Supplemental Material

Detailed Methods:

**Cell isolation and culture:** Mice were euthanized by CO₂. Blood was collected by cardiac puncture, and vascular perfusion was performed with 10 ml sterile RNase-free phosphate-buffered saline (PBS). After gallbladder removal the isolated liver was passed through a 100 µm cell strainer and the cell suspension was cleaned off extracellular matrix by centrifugation (1 min, 10 g). Cells were pelleted (8 min, 480 g) and leukocytes were isolated by density gradient centrifugation (30 min, 850 g) in 37.5% Percoll/PBS (GE Healthcare) and erythrocytes were lysed. Spleens and para-aortic lymph nodes were likewise mashed and red blood cells were lysed. CD4+ T cells were isolated by MACS (Miltenyi) using Naïve CD4+ T cell isolation kit or PE anti-CD4 antibody and anti-PE microbeads. Cells were labeled with CFSE or CellTracker violet (Molecular Probes) for adoptive transfer experiments or restimulated in vitro (50 ng/ml Phorbol 12-myristate 13-acetate, 1 µg/ml Ionomycin for 2 h; GolgiPlug (BD Biosciences) for 4 h) in serum-free X-vivo 15 medium (Lonza) or analyzed directly by flow cytometry, real-time PCR and Western blotting.

**Flow cytometry:** FACS analysis was performed on CD4+ T cells within the lymphocyte gate of forward/side scatter plots, excluding doublets and dead cells (LIVE/DEAD Fixable Aqua Dead Cell Stain Kit, Invitrogen). In some experiments granulocytes in the respective granulocyte gate of forward/side scatter plots, excluding doublets and dead cells, were analyzed. Fc receptor binding was prevented by anti-CD16/CD32 blockade (clone: 2.4G2, BD Biosciences) and unspecific binding was excluded by isotype control stainings (BD Biosciences). Intracellular staining was performed using the FoxP3 staining kit (eBioscience). Following fluorescent primary antibodies were purchased from BD Biosciences: anti-CD25 (PC61), anti-IFN-γ (XMG1.2), anti-IL-17A (TC11-18H10), anti-RORγt (Q31378), anti-GFAP (1B4), anti-LAP (TW7-16B4), anti-CD8a (53-67), anti-CD19 (1D3), anti-CD62L (MEL-14), anti-CD4 (GK1.5), anti-NK1.1 (PK136), anti-CD3ε (500A2), anti-CD11b (M1/70), anti-CD11c (HL3), purchased from BioLegend: anti-B220 (RA3-6B2), anti-Ki-67 (16A8), anti-T-bet (4B10), anti-CD86 (GL-1), anti-CD31 (390), anti-F4/80 (BM8), anti-CD146 (ME-9F1) or purchased from eBioscience: anti-Nrp-1 (3DS304M), anti-FoxP3 (FJK-16s, NRRF-30), anti-Helios (22F6). For adoptive transfer experiments of CFSE labeled cells the staining panel was designed to minimize spectral overlap. Events were acquired on CyAn ADP Analyzer (Beckman Coulter) and data were analyzed with FlowJo software version 10.0.7.

**Real-time PCR:** Liver cDNA obtained from mice fed experimental diets for 4 weeks was generated out of 1µg RNA (RNeasy kit, Qiagen). Values were normalized to Hprt1 gene transcription and fold gene induction relative to the average values of the standard chow diet fed group was calculated. FoxP3 transcripts were analyzed by quantification of amplicons using 5'-CCCCCACCAAGACACAGC-3’ (FoxP3 exon -2b), 5’-GTGAGCAGAATCCATGTGCG-3’ (FoxP3 exon -2a) and 5’-AGTCCACTTCCACCAAGCCTC-3’ (FoxP3 exon -2bΔ) in combination with the reverse primer 5’-GTTGGGCAATTGTTCTTG-3’ (FoxP3 exon1) using SybrSelect Master Mix (Applied Biosystems). Mice that were 12 weeks on experimental diet showed a significant effect of the diet on housekeeping genes, e.g. Hprt1 in liver tissues. Therefore equal amounts of mRNA from whole liver lysates and hepatic leukocytes were transcribed into cDNA and specific mRNA concentrations were calculated with: [c]=2^-ΔCt by real-time-PCR. A list of utilized gene specific Taqman probes (Applied Biosystems) is provided in (Online Table II).
Immunoblotting: Western blots of FoxP3 were performed as described before (15); intensity of FoxP3 bands was analyzed using ImageJ 1.48v (NIH) software and values are calculated relative to the corresponding band intensity of HSP70.

LDL preparation: LDL was isolated by ultracentrifugation from pooled plasma of healthy donors and kept in a native state, as described before (12).

Plasma analysis: Colorimetric assays were performed to measure plasma cholesterol (Randox Lab. Ltd.), plasma ALT (Alanine Transaminase Activity Assay Kit, Abcam) and plasma TGF-β1 (DUOSet ELISA, R&D Systems) according to manufacturers’ instructions.

Adoptive transfer: CellTracker violet labeled CD4+ splenocytes were stimulated for 10 min with 3 µg/ml anti-CD3 (SouthernBiotech, clone: C363.29B) at 37°C, washed, resuspended (3*10⁶ cells/200 µl PBS/animal) and intravenously injected via the tail vein. T cell proliferation was analyzed 4 days later in liver and spleen samples of 4 weeks SCD or WD fed recipient mice. For relocation assays, intrahepatic CD4+ T cells were isolated from 12 weeks SCD or WD fed Ldlr-/- or Ldlr+/+ mice, CFSE labeled and equal amounts per transfer experiment (3*10⁶ – 3*10⁵ cells/150 µl PBS/animal) were intravenously injected via the tail vein into each recipient Ldlr-/- mouse that has been fed WD for 12 weeks. T cell populations were analyzed 4 days after transfer and absolute cell numbers [total count PBMC]*[%CFSE+ T cells of live lymphocytes] were calculated. To eliminate confounding factors that may affect the numbers of transferred cells in the organs, we calculated cell densities [absolute cell number/mg tissue] to account for weight differences of recipient organs and cell density ratios [cell density X]/[cell density liver] to normalize spleen/aorta relocating cells to liver remigrating cells, so that cell densities are not affected by unintentional variations during the intravenous transfer.

Aorta digestion: The whole aorta was carefully dissected and adipose tissues as well as para-aortic lymph nodes were removed. The isolated aorta was then digested in 1:1 PBS/Dulbecco’s Modified Eagle Medium containing 1.5 mg/ml Collagenase type II (Worthington Biochemical Corp.) and 0.5 mg/ml Elastase (Sigma-Aldrich) for 1 h at 37°C. After digestion, single cell solutions were obtained by passage through a 100 µm cell strainer.

Study approval: All animal experiments were conducted in accordance with guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes and were approved by the Stockholm Norra regional ethical board.
Online Figure I: Intrahepatic lymphocyte populations in Western diet fed mice.
Analysis of intrahepatic lymphocyte populations of wild type mice fed cholesterol-free standard chow diet (SCD, white) or 0.15% cholesterol-containing Western diet (WD, gray) for 12 weeks. Total numbers (A) and relative population size (B) of indicated intrahepatic lymphocyte subsets (n=5). (C) Percentages of intrahepatic CD3+NK1.1+ NKT cell (n=8). (D) Cell numbers of intrahepatic CD4+FoxP3+ Treg cells (n=7) and percentages of intrahepatic FoxP3+ Treg cell populations of the total intrahepatic lymphocyte population following 4 weeks (diamonds), 8 weeks (squares) and 12 weeks (circles) of WD treatment (n=18); two-tailed Mann Whitney U test was performed. Values are expressed as mean ± SEM; *p<0.05; **p<0.01.
Online Figure II: Duration of cholesterol-containing Western diet affects the intrahepatic Treg cell population and the dietary cholesterol content affects the plasma cholesterol concentration.

(A) Representative flow cytometry analysis of intrahepatic CD4+ T cells from wild type mice fed cholesterol-free standard chow diet (SCD) or 0.15% cholesterol-containing Western diet (WD) for 4, 8 and 12 weeks. The relative gain in FoxP3+ Treg cells among CD4+ T cells in WD fed mice compared to SCD fed littermates is indicated as average increase ± standard deviation for the different time points. (B) Plasma cholesterol levels of wild type mice fed SCD (white), 0.15% (gray) or 1.125% (black) cholesterol-containing WD for 4 weeks. Each dot represents values from an individual animal; data combined from 4 independent experiments and mean values ± SEM are shown; one-way ANOVA and Bonferroni’s post hoc test was performed for statistical analysis; **p<0.01.
Online Figure III: Intrahepatic FoxP3 expression is not affected by posttranscriptional or posttranslational modifications following Western diet treatment.

(A) Representative flow cytometry analysis of intrahepatic CD4+ T cells from wild type mice fed cholesterol-free standard chow diet (SCD) or 0.15% cholesterol-containing Western diet (WD) for 4 weeks. FoxP3 was detected by two monoclonal antibodies specific for exon 1 (clone: NRRF-30) and exon 2 (clone: FJK-16s). (B) FoxP3 immunoblot (clone: eBio7979) of whole liver cell lysates of Ldlr+/+ and Ldlr-/- mice fed WD for 4 weeks (n=6); heat shock protein 70 (HSP70) was used as loading control. (C) Real-time PCR analysis of indicated alternative spliced exons from the 5' untranslated region (UTR) of FoxP3 transcripts derived from liver of SCD (white) or WD (gray) fed mice for 4 weeks (n=5). Insert depicts exon usage in SCD fed Treg cells from the spleen. Values are expressed as mean ± SEM; two-tailed Mann Whitney U test was performed for statistical analysis; *p<0.05; **p<0.01; ***p<0.01.
Online Figure IV: Phenotypic changes of intrahepatic CD4+ T cell subsets induced by hypercholesterolemia-mediated TGF-β expression.

Intrahepatic CD4+ T cell populations from Ldlr+/+ (n=5) and Ldlr−/− (n=5) mice fed cholesterol-free standard chow diet (SCD) or 0.15% cholesterol-containing Western diet (WD) for 12 weeks. (A) Representative flow cytometry data and bar graph for Helios MFI levels of intrahepatic Treg cells (gated on live CD4+FoxP3+ cells). (B) Representative flow cytometry data and bar graph for the Nrp-1+Ki-67+ subpopulation of intrahepatic FoxP3- T cells (gated on live CD4+FoxP3- cells). (C) Intrahepatic Treg cell populations plotted against hepatic TGF-β mRNA concentrations. Data out of two independent experiments are shown, values are expressed as mean ± SEM; two-tailed Mann Whitney U test was performed for statistical analysis; *p<0.05; **p<0.01; r=Spearman’s rank correlation coefficient; plain and hatched bars or circles and triangles indicate Ldlr+/+ and Ldlr−/− mice, respectively; white and gray color indicates SCD and WD, respectively.
Online Figure V

Online Figure V: Presence of human LDL particles does not influence iTreg cell differentiation *in vitro*.

FoxP3 induction was measured by flow cytometry of isolated CD4+ T cells that were stimulated *in vitro* with 1 µg/ml anti-CD3 (SouthernBiotech, clone: C363.29B) and 0.5 µg/ml anti-CD28 (eBioscience, clone: 37.51). Cells were treated for 3 days without (Th0 cell condition) or with 10 ng/ml recombinant TGF-β1 (Peprotech) (iTreg cell condition) in the presence or absence of 10 µg/ml human LDL (n=12). FoxP3 induction under iTreg cell conditions without LDL was set 100%. Values are expressed as mean ± SEM; one-way ANOVA and Bonferroni’s *post hoc* test was performed for statistical analysis.
Online Figure VI: Intrasplenic Th1 and Th17 differentiation is independent of hypercholesterolemia.

(A) Cytokine expression profiles and (B) percentages of Th17 cells (CD4+ROR-\(\gamma\)t+IL-17A+ T cells) and Th1 cells (CD4+IFN-\(\gamma\)+ T cells) of in vitro restimulated intrasplenic CD4+ lymphocytes derived from wild type (Ldlr+/+) (n=5) and Ldlr-- (n=4) mice fed cholesterol-free standard chow diet (SCD) or 0.15% cholesterol-containing Western diet (WD) for 12 weeks. One representative experiment out of two independent experiments is shown; values are expressed as mean ± SEM; one-way ANOVA and Bonferroni’s post hoc test was performed for statistical analysis; colors, shapes and patterns as described for Online Figure IV.
Online Figure VII

A

Diet: p=0.0206
Ldlr: p=0.8574

Diet: p=0.4782
Ldlr: p=0.0121

B

Diet: p=0.0206
Ldlr: p=0.8574

Diet: p=0.0640
Ldlr: p=0.9224

Diet: p=0.0090
Ldlr: p=0.9500

Diet: p=0.0379
Ldlr: p=0.9500

Online Figure VII: Differential expression of Treg/Th17-related genes in liver and hepatic leukocytes depends on diet or Ldlr genotype.

Realtime-PCR analysis of cDNA obtained from (A) whole liver lysates or (B) hepatic leukocytes fractions of wild type (Ldlr+/+) and Ldlr−/− mice fed cholesterol-free standard chow diet (SCD) or 0.15% cholesterol-containing Western diet (WD) for 12 weeks. cDNA was transcribed from equal amounts of RNA; specific mRNA concentrations were calculated with [c]= 2^∧Ct from two independent experiments; Ldlr+/+ SCD n=6, Ldlr+/+ WD n=7, Ldlr−/− SCD n=5, Ldlr−/− WD n=6; values are expressed as mean ± SEM, no detection of specific mRNA is indicated as n(values)/n(samples); two-way ANOVA and Bonferroni’s post hoc test was performed for statistical analysis, depicted P-values indicate the effect of dietary cholesterol intake (Diet) or LDLr genotype (Ldlr); colors and patterns as described for Online Figure IV.
Online Figure VIII: Differential expression of Th1-related genes, but not Th2-related genes, in liver and hepatic leukocytes depends on Ldlr genotype.

Realtime-PCR analysis of cDNA obtained from (A) whole liver lysates or (B) hepatic leukocytes fractions of wild type (Ldlr+/+) and Ldlr/- mice fed cholesterol-free standard chow diet (SCD) or 0.15% cholesterol-containing Western diet (WD) for 12 weeks. cDNA was transcribed from equal amounts of RNA; specific mRNA concentrations were calculated with $[c]=2^{-\Delta Ct}$ from two independent experiments; Ldlr+/+ SCD n=6, Ldlr+/+ WD n=7, Ldlr/- SCD n=5, Ldlr/- WD n=6; values are expressed as mean ± SEM, no detection of specific mRNA is indicated as n(values)/n(samples); two-way ANOVA and Bonferroni’s post hoc test was performed for statistical analysis, depicted $P$-values indicate the effect of dietary cholesterol intake (Diet) or LDLr genotype (Ldlr); colors and patterns as described for Online Figure IV.
Online Figure IX

Online Figure IX: Hypercholesterolemia affects CD86 expression of intrahepatic B cells in wild type mice.
CD86 expression levels of intrahepatic B cells (B220+CD19+) and CD11b+CD11c- leukocytes in wild type (Ldlr+/+) and Ldlr-/- mice in response to 12 weeks of 0.15% cholesterol-containing Western diet (WD) in relation to control group (cholesterol-free standard chow diet (SCD), set to 100%). Data represent the combination of two independent experiments (Ldlr+/+ SCD: n=6; Ldlr+/+ WD: n=7; Ldlr-/- SCD: n=5; Ldlr-/- WD: n=6); values are expressed as mean ± SEM; two-tailed Mann Whitney U test was performed for statistical analysis; *p<0.05; colors and patterns as described for Online Figure IV.
Online Figure X

Online Figure X: Prolonged Western diet induces liver injury specifically in Ldlr-/- mice.
Alanine transaminase activity (ALT) in plasma from wild type (Ldlr+/+) mice fed cholesterol-enriched diets for 4 weeks (left panel) (cholesterol-free standard chow diet (SCD, white): n=17; 0.15% cholesterol-containing Western diet (WD, gray): n=11; 1.125% cholesterol-containing Western diet (black): n=8) or for 12 weeks (middle panel) (n=8), respectively. Increasing ALT is detected in plasma of Ldlr-/- mice fed SCD (n=4), 4 weeks of WD (n=6) and 12 weeks of WD (n=15) (right panel). One-way ANOVA and Bonferroni’s post hoc test was performed for statistical analysis; values are expressed as mean ± SEM; *p<0.05.
Online Figure XI: Distribution and pro-inflammatory phenotype of hepatic T cells transferred into atherosclerotic Ldlr-/- mice.

CFSE-labeled CD4+ T cells derived from the liver of wild type (Ldlr+/+) and Ldlr-/- mice fed cholesterol-free standard chow diet (SCD) or 0.15% cholesterol-containing Western diet (WD) for 12 weeks were transferred into Ldlr-/- mice fed WD for 12 weeks (n=5). ROR-γt expression levels of transferred CD4+ T cells retrieved from (A) liver and (B) spleen. One-way ANOVA and Bonferroni's post hoc test was performed for statistical analysis. (C) Percentage of IFN-γ+ T cells among residential and transferred CD4+ T cell populations of indicated tissues after in vitro restimulation. Two-tailed Wilcoxon rank-pairs test was performed for statistical analysis. (D) Cell density of transferred CD4+ T cells retrieved from the spleen (normalized against liver remigration). Two-way ANOVA and Bonferroni’s post hoc test was performed for statistical analysis. Depicted P-value indicates the effect of dietary cholesterol intake. Values are expressed as mean ± SEM; colors and patterns as described for Online Figure IV.
## Online Table I

<table>
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