Targeting sortilin in immune cells reduces proinflammatory cytokines and atherosclerosis

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Supplementary material

Supplementary Methods

Methods

Mice

Sort1-/- mice were created as previously described (1) and backcrossed into C57BL/6 mice for more than 10 generations. *Apoe-/-* mice (B6.129P2-*Apoe*^{TmUnc1}) were obtained from Taconic. Breeding pairs were set up to produce *Sort1-/-Apoe-/-*, *Sort1-/+Apoe-/-* and *Sort1+/+Apoe-/-* mice. Genotyping was performed by PCR using primers shown in **Supplementary Table S1**. High-fat diet (HFD) contained 21% saturated fat and 0.21% cholesterol (Research Diets, D12079B). All procedures were approved by the Danish Animal Experiments Inspectorate.

Bone marrow transplantation

Sort1+/+Apoe-/- and *Sort1-/-Apoe-/-* mice (8 weeks of age) were lethally irradiated with 9.85 Gy and rescued with 10E7 age- and sex-matched unfractionated bone marrow cells from *Sort1+/+Apoe-/-* or *Sort1-/-Apoe-/-* donor mice. One donor mouse was used for each irradiated mouse to avoid compromising the independence assumption of the statistical analysis.

Successful reconstitution of the hematopoietic system with donor cells was determined by quantitative PCR in DNA isolated from whole blood cells at 4 weeks post-transplant and at the end of study. Quantitative PCR reactions were set up for the targeted and the wildtype *Sort1* alleles using Maxima SYBR Green qPCR Master Mix (Thermo Scientific) and primers indicated in **Supplementary Table S1**. PCR reactions were found to amplify with similar efficiency thereby meeting the assumptions for calculating the ratio of targeted *Sort1* (KO) over wildtype (WT) alleles in blood as $r=2^{(Ct_{WT}-Ct_{KO})}$. Chimerism for *Sort1-/-* blood cells could then be calculated as r/(r+1). DNA from blood cells of *Sort1-/-*, *Sort1-/+* and *Sort1+/+* mice were run as controls and confirmed to give <0.05%, ~50% (40-60%) and >99.9% presence of targeted *Sort1* alleles.

Tissue processing

The mice were anesthetized with 5 mg pentobarbital i.p with 10% lidocain and euthanized by withdrawal of blood from the right ventricle. The mice were flushed with Cardioplex solution, perfusion-fixed with 4% formaldehyde via the left ventricle, and then immersed in

4% formaldehyde for 6 hours. The top half of the heart including the proximal part of the aorta was either embedded in paraffin or cryoprotected in sucrose solution (25% wt/vol for 24 hours plus 50% wt/vol for 24 hours) followed by snap-freezing in OCT (Sakura Finetek). For quantification of atherosclerosis in the aortic root, sections were cut from the distal part of the heart until the commissures of the aortic valves appeared. Sections were systematically serially sampled at 80 μm intervals from this level onwards. Depending on the stage of atherosclerosis induced in the different experiments, 1, 3 or 6 levels were quantified by computer-assisted morphometry (ImageJ, NIH) in orcein-stained sections.
Aortic lesion coverage was measured *en face* using a standard method. Briefly, the aorta was cut open, stained with Oil Red O for 10 minutes at 37^{o}C, and mounted on a microscope slide with AquaTex. Slides were scanned and analysed using ImageJ.

Blood Analysis

Non-fasting total plasma cholesterol was measured using a commercially available kit (Cholesterol CHOD-PAP, Roche/Hitachi). Distribution of cholesterol across size-fractionated lipoproteins was measured in pooled samples from each group by FPLC (fast protein liquid chromatography) in the Gaubius Laboratory, TNO Biosciences, the Netherlands.

Immunoblotting

Sort1+/+ and *Sort1*-/- mice, 8-10 weeks of age, were killed by cervical dislocation. For isolation of peritoneal macrophages, the skin was removed from the abdomen and 5-10 mL culture medium (DMEM with 10% fetal bovine serum, 1% penicillin/streptomycin, 1% L-glutamine) was injected into the abdominal cavity. After 5 minutes, the fluid was retracted and the procedure was then repeated. The retracted medium was spun down and cells were resuspended in 1 mL culture medium in 24-well plates. Attached macrophages were harvested after 2 hours. Smooth muscle cells were obtained by isolating the aorta, removing the adventitia and scraping off the endothelium. Th1 cells were isolated as described in main text. All tissues and cells were lysed and homogenized. Protein levels in all samples were measured by spectrophotometry. Samples were run on NuPage 4-12% BisTris precast gels (Invitrogen). The antibody used was mouse anti-NTR3 (1:1000, BD Biosciences, 612100). Hippocampus from *Sort1*+/+ mice was used as positive control and tissues from *Sort1*-/- mice as negative controls.

Monocyte recruitment assay

Monocyte recruitment was assessed using the thioglycolate sterile peritonitis assay. Briefly, a 4% Brewer thioglycolate solution (B2551, Sigma-Aldrich) was mixed and autoclaved before storing for a minimum of 4 days at 4 degrees for optimal reactivity. Intraperitoneal injections (1 ml) was performed in mice at 8 weeks old mice, and 4 days later, the mice were anesthetized (5 mg pentobarbital i.p with 10% lidocain) and euthanized, and intraperitoneal cells were harvested with 5 ml of ice cold phosphate-buffered saline (PBS). The number of cells obtained was counted using NucleoCounter NC100.

Foam cell assays

Human LDL was isolated from healthy subjects by density gradient ultracentrifugation and fluorescently-labeled with atto-633 NHS ester (Sigma-Aldrich). LDL was aggregated as previously described (2). To obtain oxidized LDL, native LDL was incubated with copper sulphate ($CuSO_4$) (20 μM) overnight at 37^{o}C. Successful oxidation was confirmed by measuring increased migration on a 0.5% agarose gel. For foam cell formation assessment, BMM were seeded in 48-well plates (1.5x10E5 cells/well). After 24 hours, BMMs were incubated with native (1mg/ml), aggregated (1mg/ml) or oxidized (20μg/ml) LDL for 20 hours. Uptake of LDL was determined by flow cytometry (Beckman Coulter FC500).

Cell culture assays

Bone marrow cells were isolated from femurs and tibias of *Sort1*+/+ and *Sort1*-/- mice and cultured in RPMI medium supplemented with 10% FBS, 1% penicillin/streptomycin and 10% L-cell conditioned medium for 7 days to generate bone marrow derived macrophages (BMM) as previously described (3). BMMs were seeded at 1.5x10E5 cells/well in 48 well-plates, and, after 24 hours, polarized towards an M1 phenotype with LPS (γ-irradiated, L4391, Sigma-Aldrich). Where indicated sortilin propeptide was added at a concentration of 20 μM.

Untouched CD4 positive T cells were isolated from spleen mononuclear cells using negative selection with magnetic beads (Dynabeads, Invitrogen). For Th1 polarization, the cells were cultured on anti-CD3 and anti-CD28-coated wells in RPMI medium supplemented with Il-2 (20ng/ml), Il-12 (20ng/ml), anti-Il-4 (10μg/ml) and 2-mercaptoethanol (50μM) for 3 days.

Th1 cells were seeded in anti-CD3 and anti-CD28-coated 96-well plates (1x10E5 cells/well) for 6 hours before the media was collected.

Cytokine secretion into the cell culture medium was analyzed using magnetic bead-based Luminex-kits (20-plex mouse cytokine, Invitrogen) on a Luminex 100^{TM} instrument as previously described (4), or with IL-6, TNF-α and IFN-γ ELISAs (Ready-SET-Go Mouse kits, eBioscience).

For quantitative PCR, RNA was extracted from macrophages using RNeasy Micro kits (Qiagen) and converted to cDNA using RevertAid First Strand cDNA Synthesis kit (Thermo Scientific). Gene expression was assessed using Maxima SYBR Green qPCR Master Mix (Thermo Scientific) and primers listed in **Supplementary Table S1** with β2-microglobulin as the reference gene.

MAP kinase phosphorylation analysis

For assessment of MAP kinase phosphorylation upon activation of macrophages, BMMs were seeded at a concentration of 1.5x10E5 cells/well in 12-well plates and incubated for 24 hours. The cells were activated with 100 ng/ml LPS for 0, 15, 30, 60, 120, and 240 min. Hereafter the cells were lysed with TNE (10 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% nonidet P-40 (Sigma-Aldrich), pH 8.0) with complete protease inhibitor (Roche) and Phosstop (Roche), and subjected to SDS-PAGE followed by western blotting. The samples were probed for MAPK (Cell Signaling, #4695), pMAPK (Cell Signaling, #9101), and beta-actin (Sigma Aldrich, A5441). Antibodies described at www.pabmabs.com.

Surface Plasmon Resonance Analysis

Binding of ligands to sortilin was determined on a Biacore instrument (Biacore, Sweden) using CaHBS as sample and running buffer (10mM HEPES, 150nM NaCl, 1.5 mM $CaCl_2$, 1mM EGTA, 0.005% Tween-20, pH 7.4). Human soluble sortilin was immobilized on a CM5 biosensor chip at 0.057-0.073 pmol/mm^2. Recombinant human IL-6 (Millipore, IL006) was added to the chip (0-600 s) at different concentrations, ranging 100-1000 nM of IL-6. After 600 s buffer was added and IL-6 dissociated from the immobilized sortilin receptor. For the inhibition test, the GST-fused sortilin propeptide was added (200-650s), and at 650-1200 s either rhIL6, rhIL6 and GST-propeptide, or GST-propeptide was added. At 1200s, buffer was added and ligands dissociate. Studies with recombinant IFN-γ (R&D , 485−MI−100) were performed

similarly. The SPR signal is expressed in relative response units (RUs) after substraction of the RU in a control flow channel.

HEK cell experiments

HEK293 cells transfected with human full length sortilin and untransfected HEK293 cells were incubated on ice in DMEM at 4^{o}C for 15 min followed by addition of 125 nM hIL-6 (Millipore, IL006) or 125 nM hIL-6 + 20 μM sortilin propeptide. The cells were fixed with 4% paraformaldehyde in PBS, permeabilized using 0.1% saponin, blocked in 10% donkey serum, and incubated with goat anti-hIL-6 (AF406NA, R&D systems) and rabbit-anti-human sortilin (5). Secondary fluorescent-labeled antibodies (Alexa488 donkey-anti-goat and Alexa568 donkey-anti-rabbit) were used for visualization.

Co-immunoprecipitation

HEK293 cells were stably transfected with human full-length sortilin and/or hIL-6-GFP (Origene, RG202078). After reaching 90% confluency, the cells were washed and incubated in PBS pH 7.4 for 10 min after which proteins were cross-linked with 50 mM DSP (ThermoScientific, 22585) for 30 min at room temperature. The reaction was stopped with 1M Tris pH 7.5, and the cells were lysed in TNE with complete protease inhibitor (11697498001, Roche). The cleared lysate was added to beads (GammaBind G Sepharose, GE Healthcare, 71-7057-00 AH) to which IL-6 antibody (AF206NA, R&D Systems) had been coupled and incubated for 24 hrs at 4^{o}C. After 5 washes, the beads were boiled in SDS with DTE, and subjected to SDS-PAGE. After western blotting the blots were probed for sortilin (B&D Bioscience, BD612100) and IL-6 (Abcam ab6672 or R&D AF206NA).

Quantification of circulating immune cells

For calculation of absolute cell counts, CountBright Absolute Counting Beads (Invitrogen) was added to blood samples. Circulating immune cells were labelled for FACS analysis with antibodies against T cells (CD3-APC), B cells (CD19-PE) and total white blood cells (CD45-FITC, all BD Biosciences).

Supplemental References

1. Jansen P et al. Roles for the pro-neurotrophin receptor sortilin in neuronal development, aging and brain injury. *Nat Neurosci*. 2007;10(11):1449–1457.

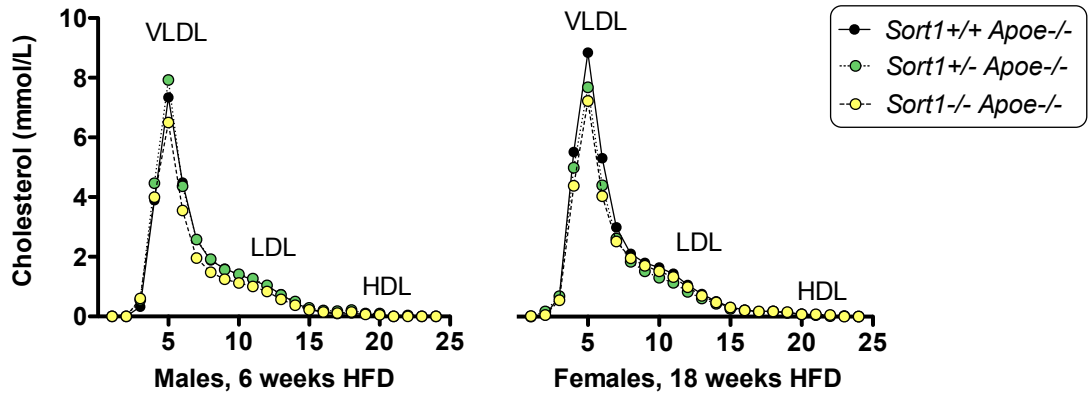
2. Moore KJ et al. Loss of receptor-mediated lipid uptake via scavenger receptor A or CD36 pathways does not ameliorate atherosclerosis in hyperlipidemic mice. *J Clin Invest*. 2005;115(8):2192–2201.

3. Weischenfeldt J, Porse B. Bone Marrow-Derived Macrophages (BMM): Isolation and Applications. *CSH Protoc*. 2008;2008:pdb.prot5080.

4. Britze A, Palmfeldt J, Gregersen N, Ovesen T. 44-plex cytokine profile of cholesteatoma. *Acta Otolaryngol*. 2014;134(1):41–50.

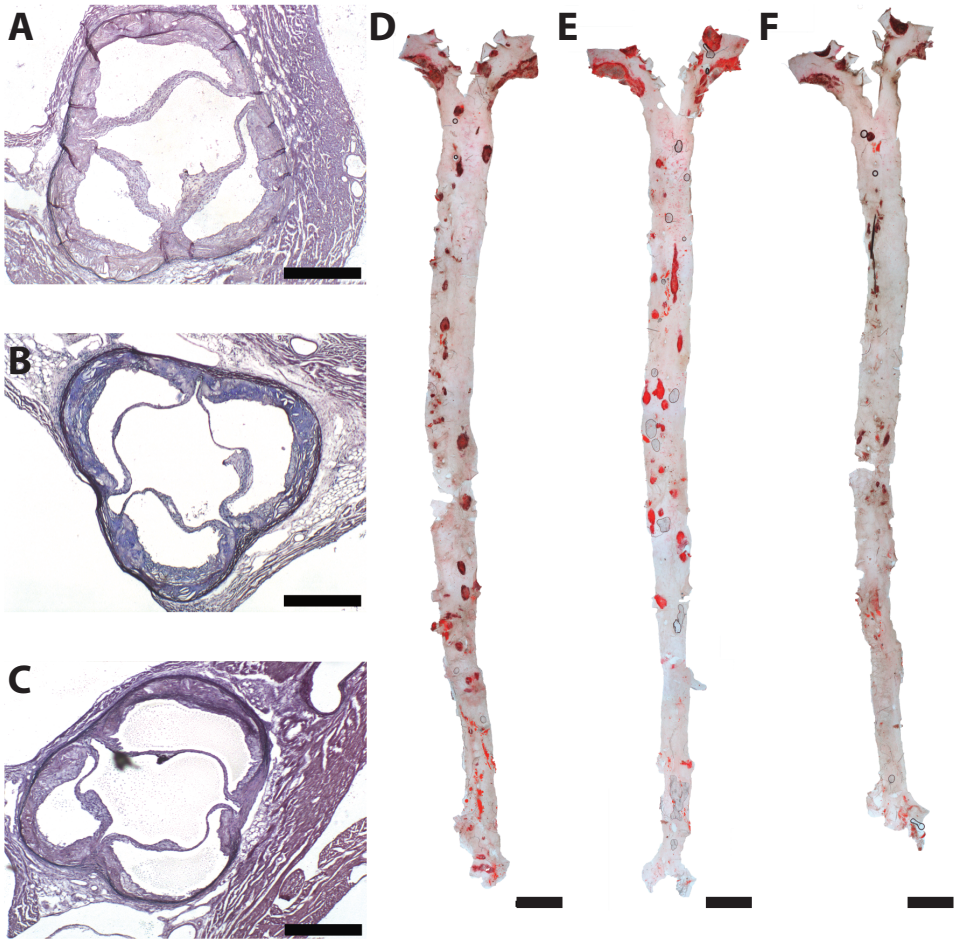
5. Petersen CM et al. Molecular identification of a novel candidate sorting receptor purified from human brain by receptor-associated protein affinity chromatography. *J Biol Chem*. 1997;272(6):3599–3605.

Supplementary Figure S1



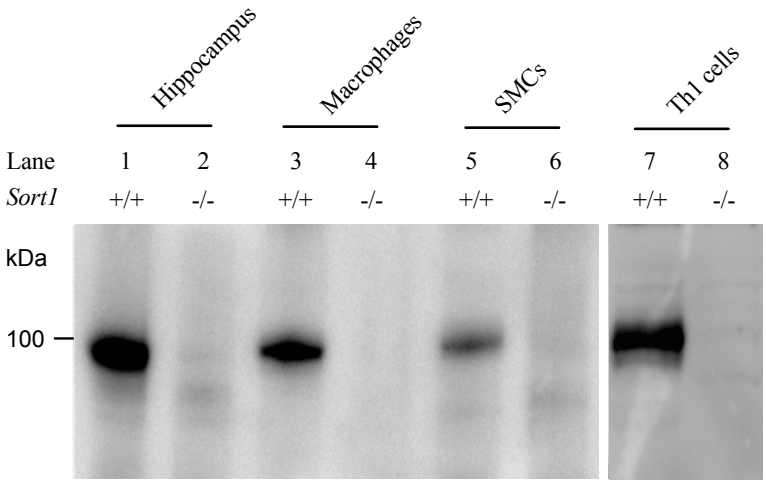
Supplementary Figure S1. Distribution of cholesterol across size-fractionated lipoprotein classes. Analysis of pooled plasma samples in male mice after 6 weeks of high-fat diet (HFD) (left) and in females after 18 weeks of HFD (right). VLDL, very-low-density lipoproteins; LDL, low-density lipoproteins and HDL, high-density lipoproteins.

Supplementary Figure S2



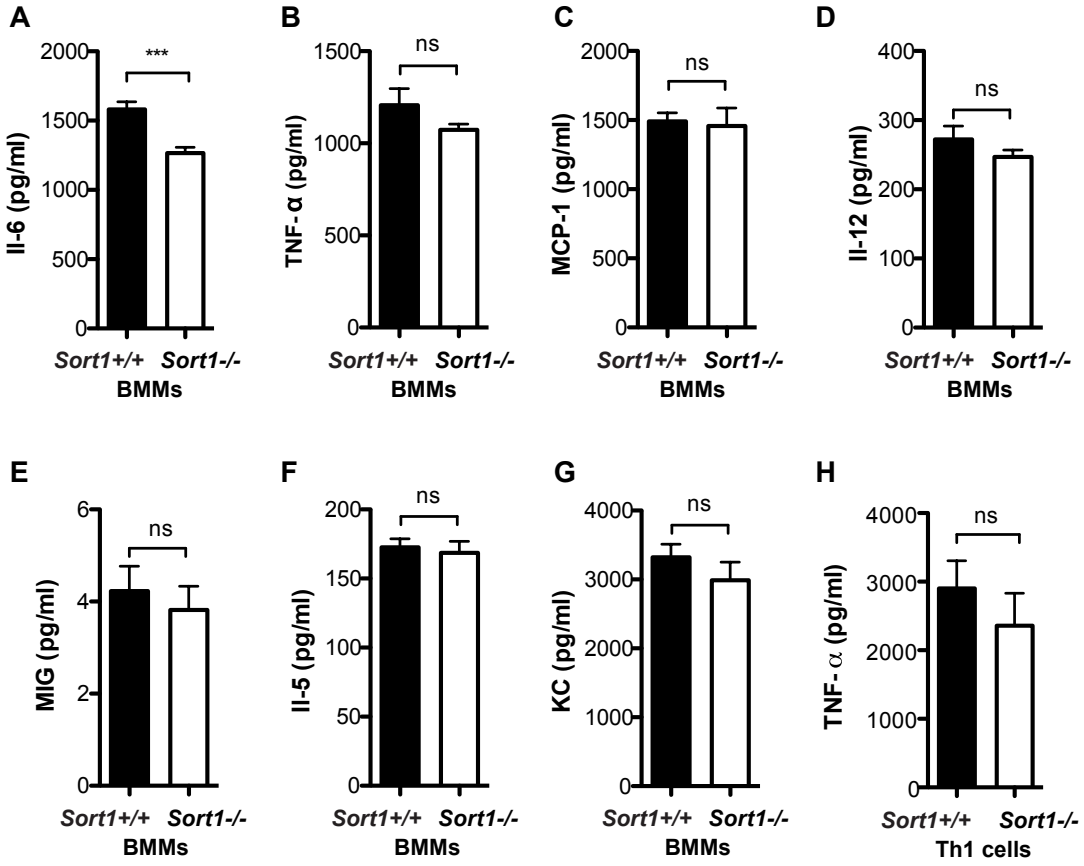
Supplementary Figure S2. Representative photographs of atherosclerosis. Orcein-stained aortic root lesions (**A-C**) and Oil Red O stained aortas (**D-F**) after 18 weeks of HFD in female *Sort1*+/+ (**A** and **D**), *Sort1*+/- (**B** and **E**), and *Sort1*-/- (**C** and **F**) *Apoe*$^{-/-}$ mice. Scale bars 500 μm (A-C) and 2 mm (D-F).

Supplementary Figure S3



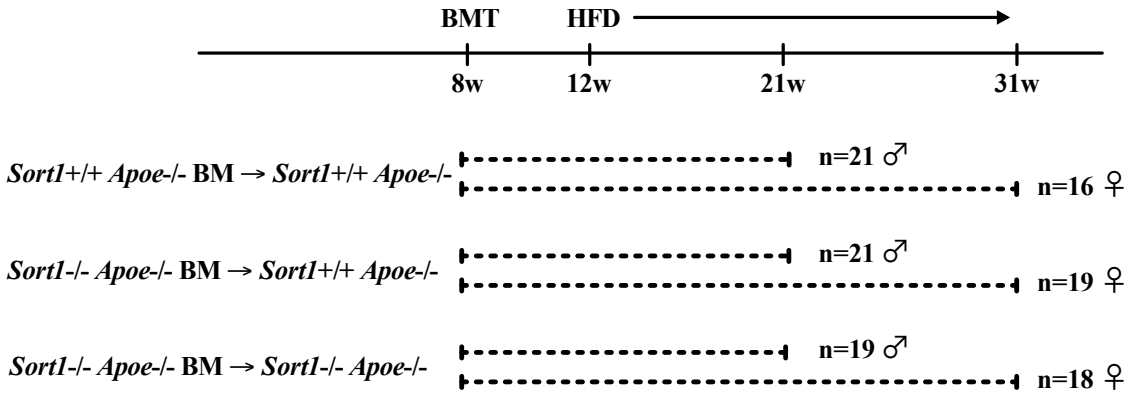
Supplementary Figure S3. Sortilin is present in major murine cell types that participate in atherosclerosis. Detection of sortilin by immunoblotting. Tissue and cell lysates from *Sort1*+/+ and *Sort1*-/- mice: Lane 1-2: Hippocampus. Lane 3-4: Peritoneal macrophages. Lane 5-6: Aortic SMCs. Lane 7-8: Isolated splenic Th1 cells analyzed in a separate blot.

Supplementary Figure S4



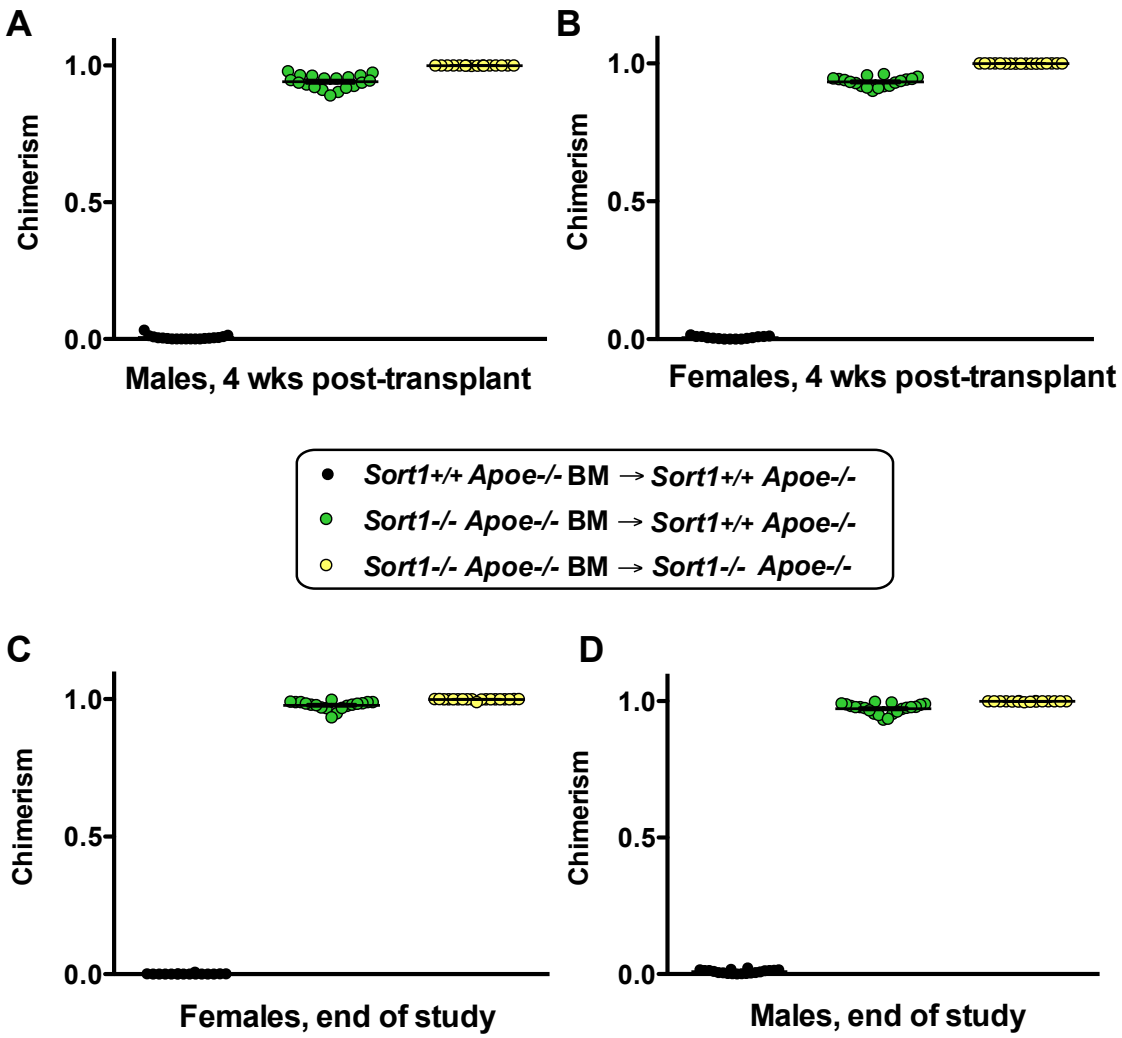
Supplementary Figure S4. Cytokine secretion from macrophages and T helper 1 cells. A-G, To screen for any difference in cytokine secretion, bone marrow-derived macrophages (BMMs) from *Sort1*+/+ (n=9) and *Sort1*-/- (n=8) mice were polarized toward an M1 phenotype with 100 ng/ml LPS for 24 hours. The secretion of several pro-inflammatory cytokines into cell media was determined with multiplex mouse cytokine Luminex assays. The secretion of IL-6 was hampered by the loss of sortilin in macrophages (**A**), while the secretion of TNF-α (**B**), MCP-1 (**C**), Il-12 (**D**), MIG (**E**), Il-5 (**F**) and KC (**G**) were not significantly affected. Bars indicate mean ± SEM. P values calculated by Student's t-test. ***indicate $p<0.0001$. **H,** Upon stimulation of T helper 1 (Th1) cells with plate-bound anti-CD3 and anti-CD28, the secretion of TNF-α was not significantly affected. Bars indicate mean ± SEM of n=6 in each group.

Supplementary Figure S5



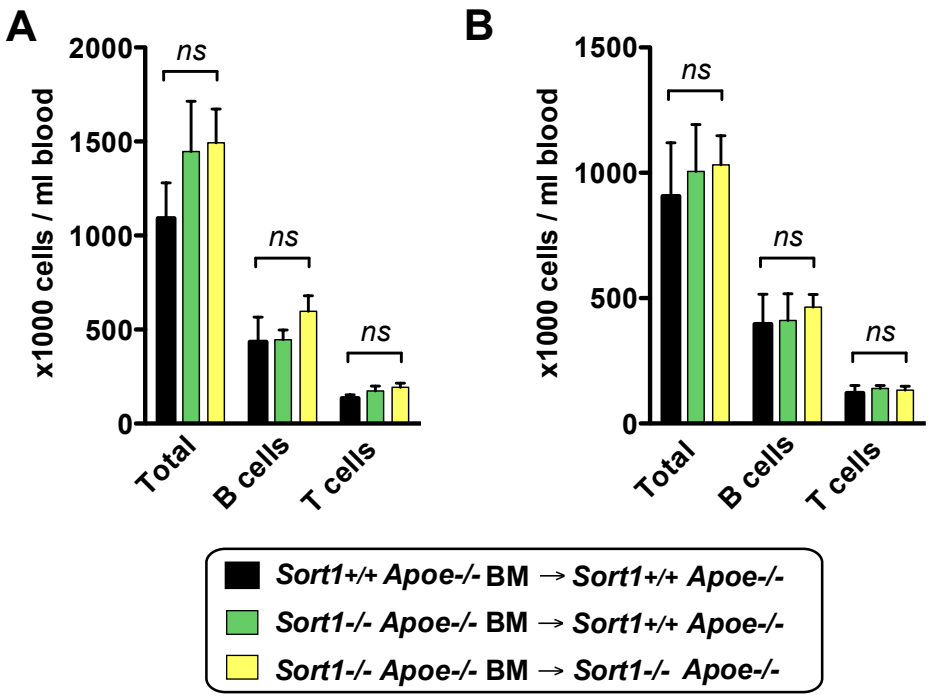
Supplementary Figure S5. Overview of bone marrow transplantation experiments. Recipient mice were lethally irradiated and rescued with age- and sex-matched bone marrow from donor mice as indicated. BM, bone marrow; BMT, bone marrow transplantion; HFD, high fat diet.

Supplementary Figure S6



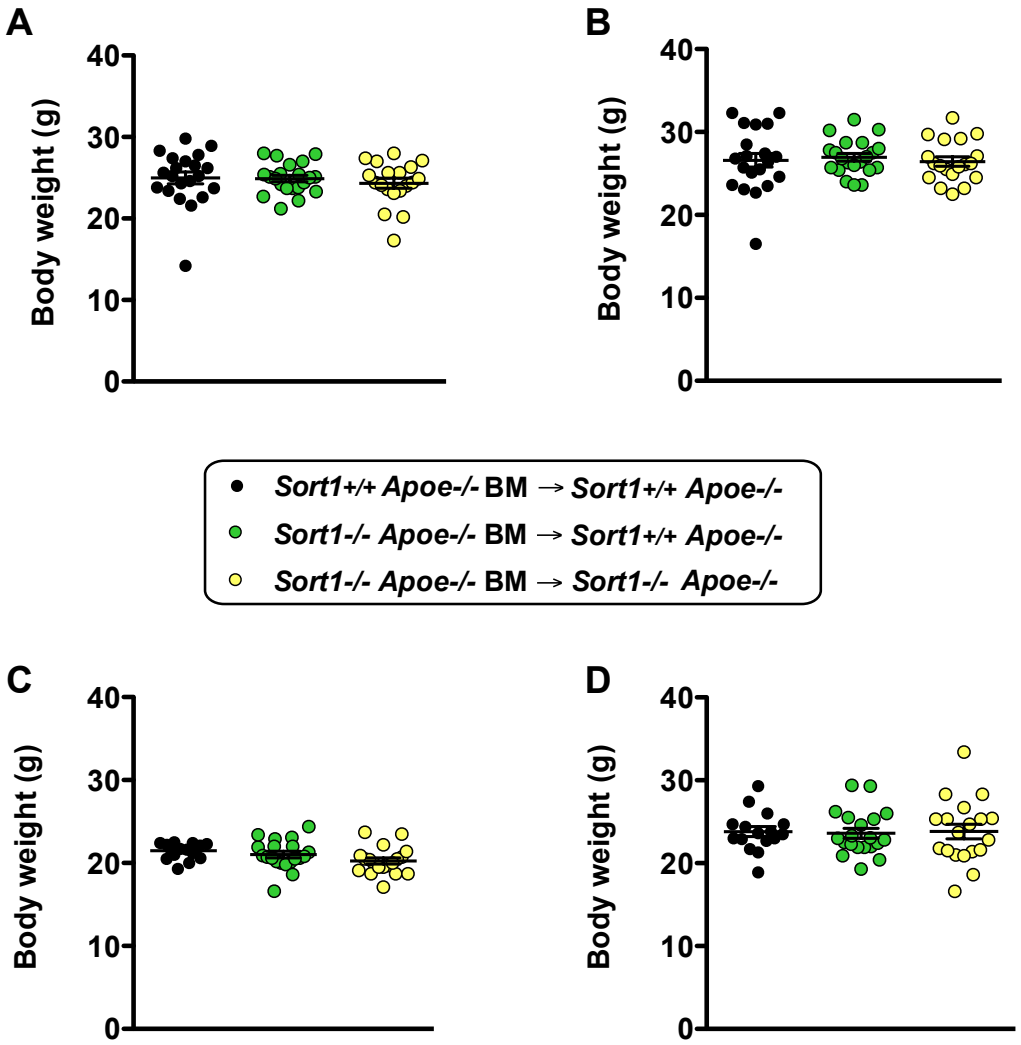
Supplementary Figure S6. Chimerism of bone marrow-transplanted mice. Chimerism, determined as the fraction of targeted *Sort1* alleles among all *Sort1* alleles in whole blood DNA, was determined 4 weeks after bone marrow (BM) transplantation and at the end of study. Chimerism in blood of *Sort1*+/+*Apoe*-/- mice transplanted with *Sort1*-/-*Apoe*-/- bone marrow was 0.941±0.006 (mean±SEM) in males (**A**) and 0.933±0.004 in females (**B**) after 4 weeks and 0.972±0.004 in males (**C**) and 0.978±0.004 in females (**D**) at the end of study. Bars indicate mean±SEM of n=16-21.

Supplementary Figure S7



Supplementary Figure S7. Normal levels of circulating immune cells in mice with sortilin-deficient bone marrow. The bar graphs show total mononuclear, B, and T cells in the blood of male (**A**) and female (**B**) bone marrow (BM)-transplanted mice as measured by flow cytometry. Bars indicate mean±SEM of n=16-21. Ns, non-significant by ANOVA.

Supplementary Figure S8



Supplementary Figure S8. Body weights of bone marrow (BM) transplanted male (**A-B**) and female (**C-D**) mice measured 4 weeks after BM transplantation (**A** and **C**) and the end of study (**B** and **D**). No significant differences detected by ANOVA. Bars indicate mean±SEM of n=16-21.

Supplementary Table S1

Supplementary Table S1. Primer sequences

Amplicon	Forward Primer	Reverse Primer
Genotyping		
Sort1 KO	5'-GTTCACACAGGAGCCCATCT-3'	5'-TGGCGGGTAATGAAAGACTC-3'
Sort1 WT	5'-GTTCACACAGGAGCCCATCT-3'	5'-TTGGGAAGACAATAGCAGGC-3'
Gene expression		
iNOS	5'-TCCTGGAGGAAGTGGGCCGAAG-3'	5'-CCTCCACGGGCCCGGTACTC-3'
Il6	5'-TGAGAAAAGAGTTGTGCAATGG-3'	5'-GGTACTCCAGAAGACCAGAGG-3'
B2m	5'-CTGCTACGTAACACAGTTCCACCC-3'	5'-CATGATGCTTGATCACATGTCTCG-3'