Liver damage promotes pro-inflammatory T-cell responses against apolipoprotein B-100

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Abstract. Plochg BFJ, Englert H, Rangaswamy C, Konrath S, Malle M, Lampalzer S, et al. Liver damage promotes pro-inflammatory T-cell responses against apolipoprotein B-100. J Intern Med. 2022;291:648–664.

Objectives. Liver-derived apolipoprotein B-100 (ApoB100) is an autoantigen that is recognized by atherogenic CD4+ T cells in cardiovascular disease (CVD). CVD is a major mortality risk for patients with chronic inflammatory liver diseases. However, the impact of liver damage for ApoB100-specific T-cell responses is unknown.

Methods. We identified ApoB100-specific T cells in blood from healthy controls, nonalcoholic fatty liver disease (NAFLD) patients, and CVD patients by activation-induced marker expression and analyzed their differentiation pattern in correlation to the lipid profile and liver damage parameters in a cross-sectional study. To assess the induction of extrahepatic ApoB100-specific T cells upon transient liver damage in vivo, we performed hydrodynamic tail vein injections with diphtheria toxin A (DTA)-encoding plasmid in human ApoB100-transgenic mice.

Results. Utilizing immunodominant ApoB100-derived peptides, we found increased ApoB100-specific T-cell populations in NAFLD and CVD patients compared to healthy controls. In a peptide-specific manner, ApoB100 reactivity in healthy controls was accompanied by expression of the regulatory T (Treg)-cell transcription factor FOXP3. In contrast, FOXP3 expression decreased, whereas expression of pro-inflammatory cytokine interleukin (IL)-17A increased in ApoB100-specific T cells from NAFLD and CVD patients. Dyslipidemia and liver damage parameters in blood correlated with reduced FOXP3 expression and elevated IL-17A production in ApoB100-specific T-cell populations, respectively. Moreover, DTA-mediated transient liver damage in human ApoB100-transgenic mice accumulated IL-17a-expressing ApoB100-specific T cells in the periphery.

Conclusion. Our results show that liver damage promotes pro-inflammatory ApoB100-specific T-cell populations, thereby providing a cellular mechanism for the increased CVD risk in liver disease patients.

Keywords: apolipoproteins, atherosclerosis, cardiovascular clinical research, immunology, liver disease

Introduction

Cardiovascular disease (CVD) accounts for the highest number of premature deaths globally [1]. CVD develops from chronic inflammation of the vasculature as a result of genetic traits and individual lifestyle choices that lead to hyperglycemia, hypercholesterolemia, and hyperlipidemia. Besides diabetes mellitus and obesity, nonalcoholic fatty liver disease (NAFLD) has been described as a major risk factor for CVD [2]. The liver is pivotal for lipid metabolism as hepatocyte-released very-low-density lipoprotein particles provide cells with cholesterol, phospholipids, and triglycerides under homeostatic conditions. In NAFLD, however,
insulin resistance and dysregulated lipolysis promote fatty acid accumulation in the liver and induce apoptosis and necrosis of hepatocytes. In response to liver damage, immune cells are activated and the homeostatic anti-inflammatory condition of “liver tolerance” is turned into a pro-inflammatory state [3]. Liver inflammation, such as virus- and (endo-)toxin-induced hepatitis, is associated with CVD [4–7], while targeting hepatocyte inflammatory signaling alleviates atherosclerosis [8].

In NAFLD patients, impaired liver function interferes with lipoprotein clearance and changes the plasma lipid profile towards elevated low-density lipoprotein (LDL) concentrations [9]. LDL deposition in the intima and innate immune responses against oxidized LDL initiate atherosclerotic lesion development and correlate with CVD risk [10,11]. LDL consists of cholesterol, phospholipids, and a single apolipoprotein B-100 (ApoB100) protein that has immunogenic properties, illustrated by the presence of ApoB100-specific CD4+ T cells in endarterectomy samples from CVD patients [12]. The impact of ApoB100-specific CD4+ T cells on atherosclerosis has been demonstrated in mice, humanized mouse models, and humans [13,14], in which distinct ApoB100-derived T-cell epitopes have been identified for the human population [14,15].

Upon recognition of the MHCII-presented T-cell epitope by the T-cell antigen receptor (TCR), the naive T cell differentiates into anti- or pro-inflammatory T-cell subsets depending on the prevalent inflammatory micromilieu. Immunosuppression by regulatory T (Treg) cells ameliorates, whereas pro-inflammatory responses by effector T helper cells aggravate both CVD and NAFLD [16,17]. Particularly, the level of IFN-γ-secreting Th1 cells and IL-17-secreting Th17 cells increase in atherosclerotic plaques [18,19] and inflamed liver [20–22]. However, Th17 cells contribute to atherosclerotic plaque stability [23,24] and to some extent Treg/Th17 cell plasticity, and mixed phenotypes in human and murine T cells have been reported [14,25–27].

Adaptive immune reactions against liver antigens affect systemic T-cell responses [28], and we previously showed in an experimental atherosclerosis model that liver damage regulates differentiation of intrahepatic T-cell subsets that migrate into the inflamed aorta [26]. Based on that, we hypothesized that NAFLD is a predisposition for the differentiation of pro-atherogenic ApoB100-specific T cells. Here we identified ApoB100-specific CD4+ T cells in peripheral blood mononuclear cells (PBMCs) from NAFLD, CVD, and healthy controls (HC) through activation-induced marker expression following stimulation with ApoB100-derived peptides [29,30]. Phenotypic characterization revealed that the FOXP3/IL-17A expression ratio in ApoB100-specific T cells decreased in NAFLD and CVD patients compared to HC. Dyslipidemia and liver parameters correlated negatively with anti-inflammatory and positively with pro-inflammatory differentiation of ApoB100-specific T cells, respectively. Consistently, we showed in human ApoB100-transgenic (hApoB100tm) mice that the population of splenic ApoB100-specific T cells increased and expressed more IL-17a in response to transient liver damage. Thus, pro-inflammatory T-cell differentiation of ApoB100-specific T cells upon liver damage may contribute to the increased CVD risk in NAFLD patients.

Materials and methods

Human samples

Blood samples from NAFLD patients diagnosed by sonography and elastography were obtained at the outpatient clinic of the hepatology section of the Department of Medicine at the University Medical Center Hamburg-Eppendorf. Excluding criteria to participate in the study were the suspicion of alcoholic etiology and diagnosed tumor diseases. Blood samples from hypercholesterolemic patients (CVD) with and without previous cardiovascular events (Table 1) were obtained from the outpatient clinic of the endocrinology section of the Department of Medicine at the University Medical Center Hamburg-Eppendorf. Blood from HC was obtained from and tested for cytomegalovirus (CMV) serology at the blood bank of the University Medical Center Hamburg-Eppendorf. Statin treatment was considered dependent on dosing strategy: low–moderate or high dose (>20 mg/d atorvastatin/simvastatin; >5 mg/d rosuvastatin). Plasma lipid levels and liver parameters were measured utilizing the hospital’s diagnostic routine laboratory at the Clinical Chemistry and Laboratory Medicine Department. Blood samples were collected during 10/2018–04/2020 and the study was closed when sample size reached a predetermined power to detect significant differences in a two-sided test of the null hypothesis (alpha = 0.05, beta = 0.2).
Table 1. **Characteristics of healthy controls (HC), nonalcoholic fatty liver disease (NAFLD), and cardiovascular disease (CVD) patients**

| Parameter                                           | HC (n = 12) | NAFLD (n = 23) | CVD (n = 24) | p-value                                      |
|-----------------------------------------------------|-------------|----------------|--------------|---------------------------------------------|
| Age (years)                                         | n.d. (18–65)| 52 (19–77)     | 44 (29–68)  | NAFLD versus CVD: 5.3 × 10^{-1}               |
| Thrombotic events (%)                               | 0           | 4.35           | 20.83        | HC versus NAFLD: 1.0 × 10^0                  |
|                                                     |             |                |              | HC versus CVD: 1.6 × 10^{-1} NAFLD versus CVD: 1.9 × 10^{-1} |
| Statin (n/dose\(^a\))                              | 12/0        | 21/0; 1/1; 1/2 | 13/0; 6/1; 5/2 | HC versus NAFLD: 1.0 × 10^0                  |
|                                                     |             |                |              | HC versus CVD: 6.4 × 10^{-3} NAFLD versus CVD: 7.8 × 10^{-3} |
|                                                     |             |                |              | HC versus CVD: 7.8 × 10^{-3}                  |
| Cytomegalovirus seropositive\(^b\) (%)              | 69.23       | 78.26          | 70.83        | HC versus NAFLD: 1.0 × 10^0                  |
|                                                     |             |                |              | HC versus CVD: 1.0 × 10^0 NAFLD versus CVD: 1.0 × 10^0 |
| Total cholesterol (mg/dl)                           | 127 (80–166)| 192 (166–265)  | 234 (109–421) | HC versus NAFLD: 1.2 × 10^{-3} |
|                                                     |             |                |              | HC versus CVD: 5.3 × 10^{-2} |
|                                                     |             |                |              | HC versus CVD: < 1.0 × 10^{-4} |
|                                                     |             |                |              | NAFLD versus CVD: 7.8 × 10^{-1} |
| Triglycerides (mg/dl)                               | 94 (36–322) | 168 (67–428)   | 120 (63–583) | HC versus NAFLD: 3.0 × 10^{-1} |
|                                                     |             |                |              | HC versus CVD: 5.0 × 10^{-1} |
|                                                     |             |                |              | HC versus CVD: 9.0 × 10^{-1} |
| Low-density lipoprotein cholesterol (mg/dl)         | 76 (38–102) | 110 (70–193)   | 171 (41–313) | HC versus NAFLD: 8.6 × 10^{-2} |
|                                                     |             |                |              | HC versus CVD: < 1.0 × 10^{-4} |
|                                                     |             |                |              | NAFLD versus CVD: 6.8 × 10^{-3} |
| High-density lipoprotein cholesterol (mg/dl)        | 39 (26–80)  | 53 (29–97)     | 59 (59–67)   | HC versus NAFLD: 4.1 × 10^{-1} |
|                                                     |             |                |              | HC versus CVD: 2.4 × 10^{-2} |
|                                                     |             |                |              | HC versus CVD: 2.4 × 10^{-2} |
| Aspartate transaminase (U/l)                        | 36 (16–100) | 37 (14–82)     | 32 (14–54)   | HC versus NAFLD: 9.8 × 10^{-1} |
|                                                     |             |                |              | HC versus CVD: 4.8 × 10^{-1} |
|                                                     |             |                |              | HC versus CVD: 2.4 × 10^{-1} |
| Alanine transaminase (U/l)                          | 17 (10–36)  | 61 (22–190)    | 45 (25–75)   | HC versus NAFLD: < 1.0 × 10^{-4} |
|                                                     |             |                |              | HC versus CVD: 6.6 × 10^{-3} |
|                                                     |             |                |              | HC versus CVD: 5.1 × 10^{-3} |
| Gamma-glutamyltransferase (U/l)                     | 32 (22–72)  | 64 (30–271)    | 22 (17–69)   | HC versus NAFLD: 3.9 × 10^{-3} |
|                                                     |             |                |              | HC versus CVD: 7.8 × 10^{-1} |
|                                                     |             |                |              | HC versus CVD: < 1.0 × 10^{-4} |
| Alkaline phosphatase (U/l)                          | 45 (18–60)  | 96 (43–278)    | 104 (43–195) | HC versus NAFLD: < 1.0 × 10^{-4} |
|                                                     |             |                |              | HC versus CVD: 3.0 × 10^{-4} |
|                                                     |             |                |              | HC versus CVD: 8.4 × 10^{-1} |

Note: Values represent the median, range (minimum to maximum), and unpaired one-way ANOVA followed by Tukey’s multiple comparison test for continuous variables or Kruskal–Wallis test for categorical variables was performed.

\(^a\)Dose 0 = not taken, dose 1 = low–moderate, dose 2 = high.

\(^b\)Patients were considered cytomegalovirus (CMV)-seronegative when less than 25% of CD4\(^+\) T cells were CMV specific and less than 25% of CMV-specific T cells expressed FOXP3, IL-17A, or IFN-\(\gamma\).

Written informed consent was obtained from each patient included in the study. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki and the regional ethics committee approved the study (PV6039). Sample group characteristics are summarized in Table 1.

Antigen-reactive human T-cell enrichment and analysis

PBMCs from blood samples were isolated by density gradient centrifugation (Histopaque-1077, Sigma-Aldrich) and stimulated with human LDL (MyBioSource), CMV (pp65, Miltenyi), synthetic oligopeptides (ThermoFisher) of ApoB100 (P1:...
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Transgenic mice

hApoB100<sup>tg</sup> mice were derived from the B6.Cg-Ldlr<sup>-tm1Her</sup> Tg(APOB100)/Kctt strain (European Mouse Mutant Archive, #09689). Genotyping was performed using 5′-TATGCATCCCCAGCTTTGG-3′, 5′-CTACCCCAACACGCCCCCTAC-3′, and 5′-ATA GATTCGCCCTTGTTGCC-3′ for Ldr and 5′-TCAT GGCTCTGAGAATGC-3′ and 5′-GAGCCCAACC TGAGAGA-3′ for APOB100. The mice used had normal LDL receptor expression and were hemizygous for the full-length human APOB100 gene with an amino acid exchange at position 2153 (glutamine to leucine) to prevent the formation of apolipoprotein B-48. All mice were treated according to the national guidelines for animal care at the animal facilities of the University Medical Center Hamburg-Eppendorf and the treatment was approved by local authorities (#56/18).

Transient liver-damage model

hApoB100<sup>tg</sup> mice of either sex 10–12 weeks old (1:1) fed a standard chow diet were randomly selected into groups with or without hepatocyte-specific transfection of diphtheria toxin A (DTA). To amplify DTA from synthetic DNA, 5′-ACTGCTGTAGCATGAATTGCCTATG-3′ and 5′-ACTGCTCTCAGAGCTTAAATCTCCTGTAG-3′ were used (Eurofins Genomics), and NheI and XhoI restriction enzymes were used to insert the sequence into the multiple cloning site of the pLIVE plasmid (Mirusbio) to enable DTA expression under the control of the mouse minimal albumin promoter. Mice anesthetized with isoflurane received a hydrodynamic tail vein injection (HDI) with a volume of 0.9% saline equivalent to 10% of the body weight of each mouse that did or did not include 5-μg pLIVE plasmid encoding for DTA. Rapid HDI administration within 4–7 s facilitated plasmid uptake and transgene expression in hepatocytes due to altered intrahepatic circulation. Four days or 2 weeks post HDI, mice were sacrificed by cervical dislocation, blood was drawn by cardiac puncture, and circulating cells were flushed by 10-ml PBS perfusion. PBMCs from the spleen and liver were isolated, as described previously [26]. Immune cell infiltration and stimulation was assessed by flow cytometry analysis using anti-CD4 (clone: GK1.5; Biologend), anti-CD11b (clone: M1/70; Biologend), anti-CD154 (Miltenyi), and anti-F4/80 (clone: REA746) antibodies in the presence of mouse Fc Block (BD Biosciences). Dead cells were excluded from the analysis using the LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (ThermoFisher). Blood parameter analysis at the mouse pathology facility of the University Medical Center Hamburg-Eppendorf and the Institute of Clinical Chemistry and Laboratory Medicine, respectively, to confirm transient liver damage by HDI-mediated DTA transfection.

Identification of LDL-specific murine T cells

Two weeks post HDI, splenic and hepatic LDL-specific T-cell populations were identified by activation-induced marker expression using the mouse CD154 Enrichment and Detection Kit (Miltenyi). In brief, PBMCs were incubated in serum-free X-VIVO 15 media with or without 10 μg/ml human LDL at 5 × 10<sup>6</sup> cells/cm<sup>2</sup>/500 μl for 12–14 h supplemented with Protein Transport Inhibitor Cocktail for the last 2 h of incubation. Magnetic separation was used to enrich antigen-specific T cells from the spleen. PBMCs...
were stained with anti-CD4 (clone: GK1.5; Biolegend) and anti-CD154 (Miltenyi) in the presence of the LIVE/DEAD Fixable Aqua Dead cell Stain Kit (ThermoFisher) and mouse Fc Block (BD Biosciences). Intracellular staining of Foxp3 (clone: FJK-16s, ThermoFisher) and IL-17a (clone: TC11-18H10, Miltenyi) was performed using the Foxp3/Transcription Factor Staining Buffer Set (ThermoFisher).

**Flow cytometry**

FACS analysis of antigen-specific T cells was performed on cells within the lymphocyte gate of forward/side scatter plots, excluding doublets and dead cells. Fc blockade (clone: Fc1.3216 and 2.4G2 for human and murine cells, respectively) and isotype controls (all BD Biosciences) were used to exclude unspecific antibody binding and staining. Data were acquired using FACSCountII and LSRFortessa cytometer (BD Biosciences) and analyzed using FlowJo v10.6.1 software (Treestar).

**MHC II binding prediction**

The MHCII binding predictions were made on 3/16/2020 using the IEDB analysis resource Consensus tool (tools.iedb.org) [32].

**Statistics**

Values are expressed as median ± interquartile range and 5–95 percentile whiskers or mean ± SEM. Percentages of antigen-specific T cells represent corrected data (subtracted with values for nonstimulated samples of the same individual). A D’Agostino and Pearson omnibus normality test was performed to assess normal distribution of data sets. An unpaired Student’s t-test was performed for statistical analysis of two groups. A one-sample t-test against a theoretical median of zero was performed to identify the accumulation of antigen-specific T cells. An unpaired one-way analysis of variance (ANOVA) followed by Dunnett’s multiple comparisons test for parametric distribution or Kruskal–Wallis test for categorical variables and a paired one-way ANOVA followed by Tukey’s multiple comparisons post hoc test were performed for comparisons of more than two groups. Correlations were calculated using Spearman’s rank test. Differences were considered significant at p-values <0.05 (two-tailed). All statistical analyses were performed using GraphPad Prism software.

**Results**

**ApoB100-specific T cells are present in HC**

To identify ApoB100-specific T-cell responses, we assessed the stimulation of specific CD4⁺ T cells in PBMC cultures incubated with antigens via activation-induced marker expression to quantify enriched antigen-specific T-cell populations (Fig. 1a). To validate our approach, we used CMV-derived peptides and analyzed CD154 induction in CMV-specific T cells from CMV-seronegative and CMV-seropositive healthy donors (Fig. S1A). We identified a CMV-specific population of 9.5% ± 2.0% naïve T cells and 37.6% ± 5.3% memory T cells above background levels in CMV-seronegative and CMV-seropositive donors, respectively. In comparison, stimulation with LDL induced CD154 expression in 1.9% ± 0.6% CD4⁺ T cells, depicting a small but significant population of LDL-specific T cells in healthy donors above background levels (Fig. S1B). As expected, more CMV-specific T cells expressing the Treg cell-associated transcription factor FOXP3 or pro-inflammatory cytokine IFN-γ were found in CMV-seropositive donors than in CMV-seronegative donors (Fig. S1C), indicating previous differentiation into anti- and pro-inflammatory T-cell subsets following CMV infection. Notably, differentiation levels of LDL-specific T cells (32.6% ± 5.8%) increased in comparison to CMV-specific T cells from CMV-seronegative donors (18.0% ± 2.5%), suggesting that a small pool of differentiated memory T cells recognizing LDL is present in blood from healthy donors.

Mature ApoB100 protein has a length of 4536 amino acids (aa), and approximately half of the many MHCII ligands are specific for LDL, which are included in the liver-derived ApoB100 isoform (within the C-terminal region aa 2153–4536) but not in the intestinal ApoB48 isoform (that consists of aa 1–2152). To compare ApoB100-specific T-cell responses elicited by liver-derived antigens, we used synthetic oligopeptides of known human TCR epitopes from ApoB100: aa 3961–3980 (P1), aa 4411–4430 (P2), and aa 3009–3023 (P3) (Fig. 1b). Stimulation with P2 and P3, but not with P1, significantly increased activation-induced CD154 expression in CD4⁺ T cells compared to non-stimulated samples (Fig. 1c), showing that P2 and P3-specific stimulation identifies ApoB100-reactive T cells in healthy donors.
Fig. 1 Analysis of specific T-cell populations recognizing ApoB100-derived oligopeptides in human peripheral blood mononuclear cells (PBMCs). (a) Schematic overview for identification of apolipoprotein B-100 (ApoB100) specific T cells, negative controls without peptide stimulation (w/o), and positive controls with cytomegalovirus (CMV) stimulation via enrichment of antigen-specific T cells detected by activation-induced marker expression (AIM). (b) Sequence and MHCII binding probability of known ApoB100-derived oligopeptides (iedb.org MHCII reference set with cutoff at <50% binding probability compared to random peptides). (c) Flow cytometry gating strategy of CD154+ enriched CD4+ T cells from healthy donors; nongated cells in gray contour plots, gated populations depicted in colored dot plots. Representative flow cytometry analysis following stimulation with CMV, without peptide (w/o) or with ApoB100-derived oligopeptides P1 (dark red), P2 (red), and P3 (orange) are shown (left panel). Individual presence of enriched peptide-specific T-cell populations above background in healthy donors (right panel). Values are corrected for CD154 background expression in samples without peptide stimulation and presented.
Analyzing individual FOXP3/IL-17A expression increased in ApoB100-specific T cells (Fig. 2a). decreased, whereas IL-17A expression significantly that in both patient groups, FOXP3 significantly and CVD patients compared to HC. We show + values indicate percentage of CD154 in the CD4 populations in healthy controls (HC), nonalcoholic fatty liver disease (NAFLD), and cardiovascular disease (CVD) patients; n = 10. (d) Representative flow cytometry analysis of enriched ApoB100-specific CD4+ T cells from NAFLD and CVD patients compared to HC (Fig. 1d). Both NAFLD patients and CVD patients displayed a significant increase of activation-induced CD154 expression in T cells stimulated with ApoB100-derived oligopeptides P2 and P3. Moreover, we found that plasma cholesterol and LDL levels correlate with P2/P3-specific T-cell populations (Fig. S2, A and B), indicating that disease-mediated abundance of LDL-derived antigens promotes specific T-cell responses.

Notably, we used the population size of CD154+ enriched CD4+ T cells for our analyses, as this approach corrects for the varying frequency of CD4+ T cells in human blood. However, the population size of CD154+ enriched CD4+ T cells after stimulation with P2/P3 or CMV correlated significantly with the calculated percentage of CD154+ T cells among the total number of PBMCs per sample (Fig. S3), validating the assessment of antigen-specific T cells by quantification of the CD154+ enriched CD4+ T-cell population.

A pro-inflammatory differentiation pattern prevails in ApoB100-specific T cells from NAFLD and CVD patients

The differentiation of anti-inflammatory FOXP3+ Treg cells and pro-inflammatory IL-17A-expressing Th17 cells from the liver and periphery has implications for inflammatory responses in atherosclerosis [26,27]. Thus, we analyzed the differentiation pattern of ApoB100-specific CD4+ T cells in NAFLD and CVD patients compared to HC. We show that in both patient groups, FOXP3 significantly decreased, whereas IL-17A expression significantly increased in ApoB100-specific T cells (Fig. 2a). Analyzing individual FOXP3/IL-17A expression ratios, we found a severe imbalance towards a pro-inflammatory phenotype in ApoB100-specific T cells from NAFLD and CVD patients (Fig. 2b). In line with previous reports, chronic inflammatory conditions in NAFLD and CVD also increased the systemic pro-inflammatory phenotype in nonspecific T cells (reduced FOXP3/IL-17A ratio in CD154- T cells: 1.6-fold in NAFLD and 3.0-fold in CVD compared to HC; data not shown). However, the pro-inflammatory differentiation pattern further exacerbated in ApoB100-specific T cells from patients (NAFLD: 7.9-fold and CVD: 8.7-fold), indicating that pro-inflammatory differentiation of ApoB100-specific T cells overlays disease-associated changes of FOXP3 and IL-17A expression in NAFLD and CVD.

Notably, FOXP3 and IL-17A co-expression was not elevated in ApoB100-specific T cells of NAFLD or CVD patients, suggesting that T-cell plasticity (e.g., Treg to Th17 cell conversion) is not increased in blood, and marker protein expression delineates separate T-cell subsets. However, we found that different T-cell epitopes foster distinct differentiation patterns of ApoB100-specific T cells. Stimulation with liver-derived P2 and P3 oligopeptides revealed that P3-specific T cells displayed less FOXP3 and more IL-17A expression, whereas P2-specific T cells mainly contributed to the increased IL-17A expression in relation to the nonspecific T-cell pool in NAFLD and CVD patients (Fig. 2c). Thus, epitope-dependent differentiation of ApoB100-specific T cells may regulate distinct T-cell responses.

To confirm that altered differentiation of ApoB100-specific T cells in NAFLD patients is linked to enhanced autoimmune reactivity towards liver-derived antigens, we analyzed the specific T-cell response against BSEP—a protein targeted by hepatic immune responses in orthotopic liver transplantation and NAFLD [33,34]. Stimulation with a BSEP-derived oligopeptide identified antigen-specific T cells in healthy donors above background levels (Fig. S4A). As expected for NAFLD
Fig. 2 ApoB100-specific T cells in nonalcoholic fatty liver disease (NAFLD) and cardiovascular disease (CVD) patients display a pro-inflammatory differentiation pattern. (a) Representative flow cytometry analysis (upper panel) and bar graphs of FOXP3 (lower left panel) and IL-17A (lower right panel) expression in ApoB100-specific T cells from healthy controls (HC), NAFLD, and CVD patients. (b) FOXP3/IL-17A expression ratio of ApoB100-specific T cells; values represent mean ± SEM. Unpaired one-way ANOVA followed by Dunnett’s multiple comparisons test was performed for statistical analysis. (c) Expression of FOXP3 (upper panels) and IL-17A (lower panels) in P2- and P3-specific T cells in comparison to the nonspecific (i.e., CD154−) T-cell pool of HC (left), NAFLD (middle), and CVD (right) samples. Values represent median ± interquartile range and 5–95 percentile whiskers, paired one-way ANOVA followed by Tukey’s multiple comparisons post hoc test was performed for statistical analysis. *p < 0.05, **p < 0.01, ***p < 0.001; color pattern as in Fig. 1.
patients, liver damage promoted BSEP-specific T cells and significantly elevated IL-17A expression in comparison to the nonspecific T-cell pool, indicating that NAFLD is associated with autoreactive liver-specific T-cell responses (Fig. S4B). Moreover, we found that BSEP-specific IL-17A+ T cells correlate negatively with ApoB100-specific FOXP3+ T cells and positively with ApoB100-specific IL-17A+ T cells, suggesting that liver damage promotes pro-inflammatory T-cell differentiation against both hepatic autoantigens (Fig. S4C).

ApoB100-specific T-cell differentiation correlates with dyslipidemia and liver damage

Reduced FOXP3 expression and elevated IL-17A production in ApoB100-specific T cells from NAFLD and CVD patients raised the question whether dyslipidemia and liver damage may have an effect on pro-inflammatory T-cell differentiation in response to ApoB100-derived epitopes. We found that increasing plasma cholesterol and LDL levels correlate with diminished FOXP3 expression in ApoB100-specific T cells (Fig. 3a) and that blood parameters associated with liver damage, such as alkaline phosphatase (ALP) and alanine transaminase (ALT), correlate with IL-17A expression in ApoB100-specific T cells (Fig. 3b). Thus, alterations of the lipid profile are associated with a reduced anti-inflammatory ApoB100-specific T-cell population, whereas liver damage is associated with an increased pro-inflammatory ApoB100-specific T-cell population, corroborating the clinical association of CVD risk and chronic inflammatory liver disease.

Liver damage promotes IL-17a expression in peripheral ApoB100-specific T cells

Our data show an association of liver damage parameters with ApoB100-specific T-cell differentiation in patients. To test whether liver damage directly promotes the generation of ApoB100-reactive T cells, we performed a transient liver-damage model in hApoB100tg mice. To this end, murine hepatocytes were transfected with a DTA-encoding plasmid via HDI in vivo (Fig. 4a). Liver-specific expression under the control of a minimal mouse albumin promoter allowed DTA-mediated liver lesion development 4 days post injection (Fig. 4b). Cell death in the liver from HDI-treated mice was assessed by histological staining and blood parameter analysis (Fig. 4 c,d; Table 2). Transfection of hepatocytes with DTA-elicited liver damage and increased liver-damage parameters compared to sham treatment without DTA. To assess liver inflammation, we analyzed the hepatic immune cell infiltration and T-cell activation using flow cytometry. We found that granulocytes accumulated in the DTA-transfected liver (Fig. 4e) and that intrahepatic CD11b+ F4/80+ cells were more abundant in livers with DTA-induced lesions compared to controls, whereas intrahepatic CD4+ T-cell populations were unchanged (Fig. 4f). Moreover, we found that CD154 expression increased specifically in hepatic, but not splenic, CD4+ T cells following DTA transfection, indicating that DTA-mediated liver damage triggers immune cell recruitment and local T-cell stimulation.

Next, we assessed the ApoB100-specific T-cell population in the liver and spleen via activation-induced marker expression 2 weeks after HDI, which enables antigen-specific T cells to proliferate and differentiate in response to DTA-mediated liver damage and eventually to migrate into the periphery (Fig. 5a). After 2 weeks, macroscopic liver lesions completely resolved and the liver histology and blood parameters of DTA-transfected and sham-treated mice were comparable (Fig. 5 b,c; Table 2). In addition, liver inflammation declined and no differences in intrahepatic CD11b+ F4/80+ cells and CD154+ CD4+ T cells were found between the DTA and control group (Fig. 5d), suggesting that HDI-mediated DTA transfection induces transient liver damage that recedes after 2 weeks. In line with this, we identified similar populations of ApoB100-specific T cells in regenerated and control livers via CD154 induction in response to LDL stimulation (Fig. 5e). However, in mice that received transient liver damage 2 weeks prior to enrichment of antigen-specific T cells, the ApoB100-specific T-cell population enhanced in the spleen. Upon DTA-mediated liver damage, we found significantly increased ApoB100-specific T-cell populations above background levels in the spleen, but not in the liver (Fig. 5f). In addition to the increased population size, the differentiation pattern of splenic ApoB100-specific T cells changed in response to DTA-mediated liver damage (Fig. 5g). ApoB100-specific T cells from mice that received DTA treatment expressed slightly more Foxp3 and significantly elevated IL-17a levels compared to ApoB100-specific T cells from sham-treated mice and nonspecific T cells (Fig. 5 h,i). Thus, transient liver damage in mice induces extrahepatic ApoB100-specific T cells with an Th17/Treg-like differentiation pattern in the spleen.
Fig. 3 ApoB100-specific T cells express less FOXP3 in correlation with dyslipidemia and more IL-17A in correlation with liver damage. (a) Inverse correlation of FOXP3 expression in ApoB100-specific T cells with plasma cholesterol (left panel) and low-density lipoprotein levels (right panel). (b) Positive correlation of IL-17A expression in ApoB100-specific T cells with alkaline phosphatase (ALP, left panel) and alanine transaminase (ALT, right panel) plasma levels. Correlation analysis was performed for statistical analysis. Lines represent linear correlation curves; p-values and Spearman’s rank correlation coefficient r are shown.

Discussion and conclusion

Here we found that liver damage gives rise to a population of ApoB100-specific CD4+ T cells that expresses a pro-inflammatory differentiation pattern. The size of this population correlated with liver damage parameters in human blood, and this finding was also corroborated by a transient liver-damage model in mice. These results may provide a rationale for the increased CVD risk in NAFLD patients and are in line with recent studies that elucidate the pro-inflammatory micromilieu of the liver and the altered antigen presentation to intrahepatic T cells in NAFLD patients [35,36]. Moreover, we previously showed that hypercholesterolemia-induced liver damage promotes the migration of hepatic T cells into the atherosclerotic aorta [26], suggesting
that enhanced differentiation of pro-inflammatory T cells in the liver increases the pool of atherogenic T cells. The association of liver damage with CVD development is further supported by clinical observations in cohorts with hepatitis and hepatotoxic acetaminophen intake [4,37]. However, this report is the first to describe the generation of pro-inflammatory ApoB100-specific T cells in response to liver damage in humans and mice.

Characteristic patterns of peripheral CD4+ T cells have been reported for NAFLD and CVD [21,23,38].
Fig. 5 Transient liver damage promotes the generation of pro-inflammatory ApoB100-specific T cells in the periphery of human ApoB100-transgenic mice. (a) Schematic overview of transient liver damage in human ApoB100-transgenic mice through hydrodynamic tail vein injection (HDI) of plasmid encoding for diphtheria toxin A (DTA) with the polyadenylation region (polyA) under the control of the mouse minimal albumin promoter (mAlb). Two weeks (2 w) after HDI, the liver and spleen were removed to assess ApoB100-specific T-cell populations following stimulation with or without (w/o) human low-density lipoprotein (LDL) via enrichment of antigen-specific T cells using activation-induced marker (AIM) expression. Representative images of (b) liver (scale bar = 5 mm) and (c) hematoxylin and eosin-stained liver sections from mice receiving HDI with or without DTA 2 weeks before; 10× magnification overview and 40× magnification focus region (insert) is shown, scale bar = 50 μm. (d) Percentage of CD11b+/F4/80+ cells among intrahepatic peripheral blood mononuclear cells (PBMCs) (left panel) and CD154 expression among intrahepatic CD4+ T cells (right panel) ex vivo from mice receiving HDI with or without DTA 2 weeks before. Unpaired Student’s t-test was performed for statistical analysis; n = 4. (e) Intrahepatic and splenic PBMCs from human ApoB100-transgenic mice, which received HDI with or without DTA 2 weeks before, were
However, independently from disease-mediated alterations, we found that the FOXP3/IL-17A expression ratio further decreased in ApoB100-specific T cells from NAFLD and CVD patients. Recent studies published by Klaus Ley’s laboratory demonstrated that the differentiation pattern of ApoB100-specific Treg cells changes in atherosclerosis towards a Th17-like phenotype and that pro-inflammatory ApoB100-specific T-cell populations are associated with CVD [14,27]. Consistently, our data show that increased ApoB100-specific T-cell populations and concomitantly higher IL-17A expression among these cells are present in CVD patients. In addition to CVD, we also observed higher percentages of ApoB100-specific T cells in NAFLD patients and revealed a moderate positive correlation with plasma cholesterol and LDL levels. Our analysis of PBMCs may even

stimulated with 10 μg/ml human LDL to enrich and identify ApoB100-specific T-cell populations via activation-induced CD154 expression. Representative flow cytometry analysis of intrahepatic CD4+ T cells and CD154+ enriched CD4+ T cells from spleen following LDL stimulation, values indicate the percentage of ApoB100-specific T cells with activation-induced CD154 expression. (f) Individual percentage of intrahepatic and splenic LDL-specific T cells above background in human ApoB100-transgenic mice receiving HDI with or without DTA 2 weeks before. Unpaired Student’s t-test was performed for statistical analysis, values are corrected for CD154 background expression in samples without LDL stimulation; n = 6. (g) Representative flow cytometry analysis of Foxp3 and IL-17a expression in ApoB100-specific T cells from spleen 2 weeks after HDI with or without DTA in comparison to the nonspecific (i.e., CD154−) T-cell population. (HDI samples with or without DTA indicated by black and white diamonds, respectively). Paired one-way ANOVA followed by Tukey’s multiple comparisons post hoc test was performed for statistical analysis; n = 6. Values represent mean ± SEM; n.s., nonsignificant, *p < 0.05, **p < 0.001.
underestimate the extent of ApoB100-specific T-cell responses as these cells may be retained at inflamed sites in the chronically diseased liver and vasculature. Elevated lipoprotein levels facilitate an enhanced T-cell response against ApoB100 through the increased antigen abundance in NAFLD patients [39], while hypercholesterolemia also promotes T-cell reactivity intrinsically in mice and humans [40–42]. Thus, impaired lipoprotein profiles potentially decrease the threshold for stimulation of ApoB100-specific T cells in both CVD and NAFLD.

Our study has shortcomings as the limited blood sample size prevented the analysis of more than three previously published immunodominant ApoB100 epitopes P1–P3 that are presented to T cells in humans, previously assigned as P265 [15], P295 [15], and P18 [14]. Incubation of ApoB100-derived oligopeptide P1 with PBMCs from HC did not stimulate T cells above background, possibly due to a slightly decreased MHCII binding capacity, whereas P2 and P3 stimulation significantly identified ApoB100-specific T cells. However, our approach reduced bias in LDL processing, limited avidity by LDL-incubated antigen-presenting cells, and allowed the analysis of different T-cell specificities independently of donor MHCII alleles as compared to tetramer stainings. Several studies emphasize the importance of T-cell responses for future vaccination strategies against atherosclerosis [15,43,44]. Here, we provide evidence that epitope-dependent analysis of ApoB100-specific T-cell subsets may help to design such an approach.

IL-17A expression in ApoB100-specific T cells correlated positively with liver damage assessed by elevated ALP and ALT plasma levels. Notably, correlations were not observed with other parameters such as gamma-glutamyltransferase and aspartate transaminase, indicating that pro-inflammatory differentiation of ApoB100-specific T cells occurred in the acute phase of liver inflammation [45,46]. In addition, using a transient liver-damage model that specifically triggered tissue damage and T-cell stimulation in the liver, we confirmed that limited liver damage promotes the differentiation of Th17/Treg-like LDL-specific T cells in mice. Moreover, we found that LDL-specific T cells emerge in the periphery subsequent to the intrahepatic T-cell response, which is in line with our previous observation that hepatic T cells relocate to the spleen and atherosclerotic aorta [26].

Particularly in NAFLD patients, we detected more IL-17A+ BSEP-specific T cells that correlated with the pro-inflammatory differentiation of ApoB100-specific T cells. BSEP has been described as an autoantigen, and BSEP expression levels correlate negatively with the NAFLD activity score [33,34]. Thus, enhanced liver damage upon disease progression may be linked to aggravated intrahepatic immune responses and increased pro-atherogenic T-cell differentiation, as liver fibrosis is a risk factor for CVD-related death in nonalcoholic steatohepatitis patients [47]. Further studies are needed to show whether NAFLD is also associated with T-cell populations that recognize other CVD-related autoantigens, for example, heat shock protein [48].

In summary, we found that pro-inflammatory differentiation of ApoB100-specific T cells is associated with hyperlipidemia and liver damage. Thus, T-cell differentiation in chronic liver disease may contribute to cardiovascular immune responses, which could provide a pathophysiological concept for subclinical atherosclerosis and increased CVD risk in NAFLD patients [2,49].

Conflict of interest

The authors declare no conflict of interest.

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Author contributions

Bastian F.J. Plochg: data curation; formal analysis; investigation; methodology; writing – original draft. Hanna Englert: formal analysis; investigation; methodology; writing – original draft.
Chandini Rangaswamy: formal analysis; investigation; methodology; writing – original draft. Sandra Konrath: formal analysis; investigation; methodology; writing – original draft. Mandy Mai: methodology. Sibylle Lampalzer: resources. Claudia Beisel: resources. Salma Wollin: resources. Maike Frye: resources; writing – review and editing. Jens Aberle: resources; writing – original draft. Johannes Kluwe: resources; writing – review and editing. Thomas Renné: funding acquisition; project administration; resources; supervision; writing – review and editing.

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in healthy donors without (w/o) or with LDL stimulation (yellow); \( n = 4 \). C) Differentiation pattern of CMV-specific T cells in CMV-seronegative (upper panel) and CMV-seropositive donors (middle panel) and LDL-specific T cells (lower panel) in healthy donors. Total percentage of FOXP3\(^+\) (dotted), IL17A\(^+\) (hatched) or IFN-\(\gamma\)\(^+\) (checkered) antigen-specific CD4\(^+\) T cells is given, green and red lines indicate fraction of anti- and pro-inflammatory differentiated T cells. Paired one-way ANOVA followed by Tukey’s multiple comparison post hoc test and paired Student’s t test was performed for statistical analysis, \( *p < 0.05, **p < 0.005 \).

**Figure S2:** Dyslipidemia correlates with ApoB100-specific T cells. Positive correlation of A) plasma cholesterol and B) LDL levels with the size of the enriched ApoB100-specific T-cell population. Correlation analysis was performed for statistical analysis; lines represent linear correlation curves, \( P \)-values and Spearman’s rank correlation coefficient \( r \) are shown.

**Figure S3:** The population size of CD154\(^+\) enriched antigen-specific CD4\(^+\) T cells corresponds with their calculated frequency among PBMCs. Enrichment of CD154\(^+\) T cells detects antigen-specific CD4\(^+\) T-cell populations that correlate significantly with their calculated frequencies among PBMCs per analyzed sample. Values from all groups stimulated with ApoB100- and CMV-derived peptides (regular and bold symbols, respectively) are depicted. Correlation analysis was performed for statistical analysis; nonlinear regression line, \( P \)-value and Spearman’s rank correlation coefficient \( r \) are shown.

**Figure S4:** Increasing IL-17A\(^+\) BSEP-specific T cells associate with NAFLD and correlate with pro-inflammatory differentiated ApoB100-specific T cells. A) Sequence and MHC II binding probability of bile salt export pump (BSEP)-derived oligopeptide (iedb.org MHCII reference set with cutoff at \(<50\%\) binding probability compared to random peptides). Representative flow cytometry analysis of CD154\(^+\) enriched CD4\(^+\) T cells stimulated without (w/o, left panel) or with BSEP (middle panel) and individual presence of enriched BSEP-specific T-cell populations in healthy controls (right panel). Values are corrected for CD154 background expression in samples without peptide stimulation and presented as median ± interquartile range and 5–95 percentile whiskers; \( n = 10 \). One-sample Wilcoxon test against theoretical median of zero was performed for statistical analysis, \( **p < 0.01 \). B) Expression of FOXP3 (upper panels) and IL-17A (lower panels) in BSEP-specific T cells from healthy controls (HC, left panels), non-alcoholic fatty liver disease (NAFLD, middle panels) and cardiovascular disease (CVD, right panels) patients; color pattern as in Figure 1. Values represent median ± interquartile range and 5–95 percentile whiskers of BSEP-specific T cells compared to the non-specific (i.e. CD154\(^-\)) T-cell pool; paired one-way ANOVA followed by Tukey’s multiple comparisons post hoc test was performed for statistical analysis, \( **p < 0.01 \). C) Negative and positive correlation of BSEP-specific IL-17A\(^+\) T cells with ApoB100-specific FOXP3\(^+\) T cells (left panel) and ApoB100-specific IL-17A\(^+\) T cells, respectively. Correlation analysis was performed for statistical analysis; lines represent linear correlation curves, \( P \)-values and Spearman’s rank correlation coefficient \( r \) are shown.