Supplementary Fig. S1  Haematopoietic sphingosine kinase 2 (SphK2) deficiency does not affect macrophage function—low-density lipoprotein receptor (LDL-R) −/− mice transplanted with SphK2 −/− (n = 11) or wild-type (WT) (n = 11) bone marrow (BM) were placed on a Western-type diet for 14 weeks and sacrificed by exsanguination. Peritoneal cells and splenocytes were isolated as described under the Material and Methods section. (A) Adherent peritoneal cells were established in culture, incubated for 24 hours in the absence or presence of lipopolysaccharide (LPS) (20.0 ng/mL) and tumor necrosis factor (TNF)α, monocyte chemoattractant protein (MCP)-1 and interleukin (IL)-12 concentrations in medium were determined using enzyme-linked immunosorbent assay (ELISA). Single peritoneal cell suspensions were stained with antibodies against F4/80, CD86 and major histocompatibility complex class II (MHCII) and analysed by flow cytometry. Data are presented as fluorescence intensity. (B) Blood or single cell suspensions from spleen were stained with antibodies against CD3, CD4 or CD8 and analysed by flow cytometry. Data are presented as relative cell numbers. Splenocytes were seeded in 96-well plates and left untreated or stimulated with concanavalin A (ConA) (10.0 µg/mL) for 96 hours. interferon (IFN)γ and IL-2 concentrations in supernatants were determined by ELISA. (C) Differential blood was assessed using routine laboratory method. Cytokine and chemokine concentrations in plasma were determined with ELISA.
Materials and Methods

Animals

B6N.129S6-Sphk2^tm1Rlp/J mice, in which part of exon 4 and exons 5 to 7 of the sphingosine kinase 2 (Sphk2) gene was replaced by a β-galactosidase (lacZ)-neomycin resistance (neo) cassette abolishing gene function, was generated as described previously. Female low-density lipoprotein receptor (LDL-R)^−/− mice on a C57Bl/6j background (B6.129S7-Ldlr^tm1Her/J) were purchased from Jackson Laboratories, Bar Harbor, Maine, United States. To induce bone marrow (BM) aplasia, LDL-R^−/− mice (6–8 weeks of age) were exposed to a single total dose of 11 Gy total body irradiation 1 day before the transplantation. BM was isolated by flushing femurs and tibias from female Sphk2^−/− and wild-type (WT) mice with phosphate-buffered saline (PBS) and single-cell suspensions were prepared by passing the cells through a 70-µm cell strainer. Irradiated recipients were transplanted with Sphk2^−/− mouse or WT mouse 10⁶ cells by intravenous injection into the tail vein. The haematological chimerism of transplanted animals was determined in genomic deoxyribonucleic acid (DNA) from blood leukocytes 4 weeks after transplantation. Thereafter, animals were put on the Western diet (0.25% cholesterol, 21% fat; Altromin, Lage, Germany) for 14 weeks (assessment of atherosclerosis) or 2 to 4 weeks (assessment of endothelial function). At the end of the treatment period, mice were sacrificed by exsanguination by heart puncture or cervical dislocation under anaesthesia and tissues were collected for further analysis. All animal experiments were approved by government authorities in charge of animal protection (LANUV).

Low-Density Lipoprotein Isolation and Labelling

LDL was isolated from the pooled plasma of healthy blood donors by a discontinuous potassium bromide gradient centrifugation (d = 1.019–1.063 g/mL). LDL was labelled with DyLight 594 fluorescent dye (Dyl, Thermo Fischer, Schwerte, Germany) according to the manufacturer’s instruction. Briefly, LDL (2.0 mg/mL, pH adjusted to 8.0 with 50 mmol/L sodium borate) was mixed with DyLight 594 reagent (1:1, v/v), incubated for 60 minutes and purified using spin columns provided by the manufacturer. The labelling efficiency was assessed by fluorescence spectroscopy.

Lipid Analysis and Lipoprotein Fractionation

Plasma total cholesterol, high-density lipoprotein cholesterol (HDL-C) and triglycerides were determined enzymatically using commercially available kits (Siemens, Eschborn, Germany). Plasma lipoproteins were fractionated using Smart chromatographic system (Pharmacia, Uppsala, Sweden) as described previously. HDL was isolated from mouse plasma by discontinuous ultracentrifugation. Sphingosine-1-phosphate (S1P) concentrations in murine plasma and HDL were determined using hydrophilic interaction liquid chromatography (HILIC, SeQuant ZIC-HILIC column) followed by tandem mass spectrometry as published previously.

Atherosclerotic Lesion Analysis

Assessment of atherosclerotic lesion size and cellularity was performed as described previously. Briefly, exsanguinated mice were subjected to in situ perfusion with saline through the left cardiac ventricle. For analysis of spontaneous atherosclerosis aortic roots were removed and embedded in OCT- cryoprotective medium. Serial cross-sections were collected, stained with Oil-Red-O for lipid and counterstained with haematoxylin (Sigma). Atherosclerotic lesion size at the aortic root was determined as the mean of 5 Oil-Red-O-stained sections through the aortic valve area, each section 50 µm apart from each other. For en face analysis, the thoracic aorta was opened longitudinally, Oil-Red-O-stained and fixed between glass slides. Images were digitally captured with a Leica DM-LB microscope equipped with JVC KY-F75 colour camera (Leica Microsystems, Wetzlar, Germany). The extent of atherosclerosis was quantified by computerized image analysis using the Leica Qwin image analysis software. Lesion size was expressed as stained area (µm²) or as percentage ratio between the area occupied by atherosclerotic plaque and the total area of aorta. For immunohistochemistry, aortic root sections were stained with antibodies directed against mouse macrophages (rat anti-mouse MOMA-2, 1:100, Millipore), vascular smooth muscle cell (rat anti-mouse actin, Sigma) or vascular cell adhesion molecule 1 (VCAM1) (1:400, rabbit anti-mouse VCAM, Abbiotec, San Diego). For labelling, secondary goat anti-rat and goat anti-rabbit antibodies conjugated to Cy3 (1:500; Dianova, Hamburg) or to AlexaFluor 488 (1:200; MoBiTec, Göttingen) were used. Following incubation conditions were used: primary antibodies at 4°C overnight, secondary antibodies at room temperature for 1 hour. Some sections were counterstained with 4',6-diamidino-2-phenylindole (Thermo Fisher) for nuclear morphology. Negative controls were performed by omitting primary antibodies. Images were analysed using a AXIOPHOT2 fluorescence microscope (Zeiss, Jena, Germany) and positive areas were quantified using the image analysis software ImageJ (National Institute of Health, Bethesda, Maryland, United States).

Assessment of Leukocyte Adhesion In Vivo

For studying leukocyte adhesion under in vivo conditions, LDL-R^−/− mice transplanted with Sphk2^−/− or WT BM were subjected to inhalation anaesthesia using isoflurane (minimum alveolar concentration 1.5 Vol. %). In vivo leukocyte staining was performed by intravenous injection of 1.0 μg/g rhodamine 6G (Sigma, Deisenhofen, Germany) and leukocyte adhesion was assessed by intra-vital microscopy of mesenterial venules as described previously. For this purpose, a small midline laparotomy was made in the lower abdomen, animals were placed on a pre-warmed inverted fluorescence microscope (Eclipse 300, Nikon, Düsseldorf, Germany) and a part of the distal ileum was gently exteriorized on a slide in a tension-free position. To avoid dehydration and hypothermia, the tissue was covered with a cover slip and continuously moistured with warmed PBS (pH 7.4). In each animal, visual fields of 5 non-branched mesenteric venules (60–140 µm diameters) were analysed.
and recorded for 30 seconds by a fluorescence camera (FView II Olympus Soft Imaging Systems, Münster, Germany). Fluorescent cells moving in close contact along the endothelium at a velocity less than that of free floating cells are considered to be rolling leukocytes. Permanently adherent cells were defined to remain stationary for more than 20 seconds. The numbers of leukocytes were normalized on 1 mm² venular surface area.

**Assessment of Vascular Permeability and Monocyte Recruitment**

To assess vascular permeability, LDL-R<sup>−/−</sup> mice transplanted with SphK2<sup>−/−</sup> or WT BM were intravenously administered with Evans blue (600 µg/animal), fluorescein isothiocyanate (FITC)-dextran (Sigma, 500 kDa; 500 µg/animal) or DyL-labelled LDL (DyL-LDL) (500 µg/animal) 15 minutes prior to injection intraperitoneally of lipopolysaccharide (LPS) (25 µg/animal). Mice were sacrificed after 3 hours and their peritoneal cavities were washed with 10 mL of ice-cold heparinized PBS. The cells were spun down and the supernatants were analyzed by flow cytometry or seeded in a 24-well plate at a density of 10<sup>5</sup>/well. After 4 hours, non-adherent cells were removed, and remaining macrophages were incubated for 24 hours in the absence or presence of LPS. Peritoneal leukocytes were immunophenotyped by flow cytometry (FACScalibur). Monoclonal antibodies for flow cytometry were from BD Bioscience (CD86) or eBioscience (F4/80 and major histocompatibility complex class II). For each fluorescence-activated cell sorting (FACS) staining, 2 × 10<sup>5</sup> cells were incubated with antibody dilutions (0.25 µg for each antibody) in PBS plus 1.0% (v/v) FCS at 4°C.

**Leukocyte Differential Count and Lymphocyte Subtyping**

Differential leukocyte count was performed manually (Pappenheim staining) in a routine hospital laboratory. Lymphocyte sub-typing was performed by flow cytometry. Briefly, whole blood was anti-coagulated with citrate, incubated for 30 minutes with FITC- or phycoerythrin-conjugated antibodies against CD3, CD4 or CD8 (5.0 µg/mL, eBioscience), and fixed for 30 minutes with 0.4% formaldehyde in PBS. Thereafter, cells were centrifuged for 10 minutes at 1,700 rpm and erythrocytes were lysed in a buffer containing 0.15 mol/L NH₄Cl, 10 mmol/L NaHCO₃, 0.1 mmol/L ethylenediaminetetraacetic acid (pH 7.4). The remaining cells were washed twice in PBS and analysed on a FACS Calibur flow cytometer.

**Tissue Harvesting and Cell Immunophenotyping**

Spleens were excised and single-cell suspensions were prepared by passing crude cell suspensions through a 70-µm mesh filter. Erythrocytes in cell suspensions were lysed by hypo-osmotic shock as described above. Peritoneal leukocytes were isolated by peritoneal lavage (ice-cold PBS) as described previously. Cells were suspended in Dulbecco-modified Eagle’s medium (DMEM) containing foetal calf serum (FCS, PAA Laboratories, Cölbe, Germany, 10.0% v/v) and 2 mmol/L glutamine and were either used for flow cytometry or seeded in a 24-well plate at a density of 0.5 × 10<sup>6</sup> cells/mL. After 4 hours, non-adherent cells were removed, and remaining macrophages were incubated for 24 hours in the absence or presence of LPS. Peritoneal leukocytes were immunophenotyped by flow cytometry (FACScalibur). Monoclonal antibodies for flow cytometry were from BD Bioscience (CD86) or eBioscience (F4/80 and major histocompatibility complex class II). For each fluorescence-activated cell sorting (FACS) staining, 2 × 10<sup>5</sup> cells were incubated with antibody dilutions (0.25 µg for each antibody) in PBS plus 1.0% (v/v) FCS at 4°C.

**Determination of Cytokine, Chemokine and Adhesion Molecule Levels**

Concentrations of cytokines (tumor necrosis factor-α, interferon γ, interleukin [IL]-2, IL-12), chemokines (monocyte chemoattractant protein 1, regulated on activation, normal T cell expressed and secreted) and soluble adhesion molecules (soluble VCAM1, soluble inter-cellular adhesion molecule 1) were quantified in plasma and/or supernatants of peritoneal leukocytes and splenocytes by commercially available enzyme-linked immunosorbent assays (R&D Systems, Wiesbaden, Germany).

**Endothelial Adhesion and Permeability Assays**

Murine endothelial cell line bEnd.5 was a generous gift of Dr. Sigrid März, Max-Planck-Institute for Molecular Medicine, Münster, and was maintained in DMEM supplemented with glucose (2.0%, v/v), sodium pyruvate (1.0%, v/v), non-essential amino acids (1.0%, v/v), FCS (20.0%, v/v) and endothelial cell growth supplement (Promocell, Heidelberg, Germany) containing epidermal growth factor and basic
fibroblast growth factor. Humane monocyte line U937 was obtained from LGC Standards (Wesel, Germany), and was maintained in DMEM with FCS (10.0%, v/v). S1P (1.0 µmol/L or 2.0 µmol/L in some experiments) was added directly to the cell culture media. For the assessment of monocyte adhesion, U937 cells were labelled for 30 minutes at 37°C with calcein-acetoxyethyl (1.0 µmol/L, Sigma) and added at several 4 × 10^6 cells/ml to a confluent bEnd.5 monolayer on cover slip in DMEM containing all components as described above except that 20% (v/v) FCS was replaced by 2% (v/v) FCS. The cover slip was kept for 30 minutes under gentle rocking as described previously. Non-adherent cells were removed thereafter by rinsing the plates three times in PBS and the number of adherent cells was counted (>5 fields per cover slip) under fluorescence microscope Leica DM-IRE. For endothelial permeability testing, bEnd.5 cells were seeded onto collagen-coated 24-well TransWell 0.4 µm or 5.0 µm pore-size culture inserts (Corning Life Sciences, Lowell, Massachusetts, United States) at a density of 10^5 cells/well and cultured for 7 days as described above to form monolayers and exposed to S1P or murine plasma (25.0% v/v) for 24 hours. After treatment, FITC-dextran (500.0 kDa) or DyL-LDL dissolved in DMEM were placed in the apical insert compartment at a final concentration of 500 or 250 µg/mL, respectively, and allowed to equilibrate for 0.5 hour. Alternatively, calcein-labelled U937 cells (4 × 10^6 cells/well) were applied apically to the insert for 4 hours. Samples were taken from basolateral chambers for fluorescence measurement. The results are expressed as the percentage of FITC-dextran or DyL-LDL influx or U937 migration across untreated bEnd.5 monolayers.

### Analysis of Gene Expression by Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction

Total RNA was isolated from RAW264.7 cells using RNAeasy Plus Purification Kit (Qiagen, Hilden, Germany) according to manufacturer protocol. RNA was eluted in water and quantified using BioPhotometer (Eppendorf, Hamburg, Germany). The entire complementary DNA (cDNA) was synthesized from 1.0 µg of total RNA using RevertAid H Minus First Strand cDNA Synthesis Kit (Fischer Scientific, Schwerte, Germany). Fully automated reverse transcription polymerase chain reaction (RT-PCR) set-up was done on a Genesis 150 workstation (TECAN, Creihsheim, Germany) and PCR products were detected using ABI7900ht sequence detection system (Applied Biosystems, Darmstadt, Germany) in a 384-well format. PCR primer sequences were as follows: VCAM1 – forward primer: TTCGGTTGTTCTGACGTGTG, reverse primer: TACCACCCATTGAGGGGAC. Relative gene expression was calculated by applying the 2^ΔΔCt method. Briefly, the threshold cycle number (Ct) of target genes was subtracted from the Ct of GAPDH (Ct_houskeeping) and raised to the 2nd power of this difference.

### General Procedures

Data are presented as means ± standard deviation for at least three separate experiments or as results representative for at least three repetitions. Comparisons between the means of two or multiple groups were performed with two-tailed Student’s t-test or one-way analysis of variance for independent samples, respectively. Pairwise comparisons were performed thereafter with Student–Newman–Keuls post hoc test. p-Values less than 0.05 were considered significant.

### References