Hypercholesterolemia Induces Differentiation of Regulatory T Cells in the Liver


Rationale: The liver is the central organ that responds to dietary cholesterol intake and facilitates the release and clearance of lipoprotein particles. Persistent hypercholesterolemia leads to immune responses against lipoprotein particles that drive atherosclerosis. However, the effect of hypercholesterolemia on hepatic T-cell differentiation remains unknown.

Objective: To investigate hepatic T-cell subsets upon hypercholesterolemia.

Methods and Results: We observed that hypercholesterolemia elevated the intrahepatic regulatory T (Treg) cell population and increased the expression of transforming growth factor-β1 in the liver. Adoptive transfer experiments revealed that intrahepatically differentiated Treg cells relocated to the inflamed aorta in atherosclerosis-prone low-density lipoprotein receptor deficient (Ldlr−/−) mice. Moreover, hypercholesterolemia induced the differentiation of intrahepatic, but not intrasplenic, Th17 cells in wild-type mice, whereas the disrupted liver homeostasis in hypercholesterolemic Ldlr−/− mice led to intrahepatic Th1 cell differentiation and CD11b+CD11c+ leukocyte accumulation.

Conclusions: Our results elucidate a new mechanism that controls intrahepatic T-cell differentiation during atherosclerosis development and indicates that intrahepatically differentiated T cells contribute to the CD4+ T-cell pool in the atherosclerotic aorta. (Circ Res. 2017;120:1740-1753. DOI: 10.1161/CIRCRESAHA.116.310054.)

Key Words: atherosclerosis ■ hypercholesterolemia ■ inflammation ■ liver ■ metabolism ■ regulatory T-cells ■ transforming growth factor beta

Atherosclerosis is a chronic inflammatory disease induced when cholesterol-containing plasma lipoproteins accumulate in the arterial wall. These lipoproteins, including very low-density lipoprotein and low-density lipoprotein (LDL), are formed in the liver, circulate throughout the vasculature, and are cleared from the blood by lipoprotein receptors in the liver. Hypercholesterolemia is a major risk factor for atherosclerotic cardiovascular disease, and lipid-lowering represents a successful strategy for disease prevention.

The liver is a unique immunological site and hepatic tolerance induction against ectopic antigens has been shown to inhibit autoimmune responses systemically.1 Hepatic tolerance is implemented by regulatory T (Treg) cell induction through transforming growth factor-β1 (TGF-β1) secretion in situ.2 The immunological homeostasis in the liver is particularly important in the context of atherosclerosis, as subacute hepatic inflammation aggravates lesion development,3 whereas disruption of inflammatory signaling pathways in hepatocytes alleviates atherosclerosis.4 However, the intrahepatic T-cell differentiation in hypercholesterolemia and its contribution to atherosclerosis has not been investigated so far.

The development of atherosclerotic lesions in the arterial wall is linked to the presence of proatherogenic CD4+ T cells as well as macrophages. Hence, impairment of T-cell homeostasis aggravates atherosclerosis, whereas inhibition of T-cell immunity abrogates the disease.5 A crucial role to suppress inflammatory processes and to sustain immune tolerance is played by CD4+ Treg cells that either originate from the thymus or differentiate in the periphery in response to TGF-β.6,7 Treg cells express the lineage-defining transcription factor FoxP3 and suppress atherosclerosis.8–10 On the contrary, disease development is aggravated by specific proinflammatory CD4+ T cells recognizing the protein component of LDL, apolipoprotein B-100, that is expressed in the liver.11,12

To elucidate the effect of plasma cholesterol concentrations on T-cell differentiation, we treated mice for different time periods with cholesterol-containing diets and used LDL receptor knockout (Ldlr−/−) mice that develop severe, diet-induced hypercholesterolemia. Subsequently, we characterized phenotype and function of differentiated T-cell subsets in the liver and assessed their migration into the artery wall by transfer experiments and relocation assays.

Our data show that dietary-induced hypercholesterolemia increases CD4+FoxP3+CD25low Treg and CD4+retinoic acid...
Liver inflammation is a risk factor for cardiovascular disease and the recognition of liver-derived antigens by proatherogenic T cells exacerbates atherosclerosis. However, the intrahepatic T-cell response in hypercholesterolemia has not been investigated. We found that dietary cholesterol intake increases the expression of transforming growth factor-β1 and induces the differentiation of Treg and Th17 cells in the liver of wild-type mice, whereas hepatocyte damage, CD11b+CD11c+ leukocyte accumulation, and Th1 cell differentiation occur in the liver of severely hypercholesterolemic Ldlr−/− mice. Adoptive T-cell transfer experiments demonstrate that hypercholesterolemia increases the migration of hepatic Treg cells into the atherosclerotic aorta of recipient Ldlr−/− mice. This work shows that hepatic T cells differentiate in response to hypercholesterolemia and indicates their contribution to the T-cell pool in the atherosclerotic aorta. These findings light on the link between chronic liver diseases, such as hepatitis and nonalcoholic fatty liver disease, and cardiovascular disease.

### Methods

**Animals**

Experiments have been carried out on in-house bred transgenic or nontransgenic hemizygous DEREG FoxP3 reporter mice on a C57BL/6J background, kindly provided by Dr T. Sparwasser (Institute for Infection Immunology, TWINCORE, Hannover, Germany) and in-house bred Ldlr−/− mice (The Jackson Laboratory), as previously described. For some experiments, cross-bred DEREGxLdlr−/− mice were used. All experimental animals were bred under standard housing conditions, were between 8 and 10 weeks of age, were genotyped, and were randomly selected into treatment groups. Mice were fed cholesterol-free standard chow diet (SCD; R70, Lantmänn, Sweden), 0.15% cholesterol-containing Western diet (WD; R638, Lantmänn, Sweden), or 1.125% cholesterol-containing WD (D12108C, Research Diets) for indicated time periods; detailed list of ingredients is provided in (Online Table I).

### Statistics

Values are expressed as mean±SEM. Nonparametric Mann–Whitney U test or parametric Student t test was used for comparisons between 2 groups as indicated. Correlations were calculated using Spearman rank test. One- and 2-way ANOVA followed by Bonferroni multiple comparison post hoc test was performed for comparisons of more than 2 groups. D’Agostino and Pearson omnibus normality test was performed to assess normal distribution of data sets. Differences were considered significant at P values <0.05 (2-tailed). All statistical analyses were performed using GraphPad Prism software.

A detailed description of additional standard methods, online figures, and figure legends is provided in the Online Supplement.

### Results

**Cholesterol-Containing Diet Increases the CD4+FoxP3+ Treg Cell Population in the Liver**

We analyzed the murine intrahepatic CD4+ T-cell compartment and observed that the liver harbors a phenotypically distinct population of FoxP3+ Treg cells that mostly lack CD25 expression compared with Treg cells from spleen (Figure 1A). Surprisingly, cholesterol-containing WD treatment for 4 weeks increased this intrahepatic Treg cell population compared with control mice receiving cholesterol-free SCD (Figure 1B and 1C). In contrast, WD treatment had no significant effect on population size or total cell numbers of intrahepatic CD3+ cells, whereas hepatocyte damage, CD11b+CD11c+ leukocyte accumulation, and Th1 cell differentiation occur in the liver of severely hypercholesterolemic Ldlr−/− mice. Adoptive T-cell transfer experiments demonstrate that hypercholesterolemia increases the migration of hepatic Treg cells into the atherosclerotic aorta of recipient Ldlr−/− mice. This work shows that hepatic T cells differentiate in response to hypercholesterolemia and indicates their contribution to the T-cell pool in the atherosclerotic aorta. These findings light on the link between chronic liver diseases, such as hepatitis and nonalcoholic fatty liver disease, and cardiovascular disease.
of the intrahepatic CD4+ T-cell population was not due to the loss of CD4+ NKT cells (Online Figure ID). These results were also confirmed in FoxP3 reporter mice (DEREG), in which fully functional CD4+FoxP3+ Treg cells are readily detectible through their green fluorescent protein expression. Analysis of intrahepatic green fluorescent protein +CD4+ T cells obtained from DEREG mice demonstrated both elevated percentages of Treg cells among CD4+ T cells and enhanced FoxP3 expression after WD treatment (Figure 1B). Moreover, these effects were even more pronounced in mice that were kept on WD for extended time periods, whereas the low percentage of CD25 expression among intrahepatic Treg cells was preserved (Figure 1C; Online Figure IIA).

Correlation Between Hypercholesterolemia and Intrahepatic Treg Cells

On the basis of our findings, we hypothesized that hypercholesterolemia may affect the residential Treg cell population in liver. To test this, we fed wild-type mice WD with different cholesterol contents and subsequently analyzed intrahepatic Treg cells. As expected, plasma cholesterol concentrations were significantly elevated in mice treated for 4 weeks with 0.15% and 1.125% cholesterol-enriched WD, respectively (Online Figure IIB). In response to higher dietary cholesterol intake, the percentage of FoxP3-expressing cells among CD4+ T cells increased, as did their proliferation (Figure 2A). To further strengthen our hypothesis that systemic hypercholesterolemia influences the intrahepatic Treg cell population, Ldlr−/− mice were fed SCD or WD to provoke even higher plasma cholesterol levels. This led to significantly expanded Treg cell populations among intrahepatic CD4+ T cells (Figure 2B). Moreover, LDLr deficiency in DEREG mice significantly increased the intrahepatic Treg cell population and decreased their CD62L expression upon WD treatment, indicating an expansion of the FoxP3+ effector cell population (Figure 2C). The intrahepatic Treg cell population correlated linearly with hypercholesterolemia in Ldlr+/+ and Ldlr−/− mice (Figure 2D and 2E). Together, this data support the notion that dietary-induced hypercholesterolemia, rather than other WD ingredients, affects the intrahepatic Treg cell population size.

The unique ability of FoxP3 to imprint the Treg cell phenotype has been demonstrated by retroviral transduction experiments. Several post-transcriptional and post-translational control mechanisms regulate the function of FoxP3. To eliminate the possibility that these modifications interfered with the detection of FoxP3, we used different antibody clones for intracellular flow cytometry, analyzed liver cell lysates in immunoblots and quantified alternatively spliced transcripts of FoxP3 mRNA. We observed homogenous FoxP3 detection by both antibodies in liver obtained from SCD and WD fed wild-type mice, a single FoxP3 protein band with higher intensity in Ldlr−/− than Ldlr+/− liver of WD fed mice and a conserved ratio of exons in the untranslated region of FoxP3 transcripts (Online Figure IIIA through IIIC). This validates our analytic approach and strongly

![Figure 1. Western diet (WD) increases the intrahepatic CD4+FoxP3+CD25low Treg cell population.](image-url)
suggests that hypercholesterolemia induces FoxP3 expression in intrahepatic T cells without changing isoforms, inducing post-translational modifications or causing alterations of the transcription start site.

**WD Increases Hepatic TGF-β1 Expression and TGF-β1–Related Phenotypic Changes of Intrahepatic T Cells**

The low expression of CD25, the conversion to CD62L− cells, and the hypercholesterolemia-induced proliferation of intrahepatic Treg cells suggest that peripheral differentiation of inducible Treg (iTreg) cells rather than accumulation of thymic Treg cells accounts for the increase in liver-resident Treg cells. To address this question in detail, we analyzed proposed markers of thymic Treg cells in intrahepatic Treg cells from normo- and hypercholesterolemic mice. Consistent with enhanced iTreg cell differentiation, we found decreased Helios expression patterns in intrahepatic Treg cells of WD fed Ldlr+/+ and Ldlr−/− mice compared with their corresponding SCD fed littermates (Online Figure IV A). In addition to

**Figure 2. Intrahepatic Treg cell population increases linearly with dietary cholesterol intake in wild-type and Ldlr−/− mice.**

Representative flow cytometry data and bar graphs for the percentage of FoxP3+ intrahepatic CD4+ T cells, Ki-67+ intrahepatic CD4+FoxP3+ Treg cells or CD62L− intrahepatic CD4+FoxP3+ Treg cells in (A) FoxP3 reporter mice (DEREG) fed increasing dietary cholesterol content for 4 wk (data combined from 3 independent experiments n=11, 11, and 6 for 0%, 0.15%, and 1.125% cholesterol, respectively), (B) Ldlr−/− mice fed cholesterol-free standard chow diet (SCD) or 0.15% cholesterol-containing Western diet (WD) for 12 wk (n=4) and (C) DEREG (Ldlr+/+) and DEREGxLdlr−/− mice fed 0.15% cholesterol-containing WD for 4 wk (n=5). Percentage of FoxP3+ Treg cells of the intrahepatic CD4+ T-cell pool obtained from (D) Ldlr+/+ (n=41) and (E) Ldlr−/− (n=13) mice fed different dietary regimens plotted against plasma cholesterol concentrations. Values are expressed as mean±SEM, 2-tailed Mann–Whitney U test was performed for statistical analysis; *P<0.05; **P<0.01; r=Spearman rank correlation coefficient; plain and hatched bars or circles and triangles indicate Ldlr+/+ and Ldlr−/− mice, respectively; white, gray, and black color indicates 0%, 0.15% and 1.125% cholesterol diet, respectively.
Helios, also Neuropilin-1 (Nrp-1) was suggested to characterize thymic Treg cells. However, Nrp-1 was stably expressed by intrahepatic Treg cells and by a subset of proliferating FoxP3- intrahepatic CD4+ T cells (Online Figure IV). Intriguingly, hypercholesterolemia increased the percentage of proliferating Nrp-1+ CD4+FoxP3- T cells in Ldlr+/+ and Ldlr−/− mice (Online Figure IVB). Because Nrp-1 is induced by and acts as receptor for TGF-β1,19,20 we reasoned that the increase of liver-resident Treg cells and the expression of Nrp-1 by proliferating liver-resident non-Treg cells may be mediated through local TGF-β1 production. Thus, we measured TGF-β1 plasma concentrations and quantified TGF-β1 mRNA in liver from SCD or WD fed mice. TGF-β1 concentrations correlated linearly with cholesterol concentrations in plasma (Figure 3A). Furthermore, TGF-β1 mRNA levels in the liver were significantly higher in WD compared...
with SCD fed mice and correlated linearly with the intrahepatic Treg cell compartment (Figure 3B; Online Figure IVC). Next, we analyzed hypercholesterolemia-induced TGF-β1 expression in liver cells by flow cytometry of the TGF-β1 propeptide, latency-associated peptide. Intracellular staining for latency-associated peptide revealed that liver cells derived from WD fed mice expressed significantly higher levels of latency-associated peptide compared with SCD fed littermates (Figure 3C). The analysis of different liver cell types showed that WD promoted latency-associated peptide expression in CD31+ liver subpopulations and moderately in CD4+ lymphocytes, but not in glial fibrillary acidic protein (GFAP+) liver cells (Figure 3D). To exclude the possibility that hypercholesterolemia also directly affects FoxP3 expression in lymphocytes, purified CD4+ T cells were cultured in the presence or absence of LDL particles. We found that the addition of LDL or oxidized LDL in vitro had no effect on FoxP3 expression in CD4+ T-cell cultures, alone or in combination with TGF-β1, indicating that hypercholesterolemia affects iTreg cell differentiation indirectly via local TGF-β1 induction rather than through an intrinsic CD4+ T-cell mechanism (Online Figure V and data not shown). Taken together, these findings demonstrate that hypercholesterolemia promotes hepatic TGF-β1 expression that is associated with increased intrahepatic iTreg cell differentiation.

Hypercholesterolemia Inhibits T-Cell Proliferation in the Liver

To assess whether hypercholesterolemia-mediated TGF-β1 induction and iTreg cell generation in the liver inhibits T-cell responses, we determined the tissue-specific capacity to support the proliferation of adoptively transferred T cells in SCD and WD fed wild-type mice. For this, CellTracker-labeled CD4+ T cells were incubated short-term with mitogenic anti-CD3 antibodies in vitro and their subsequent in vivo proliferation was assessed in liver and spleen. In both organs about 0.3% of the local T-cell population came from the adoptive transfer. Transferred T cells that entered the liver of WD fed mice were significantly inhibited in their proliferation compared with transferred cells entering the liver of SCD fed mice (Figure 4). In contrast, no differences were observed for the proliferation of transferred cells retrieved from the spleen of SCD or WD fed mice. This indicates that dietary-induced hypercholesterolemia leads to immunosuppressive conditions specifically in the liver and suggests that intrahepatic TGF-β1 and iTreg cells affect the proliferation of T cells in situ.

Hypercholesterolemia Affects the Differentiation of Intrahepatic Th17 Cells in Wild-Type and Th1 cells in Ldlr<sup>−/−</sup> Mice

Next, we sought to determine whether hepatic TGF-β1 production also affects the differentiation of local non-Treg cells. We fed Ldlr<sup>−/−</sup> and Ldlr<sup>+/−</sup> mice SCD or WD for 12 weeks to elicit strong TGF-β1-mediated T-cell differentiation in the liver and to detect a potential break of tolerance in early atherosclerosis development of WD fed Ldlr<sup>−/−</sup> mice. After in vitro restimulation, the intracellular cytokine profile revealed that increased percentages of intrahepatic CD4+ T cells differentiated in vivo into T-cell subsets compared with corresponding intraplastic CD4+ T cells (Figure 5; Online Figure VI). Intrahepatic T cells of SCD fed Ldlr<sup>−/−</sup> mice expressed mutually exclusive IL-17A and interferon-gamma (IFN-γ) in similar-sized populations that together comprised one fourth of all intrahepatic CD4+ T cells (Figure 5A, upper left). In comparison to SCD fed littermates, WD treatment resulted in a 2-fold increase of IL-17A-expressing CD4+ T cells, whereas IFN-γ expression was unaffected in Ldlr<sup>−/−</sup> mice (Figure 5A, upper right). Intrahepatic CD4+ T cells derived from SCD fed Ldlr<sup>−/−</sup> mice, however, displayed increased IFN-γ baseline expression that exceeded IL-17A expression 2-fold (Figure 5A, lower left). This imbalance was further elevated upon WD treatment, resulting in one third of all intrahepatic CD4+ T cells expressing IFN-γ, whereas IL-17A expression was barely increased (Figure 5A, lower right). In addition, we analyzed the Th17-related transcription factor ROR-γt in IL-17A<sup>+</sup> intrahepatic T cells. We revealed that dietary cholesterol intake significantly promoted Th17 cell differentiation, as defined by the CD4+ROR-γt<sup>+</sup>IL-17A<sup>+</sup> T-cell population, in the liver of Ldlr<sup>−/−</sup>, but not in Ldlr<sup>−/−</sup> mice (Figure 5B, left). However, this pattern was reversed for intrahepatic Th1 cell differentiation. Upon WD treatment, Ldlr<sup>−/−</sup> mice maintained a constant level of Th1 cells in the liver, whereas Ldlr<sup>−/−</sup> mice further increased intrahepatic Th1 cell differentiation from yet elevated Th1 cell levels under SCD condition (Figure 5B, right). In particular, the effect of hypercholesterolemia on T-cell differentiation profiles was specific for intrahepatic T cells because no changes were detected for in vivo differentiated Th17 cells (about 1%) or Th1 cells (about 12%) in the spleen (Online Figure VIB).

To further validate that dietary cholesterol intake or the Ldlr genotype correlates with intrahepatic T-cell differentiation we performed real-time polymerase chain reaction analysis of whole liver lysates and hepatic leukocytes. Corroborating our flow cytometry data, WD significantly increased TGF-β1 mRNA levels in both fractions and FoxP3 as well as ROR-γt mRNA levels in hepatic leukocytes, whereas mRNA concentrations of IL-6 correlated with the Ldlr genotype in whole liver lysates but did not reach statistical significance in hepatic leukocyte fractions (Online Figure VIIA and VIB). In contrast to diet-dependent Treg/Th17 cell-related genes, the significant increase of IL-12a mRNA concentration in whole liver lysate and IFN-γ mRNA concentration in hepatic leukocytes was associated with the Ldlr genotype, whereas Th2-related Gata-3 was unaffected by both, diet and Ldlr genotype (Online Figure VIIA and VIB).

Accumulation of CD11b<sup>+</sup>CD11c<sup>+</sup> Cells in the Liver of WD-Fed Ldlr<sup>−/−</sup> Mice

As LDLr deficiency creates intrahepatic conditions that promote Th1 cell differentiation even in the presence of hypercholesterolemia-induced TGF-β1 production, we analyzed intrahepatic antigen-presenting cells (APC), such as liver-resident CD19<sup>+</sup>B220<sup>+</sup> B cells, CD11b<sup>+</sup>CD11c<sup>−</sup>, and CD11b<sup>+</sup>CD11c<sup>+</sup> cells. In response to 12 weeks of WD, no significant changes of the analyzed subsets were observed in Ldlr<sup>−/−</sup> mice (Figure 6A and 6B). However, Ldlr<sup>−/−</sup> mice
showed a strong increase of CD11b⁺CD11c⁺ liver cells at the expense of CD11b⁺CD11c⁻ liver cells. Moreover, analysis of CD86 expression on APCs revealed that intrahepatic B cells (and to a lesser extent also CD11b⁺CD11c⁻ cells) decreased their expression of this costimulatory molecule in WD fed Ldlr⁺/⁺ mice, whereas similar CD86 levels were found in SCD and WD fed Ldlr⁻/- mice (Online Figure IX). These changes coincided with an increased release of alanine transaminase into the plasma of WD fed Ldlr⁻/- mice, reflecting hepatocyte damage, whereas the liver of wild-type mice was unaffected (Online Figure X). In summary, these results suggest that hypercholesterolemia-induced TGF-β₁ expression in the liver promotes iTreg and Th17 cell differentiation in Ldlr⁺/⁺ mice. In contrast, severe hypercholesterolemia in Ldlr⁻/- mice elicits hepatocyte damage and facilitates additional costimulatory signals by accumulating
CD11b+CD11c+ cells in the liver, which specifically restricts Th17 cell differentiation but triggers Th1 cell differentiation instead.

**Relocation of Intrahepatically Differentiated T Cells**

Because hypercholesterolemia induced differential T-cell subsets in liver, but not in spleen, we sought to assess the contribution of these T cells in atherosclerosis. To test this, intrahepatic CD4+ T cells obtained from Ldlr+/+ and Ldlr−/− mice fed SCD or WD for 12 weeks were isolated, labeled with carboxyfluorescein succinimidyl ester and transferred into Ldlr−/− mice with developing atherosclerosis. Four days after transfer, the intrahepatically differentiated and labeled cells were retrieved from different tissues and their ability to remigrate to the liver or to relocate to the spleen and aorta was assessed. We observed that in absolute numbers, transferred cells were partly remigrating to the liver but mostly relocated to the spleen (Figure 7A). The expression analysis for transcription factors of retrieved cells showed that hypercholesterolemia-induced hepatic FoxP3+ Treg cells were not remigrating into the liver but rather relocated to the spleen (Figure 7B). In both organs, transferred T cells did not maintain their hypercholesterolemia-induced ROR-γt profile (Online Figure XIA and XIB). In addition, in vitro restimulation of transferred T cells retrieved from liver and spleen demonstrated that liver-derived...
Th1 cells from *Ldlr<sup>−/−</sup>* mice did not accumulate in either organ, suggesting that proinflammatory T cells relocate elsewhere (Online Figure XIC).

**Transferred Intrahepatically Differentiated T Cells Enter the Atherosclerotic Aorta**

To assess homing of liver T cells to the atherosclerotic vasculature, we analyzed aortas of recipient *Ldlr<sup>−/−</sup>* mice fed cholesterol-free standard chow diet (SCD) or 0.15% cholesterol-containing Western diet (WD) for 12 wk. Displayed values apply for gated granulocytes. CD4<sup>+</sup> T cells could be identified (Figure 7C). Interestingly, these intrahepatically differentiated cells were relatively abundant in the aorta, where they showed a median value of 0.665% of all CD4<sup>+</sup> T cells (minimum: 0.08%, maximum: 18.5%). In contrast, the transferred cells were strongly outcompeted by residential CD4<sup>+</sup> T cells in liver, spleen, and paraaortic lymph nodes, with median values of 0.027% (minimum: 0.002%, maximum: 0.180%), 0.014% (minimum: 0.001%, maximum: 0.051%), and 0.015% (minimum: 0.004%, maximum: 0.061%), respectively. This shows that hepatic T cells have access to other tissues and particularly...
contribute to the CD4+ T-cell pool in the atherosclerotic aorta, where liver-derived antigens, such as apolipoprotein B-100, are abundant.

Next, we investigated whether dietary treatment or Ldlr genotype of the transferred hepatic T cells affected their migration into the atherosclerotic aorta. For this, the density
of cells relocating to target organs was normalized with the density of cells remigrating to the liver to compensate for unintentional variations in transferred CD4+ T-cell numbers. Hepatic CD4+ T cells originating from WD fed mice preferentially appeared in the aorta and in the spleen (Figure 7D; Online Figure XID). Moreover, hepatic CD4+ T cells that relocated to the atherosclerotic aorta expressed significantly more FoxP3 and ROR-γt than the residential CD4+ T-cell population (Figure 7E). Thus, intrahepatically differentiated Treg cells can infiltrate the inflamed aorta, where they may conceivably affect the inflammatory process in the developing atherosclerotic lesion.

**Discussion**

In this study, we show that (1) hypercholesterolemia increased the differentiation of intrahepatic iTreg and Th17 cells, whereas (2) the disruption of liver homeostasis elicits Th1 cells. (3) Induction of iTreg and Th17 cells was associated with increasing levels of TGF-β1, suggesting that this growth factor accounted for the process. Finally, (4) T cells differentiated in the hypercholesterolemic liver entered the atherosclerotic aorta, suggesting that hypercholesterolemia-induced intrahepatic T-cell subsets can affect immune responses against liver-derived antigens in atherosclerosis.

Besides their role in host defense against pathogens, immune cells can also modulate lipid metabolism. Strikingly, Treg cells have been shown to inhibit atherosclerosis not only through the suppression of adaptive immune responses but also by ameliorating the atherogenic lipidprotein profile in Ldlr−/− mice. Here, we found a linear relationship between intrahepatic Treg cell populations and plasma cholesterol concentrations. In this context, the increased differentiation and expansion of intrahepatic Treg cells seems to be a safe-guard mechanism to avoid disease development in hypercholesterolemia. This implies that elevated physiological plasma cholesterol levels could promote the induction of hepatic tolerance to liver-derived antigens, which may contribute to prevent atherosclerosis. In line with this, previous studies demonstrated that subliminal and tolerogenic immunization protocols facilitate a reduction in experimental atherosclerosis.

FoxP3+ Treg cells in secondary lymphoid organs are typically CD4+CD25high, albeit suppressive CD4+CD25− FoxP3+ Treg cells have been described. In contrast, the mouse liver harbors a FoxP3+ Treg cell population that mostly lacks CD25 expression and does not exceed 50000 cells per organ. To evaluate the suppressive capacity of this subpopulation is challenging, since the low expression of the IL-2 receptor α-chain implies that these cells have an impaired ability to compete for IL-2 in vitro assays. Therefore, we directly measured the proliferation of prestimulated responder cells in vivo to assess whether hypercholesterolemia-induced intrahepatic changes suppress cellular immune responses. By this, we found that the integral condition, specified by hepatic TGF-β1, iTreg cells, and APC maturation, inhibited the proliferation of T cells specifically in the liver of WD fed mice. Thus, hypercholesterolemia-induced iTreg cells are likely to partake in the suppression of hepatic T-cell proliferation. This property should make hepatic iTreg cells interesting targets in future vaccination strategies against atherosclerosis.

TGF-β1 is known to exert atherosclerosis-protective effects. Here, we show that TGF-β1 expression increased in the liver upon dietary-induced hypercholesterolemia. This is in line with previous studies demonstrating that hypercholesterolemia increases TGF-β1 in the plasma of atherosclerotic mice and that TGF-β1 signaling in CD4+ T cells strongly inhibits disease. In the periphery, TGF-β1 mediates the differentiation of iTreg cells in the absence of proinflammatory cytokines, whereas the presence of proinflammatory cytokines favors the differentiation of Th17 cells. This iTreg/Th17 cell balance seems to define the outcome of many chronic inflammatory diseases, including atherosclerosis. However, the priming site of differentiating T cells in atherosclerosis has been unknown so far. Here we report that intrahepatic, but not intrasplicenic, T cells responded toward atherosclerosis-prone conditions, highlighting the unique role of the liver in disease development. The liver expresses autoantigens implicated in atherogenesis, such as apolipoprotein B-100, and offers the first opportunity for potentially autoreactive T cells to encounter antigen-loaded APCs in the periphery. Hepatocytes and nonhepatocytes, such as GFAP+ hepatic stellate cells, Kupffer cells, and CD31+ liver sinusoidal endothelial cells, have been reported to synthesize or store TGF-β1. Expression of this cytokine in the liver has been related to hepatitis, fibrosis, liver regeneration, and carcinogenesis. Our current data show that hypercholesterolemia increased the TGF-β1 concentration in the liver and this was associated with increased intrahepatic iTreg/Th17 cell populations. However, causality is difficult to prove considering that the pleiotropic functions and the heterogeneous origin of the cytokine, as well as its complicated kinetics, do not allow neutralization or conditional knockout strategies.

Notably, we observed the hypercholesterolemia-mediated effect on proinflammatory T-cell populations specifically in the liver, but not in the spleen. However, WD treatment increases also Treg cell numbers in secondary lymphoid organs (manuscript in preparation). Therefore, it is possible that increased concentrations of TGF-β1 in liver and plasma elicited through hypercholesterolemia may affect T-cell differentiation systemically. However, iTreg cell differentiation in vitro via polyclonal TCR (T-cell antigen receptor) stimulation in the presence of TGF-β1 was unaffected by LDL particles and LDLr deficiency did not impair Treg cell numbers ex vivo. This suggests that the hypercholesterolemia-mediated increased differentiation of intrahepatic iTreg/Th17 cells in vivo acts indirectly through local TGF-β1 induction and APC maturation rather than by intrinsic CD4+ T-cell mechanisms. In line with this, hepatocyte damage and intrahepatic accumulation of CD11b+CD11c+ cells, a phenotype that has been attributed to infiltrating monocytes that promote Th1 differentiation upon liver injury, coincides in Ldlr−/− mice with the favored differentiation of Th1 over Th17 cells. Th1 cells are known to be hepatocyte-destructive, whereas Th17 cells have been linked to liver homeostasis, are dispensable for liver injury in experimental models, and even protect against Th1-mediated hepatocyte apoptosis. Interestingly, an increased Th1/Th17 cell ratio, as observed in Ldlr−/− mice of our study, is known to be detrimental in atherosclerosis-prone Apoe−/− mice.
A recent study by Butcher et al. found that Treg cells upon adoptive transfer migrate into the atherosclerotic aorta, where they maintain their FoxP3 expression but undergo Th1-phenotypic changes and lose suppressive capacity. In line with this, our study shows that adoptive transfer of intrahepatically differentiated T cells into atherosclerotic Ldlr−/− mice resulted in a preferential accumulation of transferred T cells in the recipient aorta. Moreover, transferred hepatic T cells that migrated into the aorta were highly differentiated and displayed elevated FoxP3 and ROR-γt expression compared with the resident T-cell population. This T-cell profile corresponds to the intrahepatic T-cell differentiation induced by hypercholesterolemia. Thus, the phenotype of hepatic T cells derived from hypercholesterolemic mice may explain their increased relocation into the aorta, whereas the remigration into the liver was nonspecific for T-cell subsets and unaffected by the treatment of donor mice. The mechanisms governing the selection of homing T cells in the aorta are unknown and may involve local expansion as well as selective recruitment by chemotactic molecules. Our results suggest that intrahepatically differentiated iTreg/Th17 cells affect the ratio of T-cell subsets in the atherosclerotic aorta. However, we cannot rule out the possibility that residual/transferred cells lost their signature cytokine expression because of plasticity, or died at the inflammation site due to exhaustion. Further investigations of intrahepatically differentiated T-cell subsets should elucidate how their migration to atherosclerotic lesions is regulated and to what extent these particular cells control the size and composition of plaques, as it has been reported in studies using global genetic targeting of Th1 and Th17 cells.

iTreg and Th17 cell differentiation is influenced by diet and metabolism. In secondary lymphoid organs, this process is influenced by bacterial metabolites of dietary fibres and sodium chloride, respectively. However, WD used in our experiments had no increased fiber or sodium chloride content compared with SCD. Moreover, LDLr deficiency further elevated iTreg cell generation in WD fed mice. Thus, we assume that intrahepatic iTreg/Th17 cell differentiation depends on dietary cholesterol rather than changes of gut microbiota activity or electrolyte homeostasis.

The standard treatment for hypercholesterolemia is the blockade of de novo cholesterol synthesis by inhibition of 3-hydroxy-3-methyl-glutaryl-CoA reductase through statins. Interestingly, statin treatment increases Treg cell populations where they maintain their FoxP3 expression but undergo Th1-phenotypic changes and lose suppressive capacity. In line with this, our study shows that adoptive transfer of intrahepatically differentiated T cells into atherosclerotic Ldlr−/− mice resulted in a preferential accumulation of transferred T cells in the recipient aorta. Moreover, transferred hepatic T cells that migrated into the aorta were highly differentiated and displayed elevated FoxP3 and ROR-γt expression compared with the resident T-cell population. This T-cell profile corresponds to the intrahepatic T-cell differentiation induced by hypercholesterolemia. Thus, the phenotype of hepatic T cells derived from hypercholesterolemic mice may explain their increased relocation into the aorta, whereas the remigration into the liver was nonspecific for T-cell subsets and unaffected by the treatment of donor mice. The mechanisms governing the selection of homing T cells in the aorta are unknown and may involve local expansion as well as selective recruitment by chemotactic molecules. Our results suggest that intrahepatically differentiated iTreg/Th17 cells affect the ratio of T-cell subsets in the atherosclerotic aorta. However, we cannot rule out the possibility that residual/transferred cells lost their signature cytokine expression because of plasticity, or died at the inflammation site due to exhaustion. Further investigations of intrahepatically differentiated T-cell subsets should elucidate how their migration to atherosclerotic lesions is regulated and to what extent these particular cells control the size and composition of plaques, as it has been reported in studies using global genetic targeting of Th1 and Th17 cells.


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Circulation Research May 26, 2017

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Hypercholesterolemia Induces Differentiation of Regulatory T Cells in the Liver
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Circ Res. 2017;120:1740-1753; originally published online April 18, 2017; doi: 10.1161/CIRCRESAHA.116.310054

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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Detailed Methods:

Cell isolation and culture: Mice were euthanized by CO₂. Blood was collected by cardia
cupuncture, 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-ROR-
γt (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′-CCCCACACGACACAGC-3′ (FoxP3 exon -2b), 5′-GTGAGACAGATCCATGTGC-
3′ (FoxP3 exon -2a) and 5′-AGTCCACTTCAACGCTC-3′ (FoxP3 exon -2bΔ) in
combination with the reverse primer 5′-GTTGGCGATGCTGTTGTTG-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^-∆Ct by realtime-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*10^6 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*10^5 – 3*10^6 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: 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-γt+IL-17A+ T cells) and Th1 cells (CD4+IFN-γ+ 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: 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^−ΔCt 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: 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 match-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

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# Online Table II

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