Supplementary Materials and Methods

Mice, Diet, and Bone Marrow Transplantation

Mice were housed under standard conditions and given free access to food and water. Experiments were performed according to Dutch laws, approved by the Committee for Animal Welfare of Maastricht University. One week before transplantation, 10-week-old female, low-density lipoprotein (LDL) receptor-deficient ($Ldlr^{-/-}$) mice were put into filter top cages and received acidified water supplemented with neomycin (100 mg/L; N1142; Sigma-Aldrich, St. Louis, MO) and polymyxin B sulphate (60,000 U/L; 21850-029; Life Technologies Cooperation, Carlsbad, CA). One day before transplantation, mice were subjected to a full-body irradiation with a lethal dose of 10 Gy. The bone marrow of 5 female wild-type mice and 5 female $Msr^{-/-}/Cd36^{-/-}$ littermates was collected and pooled. Each irradiated $Ldlr^{-/-}$ mouse received 10^7 bone marrow cells by injection into the tail vein. After a recovery period of 9 weeks, the mice were given a high-fat, high-cholesterol (HFC) diet for 7 days (n = 8 in $Msr^{+/+}/Cd36^{+/+}$ and n = 8 in $Msr^{-/-}/Cd36^{-/-}$-tranplanted [tp] mice) and 3 months (n = 7 in $Msr^{+/+}/Cd36^{+/+}$-tp group, n = 8 in $Msr^{-/-}/Cd36^{-/-}$-tp group), containing 21% butter and 0.2% cholesterol (diet 1635; Scientific Animal Food and Engineering, Villemoisson-sur-Orge, France). Chow fed mice that were killed after 9 weeks recovery were used as control group (n = 7 in both $Msr^{+/+}/Cd36^{+/+}$-tp and $Msr^{-/-}/Cd36^{-/-}$-tp mice). Blood from the tail vein was collected before the dietary period, after 7 days on the HFC diet in the short-term group, and after 3 months in the long-term group on the day of death after a 4-hour fast period. The mice were then killed by cervical dislocation. Tissues were isolated and snap frozen in liquid nitrogen and stored at −80°C or fixed in 4% formaldehyde/phosphate-buffered saline.

Plasma Lipid Analysis

Both plasma and liver lipid levels were measured with enzymatic color tests (cholesterol CHOD-PAP; 1489232; Roche, Basel, Switzerland; serum triglyceride (TG) determination kit, TR0100; Sigma-Aldrich; NEFAC, ACS-ACOD, 999-75406; Wako Chemicals, Neuss, Germany) according to the manufacturer's protocols on a Benchmark 550 Micro-plate Reader (170-6750XTU; Bio-Rad, Hercules, CA).

Liver Lipid Analysis

Approximately 50 mg of frozen liver tissue was homogenized for 30 seconds at 5000 rpm in a closed tube with 1.0-mm glass beads and 1.0 mL SET buffer (sucrose 250 mmol/L, EDTA 2 mmol/L, and Tris 10 mmol/L). Complete cell destruction was done by 2 freeze-thaw cycles and 3 times passing through a 27-gauge syringe needle and a final freeze-thaw cycle. Protein content was measured with the bicinchoninic acid (BCA) method (23225; Pierce, Rockford, IL). Cholesterol, triglycerides (TG), and free fatty acids (FFA) were measured as described above. Protocols were followed according to the manufacturers' instructions.

Liver Histology

Frozen liver sections (7 μm) were fixed in acetone and subsequently blocked for endogenous peroxidase by incubation with 0.25% of 0.03% H_2O_2 for 5 minutes. Primary antibodies used were against infiltrated macrophages and neutrophils (rat-anti-mouse Mac-1 [M1/70]) and T cells (rat-anti-mouse CD3) (both generous gifts from Prof Kraal, Free University, Amsterdam, The Netherlands), neutrophils (rat-anti-mouse Ly6-C, clone NIMP-R14) (generous gift from Prof Heeringa, Groningen, The Netherlands), Kupffer cells (KCs) (rat-anti-mouse CD68, clone FA11) (generous gift from Prof Gordon, Oxford, UK), and apoptosis (rabbit-anti-mouse cleaved caspase 3) (9661Lg; Cell Signalling, Danvers, MA). 3-Amino-9-ethylcarbazole (AEC) (A85SK-4200.S1; Bio-connect, Huissen, The Netherlands) was applied as color substrate and hematoxylin (4085.9002, Klinipath, Duiven, The Netherlands) for nuclear counterstaining. Sections were enclosed with Faramount aqueous mounting medium (S302580; DAKO, Glostrup, Denmark). For the lipid stainings, the neutral lipid marker oil red o (ORO; O0625; Sigma-Aldrich) and the fluorescent free cholesterol marker Filipin (F9765, Sigma-Aldrich) were used. The filipin staining was quantified by scoring all filipin-positive KCs between 0 and 3, where score 0 indicates not positive, and score 3 indicates extremely positive for cholesterol content inside the KCs.

Paraffin-embedded liver sections (4 μm) were stained with Hematoxylin-Eosin (HE; Hematoxilin, 4085.9002; Klinipath, Duiven, The Netherlands; and Eosin, E4382; Sigma-Aldrich), Sirius red (Direct Red 80, 43665; Sigma-Aldrich), and rabbit-anti-mouse myeloperoxidase (A0398; DAKO). Pictures were taken with a Nikon digital camera DMX1200 and ACT-1 v2.63 software (Nikon Instruments Europe, Amstelveen, The Netherlands). Immune cells were counted in 6 microscopical views (original magnification, 200×) and were noted as cells/square millimeter.

RNA Isolation and Quantitative Polymerase Chain Reaction

Total RNA was isolated from approximately 25 mg of mouse liver tissues as described previously.[1] All applications were performed according to the manufacturers' protocols. Total RNA (500 ng) from each individual mouse was converted into first-strand complementary DNA (cDNA) with the iScript cDNA synthesis kit (170-8891; Bio-Rad, Hercules, CA) according to the manufacturer's instructions. The changes in gene expression of inflammatory markers were determined by quantitative polymerase chain reaction (PCR) (qPCR) on an SDS 7900HT by using PowerSybr Green mastermix (4329001 and 4368708; both Applied Biosystems, Foster City, CA) and 10 ng of cDNA. For each gene, a standard curve was generated with a serial dilution of a liver cDNA pool. To standardize for the amount of cDNA, Cyclophillin A (Ppia) was used as the reference gene. Primer sets for the selected genes were developed with Primer Express version 2.0 (Applied Biosystems) using default settings. Primer sequences are given in Supplementary Table 1. Data from qPCR were analyzed according to the relative standard curve method.

Supplementary Table 1. Primer Sequences for Quantitative PCR

Gene	Primer forward	Primer reverse
TNF	CATCTTCTCAAAATTCGAGTGACAA	TGGGAGTAGACAAGGTACAACCC
IL-6	GCTACCAAACTGGATATAATCAGGAAA	CTTGTTATCTTTTAAGTTGTTCTTCATGTACTC
TLR-4	TATCCAGGTGTGAAATTGAAACAATT	GGGTTTCCTGTCAGTATCAAGTTTG
TLR-2	AATTGCATCACCGGTCAGAAA	GTTTGCTGAAGAGGACTGTTATGG
PPAR-α	TTCCCTGTTTGTGGCTGCTAT	TGCAACTTCTCAATGTAGCCTATGTT
PPAR-γ	TCGCTGATGCACTGCCTATG	GAGAGGTCCACAGAGCTGATT
CD68	TGACCTGCTCTCTCTAAGGCTACA	TCACGGTTGCAAGAGAAACATG
ABCA1	CCCAGAGCAAAAAGCGACTC	GGTCATCATCACTTTGGTCCTTG
ABCG1	TCGGACGCTGTGCGTTTT	CCCACAAATGTCGCAACCT
COL1A1	AACCCTGCCCGCACATG	CAGACGGCTGAGTAGGGAACA
MMP-13	ACAAAGATTATCCCCGCCTCATA	CACAATGCGATTACTCCAGATACTG
TIMP-1	CGCCTAAGGAACGGAAATTTG	GATAGATAAACAGGGAAACACTGT
TGF-β	AGCGCTCACTGCTCTTGTGA	GTCGCCCCGACGTTTG
CD36	GCCAAGCTATTGCGACATGA	AAAAGAATCTCAATGTCCGAGACTTT
SR-A	CATACAGAAACACTGCATGTCAGAGT	TTCTGCTGATACTTTGTACACACGTT
LOX1	TCCCCGTTCTGGATTGGAT	TTGCCTGATGAATATAGCTGTAAAGAAA
SR-B1	TTCTTCACTACGCGCAGTATGTG	CACTCCAAAACAAAAAGCATTTCTC

Gene	Primer forward	Primer reverse
SR-CL	TAGACGGGTCACCTGTTGATTACA	ATTGATTTCATCACACTGGAAGTCAT
SR-EC	GACGACTCCTTCTCTTCTGATCCT	TGGGCCATAGGGACCATCT
PSOX	TGCAGTCCAAAAGCGTGTGT	GTGGTGAAAACTCTTCCCATGAC
PPIA	TTCCTCCTTTCACAGAATTATTCCA	CCGCCAGTGCCATTATGG

Chimerism Determination by qPCR

For the determination of the chimerism in the transplanted mice, we have made use of the knowledge that donor bone marrow has an $Ldlr^{WT}$ origin, whereas recipient bone marrow an $Ldlr^{-/-}$ origin. Genomic DNA was isolated using the NucleoSpin Blood QuickPure DNA isolation kit (740569; Macherey Nagel, Düren, Germany).

A standard curve was generated by mixing DNA from $Ldlr^{-/-}$ and $Ldlr^{WT}$ bone marrow cells at different ratios. Chimerism was determined by quantifying the amount of $Ldlr^{-/-}$ DNA in samples from 70 μL peripheral blood. To standardize for the amount of input DNA, the nonrelevant *p50* gene was quantified. Samples were assayed in duplicate on a 7900HT real-time PCR system by using 25 ng DNA, PowerSybr Green mastermix (4329001 and 4368708; both Applied Biosystems), according to the manufacturer's instructions. $Ldlr^{-/-}$ specific primers are forward 5′-GCTGCAACTCATCCATATGCA-3′ and reverse 5′-GGAGTTGTTGACCTCGACTCTAGAG-3. p50-specific primers are forward 5′-ACCTGGGAATACTTCATGTGACTAA-3′ and reverse 5′-ACACCAGAAGTCCAGGATTATAGC-3′. A standard curve was generated by plotting the mean threshold cycle (Ct) ΔCt (Ct *p50* – Ct $Ldlr^{-/-}$) against the logarithm of the percentage $Ldlr^{-/-}$ and calculation of a regression line. Chimerism was calculated from the percentage of $Ldlr^{-/-}$ DNA in the blood samples (representing the remaining recipient bone marrow), determined by applying the mean Δ Ct of the sample to the standard curve.

Measuring Aminotranferases

The level of aminotransferases, alanine aminotransferase and aspartate aminotransferase, in plasma of each individual mouse was measured by using the Reflotron system and the test strips for alanine aminotransferase and aspartate aminotransferase measurments (Roche Diagnostics, Almere, The Netherlands), according to the manufacturer's instructions. Shortly, for each aminotransferase, 32 μL plasma was loaded on the appropriate test strip and inserted into the machine. After 2 minutes, the results were shown on the display.

Measuring Autoantibody Titers Against Modified LDL

Specific antibody titers against modified LDL in plasma were determined as described elsewhere.[2] and [3] Plasma was serially diluted and antibody binding measured by chemiluminescent enzyme-linked immunosorbent assay. A titer was defined as the reciprocal of the maximal dilution at which binding of the secondary antibody was twice as high as the background binding.

Measuring Oxysterols

The oxysterols 24S- and 27-hydroxycholesterol were measured after extraction from plasma as their trimethylsilyl ethers by highly specific and sensitive gas chromatography-mass spectrometry as described previously.[4] and [5] Deuterium labeled 24S- and 27-hydroxycholesterol were used as internal standards as an isotope dilution method.

Statistical Analysis

Data were statistically analyzed by performing 2-tailed, nonpaired, *t* tests using GraphPad Prism, version 4.03 for Windows (GraphPad Software, San Diego, CA; www.graphpad.com) for comparing $Msr^{+/+}/Cd36^{+/+}$-tp and $Msr^{-/-}/Cd36^{-/-}$-tp mice for each diet group. One-way analysis of variance test was used for comparing the different time points of high-fat feeding within the same acceptor mice. Data were expressed as the mean ± standard error of mean and considered significant at $P < .05$ ($^{*}P < .05$, $^{**}P < .01$, and $^{***}P < .001$, respectively).

Conflicts of interest The authors disclose no conflicts.

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