LDL- Reactive T Cells Regulate Plasma Cholesterol Levels and Development of Atherosclerosis in Humanized Hypercholesterolemic Mice

Running Title: Gisterå et al.; LDL- Reactive T Cells in Atherosclerosis

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Abstract

Background—Atherosclerotic cardiovascular disease is a chronic inflammatory process initiated when cholesterol-carrying low-density lipoprotein (LDL) is retained in the arterial wall. CD4+ T cells, some of which recognize peptide components of LDL as antigen, are recruited to the forming lesion, resulting in T-cell activation. Although these T cells are thought to be proatherogenic, LDL immunization reduces disease in experimental animals. These seemingly contradictory findings have hampered the development of immune-based cardiovascular therapy. The present study was designed to clarify how activation of LDL-reactive T cells impacts on metabolism and vascular pathobiology.

Methods—We have developed a T-cell receptor (TCR)-transgenic mouse model to characterize the effects of immune reactions against LDL. Through adoptive cell transfers and cross-breeding to hypercholesterolemic mice expressing the antigenic human LDL protein ApoB100, we evaluate the effects on atherosclerosis.

Results—A subpopulation of LDL-reactive T cells survived clonal selection in the thymus, developed into T follicular helper cells in lymphoid tissues upon antigen recognition, and promoted B-cell activation. This led to production of anti-LDL immunoglobulin G (IgG) antibodies that enhanced LDL clearance through immune complex formation. Furthermore, the cellular immune response to LDL was associated with increased cholesterol excretion in faeces and with reduced vascular inflammation.

Conclusions—These data show that anti-LDL immunoreactivity evokes three atheroprotective mechanisms, namely antibody-dependent LDL clearance, increased cholesterol excretion, and reduced vascular inflammation.

Key Words: Atherosclerosis; T-lymphocytes; Apolipoprotein B-100; Vaccination; Hypercholesterolemia
Clinical Perspective

What is new?

- Immune responses toward LDL are important for atherosclerosis development, but a lack of specific experimental models has limited mechanistic understanding and translation of findings into clinical therapies.
- We developed T-cell receptor transgenic mice to study LDL autoimmunity in a humanized hypercholesterolemic mouse model of atherosclerosis.
- A strong T-cell dependent B-cell response was induced by LDL, leading to production of anti-LDL IgG antibodies that enhanced LDL clearance and ameliorated atherosclerosis.

What are the clinical implications?

- This study sheds light on the pathophysiological role of LDL-reactive T cells and anti-LDL IgG antibodies, both of which are known to be present in patients with atherosclerotic cardiovascular disease.
- We show that targeting LDL-reactive T cells can enhance atheroprotective immunity and that vaccination against LDL components may be an attractive way to prevent atherosclerosis.
Introduction

Atherosclerotic cardiovascular disease is the main cause of death in the world today. It is a chronic inflammatory process initiated when cholesterol-carrying low-density lipoprotein (LDL) particles are retained in the arterial wall. LDL retention elicits a local inflammation with influx of monocytes that differentiate into macrophages, accumulate intracellular cholesterol, and produce inflammatory mediators. In parallel with macrophages, T cells are also recruited to the forming lesion. Many of them are CD4+ cells that recognize LDL as antigen, resulting in T-cell activation. T cells accumulating in atherosclerotic lesions are largely of the proinflammatory Th1 subtype and produce inflammatory cytokines such as interferon-γ (IFN-γ) and tumor necrosis factor (TNF), which can activate other cells to secrete additional mediators including interleukins, chemokines, and eicosanoids. Local production of inflammatory mediators in the atherosclerotic artery wall eventually leads to activation of acute phase responses and elevated levels of inflammatory markers such as interleukin-6 (IL-6) and C-reactive protein (CRP) in the systemic circulation. Ongoing inflammatory and hemodynamic assault on the atherosclerotic lesion may eventually cause a local dysfunction or breakdown of endothelial integrity. This, in turn, can trigger thrombus formation, local ischemia, and infarction of the end organ, as is the case in myocardial infarction and ischemic stroke. This scenario can be prevented by anti-inflammatory therapy, as recently shown in a large secondary prevention clinical trial.

The development of disease models in gene-targeted mice has permitted a dissection of the role of immunity and inflammation in atherosclerosis. By targeting key genes in cholesterol metabolism, it was possible to make mice severely hypercholesterolemic. This leads to development of atherosclerosis in this species, although it is normally resistant to this disease.
Th1 cells and their signature cytokine, IFN-γ, were found to exert proatherogenic effects in hypercholesterolemic, gene-targeted mice. Such effects were also seen when CD4+ T cells were introduced into immunodeficient Apoe−/−xscid/scid mice. Manipulation of regulatory T (Treg) cells revealed an “atheroprotective” role of this subset, whereas Th17 cells may promote collagen formation and plaque stabilization. All these studies involve genetic perturbation that affects global differentiation of T cells and the impact of antigen specific T-cell responses has remained unclear.

Immunization with LDL can elicit an atheroprotective response that inhibits lesion development. This is the case irrespective of whether antigen is administered through the parenteral or mucosal route. The atheroprotective effect appears to involve T cells, since it is associated with formation of high-titer IgG-anti-LDL. It has been ascribed to the generation of immunosuppressive Tregs producing anti-inflammatory cytokines, or to formation of anti-LDL antibodies.

During atherogenesis, periarterial and systemic B-cell responses also occur, with production of antibodies to epitopes on native and oxidized LDL particles. Both pro- and anti-atherosclerotic effects have been linked to B cells. Thus, splenectomy increases disease in hypercholesterolemic mice, whereas transfer of spleen B cells reduces it. Similarly, enhanced production of antibodies to epitopes on oxidized LDL particles attenuates disease development. Paradoxically, administration of anti-CD20 antibodies also ameliorates it.

Limited insights into the nature of the disease-associated immune response to LDL have made our understanding of the atherosclerotic process incomplete and hampered the possibilities to develop immunoprotective prevention and therapy. In other chronic inflammatory diseases, such as rheumatoid arthritis and multiple sclerosis, transgenic (tg) models in which a large
A proportion of T cells recognize the purported autoantigens, have turned out to be useful for studies of pathogenetic mechanisms and therapeutic principles\textsuperscript{30, 31}. We therefore constructed a tg mouse model in which the majority of CD4\(^+\) T cells recognize human LDL, and determined its effects on LDL turnover and atherosclerosis.

**Methods**

**Mouse strains**

Three different T-cell receptors (TCRs) were cloned from hybridomas described previously\textsuperscript{9}. The constructs were inserted into a hCD2-VA expression vector containing the promoter and locus control region of the human \textit{CD2} gene\textsuperscript{32}. The TCR \(\alpha\) and \(\beta\) constructs were microinjected into C57BL/6J embryos at the Karolinska Center for Transgene Technologies, yielding a coisogenic C57BL/6J offspring that was screened for transgene expression by PCR. The three strains were named \(BT1\) (TRAV12, TRBV31), \(BT2\) (TRAV4, TRBV31), and \(BT3\) (TRAV14, TRBV31). In subsequent experiments, C57BL/6J mice (\(B6\)) were used as controls. For LDL injections the \(BT\) strains were crossed with a \textit{Nur77-GFP} reporter mouse (C57BL/6-Tg(Nr4a1-EGFP/cre)820Khog/J, stock 016617, Jackson laboratory). For cell transfers and crosses, we used C57BL/6J-\textit{Human APOB100-tg Ldlr\textsuperscript{m1Her} (HuBL)} mice backcrossed to C57BL/6J for 10 generations\textsuperscript{3}. These mice carry the full-length human \textit{APOB100} gene, in which codon 2153 has been converted from glutamine to leucine to prevent the formation of ApoB48, thus generating only ApoB100. Mice were fed a Western diet (R638, Lantmännen) for 10 weeks\textsuperscript{9}. All experiments were performed according to institutional guidelines and were approved by the Stockholm Regional Board for Animal Ethics.
Mouse experiments

To measure T-cell activation in vivo, 10-week-old mice were injected with 100 µg LDL intraperitoneally. Sixteen hours later, spleens were harvested and T cells analyzed by flow cytometry. For adoptive T-cell transfer, 10-week-old male donors were sacrificed and spleen and lymph nodes harvested. Single cell suspensions were prepared and untouched CD4+ cells isolated by negative selection with antibodies to CD8, CD11b, CD16/32, CD45R, and Ter-119 (Dynabeads untouched mouse CD4 cells kit, Invitrogen). Cells were labeled with CellTraceViolet (Invitrogen) or directly resuspended in phosphate-buffered saline (PBS) for intravenous injection of 3*10^6 cells in the tail vein. For cell trace experiments, recipients were sacrificed 1-4 days after cell transfer. In other experiments, the recipients received the first injection at 10 weeks of age and a second injection at 15 weeks of age. They were sacrificed at 20 weeks of age, after totally 10 weeks on Western diet. All male HuBL progeny from the in-house breeding colony was included in the study and randomly assigned to receive B6, BT1, BT2, or BT3 cell transfer depending on available donors. The included mice were given a serial number to blind the following analyzes. Two mice were excluded from the study, one died before the first injection, and the second died 25 days after the first BT2 cell injection. The study was closed when the number reached a predetermined power to detect a 10 percentage points difference in lesion size (aortic arches from untreated male HuBL mice were used for the power calculation; α=0.05, β=0.2). BT1xHuBL and BT3xHuBL mice were developed through crossbreeding. Hemizygous BT1xHuBL mice (homozygous for human APOB100 and Ldlr^{tm1Her}) were then bred to HuBL mice to generate a BT1^+xHuBL study group and BT1^-xHuBL littermate
controls (HuBL). Ten-week-old male mice were either fed Western diet for 10 weeks or sacrificed for baseline analyzes.

For the vaccination study, 25-week-old male HuBL mice received subcutaneous immunizations with ApoB100 emulsified in complete Freund’s adjuvant. The immune response was boosted four weeks later with ApoB100 emulsified in incomplete Freund’s adjuvant. All mice received 100 μg protein. Control mice were immunized with PBS and adjuvant following the same protocol. All mice were sacrificed 10 weeks after the first immunization.

**Flow cytometry analysis**

Flow cytometry was performed on leukocytes isolated as single cell suspensions from spleen, thymus, or lymph nodes. Fixable Aqua Live/Dead staining was used according to manufacturer’s protocol (Invitrogen). After Fc-block (anti-CD16/32, BD Biosciences), fluorophore labeled primary IgG antibodies were employed for extracellular staining. Streptavidin Dylight 649 (Vector) was used for biotinylated primary antibodies. Intracellular staining was performed using the anti-mouse/rat Foxp3 staining set (eBioscience). All antibodies are listed in the Supplemental Methods section. Samples were acquired on a CyAn ADP flow cytometer (Beckman Coulter) and data were analyzed using FlowJo software (Tree Star).

**Tissue processing, immunohistochemistry, and lesion analysis**

Blood from sacrificed mice was collected by cardiac puncture and the vasculature perfused with sterile ribonuclease-free PBS. The aortic arch was fixed in PBS-buffered 4% formaldehyde solution for later pinning and staining with Sudan IV (Sigma-Aldrich). The rest of the aorta, para-aortic lymph nodes, and a liver lobe were dissected and snap-frozen for later RNA isolation. The heart, spleen, liver, kidney, and duodenum were dissected and preserved in OCT compound for immunohistochemistry. Lesion analysis was performed as previously described. Briefly,
hearts were serially sectioned on a cryostat, starting from the proximal part of the aortic root, and
stained with hematoxylin and Oil Red O. Kidney and liver sections were stained in the same
way. Lesion size was determined on eight sections, collected at every 100 μm of the aortic root.
For each section, images were captured in a Leica photomicroscope, and the surface areas of the
lesions and of the entire vessel were measured using Image J software (NIH). For fluorescent
staining of spleen, liver, kidney, and duodenum sections, peanut agglutinin (PNA, Vector), Nile
Red (Sigma-Aldrich), or antibodies listed in Supplemental Methods were used. Nuclei were
stained with DAPI (Sigma-Aldrich). Fluorescent micrographs were acquired with an SP2
Acusto-Optical Beam Splitter confocal laser-scanning microscope (Leica).

**Blood and plasma analyses**

Blood was collected by cardiac puncture or through tail vein bleeding in EDTA-coated tubes.

Whole blood and splenocyte single cell suspensions were analyzed on a Vet abc hemocounter
(Scil). Plasma cholesterol and triglycerides were analyzed using enzymatic colorimetric kits
(Randox) according to the manufacturer’s protocol. For lipoprotein profiling, plasma was
fractionated using a Superose 6 10/300 GL column (GE Healthcare) coupled to a Prominence
UFLC system (Shimadzu) and equilibrated with Tris-buffered saline (TBS), pH 7.4. Fractions of
200 μl were collected using a Foxy Jr fraction collector (Teldyne Isco) for subsequent detection
of cholesterol and triglycerides with abovementioned enzymatic kits.

Titers of specific antibodies to LDL, oxidized (ox)LDL, and ApoB100 were measured
with enzyme-linked immunosorbent assay (ELISA). In brief, 50 μl of the different antigens (10
μg/ml) were added to 96-well ELISA plates and incubated overnight at 4°C. Coated plates were
washed with PBS and blocked with 1% gelatin (Invitrogen) in PBS for one hour at room
temperature. Next, plates were washed and incubated for two additional hours with plasma from
individual animals, diluted in TBS with 0.1% gelatin. After washing, total IgM (Immunkemi), IgG (Vector), IgG1 (Southern Biotech), and IgG2c (BD Biosciences) levels were revealed by using biotinylated anti-mouse antibodies and HRP-streptavidin. The plates were washed, colorimetric reactions developed using tetramethylbenzidine (BD Biosciences), and absorbance measured on a microplate reader (Molecular Devices). For immune complex analysis, ELISA plates were coated with anti-ApoB100 antibodies and then incubated with mouse plasma to allow binding of LDL particles in the samples. After washing, any IgM and IgG bound to the LDL particles were detected by using biotinylated antibodies, HRP-streptavidin, and tetramethylbenzidine as described above.

For the competition ELISA, IgG antibodies (10 μg/ml) obtained from plasma of HuBL and BT3xHuBL mice were pre-incubated with increasing amounts of native LDL, oxLDL, and ApoB overnight at 4°C in glass tubes. The mixtures were then used in ELISA assays detecting IgG antibodies to LDL and oxLDL as described above.

**Statistical analysis**

Data were analyzed using Prism version 5.03 for Windows (GraphPad). Student’s t-test, 1-way analysis of variance (ANOVA) with Dunnett’s multiple comparison test, or 2-way ANOVA with Bonferroni’s post test was used for comparisons when the Shapiro-Wilk test indicated normality. The Mann-Whitney test or Kruskal-Wallis test with Dunn’s multiple comparison test was used when Gaussian distribution could not be assumed. Pearson correlation coefficient was used to assess correlations. Differences between groups were considered significant at p-values below .05 (* p≤.05, ** p≤.01, *** p≤.001). All experiments were repeated at least twice. The data, analytic methods, and study materials will be made available to other researchers for purposes of reproducing the results or replicating the procedure (available at the authors’ laboratories).
Results

**LDL-reactive TCR-transgenic mice**

A panel of CD4+ T-cell hybridomas was established from mice immunized with human LDL particles. These cells recognized epitopes in the ApoB100 protein of LDL⁹. TCR cDNA from these cells was cloned under the CD2 promoter and used for production of tg mice. Three tg strains with strong anti-LDL reactivity, termed BT1, BT2, and BT3 were used for experiments (Figure 1A, S1A-C). They all expressed tg TCR with β-chain TRBV31 together with β-chain TRAV12, 4, or 14, respectively. TRBV31 was found on >90% of all CD4+ T cells in the tg strains but only on 8% of CD4+ T cells in wild-type (wt) mice (Figure 1B, S1D-E). Nearly all these cells were naïve in the tg mice (Figure 1B, S1F-H), but exposure to human native LDL in vitro evoked a strong T-cell response (Figure 1A, S1B). The proliferative response to oxLDL was less pronounced (Figure 1A, S1C), in line with previous findings⁹.

**T-cell activation by injection of LDL**

To characterize the response to LDL antigen in vivo, we crossed the tg BT strains with a reporter mouse expressing green fluorescent protein (GFP) under Nur77, a promoter transcribed during T-cell activation³³. Injection of human LDL led to vivid T-cell activation responses of the same magnitude as those achieved after polyclonal stimulation (Figure 1C, S1I-L).

**Injection of BT1 T cells to HuBL mice**

T-cell activation in vivo was further studied by injecting BT1 T cells labeled with cell trace violet into HuBL mice (Figure S2A). These mice carry the human LDL protein, ApoB100 as a transgene³, therefore they produce humanized LDL particles similar to those used as antigen for donor mouse immunization and TCR cloning. High plasma concentrations of humanized LDL particles are found in the HuBL cross that lacks the LDL receptor. After intravenous infusion,
BT1 T cells were rapidly activated and underwent several rounds of proliferation in the *HuBL* hosts (Figure 1D, S2B-D). Induction of Foxp3+ T regulatory cells could not be observed (Figure S2E). Most BT1 cells homed to the spleen, with significant populations also in para-aortic, inguinal, and mesenteric lymph nodes (Figure 1D, S2C). No signs of proliferation or homing were observed when wt C57BL/6J (B6) T cells were injected into *HuBL* mice (Figure 1D, S2B-C). BT2 cell injections led to similar rapid proliferative responses in *HuBL* mice, whereas no proliferation occurred in *Ldlr−/−* mice that lacked transgenic production of human LDL antigen (Figure S2F). These data show that tg BT cells maintained their reactivity to human LDL and normal homing capacity after transfer into *HuBL* mice.

**Injected BT T cells promote T- and B-cell activation**

The long-term effects of a strong cellular immune response to LDL were studied in *HuBL* mice receiving BT cells twice over a 5-week period (Figure 2A, Table S1). BT cells remained detectable in the spleen five weeks after the last injection (Figure 2B). Spleens were enlarged, with an increased proportion of tg TRBV31+ effector T cells and an expanded population of T follicular helper (Tfh) cells (Figure 2C-D, S3A-J). This was accompanied by an expansion of the Th cell pool and an increased conversion of Th cells from naïve to effector/memory phenotype (Figure S3C-E). In transcript analysis of aortas, elevated levels of Foxp3, the master regulator of Treg, and to a lesser extent Tbx21, encoding the Th1-transcription factor Tbet, were found in the *HuBL/BT3* group (Table S2). This was accompanied by elevated mRNA levels of their signature cytokines, Ifng and Il10, but also the Tfh-related cytokine Il21 was found to be markedly increased (Table S2). In draining lymph nodes, the increase in Il21 mRNA was particularly striking (Table S2). In spleens, we also observed formation of germinal centers, expansion of the plasma cell pool, and production of IgG antibodies to LDL (Figure 2E-H, S3K-O). These
antibodies recognized native and oxidized forms of LDL as well as ApoB100 protein (Figure 2H-I, S4A-F). Anti-ApoB100 antibodies were of both IgG1 and IgG2c isotypes (Figure 2J-K, S4G-H). The concomitant induction of Tfh cells, formation of germinal centers, expansion of plasma cells, and increase in anti-LDL IgG demonstrates that T cells reactive to LDL protein provide help for B-cell activation, leading to anti-LDL antibody production.

**Lower plasma cholesterol levels in BT injected mice**

At the age of 15 weeks, five weeks after the first injection, strikingly lower plasma cholesterol was seen in *HuBL* mice injected with BT1 or BT3 cells (Figure 3A, Table S1). This was due to reduced levels of LDL and the very low-density lipoprotein (VLDL)/chylomicron remnant fraction (Figure 3B). The effects on plasma triglycerides were similar to those on cholesterol (Figure 3C-D). We speculated that the reduction in plasma lipids could be due to antibody-dependent elimination of lipoprotein particles from circulation. In support of this notion, LDL particles in mice injected with BT3 cells were found to be covered with antibodies, forming LDL-IgG immune complexes (Figure 3E, S4I-J). Plasma from *HuBL/BT3* chimeras enhanced FITC-oxLDL uptake into cultured macrophages, providing further support for this notion (Figure 3F-G).

**Anti-LDL IgG promotes LDL clearance**

To test whether anti-IgG antibodies promoted LDL clearance, FITC-labeled human LDL particles were mixed with anti-LDL IgG containing plasma from *HuBL/BT3* mice, or with plasma from control *HuBL/B6* mice, and injected into *HuBL* mice. FITC-LDL treated with *HuBL/BT3* plasma displayed enhanced clearance compared with FITC-LDL treated with control plasma (Figure 3H). Lipid and IgG could be detected in kidney glomeruli of *HuBL/BT3* animals, but creatinine levels remained normal (Figure S4K-M, Table S1). No increase of lipid-laden
macrophages was observed in the spleen (Figure S4N). Since the liver is a major site for clearance of IgG immune complexes\textsuperscript{34-36}, we analyzed liver extracts but could not detect any accumulation of cholesterol (Figure S4O). Therefore, further cholesterol clearance to feces is likely to occur if liver uptake of plasma lipoproteins is of importance for the cholesterol lowering effect of the LDL immune response.

**LDL-reactive T cells protect from atherosclerosis**

Atherosclerotic lesion burden was reduced by 30\% in the \textit{HuBL}/BT3 and \textit{HuBL}/BT1 animals, with a similar trend also for \textit{HuBL}/BT2 mice (Figure 3I-J). The different outcomes between the strains could be explained by different affinities and binding capacities to MHC class II-peptide complexes. A substantial downregulation of the BT2 TCR was observed in mediastinal lymph nodes (Figure 3K-L). Such a response is observed in high affinity T-cell clones and can reduce their effector functions\textsuperscript{37}. Consequently, BT1 and BT3 cells may have lower affinities but more vigorous effector functions, including B-cell help and activation of cell-mediated immunity. Our findings are in line with this notion and also suggest that B-cell activation and production of antibodies capable of clearing the antigen contributed to the lipid-lowering effect of the anti-LDL immune response.

**Thymic selection of LDL-reactive T cells**

\textit{BT1} mice were crossed with \textit{HuBL} mice in order to study the development of the cellular immune response to LDL in a humanized model that produces the antigen from birth and onwards, i.e. a situation resembling that in man. In both \textit{HuBL} and \textit{BT1xHuBL} mice, human \textit{APOB100} was mainly expressed in the gut and liver but mRNA could be detected also in the thymus, where it may aid negative selection against self-reactive T-cell clones (Figure 4A-B). A publically available dataset shows that Apob mRNA is expressed in medullary thymic epithelial
cells, i.e. the cells mainly responsible for negative thymic selection. Most of the human ApoB100 reactive, TRBV31 bright BT1 cells were eliminated in the thymus during early life, indicating that negative selection did take place against ApoB100 (Figure 4C-G, S5A). However, approximately 20% of CD4+ T cells in the periphery were TRBV31 dim in BT1xHuBL mice versus <1% in B6xHuBL animals (Figure 4H, S5B-C, Table S3). TRBV31 could therefore be used as a marker for tg cells. Detection of the tg α-chain was not possible with available antibodies, but its mRNA was overexpressed to a similar extent as that for the β-chain and the levels of the two transcripts showed a strong positive correlation (Figure S5D-E, Table S4). Among the TRBV31 dim cells, 10-15% were Tfh and Th1 effector cells (versus 2-6% in B6xHuBL mice), with a modest contribution of Foxp3+ regulatory T cells (Figure 5A-C, S5F-H, Table S4). Transcript analysis of draining lymph nodes also showed a significant, 36% reduction in IL-6 mRNA in the BT1xHuBL cross, implying reduced inflammatory activation (Table S4).

**Anti-LDL immunity protects against atherosclerosis**

BT1xHuBL mice had increased plasma levels of anti-LDL IgG, including antibodies to oxLDL, native LDL, and ApoB100, mainly of the IgG1 isotype (Figure 5D-G, S5I-P). Similar to the cell transfer experiments, immune complex formation with LDL-[anti-LDL IgG] complexes was detected also in these animals (Figure 5H, S5Q-R). It was associated with significantly reduced plasma cholesterol, VLDL, and LDL (Figure 5I-J, S5S-U). ApoB expression was not different in liver or gut (Figure S6A-B, Table S4), and cholesterol levels were decreased in liver extracts (Figure S6C-D).

Atherosclerotic lesions were substantially reduced, by approximately 50%, in BT1xHuBL mice (Figure 5K-N, S6E-F). This was accompanied by reduced expression of VCAM-1, a marker of vascular NF-κB activation, without any other significant effects on lesion composition.
(S6G-M). The disease burden was proportional to cholesterol levels (Figure S6N) and showed a significant, negative correlation to immune complex concentration (Figure S6O).

A similar, atheroprotective effect was achieved when HuBL mice were immunized with ApoB preparations. This treatment also led to induction of IgG-anti-LDL antibodies, reduced plasma cholesterol and reduced atherosclerosis (Figure 6A-D, S7A-E).

**Clearance of lipoproteins by a humoral response in BT3xHuBL mice**

Since BT3 cells induced the highest titers of anti-LDL antibodies, we crossed the BT3 line with HuBL mice and investigated the phenotype of the offspring. The thymus of BT3xHuBL mice showed more pronounced signs of negative selection compared to BT1xHuBL mice (Figure S8A-B). In the periphery, a reduction of T-helper cells was observed, but most of them were TRBV31+ effector/memory cells (Figure 7A, S8C-G). A significant proportion of the TRBV31dim cells had differentiated into Tfh cells (Figure 7B). The enlarged spleen showed signs of ongoing inflammation and had increased germinal center B cells and plasma cells that produced high titer anti-LDL IgG (Figure 7C-D, S8H-O, Table S5). The B-cell response was further characterized by a competition assay in which purified IgG antibody binding to immobilized antigens was competed with soluble LDL, oxLDL, or ApoB100. This assay showed overlapping specificities between anti-LDL and anti-oxLDL antibodies (Figure 7E-F). LDL, oxLDL, and ApoB100 protein could all compete for binding. OxLDL was the most efficient competitor, indicating the presence of oxidation specific epitopes. The pattern was similar to HuBL IgG (Figure S8P-Q).

In the circulation of BT3xHuBL mice, anti-LDL IgG formed immune complexes that were accompanied by lower plasma cholesterol and triglycerides and a significant protection from atherosclerosis (Figure 7G-J, S8R-S). Injection of IgG from these mice reduced plasma
ApoB concentrations in recipients (Figure 7K, S8C). These mice also displayed increased accumulation of lipids and IgG1 in the liver (Figure 7L-M, S8T-U). Further clearance of cholesterol to feces was also detected (Figure 7N). Lipoprotein production remained unaltered, as judged by ApoB expression in liver and gut (Figure S8V-X).

**Discussion**

Our data provide insights into mechanisms of atheroprotective immunity. They show that a subpopulation of LDL-reactive T cells survives clonal selection and is able to elicit adaptive immune reactions to LDL. Such reactions were mounted both to injected, autologous LDL and as a response to endogenously produced LDL in the humanized mouse. Therefore, LDL reactive T cells can mount autoimmune reactions to lipoprotein particles.

When naïve LDL reactive T cells were injected into mice producing human LDL, immune activation occurred in secondary lymphoid organs including spleen and draining lymph nodes. Although direct evidence is not available in mouse models, it is likely that recall activation of effector/memory T cells occurs in the diseased artery, as is the case in man.

The cellular immune response to LDL had important functional consequences, the net effect of which was a reduction of atherosclerosis. By providing B-cell help, it triggered formation of a set of anti-LDL antibodies that can enhance LDL clearance from the circulation. Furthermore, the immune response to LDL was associated with increased cholesterol excretion and with signs of reduced vascular inflammation. It is likely that all these effects synergized to inhibit disease development. These findings should be helpful in the development of immunotherapy against atherosclerotic cardiovascular disease.
The LDL-reactive T cells provided help for activation of LDL-reactive B cells. This process initiated germinal center reactions, with plasma cell formation and production of anti-LDL antibodies. Anti-LDL antibodies formed immune complexes with LDL that were detected in peripheral blood. Formation of immune complexes significantly increased clearance of LDL particles from the circulation, thus reducing plasma cholesterol levels, which is in line with findings made by Klimov et al. in the 1980s\textsuperscript{40}. Statistical analysis showed that LDL-[anti-LDL IgG] immune complexes, plasma cholesterol, and atherosclerotic lesion size were correlated, suggesting that these factors were dependent on each other. Our data point to the liver as the major site of elimination of lipoprotein-derived lipid, however, the detection of antibodies and lipid in kidneys in one of the strains warrants further investigation.

Previous experiments to functionally assess the role of LDL-reactive T cells in atherogenesis have pointed to major roles for Th1 and Treg cells. We were not able to detect a decisive shift of these cell types but the finding of reduced IL-6 expression in para-aortic lymph nodes of mice carrying strong LDL immunoreactivity suggests that local anti-inflammatory effects contributed to atheroprotection.

The most striking finding in our study was the induction of atheroprotective humoral immunity to LDL. The notion that LDL-reactive B cells mount atheroprotective immunity is in line with previous findings that B cells carry atheroprotective immunity\textsuperscript{25,26}, that atheroprotection is associated with formation of IgG antibodies\textsuperscript{22}, and that disease is increased in mice lacking inhibitory and decreased in animals lacking activating Fc receptors\textsuperscript{41,42}. Our current data extend these findings by demonstrating that humoral immunity to LDL is induced by Tfh effector cells that trigger B-cell activation, germinal center formation, and production of high-
affinity antibodies to LDL. Furthermore, our data show that antibody-mediated clearance of LDL antigen contributed to the atheroprotective effect of LDL immunity.

The observed, incomplete tolerance to LDL and ApoB did not involve any substantial induction of natural Tregs but was due to clonal elimination in the thymus and peripheral anergy of cells escaping positive selection. This finding does not rule out that peripheral tolerance mechanisms involving Treg may be important in atherosclerosis and it should be kept in mind that the present experimental design involves transgenic TCR with high affinity to antigen. It is possible that T cells with lower affinity to antigen may play a significant role under “normal” conditions that do not involve genetically modified immune responses.

We have previously shown that induction of antibodies that block the immunological synapse of ApoB100-reactive T cells can reduce atherosclerosis. It was, therefore, surprising that the net effect of a strong cellular immune response against the same antigen is atheroprotective. The two experiments are, however, not directly comparable since synapse blockade likely inhibits all downstream effects of an immune response, whereas antigen activation of an antigen-specific T cell triggers a specific effector response that depends on the precise conditions at the time of activation, including the presence of specific metabolites, cytokines, and costimulatory factors. Furthermore, blockade of the immunological synapse with an antibody to the pertinent TCR may in itself exert immunomodulatory effects. Further studies are needed to elucidate the mechanisms leading to these results.

Our data clarify observations of antibody induction and plasma lipoprotein reduction in several experiments employing immunization or anti-LDL IgG administration to control atherosclerosis. The finding that T-cell reactions to LDL involve development of Tfh cells, germinal center formation, and antibody-dependent LDL clearance support and extend the
recent report that formation of tertiary lymphoid structures protects against atherosclerosis\textsuperscript{47}. They also shed light on the observation that disturbed T-cell migration leads to hypercholesterolemia, reduced anti-oxLDL antibodies, and increased atherosclerosis in mice\textsuperscript{48} and that antibodies to oxLDL are inversely correlated with particle concentration in humans\textsuperscript{49}. They are, however, seemingly at odds with studies showing proatherosclerotic effects of Tfh cells\textsuperscript{50} and B2 cells\textsuperscript{28}. In these reports, disease development was studied under conditions when global immunoregulatory networks were disrupted by mutations in the major histocompatibility complex and by administration of cytolytic antibodies, respectively. In contrast, our current data address the disease-associated autoimmunity to LDL and identify an atheroprotective mechanism elicited by expansion of LDL-reactive T cells. Such T cells have been cloned from human atherosclerotic lesions\textsuperscript{8} and are, therefore, active in clinical disease.

In humans, anti-LDL titers are generally low and studies have shown weak or no associations between them and clinical cardiovascular events. It is, therefore, of interest to enhance immune responses in models in order to assess their pathophysiological consequences. It will also be important to develop and apply high-resolution imaging to measure lesion size in man and determine its association with anti-LDL antibodies.

The models used in this study exaggerate hyperlipidemia as well as cellular immune reactivity and made it possible to study effects of a strong immune response in a hyperlipidemic host within a reasonable time frame. They allowed us to analyze immune responses that may not be detectable in models with less profound immune reactivity towards LDL. It will now be important to follow these aspects of anti-LDL immunity in other models, expand the vaccination studies to characterize LDL-specific T-cell responses, and eventually translate the findings into human disease.
In conclusion, this study shows that T cells reactive to LDL survive clonal selection and can mount atheroprotective immune responses that involve humoral immunity, reduction of plasma cholesterol and reduced lesion formation. By targeting LDL-reactive T cells, immunization with LDL protein can enhance such atheroprotective immunity. This may be an attractive way of inhibiting or preventing atherosclerotic cardiovascular disease.

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Disclosures


References


Figure Legends

Figure 1. T-cell phenotype in BT transgenic mice.

(A) Splenocytes incubated in triplicates with different stimuli for 60 hours in vitro. The mean proliferation is expressed as counts per minute (CPM) for unstimulated cells and cells stimulated with 10 µg/ml mouse LDL, human LDL, or oxidized human LDL (B6 n=2-3, BT1 n=3-4, BT2 n=2-4, BT3 n=2-4, 2-way ANOVA with Bonferroni’s post test, dots represent individual mice, bars show mean ± SEM).

(B) Representative flow cytometry plots of expression of TRBV31 and CD62L in CD4+ T-helper cells.

(C) Flow cytometry histogram of Nur77-GFP expression in splenic TRBV31+CD3+CD4+ T-helper cells 16 hours after injection (inj) of 100 µg LDL intraperitoneally, showing one representative experiment of three.

(D) CD4+ T-cell proliferation in HuBL mice. Proliferation of CD4+ T-helper cells labeled with CellTraceViolet transferred to HuBL recipients that express the antigen for BT1 cells. (HuBL/B6 n=3, HuBL/BT1 n=3, each dot represents a separate organ).

See also Figures S1 and S2.

Figure 2. BT cells recognize LDL, develop into Tfh cells, and promote anti-LDL antibody production.

(A) Experimental design of T-cell transfer experiment.

(B) Proportion TRBV31+ of T-helper cells in spleen (HuBL/B6 n=14, HuBL/BT1 n=11, HuBL/BT2 n=10, HuBL/BT3 n=11, 1-way ANOVA with Dunnett’s post test)
(C) Spleen weight (Kruskal-Wallis test with Dunn’s post test).

(D) Flow cytometry plots of Tfh cell staining showing percentage of PD1+CXCR5+ Tfh cells in the CD44+CD62L-TRBV31+ Th population.

(E) Immunofluorescence micrographs showing B cells (B220+, blue), germinal centers (PNA+, pink), and macrophages (F4/80+, green) in spleen, with a 100 µm scale bar.

(F) Flow cytometry plots showing GL7+CD95+ germinal center B cells in the IgD^low^CD19^+^B220^+^ B-cell population.

(G) Flow cytometry plots of CD138^+^ plasma cells in the B220^low^ lymphocyte population.

(H) Anti-native LDL IgG antibodies (1:150 dilution, Kruskal-Wallis test with Dunn’s post test).

(H-K) Optical density at 450 nm is shown on y-axis (HuBL/B6 n=12, HuBL/BT1 n=11, HuBL/BT2 n=10, HuBL/BT3 n=10).

(I) Anti-oxLDL IgG (1:150 dilution, 1-way ANOVA with Dunnett’s post test).

(J-K) Anti-ApoB100 IgG1 and IgG2c (1:15 dilution, Kruskal-Wallis test with Dunn’s post test). Dots represent individual mice, bars show mean ± SEM. See also Figures S3 and S4.

Figure 3. T cell reactivity to LDL leads to reduced plasma lipids, formation of immune complexes, and reduced atherosclerosis.

(A) Plasma cholesterol at 15 weeks of age, after 5 weeks on Western diet (HuBL/B6 n=11, HuBL/BT1 n=7, HuBL/BT2 n=10, HuBL/BT3 n=7, 1-way ANOVA with Dunnett’s post test).

(B) Lipoprotein cholesterol profile (CR=Chylomicron remnants, HDL=High-density lipoprotein, HuBL/B6 n=5, HuBL/BT3 n=5, 2-way ANOVA with Bonferroni’s post test, curve shows mean values).

(C) Plasma triglycerides (same statistics as in A).
(D) Lipoprotein triglyceride profile (same statistics as in B).

(E) Circulating immune complexes with LDL and anti-LDL IgG (optical density 450 nm, 
\(HuBL/\)B6 \(n=12, \ HuBL/\)BT1 \(n=11, \ HuBL/\)BT2 \(n=10, \ HuBL/\)BT3 \(n=10, \) 1:100 dilution, 1-way ANOVA with Dunnett’s post test).

(F) Uptake of oxLDL measured in RAW264.7 macrophages after 24 hours incubation with 25 
µg/ml FITC-labeled oxLDL (No plasma \(n=6, \ HuBL/\)B6 plasma \(n=6, \ HuBL/\)BT3 plasma \(n=6, \) 1:100 plasma dilution, Kruskal-Wallis test with Dunn’s post test).

(G) Representative micrographs of hemtoxylin and Oil Red O stained RAW264.7 macrophages 
after 24 hours incubation with 25 µg/ml oxLDL.

(H) Clearance of injected FITC-LDL particles pre-treated with either \(HuBL/\)B6 or \(HuBL/\)BT3 
plasma (\(HuBL/\)B6 plasma \(n=9, \ HuBL/\)BT3 plasma \(n=8, \) 2-way ANOVA with Bonferroni’s post 
test, graph shows mean ± SEM).

(I) Atherosclerotic lesion burden in the aortic arch (\(HuBL/\)B6 \(n=14, \ HuBL/\)BT1 \(n=10, \) 
\(HuBL/\)BT2 \(n=10, \ HuBL/\)BT3 \(n=11, \) 1-way ANOVA with Dunnett’s post test).

(J) En face preparations of aortic arches with lipid-laden plaques stained with Sudan IV (orange 
color).

(K) Flow cytometry histogram of TRBV31 expression in CD3⁺CD4⁺TRBV31⁺ lymphocytes to 
detect TCR downregulation in mediastinal lymph nodes.

(L) Mean fluorescence intensity (MFI) in T-helper cells (\(HuBL/\)B6 \(n=14, \ HuBL/\)BT1 \(n=12, \) 
\(HuBL/\)BT2 \(n=10, \ HuBL/\)BT3 \(n=12, \) 1-way ANOVA with Dunnett’s post test).

Dots represent individual mice, bars show mean ± SEM. See also Figure S4.
Figure 4. Survival of TRBV31$^{\text{dim}}$ T cells in the BT1xHuBL cross.

(A) Human APOB mRNA expression in thymus, expression was not detected in the BT1 strain lacking the human APOB transgene (Ct values, HuBL n=1, BT1xHuBL n=3, BT1 n=4).

(B) Hprt mRNA levels in thymus, used as house-keeping gene.

(C) Flow cytometry plots showing expression of CD4 and CD8 in thymocytes.

(D) Number of cells in single-cell suspensions of thymus (HuBL n=8, BT1xHuBL n=4, BT1 n=4).

(E) CD4$^+$CD8$^-$ double negative, CD4$^+$CD8$^+$ double positive, CD4 single positive, and CD8 single positive TRBV31$^+$ thymocytes (HuBL n=3, BT1xHuBL n=3, BT1 n=3, 2-way ANOVA with Bonferroni’s post test).

(F) Proliferation of splenocytes from the indicated strains, stimulated with 10 µg/ml human LDL or anti-CD3/anti-CD28 (HuBL n=7, BT1xHuBL n=5, BT1 n=4). Stimulation index is calculated as fold change of CPM over unstimulated cells.

(G) Flow cytometry plots showing TRBV31 expression by CD4-single positive thymocytes in 10-week-old animals.

(H) Flow cytometry plots of TRBV31 expression in spleen CD4$^+$ T cells, 10-week-old animals. Dots represent individual mice, bars show mean ± SEM. See also Figure S5.

Figure 5. Induction of anti-LDL antibodies and protection against atherosclerosis in the BT1xHuBL cross.

(A) Design of diet experiment with compound mutant mice.

(B) Representative flow cytometry plots of Tfh cell staining in the TRBV31$^{\text{bright}}$ and TRBV31$^{\text{dim}}$ populations.

(C) PD1$^+$CXCR5$^+$ Tfh cells in the CD44$^+$CD62L$^-$TRBV31$^+$ Th population. (HuBL n=7,
**BT1xHuBL** n=8, 1-way ANOVA with Bonferroni’s post test).

(D) Plasma anti-LDL IgG (**HuBL** n=9, **BT1xHuBL** n=12, 1:15 dilution, Student’s *t*-test,).

(D-H) Optical density at 450 nm is shown on *y*-axis.

(E) Anti-oxLDL IgG (**HuBL** n=10, **BT1xHuBL** n=12, 1:15 dilution, Student’s *t*-test)

(F-G) Anti-ApoB100 IgG1 and IgG2c (**HuBL** n=10, **BT1xHuBL** n=12, 1:15 dilution, Mann-Whitney test).

(H) Circulating immune complexes with LDL and anti-LDL IgG (**HuBL** n=10, **BT1xHuBL** n=12, 1:100 dilution, Student’s *t*-test).

(I-J) Plasma cholesterol and triglycerides at 20 weeks of age (**HuBL** n=10, **BT1xHuBL** n=12, Student’s *t*-test).

(K) En face preparations of aortic arches with lipid-laden plaques stained with Sudan IV (orange color).

(L) Atherosclerotic burden in in aortic arch (**HuBL** n=10, **BT1xHuBL** n=12, Student’s *t*-test).

(M) Mean lesion area in the aortic root (**HuBL** n=7, **BT1xHuBL** n=8, Student’s *t*-test).

(N) Micrographs show Oil Red O staining of neutral lipids (red color) in cross-sections of the aortic root.

Dots represent individual mice, bars show mean ± SEM. See also Figures S5 and S6.

**Figure 6. Lipid-lowering and atheroprotective ApoB100 vaccination.**

(A) Experimental design of ApoB100 vaccination in **HuBL** mice.

(B) Anti-ApoB100 IgG titers, optical density at 450 nm on *y*-axis (**HuBL** mice; PBS-adjuvant n=6, ApoB100-adjuvant n=5, 2-way ANOVA with Bonferroni’s multiple comparison test).

(C) Plasma cholesterol levels at 35 weeks of age (**HuBL** mice; PBS-adjuvant n=6, ApoB100-
adjuvant n=5; Mann-Whitney test).

(C-D) Triangles represent individual mice and bars show mean ± SEM.

(D) Mean atherosclerotic lesion area in the aortic root (HuBL mice; PBS-adjuvant n=5, ApoB100-adjuvant n=4; Mann-Whitney test). See also Figure S7.

Figure 7. Lipid-lowering antibodies and reduced atherosclerosis in BT3xHuBL mice.

(A) TRBV31+ T-helper cells in spleen (HuBL n=16, BT3xHuBL n=7, Mann-Whitney test).

(B) PD1+CXCR5+ Tfh cells in the CD44+CD62L-TRBV31+ Th population (HuBL n=7, BT3xHuBL n=8, 1-way ANOVA with Bonferroni’s post test).

(C) GL7+CD95+IgDlow germinal center B cells (HuBL n=11, BT3xHuBL n=12, Student’s t-test).

(D) CD138+ plasma cells (HuBL n=11, BT3xHuBL n=12, Mann-Whitney test).

(E) Competition assay for evaluation of anti-LDL IgG specificity in total IgG isolated from BT3xHuBL mice (n=4, 2-way ANOVA with Bonferroni’s post test, significant competition by all three competitors).

(F) Competition assay for evaluation of anti-oxLDL IgG specificity in total IgG isolated from BT3xHuBL mice (n=4, 2-way ANOVA with Bonferroni’s post test, significant competition by all three competitors).

(G) Plasma cholesterol (HuBL n=16, BT3xHuBL n=12, Student’s t-test).

(H) Plasma triglycerides (HuBL n=16, BT3xHuBL n=12, Mann-Whitney test).

(I) Atherosclerotic burden in in aortic arch (HuBL n=16, BT3xHuBL n=12, Student’s t-test).

(J) En face preparations of the aortic arch with lipid-laden plaques stained with Sudan IV (orange color).

(K) ApoB measured in plasma at different time points after infusion of 200 μg IgG antibodies
(HuBL mice; HuBL IgG n=4, BT3xHuBL IgG n=6, 2-way ANOVA, braces indicate significance level for treatment comparison, graph shows mean ± SEM).

(L) Micrographs of Oil Red O-stained liver sections with a 500 µm scale bar.

(M) Immunofluorescence micrographs showing IgG1 (green) and cell nuclei (DAPI+, blue) in liver, with a 100 µm scale bar.

(N) Cholesterol measured in lipid extracts from feces (HuBL n=8, BT3xHuBL n=5, Student’s t-test, each dot represent one cage).

(A-D) and (G-I) Dots represent individual mice, bars show mean ± SEM. See also Figure S8.
A  Immunization: Complete Freund’s adjuvant  Incomplete Freund’s adjuvant

HuBL

Standard chow

25 wks  29 wks  35 wks

Adjuvant

ApoB100

B

C

D

Anti-ApoB100 IgG

Cholesterol (mmol/l)

Adjuvant  ApoB100

Lesions in aortic root (um²)

Adjuvant  ApoB100

***

1:50

1:500

1:5000

1.5x10⁵

1.0x10⁵

5.0x10⁴

*
SUPPLEMENTAL MATERIAL

LDL-reactive T cells regulate plasma cholesterol levels and development of atherosclerosis in humanized hypercholesterolemic mice

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The PDF file includes:

Supplemental Figures
Figure S1. Phenotype of BT transgenic mice.
Figure S2. CD4+ BT-cell proliferation in HuBL mice.
Figure S3. Cell populations in spleens of HuBL mice injected with CD4+ BT cells.
Figure S4. Antibodies and lipid distribution in HuBL mice injected with CD4+ BT cells.
Figure S5. T-helper cell phenotype and antibodies in BT1xHuBL mice.
Figure S6. Lipid distribution and atherosclerotic plaque composition in BT1xHuBL mice.
Figure S7. Phenotype of ApoB100-vaccinated HuBL mice.
Figure S8. Characterization of BT3xHuBL mice.

Supplemental Tables
Table S1. Phenotype of HuBL mice injected with CD4+ BT cells.
Table S2. mRNA levels in organs of HuBL mice injected with CD4+ BT cells.
Table S3. Phenotype of HuBL vs. BT1xHuBL mice.
Table S4. mRNA levels in organs of HuBL vs. BT1xHuBL mice.
Table S5. Phenotype of HuBL vs. BT3xHuBL mice.

Supplemental Methods
List of antibodies used for flow cytometry analysis.
Figure S1. Phenotype of BT transgenic mice.
(A) Experimental setup for phenotypic analysis.
(B) IFN-γ in supernatant after stimulation of splenocytes with 10 µg/ml human LDL, 10 µg/ml human ApoB100, and anti-CD3/anti-CD28, measured by ELISA (B6 n=6, BT1 n=4, 2-way ANOVA with Bonferroni’s post test).
(C) BT1 splenocytes incubated with native LDL or oxLDL for 60 hours in vitro (n=4, 2-way ANOVA with Bonferroni’s post test, dots show mean ± SEM).
(D) Percentage of CD3+CD4+ T-helper cells in the spleen (B6 n=8, BT1 n=8, BT2 n=4, BT3 n=8).
(E) TRBV31+ T-helper cells in the spleen (B6 n=9, BT1 n=9, BT2 n=7, BT3 n=11).
(F) Naïve CD62L+ cells in the TRBV31+ T-helper cell population in the spleen.
(G) Tbet+ Th1 cells in the TRBV31+ T-helper cell population in the spleen.
(H) Foxp3+ regulatory T cells in the TRBV31+ T-helper cell population in the spleen.
(I) Experimental design of LDL injection into Nur77-GFP reporter mice.
(J) Nur77-GFP expression in TRBV31+ T-helper cells (Negative control n=2, BT1 n=4, BT2 n=3, BT3 n=3, Positive control n=2). Uninjected Nur77-GFP+ mice were used as negative controls (-). Splenocytes from Nur77-GFP+ mice treated with anti-CD3 and anti-CD28 in vitro overnight were used as positive controls (+).
(K) CD69 expression in TRBV31+ T-helper cells after LDL injection.
(K-L) Experimental design according to I-J (Negative control n=3, BT1 n=4, BT2 n=3, BT3 n=3, Positive control n=1).
(L) CD25 expression in TRBV31+ T-helper cells after LDL injection.
Figure S2. CD4⁺ BT-cell proliferation in HuBL mice.
(A) Experimental design of labelled CD4⁺ cell transfer into HuBL mice.
(B) Flow cytometry plots showing proliferation of CD4⁺ T-helper cells labelled with CellTraceViolet transferred to HuBL recipients that expresses the antigen for BT1 cells. The upper panel shows splenic T-helper cells 1-3 days after the transfer of CD4⁺ T cells from B6 mice, and the lower panel shows transfer of CD4⁺ cells from BT1 mice 1-4 days after the transfer.
(C) The relative number of CellTraceViolet-labelled CD4⁺ T cells from B6 and BT1 mice in different organs 1-4 days after the transfer into HuBL mice.
(D) Purity of the isolated CD4⁺ cells being transferred after negative selection (B6 n=9, BT1 n=9, BT2 n=7, BT3 n=11).
(E) Plots showing proliferation of BT1 CD4⁺ T cells transferred into HuBL mice with staining of Foxp3 on the y-axis. No induction of regulatory T cells was seen during this initial response to the antigen.
(F) Histogram showing proliferation of CellTraceViolet-labelled BT2 CD4⁺ T cells injected into an Ldlr⁻/⁻ recipient, without the antigen (gray line), and a HuBL recipient, with transgenic supply of human ApoB (black line). The samples are gated on CD3⁺CD4⁺CellTraceViolet⁺ T-helper cells in the inguinal lymph nodes 72 hours after injection. The x-axis shows fluorescence intensity and the y-axis the number of events.
Figure S3. Cell populations in spleens of HuBL mice injected with CD4\(^+\) BT cells.

(A) Total splenocyte count after meshing the organ through a cell strainer (HuBL/B6 n=15, HuBL/BT1 n=12, HuBL/BT2 n=10, HuBL/BT3 n=11).

(A-N) 1-way ANOVA with Dunnett’s post test, dots represent individual mice, bars show mean ± SEM.

(B-C) CD3\(^+\)CD4\(^+\) T-helper cells.

(B-N) HuBL/B6 n=11, HuBL/BT1 n=7, HuBL/BT2 n=10, HuBL/BT3 n=11.

(D) CD62L\(^-\)CD44\(^-\) naïve T-helper cells.

(E) CD44\(^+\)CD62L\(^+\) effector/memory T-helper cells.

(F) TRBV31\(^+\) T-helper cells.

(G) Tbet\(^+\)TRBV31\(^+\) Th1 cells.

(H) Foxp3\(^+\)TRBV31\(^+\) regulatory T-helper cells.

(I-J) PD1\(^+\)CXCR5\(^+\)CD44\(^+\)CD62L\(^+\) Tfh cells.

(K) CD19\(^+\)B220\(^{low}\) B1 cells.

(L) CD19\(^+\)B220\(^{high}\) B2 cells.

(M) GL7\(^+\)CD95\(^+\)IgD\(^{low}\) germinal center B cells.

(N) CD138\(^+\)CD28\(^+\) plasma cells.

(O) Immunofluorescence micrographs of spleen showing B cells (B220\(^+\), green) and plasma cells (CD138\(^+\), blue), with a 100 \(\mu\)m scale bar.
Figure S4. Antibodies and lipid distribution in HuBL mice injected with CD4+ BT cells.

(A-B) Anti-native LDL IgM and IgG. (A-J) Graphs show mean ± SEM, titers are displayed on the x-axis, and the y-axis shows optical density at 450 nm. (HuBL/B6 n=12, HuBL/BT1 n=11, HuBL/BT2 n=10, HuBL/BT3 n=10, 2-way ANOVA with Bonferroni’s post test, ns=not significant).

(C-D) Anti-oxLDL IgM and IgG.

(E-H) Anti-ApoB100 IgM, IgG, IgG1, and IgG2c.

(I-J) Immune complexes between LDL and IgM or IgG, respectively.

(K) Micrographs of Oil Red O-staining in glomeruli of kidneys, with a 50 µm scale bar. Nuclei counterstained with hematoxylin.

(L) Immunofluorescence micrographs showing IgG1 (green), C3 (red), and nuclei (DAPI+, blue) in glomeruli of kidneys, with a 50 µm scale bar.

(M) Immunofluorescence micrographs showing IgG2c (green), C3 (red), and nuclei (DAPI+, blue) in glomeruli of kidneys, with a 50 µm scale bar.

(N) Fluorescent micrographs showing lipids (Nile Red+, pink) and macrophages (F4/80+, blue) in spleen, with a 100 µm scale bar.
Gisteră et al.

(O) Cholesterol content in liver tissue extracts (HuBL/B6 n=14, HuBL/BT1 n=12, HuBL/BT2 n=9, HuBL/BT3 n=12, Kruskal-Wallis test with Dunn’s post test).
Figure S5. T-helper cell phenotype and antibodies in BT1xHuBL mice.

(A) CD4 CD8^+ double negative, CD4^+CD8^+ double positive, CD4 single positive, and CD8 single positive TRBV31^+ thymocytes (HuBL n=3, BT1xHuBL n=3, BT1 n=3, 2-way ANOVA with Bonferroni’s post test).

(A-H) Dots represent individual mice, bars show mean ± SEM.

(B) Proportion of TRBV31^bright T-helper cells among CD4^+ T cells in the spleen (HuBL n=10, BT1xHuBL n=13, Student’s t-test).

(C) TRBV31^dim T-helper cells (HuBL n=10, BT1xHuBL n=13, Student’s t-test).

(D) Correlation between Trav12 and Trbv31 mRNA levels in para-aortic lymph nodes in BT1xHuBL mice (n=12, Pearson correlation).

(E) Correlation between Trav12 and Trbv31 mRNA levels in para-aortic lymph nodes in HuBL mice (n=10, Pearson correlation).

(F) CD62L^+ naïve T-helper cells, the circles show the TRBV31^bright population and the closed diamonds the TRBV31^dim population that is missing in the HuBL mice as shown in C (HuBL n=10, BT1xHuBL n=11, 1-way ANOVA with Bonferroni’s post test).

(G) Tbet^+TRBV31^+ T-helper cells (HuBL n=9, BT1xHuBL n=10, 1-way ANOVA with Bonferroni’s post test).

(H) Foxp3^+ regulatory T-helper cells (HuBL n=10, BT1xHuBL n=13, 1-way ANOVA with Dunnett’s post test).

(I-J) Anti-native LDL IgM and IgG.

(I-R) Graphs show mean ± SEM, titers are displayed on the x-axis, and the y-axis shows optical density at 450 nm.
Gisteră et al.

(HuBL n=10, BT1xHuBL n=12, 2-way ANOVA with Bonferroni’s post test).
(K-L) Anti-oxLDL IgM and IgG.
(M-P) Anti-ApoB100 IgM, IgG, IgG1, and IgG2c.
(Q-R) Immune complexes between LDL and IgM or IgG, respectively.
(S-T) Lipoprotein cholesterol and triglyceride profiles (HuBL n=5, HuBLxB1T1 n=5, 2-way ANOVA with Bonferroni’s post test, curve shows mean values).
(U) Correlation between plasma cholesterol and LDL-IgG immune complexes in BT1xHuBL mice (n=12, Pearson correlation).
Figure S6. Lipid distribution and atherosclerotic plaque composition in BT1xHuBL mice.
(A) Immunofluorescence micrographs showing human ApoB (green) and nuclei (DAPI+, blue) in duodenum, with a 100 µm scale bar.
(B) Immunofluorescence micrographs showing human ApoB (green) and nuclei (DAPI+, blue) in liver, with a 100 µm scale bar.
(C) Micrographs of Oil Red O-stained liver sections with a 500 µm scale bar.
(D) Cholesterol content in liver tissue extracts (HuBL n=10, BT1xHuBL n=15, Mann-Whitney test).
(E-G, J-O) Dots represent individual mice, bars show mean ± SEM.
(E) Lesions in the innominate artery analyzed in en face preparations (HuBL n=8, BT1xHuBL n=9, Student’s t-test).
(F) Quantification of the Oil Red O-stained lesion area in eight consecutive sections, 100 – 800 µm from the aortic root (HuBL n=7, BT1xHuBL n=8, 2-way ANOVA with Bonferroni’s post test, braces indicate significance level for strain comparison).
(G) Quantification of immunohistochemical staining of CD4+ T cells in atherosclerotic lesions in aortic root sections.
(H) Quantification of immunohistochemical staining of CD8+ T cells in atherosclerotic lesions in aortic root sections.
(I) Quantification of CD68+ stained area in atherosclerotic lesions in aortic root sections.
(J) Quantification of VCAM-1+ stained area in atherosclerotic lesions in aortic root sections.
(K) Micrographs of VCAM-1 stained (brown) atherosclerotic lesions in the aortic root with a 100 µm scale bar, dotted lines delineate lesion area. Hematoxylin counterstaining visualizes nuclei (blue).
(L) Quantification of immunohistochemical staining of IAα+ cells in atherosclerotic lesions in aortic root sections.
(M) Quantification of α-SM-actin+ stained area in atherosclerotic lesions in aortic root sections.
(N) Correlation between lesions in innominate artery and plasma cholesterol in BT1xHuBL mice (n=12, Pearson correlation).
(O) Correlation between lesions in innominate artery and LDL-IgG immune complexes in BT1xHuBL mice (n=9, Pearson correlation).
Figure S7. Phenotype of ApoB100-vaccinated HuBL mice.

(A) Body weight (HuBL mice; PBS-adjuvant n=6, ApoB100-adjuvant n=5, triangles represent individual mice, bars show mean ± SEM).

(B-D) Anti-ApoB100 IgM, IgG1, and IgG2c. Graphs show mean ± SEM, titers are displayed on the x-axis, and the y-axis shows optical density at 450 nm (HuBL mice; PBS-adjuvant n=6, ApoB100-adjuvant n=5, 2-way ANOVA with Bonferroni’s post test).

(E) Quantification of Oil Red O-stained lesion area in eight consecutive sections, 100 – 800 µm from the aortic root (HuBL mice; PBS-adjuvant n=5, ApoB100-adjuvant n=4, 2-way ANOVA with Bonferroni’s post test, graph shows mean ± SEM, braces indicate significance level for treatment comparison).
Figure S8. Characterization of BT3xHuBL mice.
(A) Number of cells in thymus counted after preparation of single cell suspension (HuBL n=5, BT3xHuBL n=9, BT3 n=2, 1-way ANOVA with Bonferroni’s post test).
(B) Representative flow cytometry plots showing expression of CD4 and CD8 in thymocytes.
(C) Design of diet experiment with compound mutant mice and purified IgG transfer experiment.
(D) CD3⁺CD4⁺ T-helper cells in spleen (HuBL n=16, BT3xHuBL n=12, Mann-Whitney test).
(E) Proportion CD44⁺CD62L⁻ effector/memory of T-helper cells in spleen (HuBL n=11, BT3xHuBL n=12, Student’s t-test).
(F) Proportion Tbet⁺ Th1 of TRBV31⁺ T-helper cells in spleen (HuBL n=16, BT3xHuBL n=7, Mann-Whitney test).
(G) Proportion Foxp3⁺ T regulatory cells of TRBV31⁺ T-helper cells in spleen (HuBL n=16, BT3xHuBL n=7, Student’s t-test).
(H-I) Anti-native LDL IgM and IgG in plasma.
(H-O and R-S) Graphs show mean ± SEM, titers are displayed on the x-axis and the y-axis shows optical density at 450 nm (HuBL n=11, BT3xHuBL n=16, 2-way ANOVA with Bonferroni’s post test).
(J-K) Anti-oxLDL IgM and IgG in plasma.
(L-O) Anti-ApoB100 IgM, IgG, IgG1, and IgG2c in plasma.
(P) Competition assay for evaluation of anti-LDL IgG specificity in total IgG isolated from HuBL mice (n=4, 2-way
ANOVA with Bonferroni’s post test, significant competition by all three competitors).

(Q) Competition assay for evaluation of anti-oxLDL IgG specificity in total IgG isolated from HuBL mice (n=4, 2-way ANOVA with Bonferroni’s post test, significant competition by all three competitors).

(R-S) Immune complexes between LDL and IgM or IgG, respectively.

(T) Quantification of average Oil Red O+ lipid droplet size in liver sections (HuBL n=9, BT3xHuBL n=5, Mann-Whitney test).

(U) Quantification of IgG1+ stained area in liver sections (HuBL n=13, BT3xHuBL n=6, Student’s t-test).

(V) Immunofluorescence micrographs showing human ApoB (green) and nuclei (DAPI+, blue) in liver, with a 100 µm scale bar.

(X) Immunofluorescence micrographs showing human ApoB (green) and nuclei (DAPI+, blue) in duodenum, with a 100 µm scale bar.
Table S1. Phenotype of *HuBL* mice injected with CD4⁺ BT cells*

<table>
<thead>
<tr>
<th></th>
<th><em>HuBL/B6</em></th>
<th><em>HuBL/BT1</em></th>
<th><em>HuBL/BT2</em></th>
<th><em>HuBL/BT3</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Body weight</strong> (g)</td>
<td>28.5 ± 0.7 n=15</td>
<td>30.5 ± 0.9 n=12</td>
<td>26.3 ± 0.6 n=10</td>
<td>28.1 ± 1.4 n=12</td>
</tr>
<tr>
<td><strong>Plasma ApoB†</strong> (g/l)</td>
<td>7.35 ± 0.44 n=7</td>
<td>5.86 ± 0.26 n=8</td>
<td>6.43 ± 0.69 n=10</td>
<td>4.30 ± 0.60 n=5</td>
</tr>
<tr>
<td><strong>Plasma Cholesterol</strong> (mmol/l)</td>
<td>45.4 ± 3.1 n=14</td>
<td>34.3 ± 3.0 n=11</td>
<td>35.2 ± 6.4 n=9</td>
<td>28.0 ± 2.6 n=11</td>
</tr>
<tr>
<td><strong>Plasma Triglycerides</strong> (mmol/l)</td>
<td>8.62 ± 0.62 n=14</td>
<td>8.99 ± 0.73 n=11</td>
<td>7.54 ± 0.89 n=9</td>
<td>6.40 ± 0.71 n=11</td>
</tr>
<tr>
<td><strong>Plasma Creatinine</strong> (µmol/l)</td>
<td>88.9 ± 16.3 n=12</td>
<td>64.2 ± 14.7 n=8</td>
<td>70.7 ± 10.0 n=9</td>
<td>57.3 ± 11.0 n=9</td>
</tr>
<tr>
<td><strong>Blood WBC‡</strong> (10⁹/l)</td>
<td>12.1 ± 0.9 n=15</td>
<td>12.2 ± 1.6 n=12</td>
<td>13.8 ± 1.6 n=10</td>
<td>14.2 ± 1.6 n=12</td>
</tr>
<tr>
<td><strong>Blood Lymphocytes</strong> (10⁹/l)</td>
<td>7.56 ± 0.35 n=15</td>
<td>7.83 ± 0.52 n=12</td>
<td>9.59 ± 0.73 n=10</td>
<td>10.1 ± 0.78 n=12</td>
</tr>
<tr>
<td><strong>Blood Monocytes</strong> (10⁹/l)</td>
<td>0.47 ± 0.06 n=15</td>
<td>0.43 ± 0.04 n=12</td>
<td>0.49 ± 0.06 n=10</td>
<td>0.54 ± 0.06 n=12</td>
</tr>
<tr>
<td><strong>Blood Granulocytes</strong> (10⁹/l)</td>
<td>4.10 ± 0.61 n=15</td>
<td>3.91 ± 0.34 n=12</td>
<td>3.71 ± 0.38 n=10</td>
<td>3.57 ± 0.47 n=12</td>
</tr>
<tr>
<td><strong>Spleen Lymphocytes</strong> (10⁶)</td>
<td>117 ± 9.5 n=15</td>
<td>151 ± 12 n=12</td>
<td>146 ± 14 n=10</td>
<td>241 ± 26 n=12</td>
</tr>
<tr>
<td><strong>Spleen Monocytes</strong> (10⁶)</td>
<td>3.58 ± 0.56 n=15</td>
<td>4.17 ± 0.52 n=12</td>
<td>4.00 ± 0.60 n=10</td>
<td>8.97 ± 1.5 n=12</td>
</tr>
<tr>
<td><strong>Spleen Granulocytes</strong> (10⁶)</td>
<td>15.3 ± 2.4 n=15</td>
<td>20.7 ± 2.3 n=12</td>
<td>19.4 ± 2.8 n=10</td>
<td>37.3 ± 6.5 n=12</td>
</tr>
</tbody>
</table>

* After a western diet period of 10 weeks
† After a western diet period of five weeks.
‡ WBC=White blood cells
Gistera et al.

Table S2. mRNA levels in organs of HuBL mice injected with CD4⁺ BT cells*

<table>
<thead>
<tr>
<th>Organ</th>
<th>mRNA</th>
<th>HuBL/B6</th>
<th>HuBL/BT1</th>
<th>HuBL/BT2</th>
<th>HuBL/BT3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aorta</td>
<td>Trbv31</td>
<td>1.12 ± 0.15 n=14</td>
<td>3.08 ± 0.61 n=12</td>
<td>1.77 ± 0.22 n=9</td>
<td>15.1§ ± 2.0 n=10</td>
</tr>
<tr>
<td></td>
<td>Tbx21</td>
<td>1.14 ± 0.17 n=14</td>
<td>1.71 ± 0.23 n=10</td>
<td>1.04 ± 0.12 n=9</td>
<td>2.68‡ ± 0.35 n=10</td>
</tr>
<tr>
<td></td>
<td>Gata3</td>
<td>1.09 ± 0.12 n=14</td>
<td>1.28 ± 0.16 n=10</td>
<td>0.88 ± 0.09 n=9</td>
<td>1.21 ± 0.25 n=10</td>
</tr>
<tr>
<td></td>
<td>Rorc</td>
<td>1.38 ± 0.33 n=14</td>
<td>1.77 ± 0.31 n=10</td>
<td>0.90 ± 0.15 n=9</td>
<td>0.83 ± 0.17 n=10</td>
</tr>
<tr>
<td></td>
<td>Foxp3</td>
<td>1.20 ± 0.20 n=14</td>
<td>4.23† ± 0.96 n=10</td>
<td>4.33‡ ± 0.74 n=9</td>
<td>9.97§ ± 1.89 n=10</td>
</tr>
<tr>
<td></td>
<td>Il6</td>
<td>1.23 ± 0.22 n=14</td>
<td>1.38 ± 0.28 n=12</td>
<td>1.39 ± 0.21 n=6</td>
<td>2.68 ± 0.82 n=8</td>
</tr>
<tr>
<td></td>
<td>Il10</td>
<td>1.11 ± 0.13 n=14</td>
<td>1.82 ± 0.24 n=10</td>
<td>1.22 ± 0.23 n=9</td>
<td>2.35§ ± 0.20 n=10</td>
</tr>
<tr>
<td></td>
<td>Ifng</td>
<td>1.15 ± 0.18 n=14</td>
<td>2.60† ± 0.44 n=10</td>
<td>1.45 ± 0.20 n=9</td>
<td>12.5§ ± 3.13 n=10</td>
</tr>
<tr>
<td></td>
<td>Il21</td>
<td>0.71 ± 0.46 n=14</td>
<td>5.91 ± 2.14 n=10</td>
<td>5.01 ± 1.23 n=9</td>
<td>36.4§ ± 13.7 n=10</td>
</tr>
<tr>
<td>Para-aortic LN</td>
<td>Trbv31</td>
<td>1.67 ± 0.43 n=14</td>
<td>3.20† ± 0.53 n=12</td>
<td>1.42 ± 0.26 n=10</td>
<td>2.57 ± 0.44 n=12</td>
</tr>
<tr>
<td></td>
<td>Tbx21</td>
<td>1.14 ± 0.18 n=14</td>
<td>1.37 ± 0.16 n=12</td>
<td>1.24 ± 0.18 n=10</td>
<td>1.28 ± 0.20 n=12</td>
</tr>
<tr>
<td></td>
<td>Gata3</td>
<td>1.19 ± 0.20 n=14</td>
<td>0.73 ± 0.10 n=12</td>
<td>0.80 ± 0.16 n=10</td>
<td>0.59† ± 0.06 n=12</td>
</tr>
<tr>
<td></td>
<td>Rorc</td>
<td>1.17 ± 0.16 n=14</td>
<td>0.83 ± 0.07 n=12</td>
<td>0.69 ± 0.09 n=10</td>
<td>0.78† ± 0.24 n=12</td>
</tr>
<tr>
<td></td>
<td>Foxp3</td>
<td>1.16 ± 0.16 n=14</td>
<td>1.10 ± 0.13 n=12</td>
<td>1.30 ± 0.21 n=10</td>
<td>1.20 ± 0.17 n=12</td>
</tr>
<tr>
<td></td>
<td>Il4</td>
<td>1.12 ± 0.12 n=14</td>
<td>8.45§ ± 1.14 n=12</td>
<td>6.67‡ ± 0.98 n=10</td>
<td>9.69§ ± 1.70 n=12</td>
</tr>
<tr>
<td></td>
<td>Il10</td>
<td>1.27 ± 0.27 n=14</td>
<td>1.45 ± 0.26 n=12</td>
<td>0.74 ± 0.17 n=10</td>
<td>0.88 ± 0.13 n=12</td>
</tr>
<tr>
<td></td>
<td>Ifng</td>
<td>1.29 ± 0.27 n=14</td>
<td>2.56† ± 0.38 n=12</td>
<td>1.28 ± 0.17 n=10</td>
<td>2.49§ ± 0.35 n=12</td>
</tr>
<tr>
<td></td>
<td>Il21</td>
<td>1.12 ± 0.13 n=14</td>
<td>6.02‡ ± 1.10 n=12</td>
<td>9.36§ ± 1.93 n=10</td>
<td>12.8§ ± 2.05 n=12</td>
</tr>
<tr>
<td>Liver</td>
<td>APOB</td>
<td>0.99 ± 0.10 n=13</td>
<td>0.92 ± 0.07 n=12</td>
<td>1.03 ± 0.10 n=10</td>
<td>0.80 ± 0.07 n=12</td>
</tr>
<tr>
<td></td>
<td>Col1a1</td>
<td>1.56 ± 0.42 n=14</td>
<td>0.69 ± 0.15 n=12</td>
<td>1.88 ± 0.75 n=10</td>
<td>1.95 ± 0.71 n=11</td>
</tr>
<tr>
<td></td>
<td>Col3a1</td>
<td>1.57 ± 0.45 n=14</td>
<td>1.46 ± 0.59 n=12</td>
<td>1.54 ± 0.35 n=9</td>
<td>2.67 ± 0.75 n=11</td>
</tr>
<tr>
<td></td>
<td>Tnf</td>
<td>1.65 ± 0.59 n=14</td>
<td>0.49 ± 0.07 n=12</td>
<td>2.77 ± 0.91 n=10</td>
<td>1.76 ± 0.54 n=11</td>
</tr>
<tr>
<td></td>
<td>Tgfb1</td>
<td>1.43 ± 0.31 n=14</td>
<td>1.17 ± 0.30 n=12</td>
<td>1.75 ± 0.45 n=9</td>
<td>1.82 ± 0.38 n=12</td>
</tr>
</tbody>
</table>

* Data calculated as relative expression using the 2−ΔΔCT formula, normalized to the house-keeping gene Hprt
† p ≤ .05, Kruskal-Wallis test with Dunn’s multiple comparison test vs. HuBL/B6 group
‡ p ≤ .01, Kruskal-Wallis test with Dunn’s multiple comparison test vs. HuBL/B6 group
§ p ≤ .001, Kruskal-Wallis test with Dunn’s multiple comparison test vs. HuBL/B6 group
|| LN=lymph nodes
Table S3. Phenotype of *HuBL* vs. *BT1xHuBL* mice*

<table>
<thead>
<tr>
<th></th>
<th><em>HuBL</em></th>
<th><em>BT1xHuBL</em></th>
<th>Significance (Student’s <em>t</em>-test)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Body weight (g)</strong></td>
<td>32.0 ± 0.5 n=10</td>
<td>31.3 ± 0.7 n=13</td>
<td>n.s.†</td>
</tr>
<tr>
<td><strong>Blood WBC‡ (10⁹/l)</strong></td>
<td>12.2 ± 0.9 n=10</td>
<td>12.4 ± 1.6 n=13</td>
<td>n.s.</td>
</tr>
<tr>
<td><strong>Blood Lymphocytes (10⁹/l)</strong></td>
<td>8.21 ± 0.60 n=10</td>
<td>8.20 ± 1.07 n=13</td>
<td>n.s.</td>
</tr>
<tr>
<td><strong>Blood Monocytes (10⁹/l)</strong></td>
<td>0.42 ± 0.04 n=10</td>
<td>0.45 ± 0.07 n=13</td>
<td>n.s.</td>
</tr>
<tr>
<td><strong>Blood Granulocytes (10⁹/l)</strong></td>
<td>3.55 ± 0.33 n=10</td>
<td>3.80 ± 0.54 n=13</td>
<td>n.s.</td>
</tr>
<tr>
<td><strong>Plasma Creatinine (µmol/l)</strong></td>
<td>84.7 ± 11.5 n=14</td>
<td>92.2 ± 8.45 n=15</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

* After a western diet period of 10 weeks
† n.s.=not significant
‡ WBC=White blood cells
Table S4. mRNA levels in organs of *HuBL* vs. *BTxHuBL* mice*

<table>
<thead>
<tr>
<th>Organ</th>
<th>mRNA</th>
<th><em>HuBL</em></th>
<th><em>BTxHuBL</em></th>
<th>Significance (Mann-Whitney test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aorta</td>
<td>Trbv31</td>
<td>1.44 ± 0.39 n=10</td>
<td>4.50 ± 0.24 n=11</td>
<td><em>p</em> = .0003</td>
</tr>
<tr>
<td></td>
<td>Tbx21</td>
<td>1.16 ± 0.24 n=10</td>
<td>0.99 ± 0.13 n=11</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>Gata3</td>
<td>1.09 ± 0.15 n=10</td>
<td>1.19 ± 0.12 n=11</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>Rorc</td>
<td>1.06 ± 0.12 n=10</td>
<td>1.13 ± 0.24 n=11</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>Foxp3</td>
<td>1.11 ± 0.18 n=10</td>
<td>0.66 ± 0.05 n=10</td>
<td><em>p</em> = .02</td>
</tr>
<tr>
<td></td>
<td>Il5</td>
<td>1.06 ± 0.10 n=10</td>
<td>1.15 ± 0.09 n=11</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>Il6</td>
<td>1.29 ± 0.24 n=10</td>
<td>1.19 ± 0.18 n=11</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>Il10</td>
<td>1.06 ± 0.24 n=10</td>
<td>0.68 ± 0.05 n=11</td>
<td><em>p</em> = .01</td>
</tr>
<tr>
<td></td>
<td>Foxp3</td>
<td>1.11 ± 0.18 n=10</td>
<td>0.66 ± 0.05 n=10</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>Il5</td>
<td>1.06 ± 0.10 n=10</td>
<td>1.15 ± 0.09 n=11</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>Il6</td>
<td>1.29 ± 0.24 n=10</td>
<td>1.19 ± 0.18 n=11</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>Il10</td>
<td>1.06 ± 0.24 n=10</td>
<td>0.68 ± 0.05 n=11</td>
<td><em>p</em> = .01</td>
</tr>
<tr>
<td></td>
<td>Ifng</td>
<td>1.31 ± 0.41 n=10</td>
<td>0.93 ± 0.10 n=11</td>
<td>n.s.</td>
</tr>
<tr>
<td>Para-aortic LN†</td>
<td>Trav12</td>
<td>1.26 ± 0.22 n=10</td>
<td>5.56 ± 0.57 n=12</td>
<td><em>p</em> &lt; .0001</td>
</tr>
<tr>
<td></td>
<td>Trbv31</td>
<td>1.04 ± 0.10 n=10</td>
<td>3.06 ± 0.30 n=12</td>
<td><em>p</em> = .0003</td>
</tr>
<tr>
<td></td>
<td>Tbx21</td>
<td>1.06 ± 0.12 n=10</td>
<td>0.96 ± 0.04 n=12</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>Gata3</td>
<td>1.02 ± 0.07 n=10</td>
<td>0.98 ± 0.06 n=12</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>Rorc</td>
<td>1.04 ± 0.09 n=10</td>
<td>1.31 ± 0.13 n=12</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>Foxp3</td>
<td>1.04 ± 0.11 n=10</td>
<td>0.88 ± 0.07 n=12</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>Il5</td>
<td>1.05 ± 0.11 n=10</td>
<td>1.69 ± 0.52 n=12</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>Il6</td>
<td>1.11 ± 0.21 n=10</td>
<td>0.71 ± 0.04 n=12</td>
<td><em>p</em> = .02</td>
</tr>
<tr>
<td></td>
<td>Il10</td>
<td>1.01 ± 0.05 n=10</td>
<td>0.99 ± 0.06 n=12</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>Ifng</td>
<td>1.09 ± 0.18 n=10</td>
<td>0.90 ± 0.06 n=12</td>
<td>n.s.</td>
</tr>
<tr>
<td>Liver</td>
<td>APOB</td>
<td>1.05 ± 0.12 n=10</td>
<td>1.10 ± 0.12 n=14</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>Col1a1</td>
<td>1.26 ± 0.27 n=10</td>
<td>1.03 ± 0.19 n=14</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>Col3a1</td>
<td>1.32 ± 0.35 n=10</td>
<td>1.38 ± 0.23 n=14</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>Tnf</td>
<td>1.03 ± 0.59 n=10</td>
<td>0.95 ± 0.08 n=14</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>Tgfb1</td>
<td>1.26 ± 0.86 n=10</td>
<td>1.21 ± 0.71 n=14</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>Ifng</td>
<td>1.77 ± 0.59 n=10</td>
<td>2.22 ± 0.45 n=14</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

* Data calculated as relative expression using the 2^ΔΔCt formula, normalized to the house-keeping gene Hprt
† LN=lymph nodes
Table S5. Phenotype of *HuBL* vs. *BT3xHuBL* mice*

<table>
<thead>
<tr>
<th></th>
<th><em>HuBL</em></th>
<th><em>BT3xHuBL</em></th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30.2 ± 0.5 n=16</td>
<td>24.6 ± 0.7 n=12</td>
<td><em>p = .003</em></td>
</tr>
<tr>
<td><strong>Significance</strong></td>
<td>(Student’s <em>t</em>-test)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Body weight</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(g)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Blood WBC†</strong></td>
<td>12.1 ± 0.7 n=16</td>
<td>15.1 ± 1.1 n=12</td>
<td><em>p = .03</em></td>
</tr>
<tr>
<td>(10⁹/l)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Blood Lymphocytes</strong></td>
<td>7.64 ± 0.55 n=16</td>
<td>7.62 ± 2.51 n=12</td>
<td>n.s.‡</td>
</tr>
<tr>
<td>(10⁹/l)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Blood Monocytes</strong></td>
<td>0.51 ± 0.04 n=16</td>
<td>0.74 ± 0.07 n=12</td>
<td><em>p = .004</em></td>
</tr>
<tr>
<td>(10⁹/l)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Blood Granulocytes</strong></td>
<td>3.91 ± 0.66 n=16</td>
<td>6.73 ± 0.90 n=12</td>
<td><em>p = .006</em></td>
</tr>
<tr>
<td>(10⁹/l)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Spleen weight</strong></td>
<td>109 ± 5 n=11</td>
<td>175 ± 16 n=12</td>
<td><em>p = .002</em></td>
</tr>
<tr>
<td>(mg)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Spleen Lymphocytes</strong></td>
<td>141 ± 45 n=11</td>
<td>152 ± 38 n=12</td>
<td>n.s.</td>
</tr>
<tr>
<td>(10⁶)</td>
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<tr>
<td><strong>Spleen Monocytes</strong></td>
<td>3.64 ± 0.59 n=11</td>
<td>9.17 ± 1.09 n=12</td>
<td><em>p = .001</em></td>
</tr>
<tr>
<td>(10⁶)</td>
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<tr>
<td><strong>Spleen Granulocytes</strong></td>
<td>15.3 ± 1.6 n=11</td>
<td>48.0 ± 9.3 n=12</td>
<td><em>p = .0007</em></td>
</tr>
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<td>(10⁶)</td>
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<tr>
<td><strong>Plasma ALT§</strong></td>
<td>23.6 ± 5.3 n=11</td>
<td>28.9 ± 7.1 n=16</td>
<td>n.s.</td>
</tr>
<tr>
<td>(U/l)</td>
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* After a western diet period of 10 weeks
† WBC=White blood cells
‡ n.s.=not significant
§ ALT=Alanine transaminase
Supplemental Methods

Lipoprotein preparations

LDL (density 1.019 – 1.063 g/ml) was isolated by ultracentrifugation from pooled plasma of healthy donors and dialyzed extensively against PBS\(^1\). One mM EDTA was added to prevent oxidation. Using the same procedure, mouse LDL was prepared from plasma of Ldlr\(^{-/-}\) mice. Highly oxidized LDL was obtained by incubating LDL (1 mg/ml protein content) with 20 μM CuSO\(_4\) for 18 hours at 37°C. Soluble ApoB100 was isolated as previously described\(^2\). Briefly, protein was precipitated from LDL, resuspended in sodium dodecyl sulfate, filtered on a PD-10 column (GE Healthcare), and further purified on a Superdex-200 size exclusion column (0.5 ml/min, in Tris-HCl buffer, pH 7.4). FITC-LDL and FITC-oxLDL was prepared as previously described\(^3\).

Macrophage oxLDL uptake assay

RAW264.7 mouse macrophage-like cells (ATCC) were used to evaluate uptake of oxLDL. Two-hundred thousand cells per well were seeded into 48-well cell culture plates (Corning) in 1% FBS RPMI 1640 medium (Thermo Fisher Scientific) with 25 µg/ml FITC-labeled oxLDL. Plasma was dialyzed against PBS and added at a dilution of 1:100. Cellular FITC signals were recorded with an IncuCyte ZOOM live-cell imaging and analysis platform (Essen Bioscience). After 24 hours incubation at 37°C in a humid 5% CO\(_2\) atmosphere, four images per well from six replicates were taken using a 20x objective and subsequently analyzed using the IncuCyte Basic Software. The cells were then washed, fixed in PBS-buffered 4% formaldehyde solution, and stained with hematoxylin and Oil Red O. Photomicrographs were acquired using a 40x objective and a Leica DMIL inverted microscope.

Lipoprotein clearance studies

For lipoprotein clearance studies, 100 μg FITC-LDL was mixed with 70% mouse plasma from HuBL mice injected with either B6 or BT3 CD4+ T cells, and injected into the tail vein of male HuBL mice. Blood samples were collected in EDTA-coated tubes 1, 5, 15, 30, and 60 minutes after the injection. IgG purification was performed using Protein G resin (GE Healthcare). After dialysis against PBS, the protein concentration was determined with a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific). Two-hundred μg of the isolated IgGs were infused into 10-week-old male HuBL recipients and blood samples were collected before and at 15, 60, and 360 minutes after the infusion.

RNA isolation, cDNA synthesis, and real-time PCR

RNA was isolated from aorta, para-aortic lymph nodes, liver, and thymus using RNeasy kit (Qiagen). Total RNA quality was analyzed on a BioAnalyzer instrument (Agilent Technologies) and was quantified by 260 nm absorbance measurement using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific). Reverse transcription was performed with high-capacity cDNA reverse transcription kit and amplification was performed by real time-PCR using TaqMan Universal Master Mix and pre-manufactured primers and probes for the genes of interest and for hypoxanthine guanine phosphoribosyl transferase (Hprt, assay-on-demand), in an ABI 7900HT Fast Real-Time PCR System (all Applied Biosystems). For Trav12 and Trbv31 mRNA, specific primers and probe were used as previously described\(^2\). Data were analyzed using relative expression with the formula \(2^{-\Delta\Delta Ct}\), where \(\Delta\Delta Ct = \Delta Ct (sample) – \Delta Ct (mean Ct values of the controls)\), and \(\Delta Ct\) is the Ct of the housekeeping gene (Hprt) subtracted from the Ct of the target gene.

Immunohistochemistry and immunofluorescent analysis

Primary antibodies to CD4 (clone H129.19), CD8 (clone 53-6.7), VCAM-1 (clone 429; all from BD Biosciences), α-SM-actin (ab5694, Abcam), and CD68 (clone FA-11, AbD Serotec) were applied to acetone-fixed cryosections, followed by biotinylated rabbit anti-rat IgG. The primary antibody for IA\(^b\) (clone KH74, BD Biosciences) was a biotin-conjugated IgG. Staining was visualized with Vectastain ABC kit and diaminobenzidine (Vector). Quantification of immunohistochemical staining was documented as the ratio of thresholded chromogen
area using QWin Standard Y 2.8 computerized analysis (Leica) or calculated as the number of stained cells divided by the intimal lesion area. For fluorescent staining of spleen, liver, kidney, and duodenum sections, antibodies against B220 (clone RA3-6B2), F4/80 (clone BM8; both from BioLegend), IgG1 (1070, Southern Biotech), IgG2c (clone 5.7), CD138 (clone 281-1; both from BD Biosciences), C3 (A0062, Dako), and ApoB (20-AG40, Fitzgerald) were used. Horse anti-goat IgG (Dylight 488, Vector) was used as secondary antibody for ApoB staining.

**Plasma analysis**

Plasma was separated through centrifugation of whole blood, 1500g for 15 minutes. Plasma creatinine levels were analyzed using a colorimetric assay (Cayman Chemical Company) relying on the Jaffe’ reaction. Plasma alanine transaminase activity was measured using a colorimetric assay kit (Abcam). Plasma ApoB levels were measured with a human ApoB ELISA development kit (Mabtech). For FITC-LDL detection, plasma was diluted 1:25 in PBS and FITC fluorescence was analyzed using a Wallac 1420 Victor2 reader (Perkin Elmer).

**Cholesterol extraction from liver and feces**

Feces was collected during eight hours the day before sacrifice of the mice. Upon sacrifice, a piece of the liver was snap-frozen. One-hundred mg tissue was homogenized in methanol, and lipids were extracted by chloroform separation. After drying the extracts, they were redissolved and cholesterol content were measured with a colorimetric kit (Randox).

**Proliferation assay**

Splenocytes were isolated by meshing spleens from individual mice through a 100 µm cell strainer followed by osmotic lysis of red blood cells (EL buffer; Qiagen). Five-hundred thousand splenocytes were incubated in 96-well plates with 200 µl serum-free RPMI 1640 medium containing ITS Premix (Corning), 0.1% bovine serum albumin, nonessential amino acids, L-glutamine, 1 mM sodium pyruvate, and 50 µM β-Mercaptoethanol for 60 hours at 37°C in a humid 5% CO2 atmosphere. Triplicates were used for all samples. The splenocytes were stimulated with 10 µg/ml human LDL, 10 µg/ml mouse LDL, 0.6 µg/ml Concanavalin A (Con A), or 1 µg/ml rat-anti mouse CD3 antibody (clone C363.29B, Southern Biotech) together with 2 µg/ml rat-anti mouse CD28 antibody (clone 37.51, eBioscience). One µCi 3H-thymidine (Perkin Elmer) was added after 48 hours and DNA replication was measured in a scintillation counter (Wallac). Results are expressed as counts per minute (CPM) or stimulation index, calculated as CPM of the stimulated cells subtracted with the CPM of unstimulated cells from the same animal, and then divided with the CPM of unstimulated cells. IFN-γ levels in supernatants were measured by a commercial ELISA kit (Mabtech).

**Supplemental References**

List of antibodies used for flow cytometry analysis.

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<th>Target</th>
<th>Clone</th>
<th>Company</th>
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