Supplemental Material

Myeloid cannabinoid CB1 receptor deletion confers atheroprotection in male mice by reducing macrophage proliferation in a sex-dependent manner

(Wang et al.)

Content:

Supplemental methods

Plaque histology and immunofluorescence

Hearts were embedded in Tissue-Tek O.C.T. compound (Sakura) for cutting 5 µm cross-sections at the level of the aortic roots. Aortic arches were fixed in 4% PFA and embedded in paraffin for longitudinal sectioning (5 µm). Aortic root sections were stained with ORO for lesion size quantification. Per mouse, 8 sections with a distance of 50 µm from each other were analyzed, and mean values per heart calculated. Aortic arch slides were deparaffinized and stained with hematoxylin and eosin for lesion size quantification relative to the total inner vessel area. Plaque collagen content was assessed by Masson's trichrome staining (stain kit Sigma HT15) of deparaffinized aortic arch sections. Per arch, 4 sections at 50 µm distance from each other were analyzed. Digital images were acquired with a brightfield microscope (DM6000B) connected to a digital camera (DMC6200, Leica). The lesion size was quantified using Leica Application Suite LAS V4.3 software.

Immunostainings were performed on 5 µm thick cryosections of the aortic root. After fixation with pre-cooled acetone and blocking nonspecific binding (1% BSA in PBS), sections were incubated with antibodies against CD68, iNOS, Ki67 and Ly6G (Supplementary Table 1-2). Negative staining controls were performed with corresponding isotypes. The primary antibodies were detected with anti-rat Cy3 and anti-rabbit AF488 conjugated secondary antibodies (Supplementary Table 3). For staining of lipid droplets within macrophages, CD68 staining was followed by Nile Red (N3013, Sigma-Aldrich) staining for 5 minutes. Nuclei were counterstained with Hoechst 33342. Images were taken with a fluorescence microscope (DM6000B) connected to a monochrome digital camera (DFC365FX, Leica) equipped with Thunder technology for computational clearing, and analyzed with the Leica Application Suite LAS V4.3 software or ImageJ software. Per heart, 3 aortic root sections were analyzed, and mean values per section calculated.

In situ hybridization

To determine the presence of CB1 in macrophage, fluorescence *in situ* hybridization was performed by using a murine *Cnr1* probe (VB6-17606, Affymetrix). Aortic root cryosections were collected on RNAse free slides (pretreated with RNAse ZAP). RNase-free requirements were maintained up to post-hybridization steps. The sections were prefixed in 4% PFA for 5 min at RT and washed 3 times with PBS for 5 min. The tissue sections were treated with pre-warmed 10 µg/ml proteinase K (diluted in PBS) for 5 min at RT, followed by post-fixing with 100% ethanol for 1 min. After washing 3 times for 10 min with PBS, the sections were transferred to an RNAse free chamber. The ViewRNA™ Cell Plus Assay-Kit (Invitrogen) was used for target probe hybridization by incubating the sections at 40° C for 2 h. The hybridization probe was mixed with probe set diluent to a final concentration of 5 µg/ml. Following the hybridization step, the sections were washed 3 times with wash buffer at RT for 5 min. Signal amplification was performed by incubating with the pre-amplifier mix for 30 min at 40° C, washing and incubating with the amplifier mix for 1 h at 40°C. After washing, the sections were incubated for 1 h at 40°C with the working label probe mix. Following the in-situ hybridization, the sections were washed, incubated with blocking buffer and stained with CD68 antibody and Hoechst as described above. Images were taken using a Leica DM 6000B microscope.

In vitro assays with bone marrow-derived macrophages

Bone marrow cells were collected from tibia and femur by centrifugation (8600x g for 1min at 4 °C) and then subjected to red blood cell lysis using ACK buffer. After washing, the cells were resuspended in RPMI-1640 (Gibco Life Technologies) medium supplemented with 10% FCS, 2 mM L-glutamine, penicillin (100 U/mL), streptomycin (100 µg/mL) and 10 % filtered L-929 cell-conditioned medium as a source of M-CSF. The cells were plated into 12-well-plates, and the medium was changed every 2–3 days for a total of 7-10 days to allow differentiation into bone marrow-derived macrophages (BMDMs).

For immunostainings, cells were plated on sterile cover slips in 24-well plates. In some experiments, BMDMs were treated with estradiol (10 nM), tamoxifen (1 µM), LPS (10 ng/mL), CB1 agonist ACEA (1 µM), or CB1 antagonist AM281 (10 µM). In case of combined LPS treatment with CB1 blocking, AM281 was added 15 min prior to LPS. The p53 inhibitor pifithrin-α (PFT-α; 25 µM) was added 5 min before stimulating the cells with ACEA or AM281. For reactive oxygen species (ROS) detection, BMDM were incubated with DHR123 (2.5 µg/ml; 851000, Cayman) in RPMI 1640 (plus Glutamine) containing 0.1% BSA and 0.1 mM HEPES for 20 min at 37°C. Then, the samples were immediately acquired with a FACSCantoII to detect cellular ROS levels as green fluorescence intensities. For mitochondrial staining, BMDMs were incubated with prewarmed (37°C) staining solution containing MitoTracker (ThermoFisher) at a concentration of 50 nM for 30 min and subsequently measured with a FACS Canto-II. For assessing oxLDL uptake, BMDMs were incubated with 1 µg/ml Dil-oxLDL in complete RPMI medium for 30min. After loading, cells were washed twice with prewarmed PBS before detaching with prewarmed citrate buffer (135mM KCl, 15 mM sodium citrate). The amount of intracellular Dil-oxLDL was measured with a FACS Canto-II. Cell proliferation rates were measured by intracellular Ki67 staining and flow cytometry as described above. IL1β in BMDM supernatants was measured by DuoSet ELISA (R&D Systems) with a Tecan Infinite F200 PRO microplate reader. For detection of nuclear p53, cells grown on cover slips and treated for 1h with ACEA, AM281 or vehicle were fixed with acetone and subsequently immunostained with primary antibody against phospho-p53 and subsequently with anti-rabbit-AF488. Images were taken with a fluorescence microscope (DM6000B) connected to a monochrome digital camera (DFC365FX, Leica) equipped with Thunder technology for computational clearing, and analyzed with the Leica Application Suite LAS V4.3 software or ImageJ software. Throughout all experiments, each independent biological replicate was generated with separate BMDM donor mice.

Droplet digital PCR

Total RNA from sorted cells was isolated, reverse transcribed and droplet digital PCR (ddPCR) was performed with the QX200 ddPCR™ system (Bio-Rad). The reaction mixture was prepared by combining 2× ddPCR Mastermix (Bio-Rad), 20× primer and probes (final concentrations of 900 nM and 250 nM, respectively; Integrated DNA Technologies) and cDNA template in a final volume of 20 µl (Supplementary Table 5). Then, each ddPCR reaction mixture was loaded into the sample well of an eight-channel disposable droplet generator cartridge (Bio-Rad). A volume of 60 µl of droplet generation oil (Bio-Rad) was loaded into the oil well for each channel. The cartridge was placed into the droplet generator (Bio-Rad), and the droplets were collected and transferred to a 96-well PCR plate. The plate was heat-sealed with a foil seal, placed on the thermal cycler and amplified to the endpoint (40 cycles). Subsequently, the 96-well PCR plate was loaded on the droplet reader (Bio-Rad) and the analysis was performed with the QuantaSoft analysis software (Bio-Rad).

RNA sequencing

Bulk RNA-sequencing was performed using the prime-seq protocol that can be found on protocols.io (dx.doi.org/10.17504/protocols.io.s9veh66).[1] Briefly, BMDMs were lysed in 100 µL of RLT+, 1% beta-mercaptoethanol and 50 µL of lysate was used for RNA-sequencing. The samples were proteinase K and DNase I digested and then cDNA synthesis was performed using uniquely barcoded oligodT primers. Samples destined for the same library were pooled and pre-amplification was then performed using 11-14 cycles, depending on the initial input per library. The cDNA was quantified using the PicoGreen dsDNA assay kit (Thermo Fisher, P11496) and qualified using the Bioanalyzer HS DNA chip (Agilent, 5067-4626). Libraries were then constructed with the NEB Next Ultra II FS kit (E6177S, NEB) using the prime-seq specifications. The libraries were quantified and qualified using the HS DNA chip on the Bioanalyzer and sequenced on an Illumina Hiseq 1500 at an average depth of 12.7 million reads per sample.

Reads were demultiplexed using deML and then filtered, aligned to the mouse genome mm10 using STAR 2.5.2b with default settings. BAM files were indexed and filtered on MAPQ > 15 with SAMtools 1.3.1. Raw tag counts and RPKM (reads per kilo base per million mapped reads) values per gene were summed using HOMER2's analyzeRepeats.pl script with default settings and the -noadj or -rpkm options for raw counts and RPKM reporting, respectively. Datasets were filtered to select genes expressed in at least 10% of samples. Differential gene expression analysis was calculated by using DESeq2 (Version 1.37.4) Bioconductor package in R version 4.2.0 (2022-04-22) under Ubuntu 20.04.3 LTS system, based on the negative binomial distribution.[2] Size factors and dispersion of samples were estimated first, then Negative Binomial GLM fitting and Wald statistics calculation was performed by DESeq2. Differential expression genes were identified by criteria: adjusted P value < 0.10 and fold changes > 1.5, based on the calculation results of DESeq2. Gene ontology enrichment analysis was performed using the enrichplot (Version 1.17.2) Bioconductor package[3] with differential expression genes (only filtered by adjusted P value < 0.10). Heatmap was drawn by pheatmap package (Version 1.0.12). Volcano plot was drawn by ggplot2 package (Version 3.4.0). Cneplots and dotplots were drawn by enrichplot packge (Version 1.17.2). The Gene set enrichment analysis (GSEA) was done on GSEA software v4.3.2 for Windows with M2 curated gene sets and M5 ontology gene sets from mouse collections and C2 gene sets from human collections.[4, 5] The permutation type was gene_set. The significant pathways were chosen by following criteria: normalized enrichment score (NES) < -1 or > 1, false discovery rate (FDR) < 25% and nominal P value < 0.05.

Phospho kinase array

BMDMs isolated from 4 donor mice were treated with ACEA for 60 min and then washed once in ice-cold PBS, and lysed for 15 min on ice using M-PER Mammalian Extraction Buffer containing Halt Phosphatase Inhibitor and EDTA-free Halt Protease Inhibitor Cocktail (1:100 each; Thermo Scientific). Lysates were centrifuged for 15 min at 16,000 × g at 4°C. Protein

quantification was performed with Pierce™ Coomassie Plus (Bradford) Assay according to the manufacturer's instructions. Serine-Threonine kinase (STK) profiles were determined using the PamChip® peptide Ser/Thr Kinase assay microarray systems on PamStation®12, respectively (PamGene International). Each STK-PamChip® array contains 144 individual phospho-site(s). Per array, 1.0 µg of protein and 400 µM ATP were applied together with an antibody mix to detect the phosphorylated Ser/Thr. The spot intensity was quantified (and corrected for local background) using the BioNavigator software version 6.3 (PamGene International). Upstream Kinase Analysis, a functional scoring method (PamGene), was used to rank kinases based on combined specificity scores (based on peptides linked to a kinase, derived from six databases) and sensitivity scores (based on treatment-control differences). The heatmap contains the kinases with a median final scores >1.2 and p-values were adjusted for multiple comparisons by the false discovery rate (FDR). Pathway enrichment analysis was implemented on the differentially expressed kinases (median final scores >1.2 and adjusted p-value <0.05) with the use of the R packages ReactomePA.[3] The biological complexities in which these kinases correlate with multiple annotation categories were visualized in a network plot with the help of the R package Enrichplot Version 1.4.0. 2019 (10.18129/B9.bioc.enrichplot).

Extracellular flux analysis

Mitochondrial respiration was assessed using the Seahorse Cell Mito Stress Test in a Seahorse Analyzer XFe24 (Agilent) with some adjustments to the manufacturer's protocol. Briefly, $4x10^5$ BMDM cells were transferred to a 24-well Seahorse plate after 7 days of differentiation. BMDM were incubated with CB1 agonist ACEA (1 µM), CB1 antagonist AM281 (10 µM), LPS (10 ng/ml), or vehicle for 48h. Prior to the assay, the culture medium was replaced with Seahorse medium (XF RPMI medium, pH 7.4, supplemented with 10 mM glucose, 1 mM pyruvate and 2 mM L-glutamine) and the cells were incubated for 60 min at 37 °C without CO_2. The oxygen consumption rate (OCR) was measured (3 min mix, 2 min wait

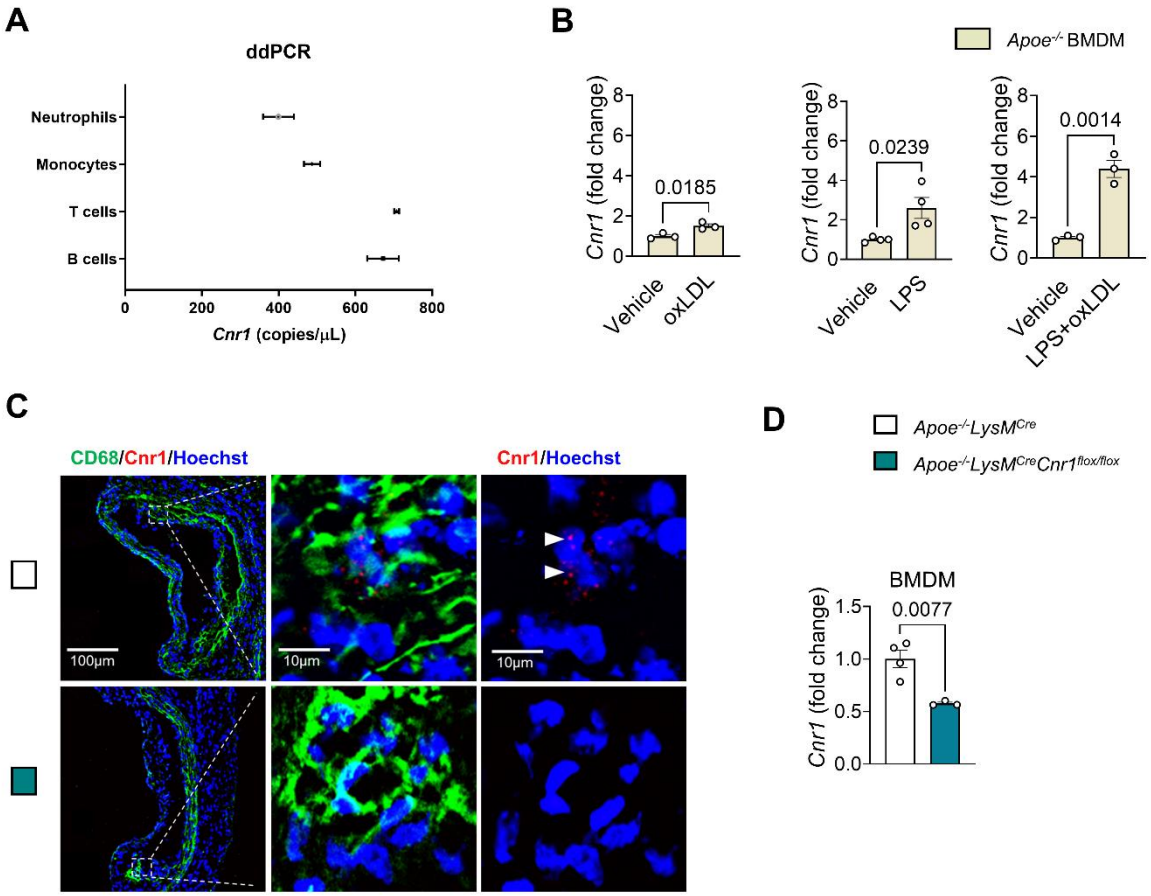
and 3 min measure cycles) under basal conditions and after sequential injection of oligomycin (1 µM; OM), fluoro-carbonyl cyanide phenylhydrazone (1 µM; FCCP), and antimycin A (1 µM; AA) plus rotenone (1 µmol/L; ROT; all from Sigma-Aldrich).

Human carotid artery plaques

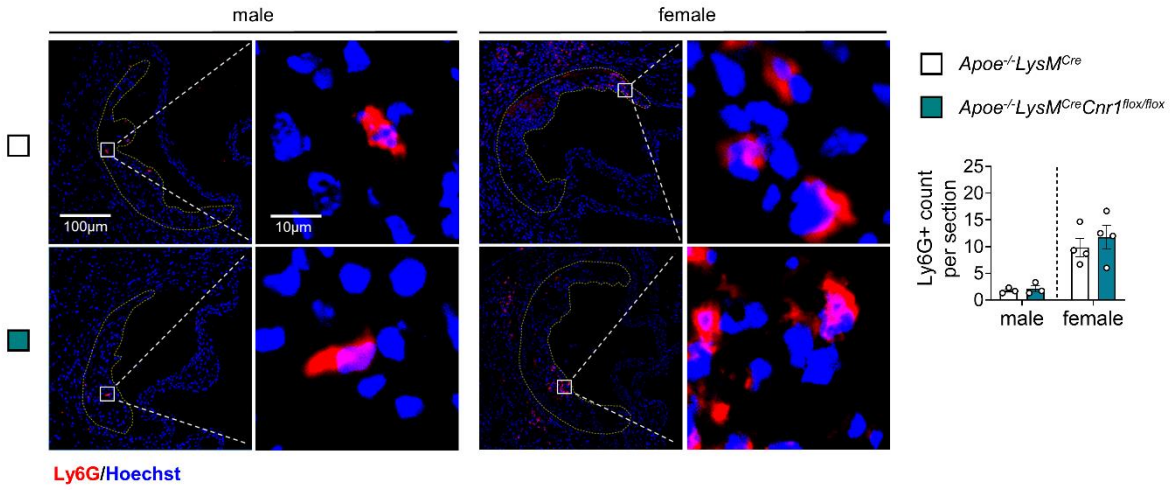
Human carotid artery plaques were harvested during carotid artery endarterectomy (CEA) surgery, transported to the laboratory and snap frozen. CEA was performed due to advanced atherosclerotic lesion formation and stenosis in the carotid arteries. The patients' characteristics are summarized in Supplemental Table 6. The tissue homogenization was performed in 700 µl Qiazol lysis reagent and total RNA was isolated using the miRNeasy Mini Kit (Qiagen, Netherlands) according to manufacturer´s instruction. RNA concentration and purity were assessed using NanoDrop. RIN number was assessed using the RNA Screen Tape (Agilent, USA) in the Agilent TapeStation 4200. Normalized gene expression levels obtained by RNA sequencing were used for linear regression analysis using the "ggcorrplot" R package(v0.1.4). A two-tailed $P<0.05$ was considered to be statistically significant. All patients provided written informed consent in accordance with the Declaration of Helsinki. The study has been approved by the local Ethics Committee of the Klinikum rechts der Isar of the Technical University Munich (diary numbers 2799/10 and 2799/10S).

References

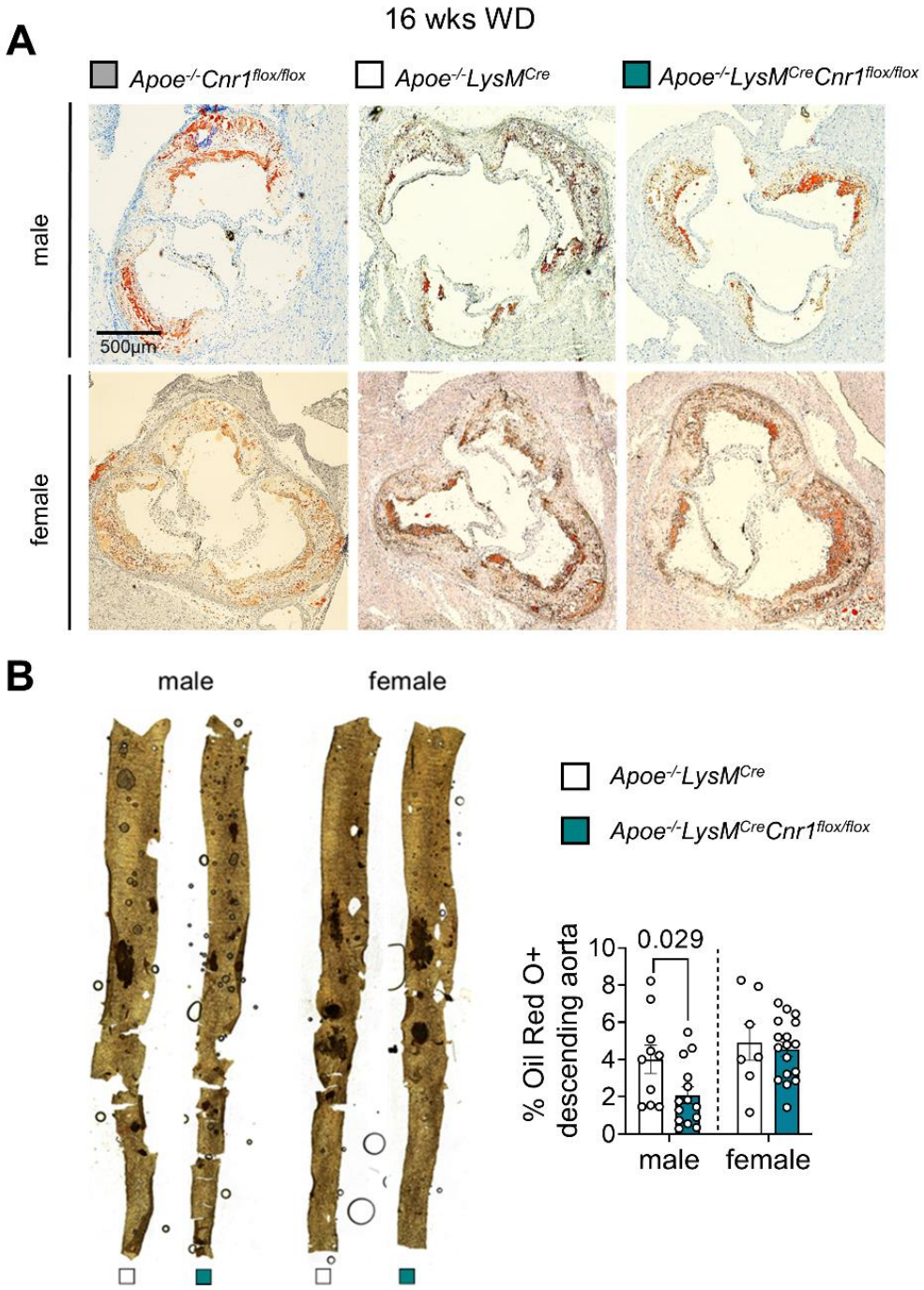
1. Janjic A, Wange LE, Bagnoli JW, Geuder J, Nguyen P, Richter D, Vieth B, Vick B, Jeremias I, Ziegenhain C, Hellmann I, Enard W. Prime-seq, efficient and powerful bulk RNA sequencing. *Genome biology* 2022;**23**:88.
2. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome biology* 2014;**15**:550.
3. Yu G, He QY. ReactomePA: an R/Bioconductor package for reactome pathway analysis and visualization. *Mol Biosyst* 2016;**12**:477-479.
4. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, Paulovich A, Pomeroy SL, Golub TR, Lander ES, Mesirov JP. Gene set enrichment analysis: A knowledge-based approach for interpreting genome-wide expression profiles. *Proceedings of the National Academy of Sciences* 2005;**102**:15545-15550.
5. Mootha VK, Lindgren CM, Eriksson K-F, Subramanian A, Sihag S, Lehar J, Puigserver P, Carlsson E, Ridderstråle M, Laurila E, Houstis N, Daly MJ, Patterson N, Mesirov JP, Golub TR, Tamayo P, Spiegelman B, Lander ES, Hirschhorn JN, Altshuler D, Groop LC. PGC-1α-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. *Nature Genetics* 2003;**34**:267-273.



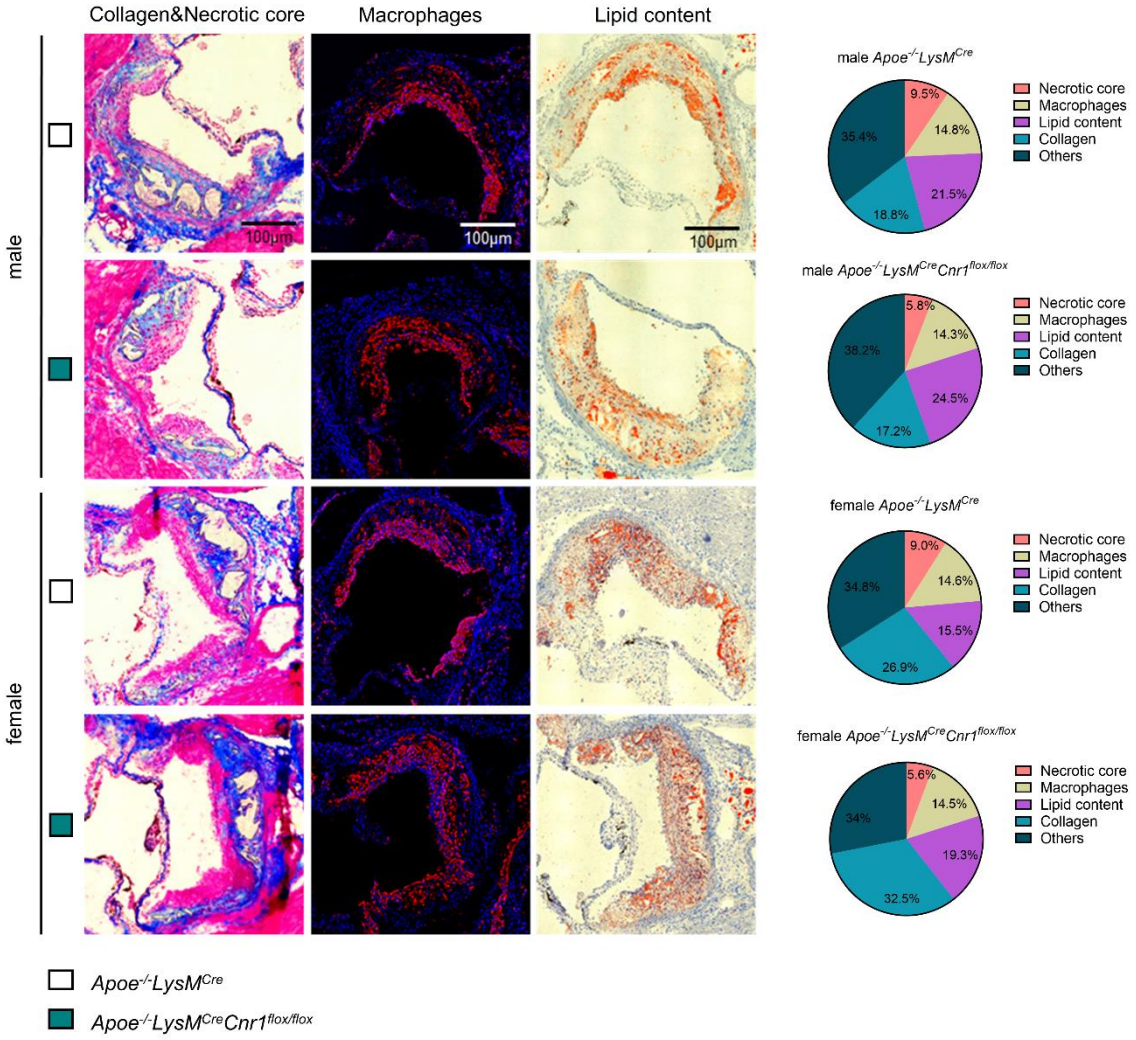
Supplemental Figure 1. Detection of murine *Cnr1* expression in myeloid cell subsets and atherosclerotic plaques. (**A**) ddPCR analysis of *Cnr1* expression in sorted myeloid cell subsets of male mice (n=3). (**B**) qPCR analysis of *Cnr1* expression in vehicle, oxLDL (1 µg/ml), LPS (10 ng/mL) or oxLDL (1 µg/ml) + LPS (10 ng/mL)-treated BMDMs from *Apoe*$^{-/-}$ male mice (n=3-4). (**C**) Fluorescence *in situ* hybridization of *Cnr1* co-immunostained for CD68 (green) and Hoechst33342 (nuclei, blue) in aortic root sections of male *Apoe*$^{-/-}$ mice subjected to 4 weeks WD. Scale bar: 100 µm (left) and 10 µm (right). (**D**) qPCR analysis of *Cnr1* expression in BMDMs from male *Apoe*$^{-/-}$*LysM*^{Cre} and compared with *Apoe*$^{-/-}$*LysM*^{Cre}*Cnr1*$^{flox/flox}$ mice (n=3-4). Each dot represents one mouse and all data are shown as mean ± s.e.m. Two-sided unpaired Student's t-test (**B** and **D**) was used to determine the *P* value.



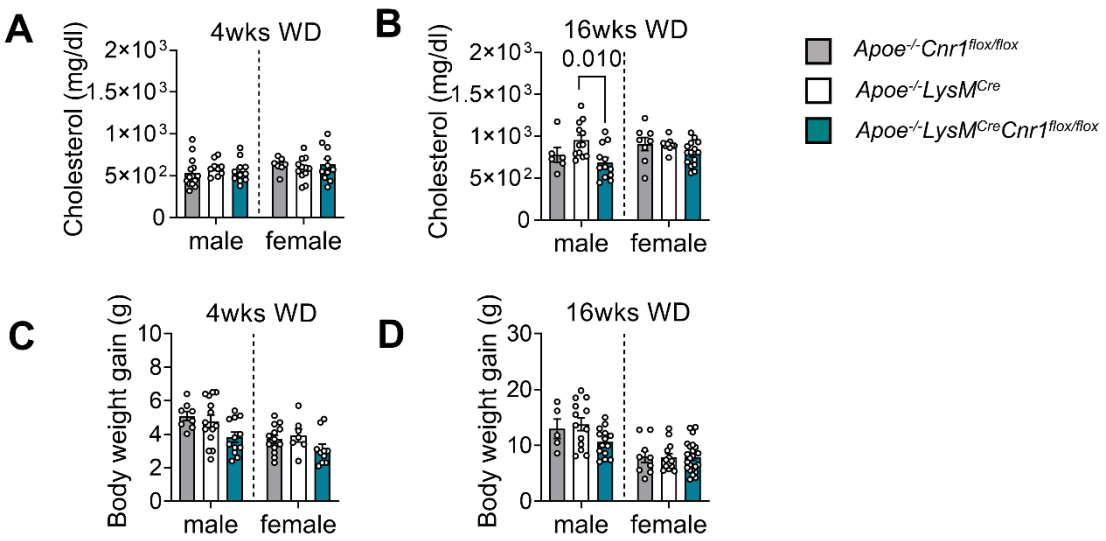
Supplemental Figure 2. Impact of myeloid *Cnr1* deficiency on neutrophil recruitment at early stage of atherosclerosis. Representative immunostainings and quantification of neutrophils (Ly6G, red) in aortic root sections of male and female *Apoe*$^{-/-}$*LysM*^{Cre} and *Apoe*$^{-/-}$*LysM*^{Cre}*Cnr1*$^{flox/flox}$ mice (n=3-4) after 4 weeks of WD; nuclei are counterstained with Hoechst33342 (blue). Scale bar: 100 µm (left) and 10 µm (right). Each dot represents one mouse and all data are expressed as mean ± s.e.m. Two-sided unpaired Student's t-test. Male and female were analyzed independently.



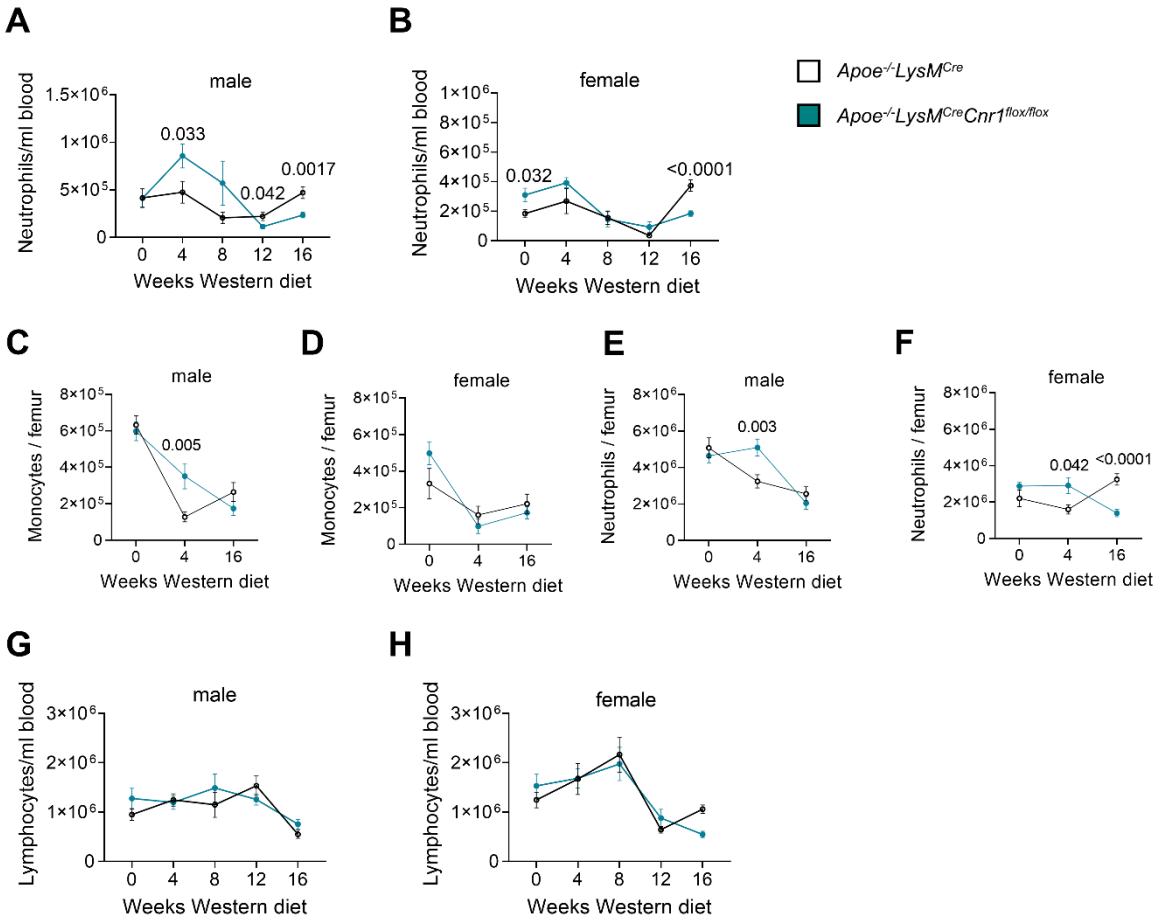
Supplemental Figure 3. Impact of myeloid *Cnr1* deficiency on advanced stage of atherosclerosis. (**A**) Representative Oil-Red-O stains of aortic roots of male and female *Apoe*$^{-/-}$*Cnr1*$^{flox/flox}$, *Apoe*$^{-/-}$*LysM*^{Cre} and *Apoe*$^{-/-}$*LysM*^{Cre}*Cnr1*$^{flox/flox}$ mice after 16 weeks of WD. Scale bar: 500 µm. (**B**) Plaque area within descending thoraco-abdominal aortas after 16 weeks WD determined by en face preparations stained with Oil-red-O, and calculated as percentage of plaque per total vessel area (n=7-17). Each dot represents one mouse and all data are expressed as mean ± s.e.m. Two-sided unpaired Student's t-test (**male**) or Welch's t test (**female**). Male and female were analyzed independently.



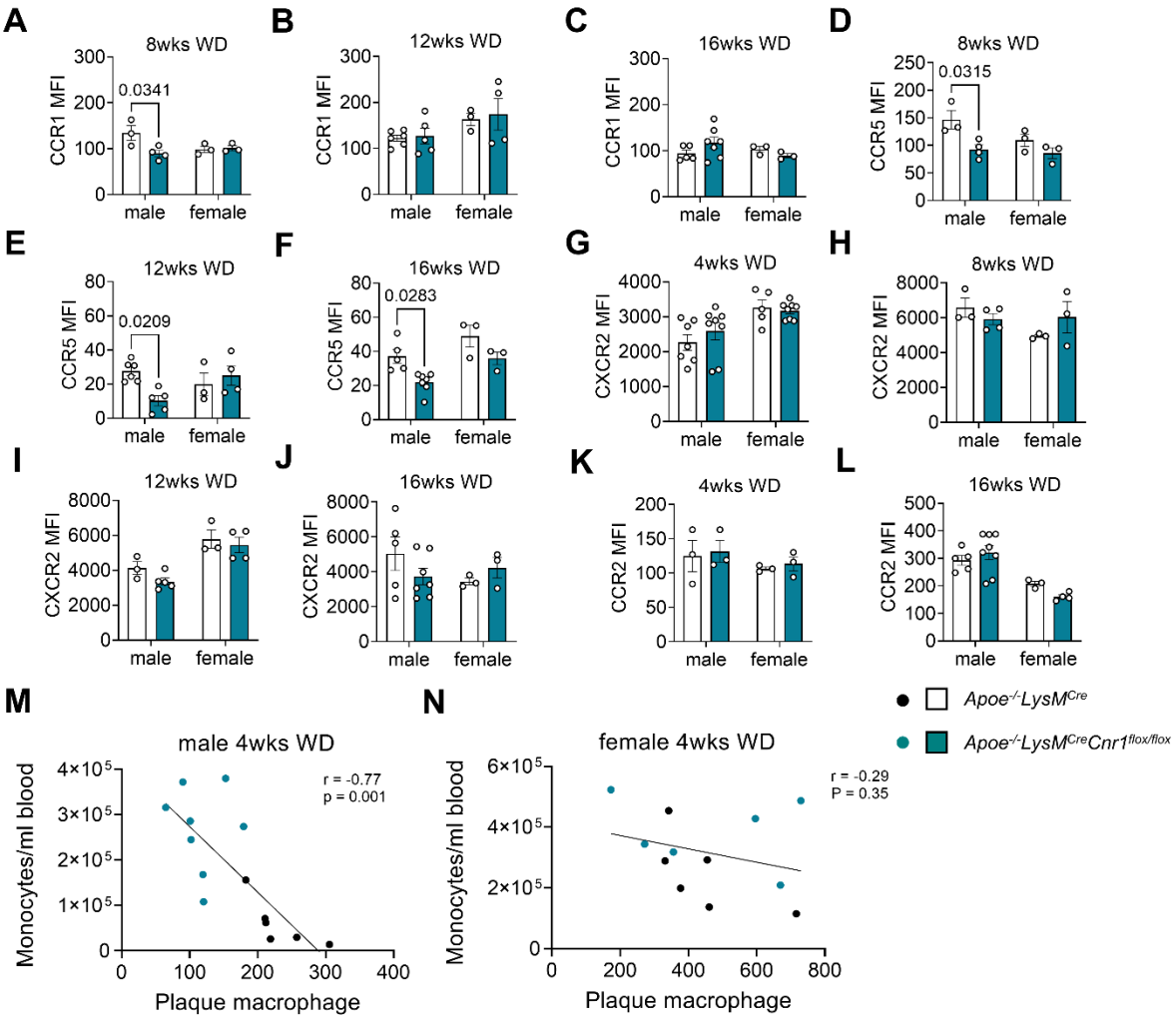
Supplemental Figure 4. Impact of myeloid *Cnr1* deficiency on advanced plaque composition. Representative male and female aortic root plaque images stained with Oil-red-O and Masson staining's for lipid or collagen content, respectively, and quantification of the necrotic core area after 16 weeks WD. Scale bar: 100 μm. Necrotic core and collagen content per total plaque area (n=6-10) were calculated. The accumulation of lesional macrophages was determined by CD68 staining (n=6-10).



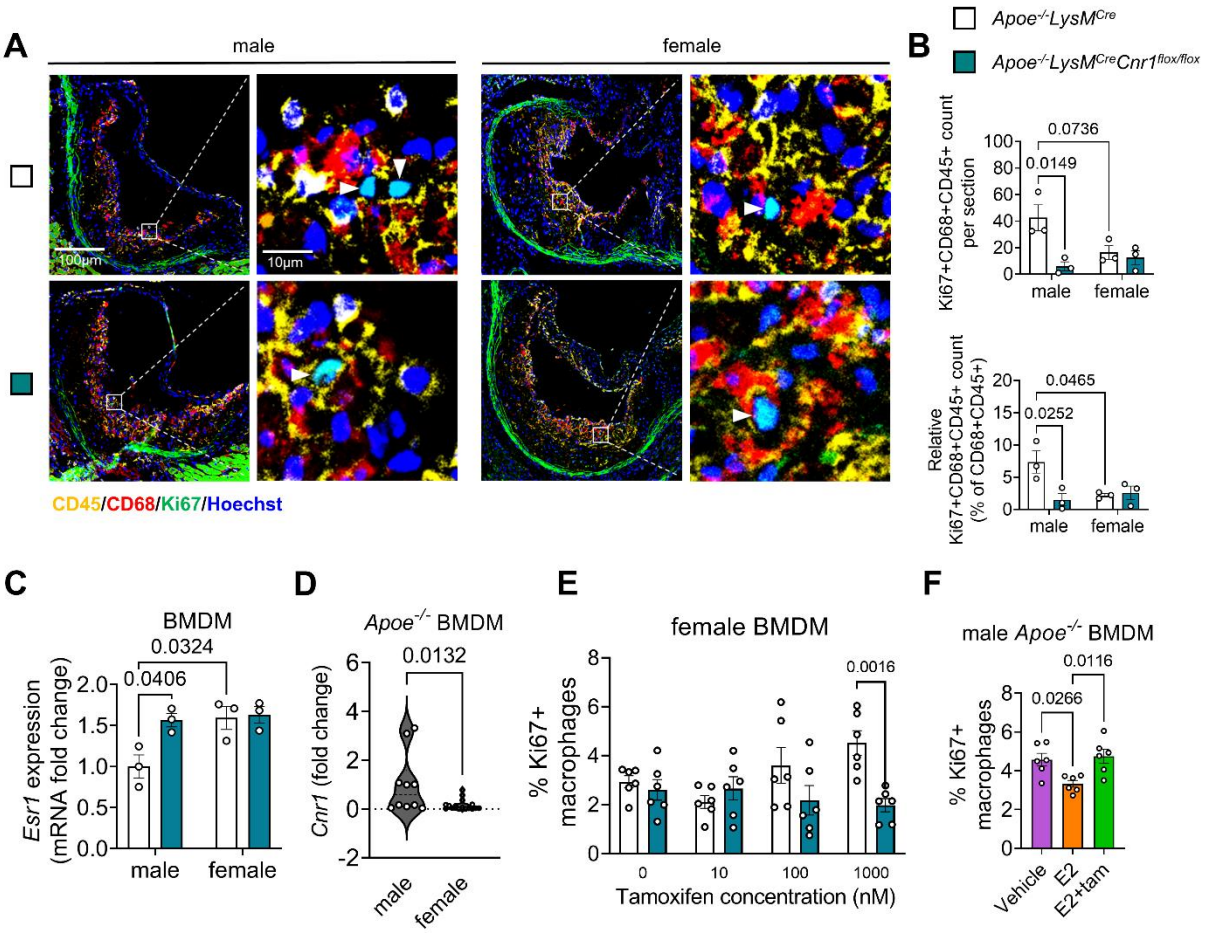
Supplemental Figure 5. Impact of myeloid *Cnr1* deficiency on metabolic parameters. (**A-B**) Plasma cholesterol levels after 4 and 16 weeks Western diet (WD) in male and female *Apoe*$^{-/-}$*Cnr1*$^{flox/flox}$, *Apoe*$^{-/-}$*LysM*^{Cre} and *Apoe*$^{-/-}$*LysM*^{Cre}*Cnr1*$^{flox/flox}$ mice (n=6-14). (**C-D**) Body weight gain after 4 and 16 weeks WD relative to the initial body weight in male and female *Apoe*$^{-/-}$*Cnr1*$^{flox/flox}$, *Apoe*$^{-/-}$*LysM*^{Cre} and *Apoe*$^{-/-}$*LysM*^{Cre}*Cnr1*$^{flox/flox}$ mice (n=5-23). Each dot represents one mouse and all data are expressed as mean ± s.e.m. One-way ANOVA followed by Dunnett T3 test (**A-D**). Male and female were analyzed independently.



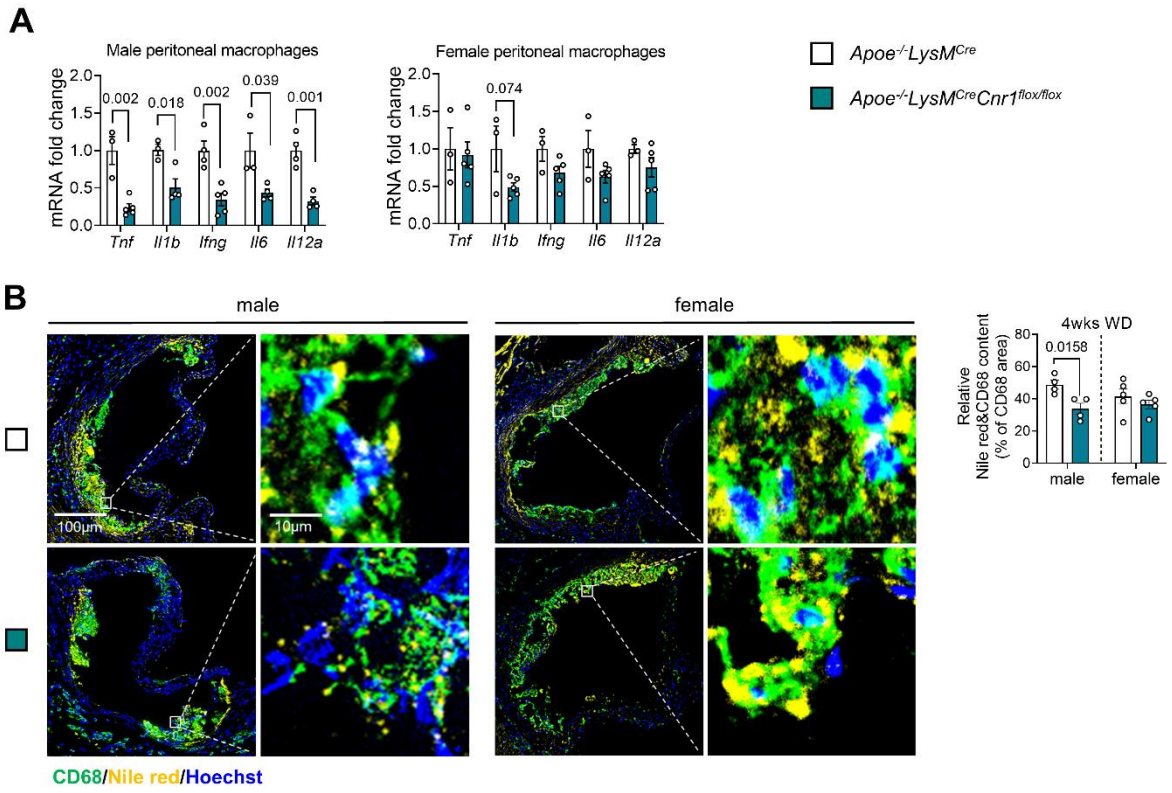
Supplemental Figure 6. Impact of myeloid *Cnr1* deficiency on circulating and bone marrow leukocyte counts. Number of circulating neutrophils in male (m) and female (f) mice assessed by flow cytometry after 0 (n=9-10), 4 (n=18-19 m; n=6-10 f) 8 (n=10 m; n=5-10 f), 12 (n=7-8 m; n=5-6 f) and 16 (n=14-15 m; n=15-23 f) weeks of Western diet (WD) (**A** and **B**). Number of femural monocytes gated as CD45+ CD11b+ CD115+ (**C** and **D**) and neutrophils gated as CD45+ CD11b+ Ly6G+ (**E** and **F**) assessed by flow cytometry after 0, 4 and 16 weeks of WD. Number of circulating lymphocytes assessed by flow cytometry after 0, 4, 8, 12 and 16 weeks of WD (**G** and **H**). Each dot represents one mouse and all data are expressed as mean ± s.e.m. Two-sided unpaired Student's t-test (**A-H**). Male and female were analyzed independently.



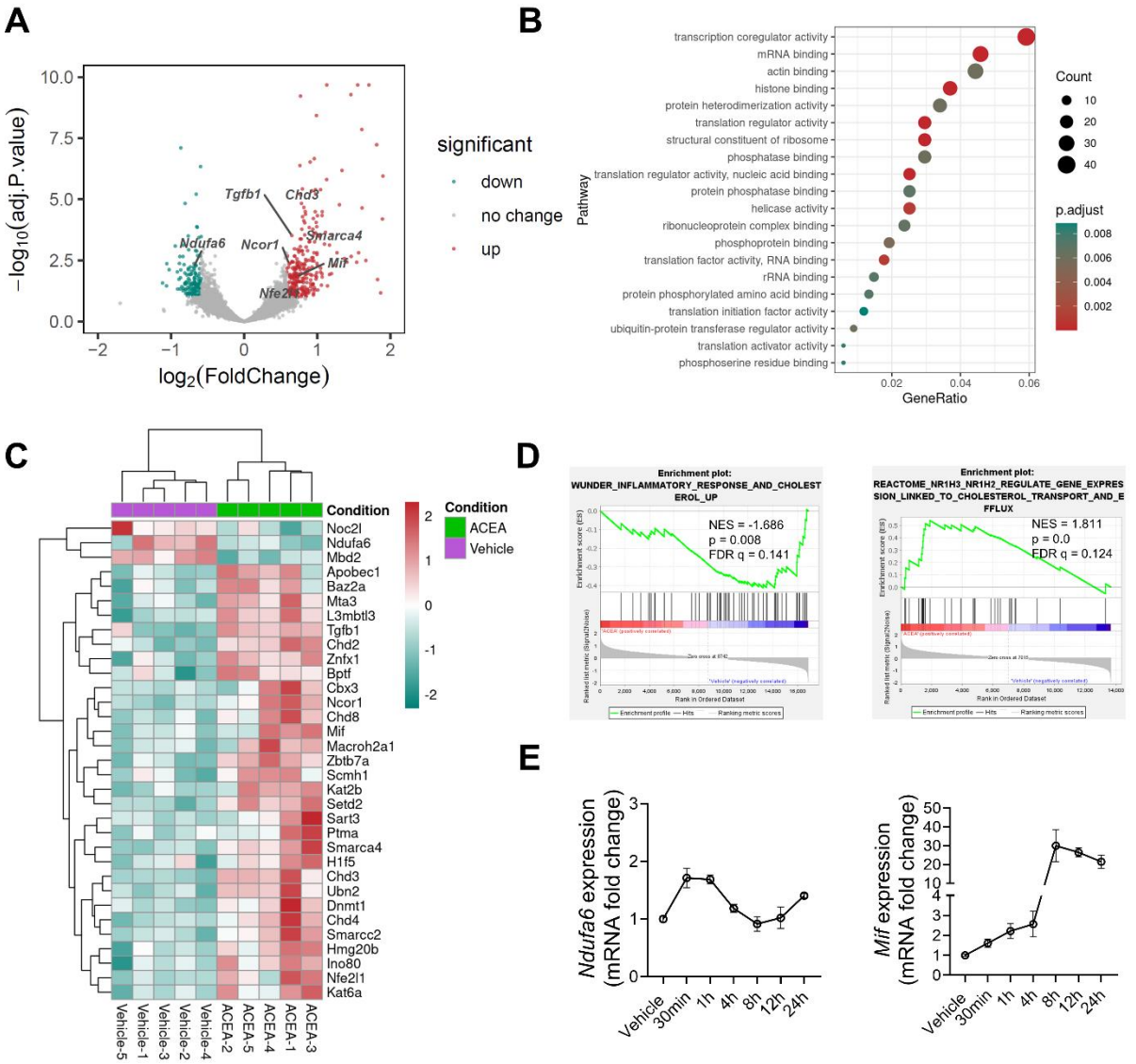
Supplemental Figure 7. Impact of myeloid *Cnr1* deficiency on circulating monocyte chemokine receptor surface levels. (**A-F**) Flow cytometric analysis of CCR1 and CCR5 surface expression on circulating monocytes after 8, 12 and 16 weeks of WD (n=3-7). (**G-J**) Flow cytometric analysis of CXCR2 surface expression on circulating neutrophils after 4, 8, 12 and 16 weeks of WD (n=3-7). (**K**, **L**) Flow cytometric analysis of CCR2 surface expression on circulating monocytes after 4 and 16 weeks of WD (n=3-7). (**M**, **N**) Pearson correlation analysis between plaque macrophages and circulating monocytes after 4 weeks WD. Each dot represents one female or male mouse and all data are expressed as mean ± s.e.m. Two-way ANOVA followed by Tukey test (**A**-**L**).



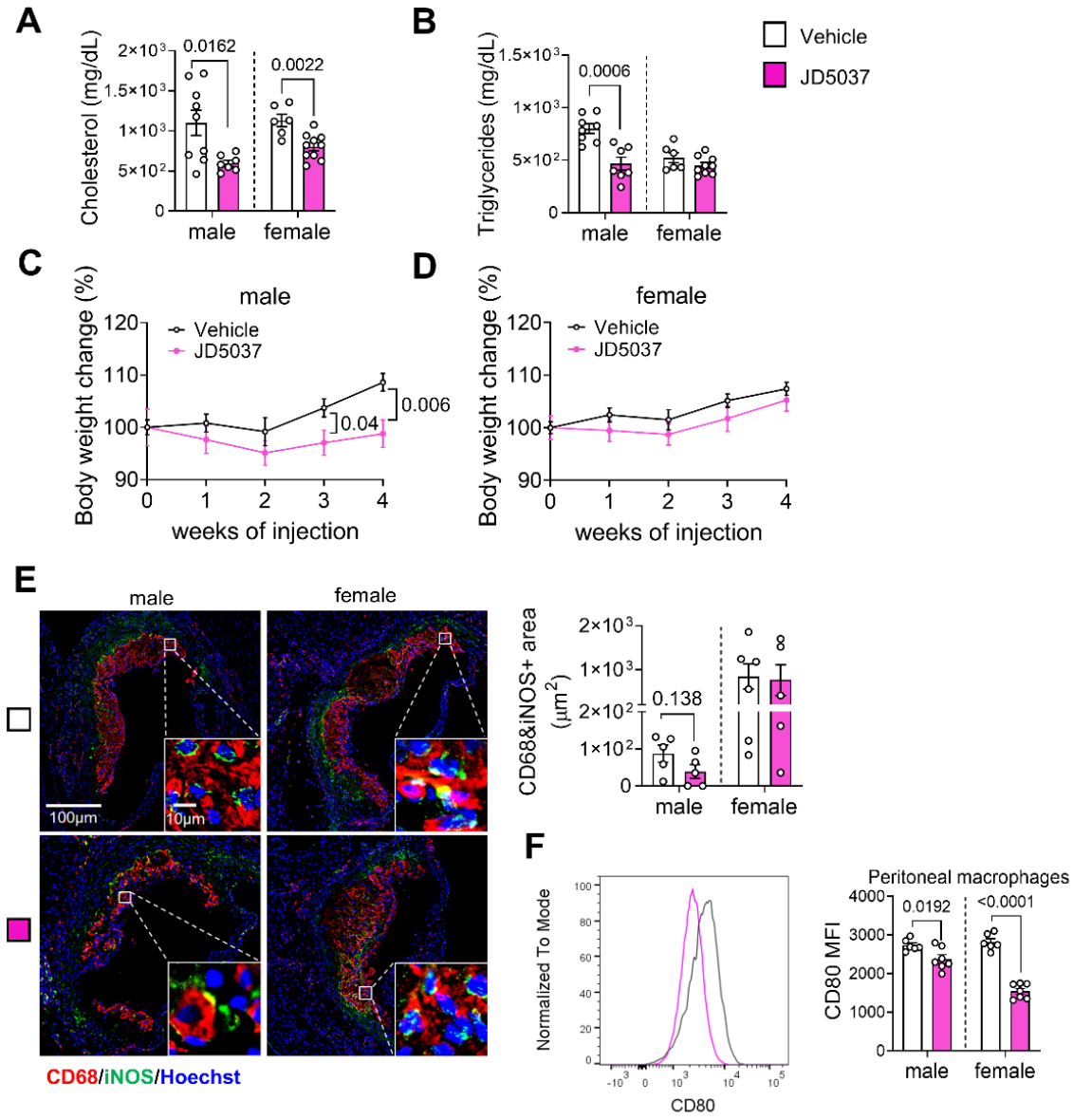
Supplemental Figure 8. Impact of myeloid *Cnr1* deficiency on advanced stage plaque macrophage proliferation and role of estrogen receptor α signaling. (A) Representative images of proliferating (Ki67+, green and marked with white arrowheads) macrophages (CD45+, yellow and CD68+, red) in male and female aortic root plaques after 16 weeks Western diet (WD); nuclei were counterstained with Hoechst 33342 (blue). scale bar: 100 µm (left) and 10 µm (right). (**B**) Total and relative counts of proliferating plaque macrophages (n=3). (**C**) Gene expression levels in untreated male and female *Apoe*$^{-/-}$*LysM*^{Cre} and *Apoe*$^{-/-}$*LysM*^{Cre}*Cnr1*$^{flox/flox}$ BMDMs (n=3). (**D**) Gene expression levels in male and female *Apoe*$^{-/-}$ BMDMs (n=3). (**E**) Proliferation rates in female *Apoe*$^{-/-}$*LysM*^{Cre} and *Apoe*$^{-/-}$*LysM*^{Cre}*Cnr1*$^{flox/flox}$ BMDM treated with vehicle or tamoxifen for 24 h (n=6). (**F**) Proliferation rates in male *Apoe*$^{-/-}$ BMDMs treated with vehicle, 10 nM estradiol (E2) and 1 µM tamoxifen (tam) for 24 h (n=6). Each dot represents one mouse and all data are expressed as mean ± s.e.m. Two-sided unpaired Student's t-test (**D**, **E**), one-way ANOVA followed by Tukey test (**F**), two-way ANOVA followed by Tukey test (**B**, **C**).



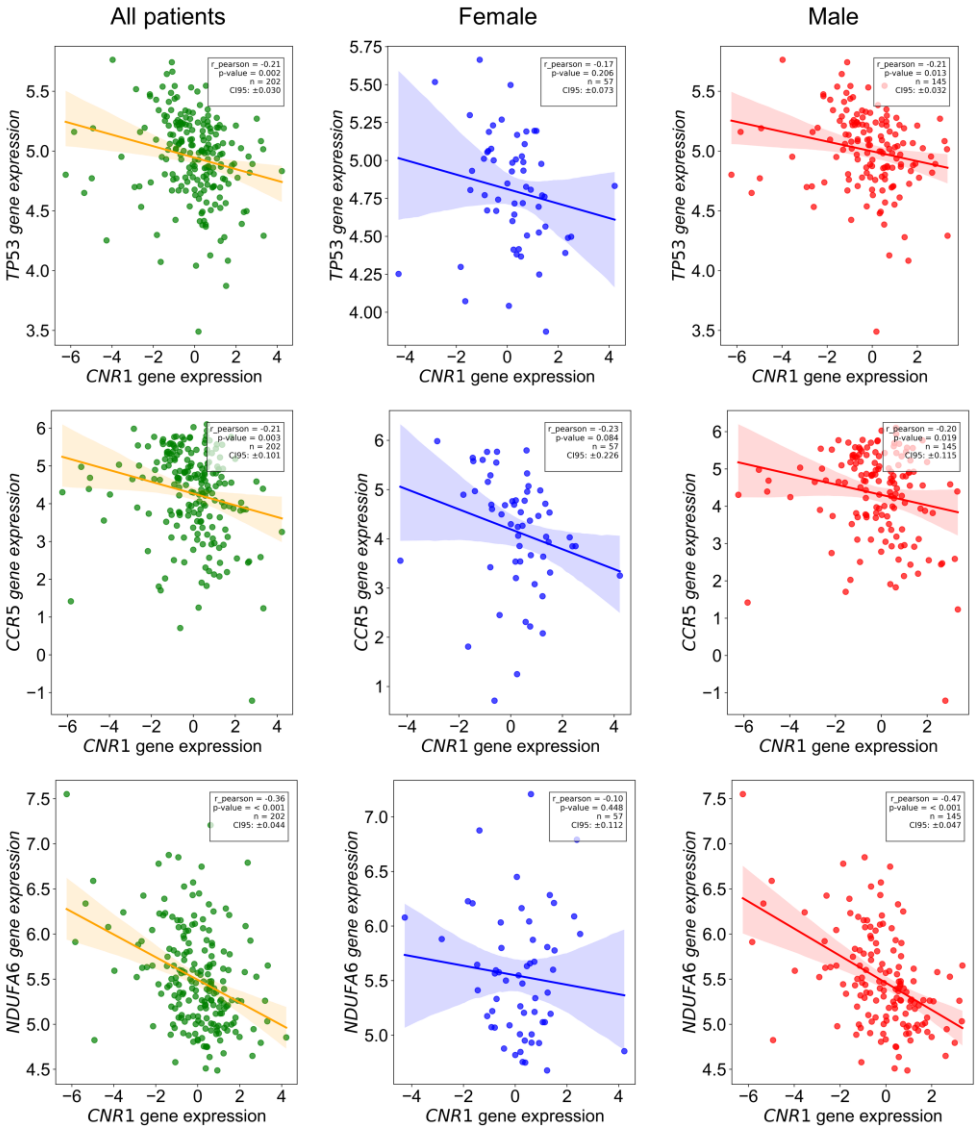
Supplemental Figure 9. Impact of myeloid *Cnr1* deficiency on peritoneal macrophage inflammatory gene expression and plaque macrophage lipid droplet accumulation. (**A**) Expression levels of pro-inflammatory genes in peritoneal macrophages of male and female *Apoe*$^{-/-}$*LysM*^{Cre} and *Apoe*$^{-/-}$*LysM*^{Cre}*Cnr1*$^{flox/flox}$ mice after 4 weeks Western diet (WD) (n=3-5). (**B**) Nile Red staining (yellow) of lipid droplets within aortic root plaque macrophages (CD68, green) of male and female *Apoe*$^{-/-}$*LysM*^{Cre} and *Apoe*$^{-/-}$*LysM*^{Cre}*Cnr1*$^{flox/flox}$ mice after 4 weeks of WD (n=4-5). Nuclei were stained with Hoechst33342 (blue); Scale bar: 100 µm (left) and 10 µm (right). The accumulation of lipid droplets in macrophages was quantified as CD68 and Nile red double positive staining. Each dot represents one mouse and all data are expressed as mean ± s.e.m. Two-sided unpaired Student's t-test. Male and female were analyzed independently.



Supplemental Figure 10. Impact of CB1 stimulation on the transcriptomic profile of BMDM. (**A**) Volcano plot of bulk RNA sequencing data showing differentially expressed genes (DEGs) in male *Apoe*$^{-/-}$ BMDMs stimulated with 1 µM ACEA for 24 h versus vehicle (n=5 separate BMDM donor mice). (**B**) Pathway analysis showing top molecular functions regulated in CB1-stimulated male BMDM. (**C**) Heat map showing chromatin modification pathway-related DEGs and selected genes highlighted in (**A**). (**D**) Gene set enrichment analysis (GSEA) identifies CB1-regulated gene sets associated with inflammatory response and cholesterol. Enrichment plots of significantly enriched gene sets with normalized enrichment score (NES), nominal P value and false discovery rate (FDR) q-value are shown. (**E**) qPCR analysis of male *Apoe*$^{-/-}$ BMDMs stimulated with 1 µM ACEA for the indicated time points, normalized to vehicle (time point 0). Data in (**E**) are expressed as mean ± s.e.m.



Supplemental Figure 11. Impact of peripheral CB1 antagonist JD5037 on metabolic parameters, plaque macrophage accumulation and phenotype. (**A**-**B**) Plasma cholesterol, triglyceride levels and (**C-D**) body weight progression during the treatment period in male and female *Ldlr*$^{-/-}$ mice (n=6-10) after a total of 8 weeks WD diet, and receiving daily injections of vehicle- or peripheral CB1 antagonist JD5037 for the last 4 weeks of diet. (**E**) Double immunostaining and quantification of iNOS (green) and CD68 (red)-positive macrophages in aortic root lesions of male and female *Ldlr*$^{-/-}$ mice (n=5-6) treated with vehicle or JD5037. Nuclei were stained with Hoechst33342 (blue). Scale bar: 100 µm (left) and 10 µm (right). (**F**) Surface expression of the pro-inflammatory macrophage marker CD80 on peritoneal macrophages, assessed at the study endpoint (n=6-7). Each dot represents one mouse and all data are expressed as mean ± s.e.m. Two-sided unpaired Student's t-test (**A-F**). Male and female were analyzed independently.



Supplemental Figure 12. Correlation analysis between CNR1 and key markers of cell cycle control, inflammation and mitochondrial oxidative metabolism in human plaques. Bulk RNA sequencing data from human carotid plaques were used for linear regression analysis, based on normalized gene expression levels of selected genes. Scatter plots and regression lines are shown (n = 202 samples). Middle and right graphs show scatter plots for female (n = 57) or male patients (n = 145) only. Pearson correlation coefficients (r) and P values are presented in the graphs.

Supplemental Table 1: Antibodies used for immunostaining

Antigen	Source	Dilution	Reference	Provider
CD45	Rat	1:100	MCA1031G	Bio-Rad
CD68	Rabbit	1:200	PA5-78996	ThermoFisher
CD68	Rat	1:400	MCA1957GA	Bio-Rad
Ki67	Rat	1:50	11-5698-82	eBioscience
Ly6G	Rat	1:100	551459	BD
NOS(pan)	Rabbit	1:100	2977	Cell Signaling
Phospho-p53	Rabbit	1:100	12571s	Cell Signaling

Supplemental Table 2: Isotype controls

Immunoglobulin	Reference	Provider
Normal rat IgG	6-001-A	RD system
Normal rabbit IgG	315-005-003	JIR
Rat IgG2a FITC	553929	BD

The working concentrations of normal IgGs for isotype controls was similar to the concentration used for primary antibodies. IgG = Immunoglobulin G; JIR = Jackson ImmunoResearch.

Supplemental Table 3: Secondary antibodies

Antigen	Source	Conjugation	Dilution	Reference	Provider
anti-rabbit	donkey	AF488	1:100	711-545-152	JIR
anti-rabbit	donkey	AF647	1:400	711-606-152	JIR
anti-rat	donkey	AF488	1:300	712-546-150	JIR
anti-rat	donkey	Cy3	1:300	712-165-153	JIR

Supplemental Table 4: Murine FACS antibodies.

Antigen	Conjugation	Dilution	Reference	Provider
CCR1	PE	1:50	FAB5986P	R&D
CCR5	PE-Cy7	1:100	107017	Biolegend
CD3	FITC	1:500	555274	BD
CD11b	PB	1:500	101224	BioLegend
CD11b	APC	1:500	17-0112-82	invitrogen
CD11b	APC-Cy7	1:500	101225	Biolegend
CD11b	PerCP	1:500	101230	Biolegend
CD16/32	purified	1:1000	553142	BD
CD19	PE	1:500	12-0193-82	eBioscience
CD38	PE-Cy7	1:500	102717	Biolegend
CD45.2	APC	1:500	558702	BD
CD45.2	BV510	1:500	109837	Biolegend
CD45.2	FITC	1:500	553772	BD
CD80	PerCP/Cy5.5	1:200	104721	Biolegend
CD115	APC	1:500	17-115-282	eBioscience
F4/80	APC	1:500	123116	Biolegend
F4/80	PB	1:500	123123	Biolegend
Ki67	FITC	1:200	11-5698-82	eBioscience
Ly6C	BV510	1:500	128033	Biolegend
Ly6G	APC-Cy7	1:500	127623	Biolegend
Live/dead	Zombie NIR	1:400	423105	Biolegend
Mitotracker green	FITC	100nM	M7514	Invitrogen

APC = Allophycocyanin; BV = Brilliant violet; Cy = Cyanine; FC = Flow Cytometry; FITC = Fluorescein isothiocyanate; IF=Immunofluorescence; PB = Pacific Blue; PE = Phycoerythrin; PerCP = Peridinin chlorophyll.

Supplemental Table 5: Primers for qPCR and ddPCR analysis

Murine gene	5'-3' primer sequence or Assay ID
Ccr1	Mm00438260_s1
Ccr5	Fw AATATTTCCTTGAAAGTATTTTTAGCCGT
	Rev TTAAAACTCTTTTGATTGAGAGTAAGCA
	Probe *FAM*-AGATGTTATG TCCAAGCATG CAGTTTCGGA-*TAMRA*
Cd74	Mm00658576_m1
Chil3	Fw GGA AGC CCT CCT AAG GAC AAA
	Rev GAA TGT CTT TCT CCA CAG ATT CTT
	Probe *FAM*-TGT TCT GGT GAA GGA AAT GCG TAA-*TAMRA*
Cnr1	Fw ATGCGAAGGGGTTCCCTC
	Rev ATGGTACGGAAGGTGGTATCT
	Probe *FAM*-TGGCACCTCTTTCTCAGTCACGTTGAGC-*TAMRA*
Cnr1 (ddPCR)	Fw ATGCGAAGGGGTTCCCTC
	Rev ATGGTACGGAAGGTGGTATCT
	Probe 5'6-FAM-TGGCACCTC/ZEN/TTTCTCAGTCACGTTGAGC-3IABkFQ
Cx3cr1	Mm02620111_s1
Esr1	Mm00433149_m1
Hprt	Fw GACCGGTCCCGTCATGC
	Rev TCATAACCTGGTTCATCATCGC
	Probe *VIC*-ACCCGCAGTCCCAGCGTCGTG-*TAMRA*
Hprt (ddPCR)	Fw GACCGGTCCCGTCATGC
	Rev TCATAACCTGGTTCATCATCGC
	Probe 5HEX-ACCCGCAGT/ZEN/CCCAGCGTCGTG-3IABkFQ
Ifng	Mm01168134_m1
Il1b	Mm00434228_m1
Il6	Mm00446190_m1
Il12a	Mm00434165_m1
Irf5	Mm00496477_m1
Mif	Mm01611157_gH
Ncor1	Mm01333102_m1
Ndufa6	Mm01303455_g1
Nos2	Mm00440502_m1
Tnf	Mm00443258_m1

Self-designed primers and probes for qPCR were purchased from MWG-Biotech AG and TaqMan Gene Expression Arrays obtained from Life Technologies. ddPCR = digital droplet PCR; Fwd = Forward; Rev = Reverse.

Supplemental table 6: Patient characteristics

Patient characteristics		Total Number (n= 202)
Age (mean, range), years		72.4 (53-93)
Gender	male	145 (71.8 %)
	female	57 (28.2 %)
BMI (Mean)		26.3
ASA Stadium (Mean)		2.6
Hypertension		151 (74.8 %)
CHD		46 (22.8 %)
Diabetes		15 (7.4 %)
Dyslipidemia		115 (56.9 %)
Dialysis		0
Smoking	active smoker	22 (10.9 %)
	ex smoker	63 (31.2 %)
	never	111 (56%)
	n/a	6
State at presentation	symptomatic	79 (39.1 %)
	asymptomatic	123 (60.9 %)
Type of symptoms	TIA	23 (11.4 %)
	amaurosis fugax	24 (9.4 %)
	apoplex	19 (11.9 %)
Degree of stenosis (mean, range), %		81.8 (40-100)

TIA = transient ischemic attack