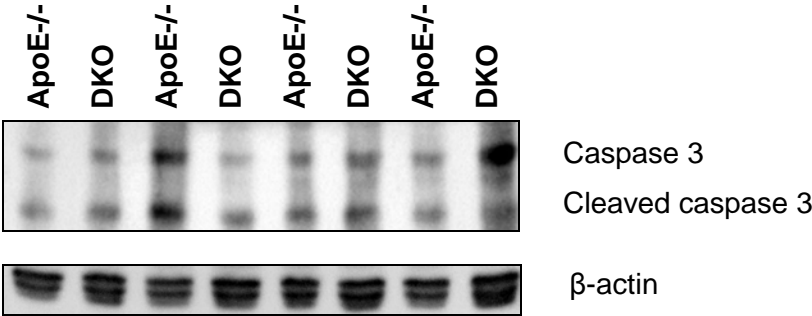


Figure S4. Comparable gelatinolytic activity in ApoE^{-/-} and DKO mice. Five million neutrophils/ml of medium were cultivated for 24 h. Conditioned medium (50 μl) was subjected to SDS-PAGE under non-denaturing conditions. Gelatinolytic activity was visualized with 0.05% Coomassie blue solution and quantified with inverted densitometry using ImageJ. Data represent means (n=3) + SD.

Supplementary Figure S5

A



B

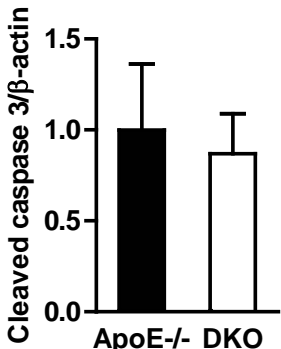


Figure S5: Comparable apoptosis in aortae of ApoE^{-/-} and DKO mice. (A) Western blot analysis of caspase 3 protein expression in aortae of ApoE^{-/-} and DKO mice. (B) Densitometric quantification of cleaved caspase 3/ β -actin (loading control) represent means (n=4) + SD.

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