UNIVERSITÄTSKLINIKUM HAMBURG-EPPENDORF

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Leberschäden fördern proinflammatorische T-Zell-Reaktionen gegen Apolipoprotein B100

Dissertation

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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 (*hApoB100*^{tg}) 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}
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: $<1.0 \times 10^{-4}$NAFLD versus CVD: 5.3×10^{-2}
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}NAFLD 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: $<1.0 \times 10^{-4}$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^{-3}NAFLD 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}NAFLD versus CVD: 2.4×10^{-1}
Alanine transaminase (U/l)	17 (10–36)	61 (22–190)	45 (25–75)	HC versus NAFLD: $<1.0 \times 10^{-4}$HC versus CVD: 6.6×10^{-2}NAFLD 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}NAFLD versus CVD: $<1.0 \times 10^{-4}$
Alkaline phosphatase (U/l)	45 (18–60)	96 (43–278)	104(43–195)	HC versus NAFLD: $<1.0 \times 10^{-4}$HC versus CVD: 3.0×10^{-4}NAFLD 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-γ.

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:

amino acids [aa] 3961–3980; P2: aa 4411–4430; P3: aa 3009–3023), and of bile salt export pump (BSEP: aa 534–554) or without additional antigen. Antigen incubation (10 μg/ml) was performed with 5×10^6 PBMCs in serum-free X-VIVO 15 media (Lonza) supplemented with 1 μl/ml CD40 (Miltenyi) at 37°C, 5% CO_2 for 12–14 h enabling stimulation (but not proliferation) of antigen-specific T cells. For the last 2 h of incubation, vesicle transport was inhibited with a 2 μl/ml Protein Transport Inhibitor Cocktail (ThermoFisher). T cells were labelled with antibodies against CD4 (clone: SK3) and CD154 (clone: REA746) in the presence of the LIVE/DEAD Fixable Aqua Dead cell Stain Kit (ThermoFisher) and human Fc Block (BD Biosciences). Antigen-specific T cells were labelled and enriched using biotin-conjugated anti-CD154 antibodies of the CD154 MicroBead Kit (Miltenyi) according to the manufacturer's instructions and stained with antibiotin antibodies (clone: REA746). Intracellular staining of FOXP3 (clone: 236A/E7, which recognizes all isoforms of the transcription factor) [31], CD154 (clone: 24–31), IL-17A (clone: Bio64DEC17), and IFN-γ (clone 4S.B3) was performed using the Foxp3/Transcription Factor Staining Buffer Set (ThermoFisher). Fluorescence-labelled antibodies were purchased from ThermoFisher (anti-CD4, anti-FOXP3, anti-IL-17A), Biolegend (anti-IFN-γ, anti-CD154), and Miltenyi (antibiotin).

Transgenic mice

hApoB100^{tg} mice were derived from the B6.Cg-Ldlr <tm1Her> Tg(APOB100)/Kctt strain (European Mouse Mutant Archive, #09689). Genotyping was performed using 5′-TATGCATCCCCAGTCTTTGG-3′, 5′-CTACCCAACCAGCCCCTTAC-3′, and 5′-ATA GATTCGCCCTTGTGTCC-3′ for *Ldlr* and 5′-TCAT GGGCTTGGAGAATGC-3′ and 5′-GAGCCCACCC TGCAGAGA-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^{tg} 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′-ACTGCTGCTAGCATGGATCCTGATG-3′ and 5′-ACTGCTCTCGAGTTAGAGCTTTAAATCTCTGTAG-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; Biolegend), anti-CD11b (clone: M1/70; Biolegend), anti-CD154 (Miltenyi), and anti-F4/80 (clone: BM8; ThermoFisher) 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). DTA-induced liver inflammation was monitored by hematoxylin and eosin staining of liver sections and 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 5×10^6 cells/cm^2/500 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 FACSCantoII 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.

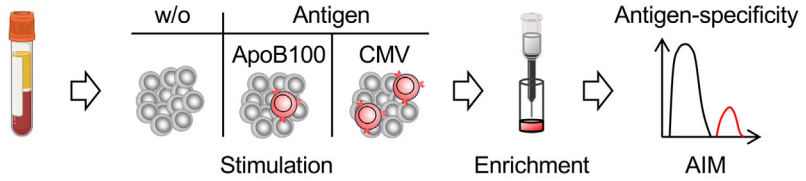
Results

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% naive 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 nonstimulated samples (Fig. 1c), showing that P2- and P3-specific stimulation identifies ApoB100-reactive T cells in healthy donors.

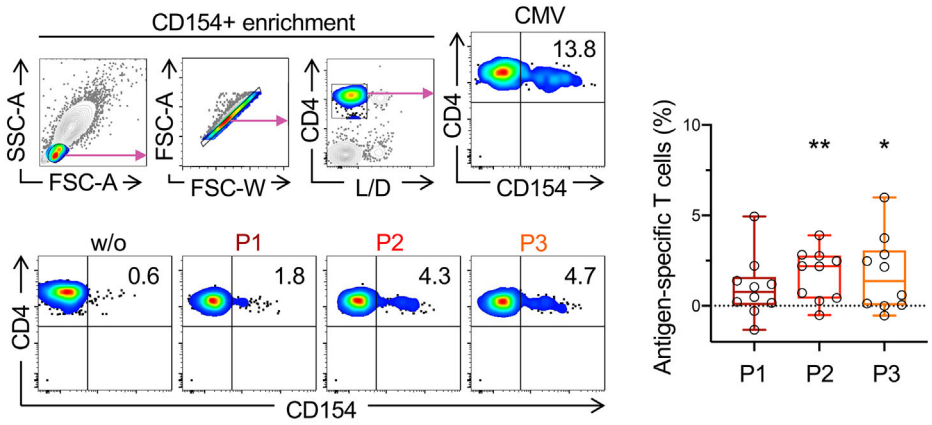
(a)



(b)

ApoB100 antigen	Oligopeptide sequence	MHCII binding	Reference
P1	3961-LRYQKDKKGISTSAASPAVG-3980	19/27	[15]
P2	4411-SQVEQFLHRNIQEYLSILTD-4430	25/27	[15]
P3	3009-SLFFSAQPFEITAST-3023	26/27	[14]

(c)



(d)

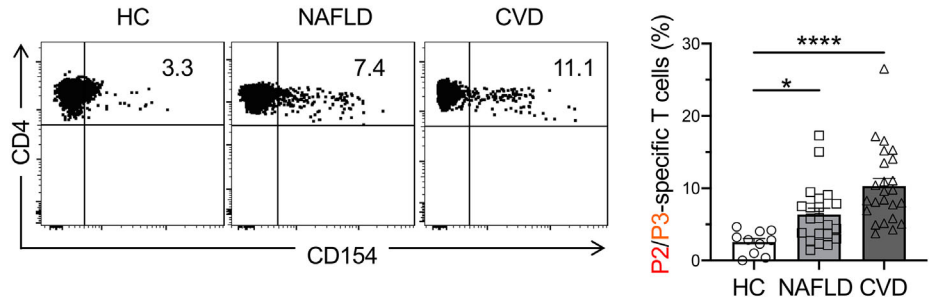


Fig. 1 *Analysis of specific T-cell populations recognizing ApoB100-derived oligopeptides in human peripheral blood mononuclear cells (PBMCs). (a) Schematic overview for identification of apolipoprotein B-100 (ApoB100) specific T cells, negative controls without peptide stimulation (w/o), and positive 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*

ApoB100-specific T cells accumulate in NAFLD and CVD patients

Next, we compared the population size of ApoB100-specific T cells in blood from healthy donors to that from patients with clinically diagnosed NAFLD and from patients with CVD. We found that the well-known risk for CVD in NAFLD patients is associated with accumulation of ApoB100-specific $CD4^+$ T cells 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 non-specific 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

*as median ± interquartile range and 5–95 percentile whiskers; one-sample Wilcoxon test against theoretical median of zero was performed for statistical analysis; n = 10. (d) Representative flow cytometry analysis of enriched ApoB100-specific T-cell populations in healthy controls (HC), nonalcoholic fatty liver disease (NAFLD), and cardiovascular disease (CVD) patients; values indicate percentage of CD154 in the $CD4^+$ T-cell population (left panel). Individual percentage of the enriched P2- and P3-specific T-cell population above background in human PBMCs assessed by flow cytometry analysis from NAFLD (light gray bars, n = 23) and CVD (dark gray bars, n = 24) patients compared to HC (white bars, n = 10). Values represent mean ± SEM; unpaired one-way ANOVA followed by Dunnett's multiple comparisons test was performed for statistical analysis; *p < 0.05, **p < 0.01, ****p < 0.0001.*

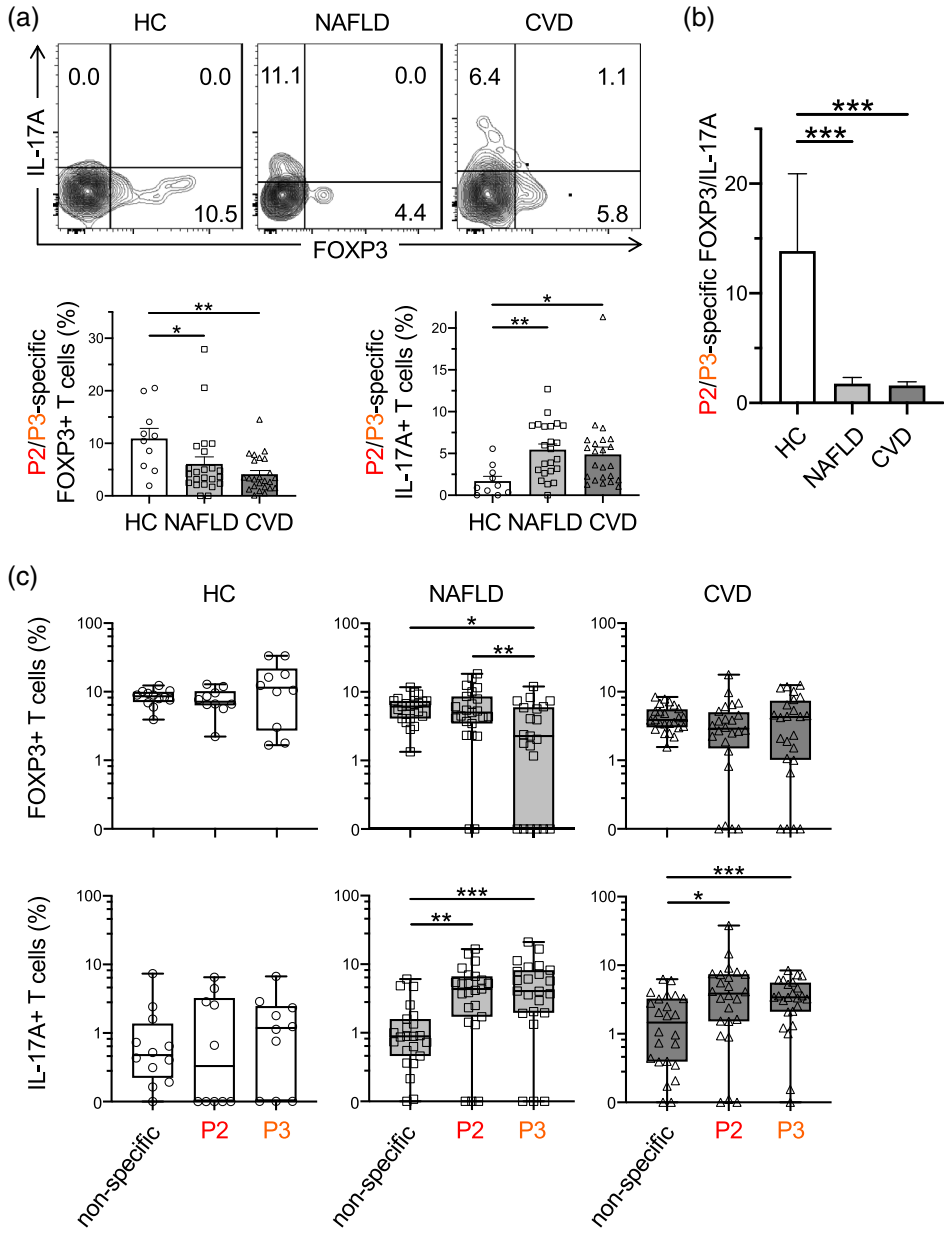


Fig. 2 *ApoB100-specific T cells in nonalcoholic fatty liver disease (NAFLD) and cardiovascular disease (CVD) patients display a pro-inflammatory differentiation pattern. (a) Representative flow cytometry analysis (upper panel) and bar graphs of FOXP3 (lower left panel) and IL-17A (lower right panel) 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 *hApoB100*^{tg} 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.

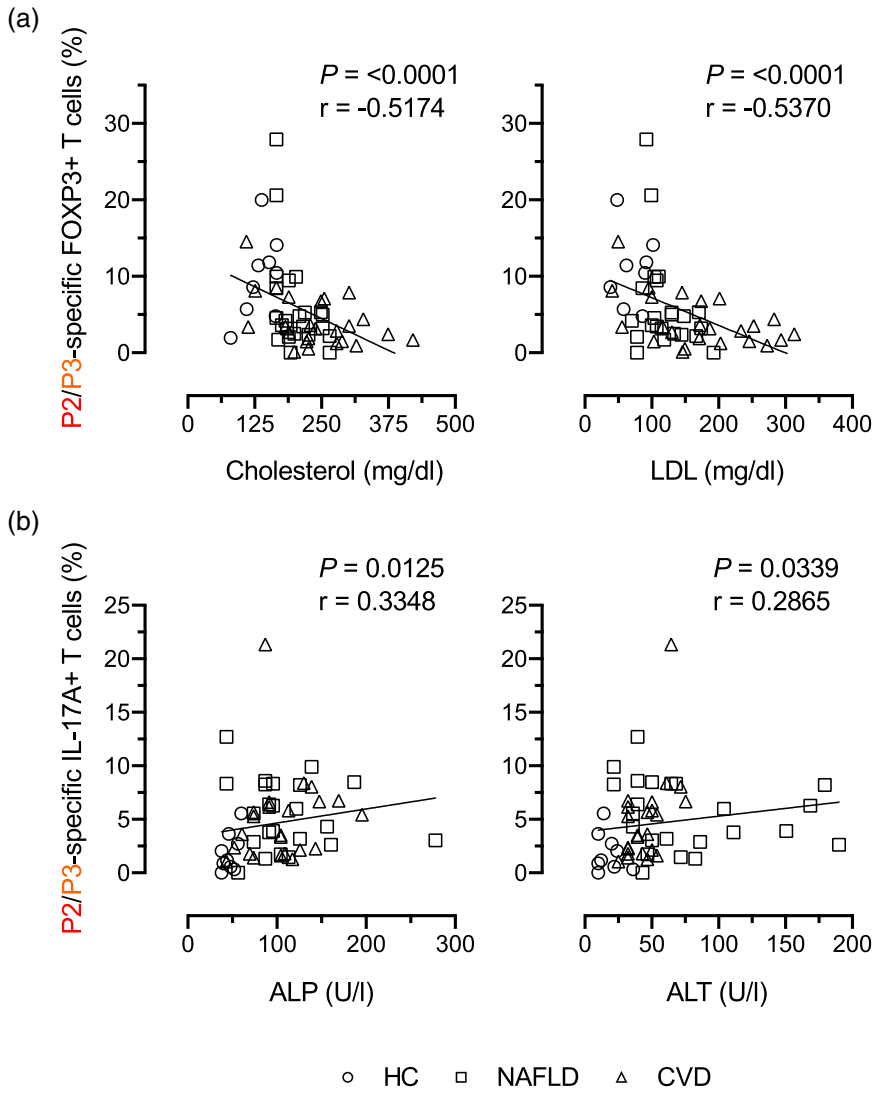


Fig. 3 *ApoB100-specific T cells express less FOXP3 in correlation with dyslipidemia and more IL-17A in correlation with liver damage. (a) Inverse correlation of FOXP3 expression in ApoB100-specific T cells with plasma cholesterol (left panel) and low-density lipoprotein levels (right panel). (b) Positive 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

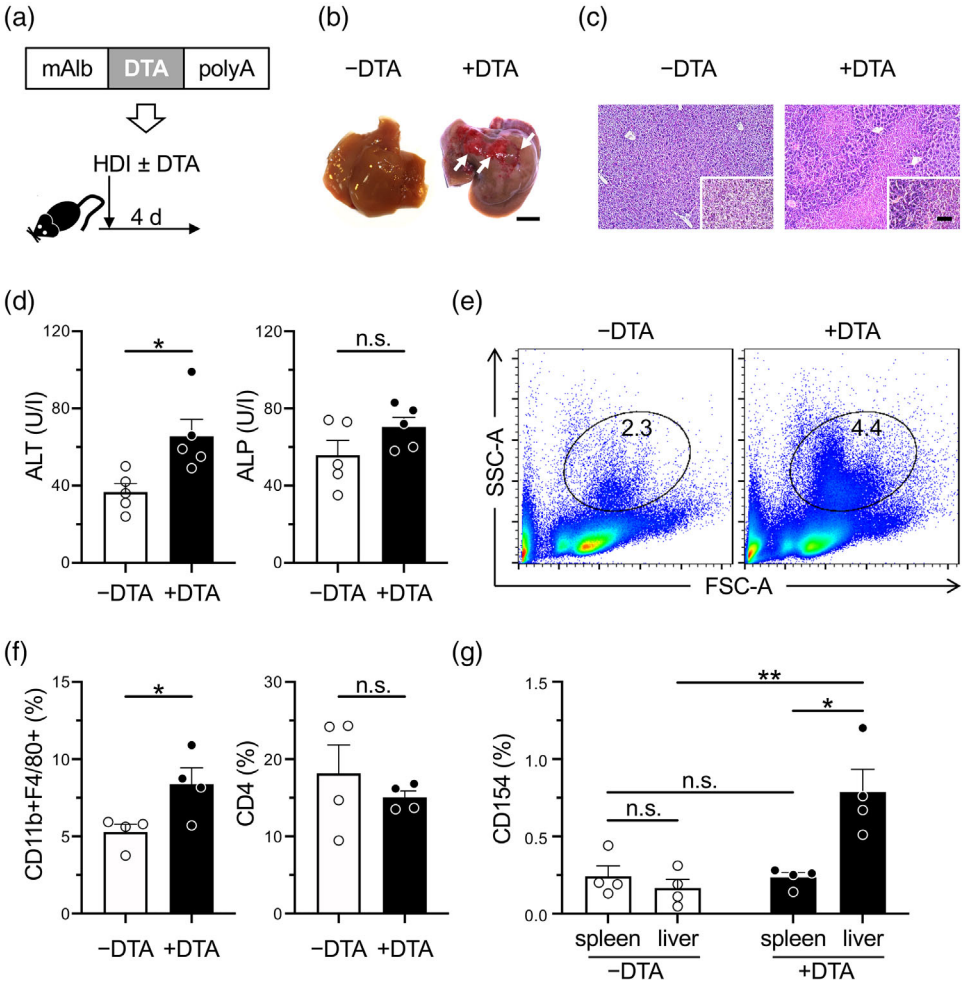


Fig. 4 *Diphtheria toxin A (DTA) transfection causes liver damage and induces liver inflammation in vivo. (a) Schematic overview of experimental liver damage assessed 4 days (4 d) after hydrodynamic tail vein injection (HDI) of plasmid encoding for DTA with polyadenylation region (polyA) under the control of the mouse minimal albumin promoter (mAlb) in human ApoB100-transgenic mice. (b) Representative images of liver lesions (scale bar = 5 mm) and (c) hematoxylin and eosin-stained liver sections from mice receiving HDI with or without DTA 4 days before; 10× magnification overview and 40× magnification focus region (insert) is shown, scale bar = 50 μm. (d) Plasma levels of liver damage parameter alanine transaminase (ALT, left panel) and alkaline phosphatase (ALP, right panel) from mice receiving HDI with or without DTA 4 days before; n = 5 data from two independent experiments. (e) Representative flow cytometry analysis of intrahepatic peripheral blood mononuclear cells (PBMCs) from mice receiving HDI with or without DTA 4 days before; values indicate the percentage of the depicted granulocyte gate. (f) Percentage of CD11b$^+$F4/80$^+$ cells (left panel) and CD4$^+$ T cells (right panel) among intrahepatic PBMCs and (g) CD154 expression of CD4$^+$ T cells ex vivo in liver and spleen from mice receiving HDI with or without DTA four days before; data from one of two independent experiments is shown, n = 4. Unpaired Student's t-test was performed for statistical analysis; values represent mean ± SEM; n.s., nonsignificant, *p < 0.05, **p < 0.01.*

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].

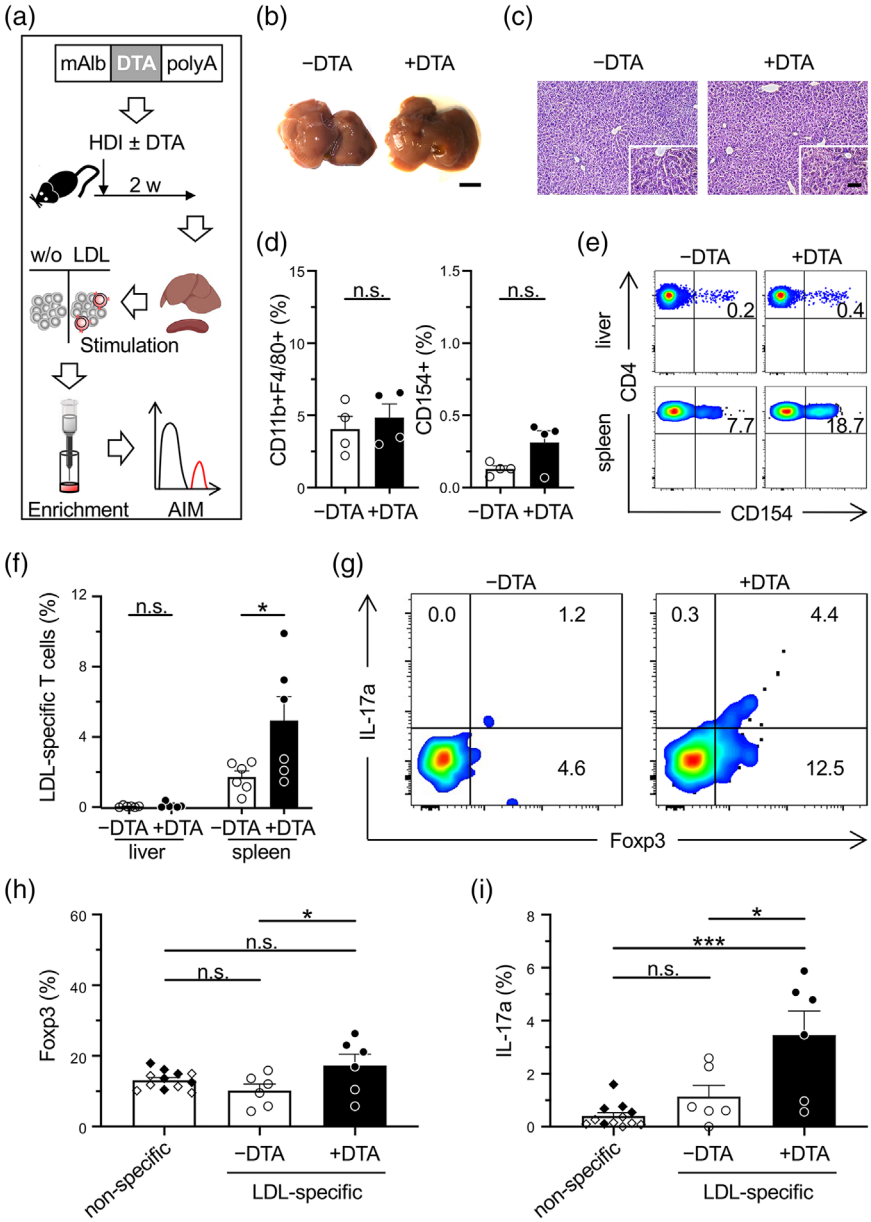


Fig. 5 *Transient liver damage promotes the generation of pro-inflammatory ApoB100-specific T cells in the periphery of human ApoB100-transgenic mice. (a) Schematic overview of transient liver damage in human ApoB100-transgenic mice through hydrodynamic tail vein injection (HDI) of plasmid encoding for 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*

Table 2. *Blood parameter of mice after hydrodynamic tail vein injection (HDI) with or without diphtheria toxin A (DTA) encoding plasmid*

	Mouse plasma 4 days after HDI		
Parameter	−DTA ($n = 5$)	+DTA ($n = 5$)	*p*-value
Alanine transaminase (U/l)	36 (24–50)	59 (49–99)	1.87×10^{-2}
Aspartate transaminase (U/l)	78 (38–142)	241 (108–343)	1.16×10^{-2}
Glutamate dehydrogenase (U/l)	19 (7–636)	623 (407–1087)	1.30×10^{-2}
Alkaline phosphatase (U/l)	73 (35–108)	72 (58–83)	8.21×10^{-1}
Total cholesterol (mg/dl)	67 (30–75)	76 (61–96)	1.12×10^{-1}
Triglycerides (mg/dl)	74 (52–89)	124 (86–196)	1.93×10^{-2}
High-density lipoprotein (HDL) cholesterol (mg/dl)	31 (20–38)	35 (20–41)	9.05×10^{-1}
Low-density lipoprotein (LDL) cholesterol (mg/dl)	16 (0–30)	18 (9–43)	4.64×10^{-1}
	Mouse plasma 2 weeks after HDI		
Parameter	−DTA ($n = 4$)	+DTA ($n = 4$)	*p*-value
Alanine transaminase (U/l)	47 (28–55)	34 (22–43)	1.91×10^{-1}
Aspartate transaminase (U/l)	47 (39–116)	68 (27–264)	4.74×10^{-1}
Glutamate dehydrogenase (U/l)	28 (24–33)	17 (12–20)	1.01×10^{-2}
Alkaline phosphatase (U/l)	59 (47–63)	57 (53–60)	9.53×10^{-1}
Total cholesterol (mg/dl)	98 (88–216)	82 (67–158)	4.80×10^{-1}
Triglycerides (mg/dl)	90 (68–174)	88 (77–131)	7.40×10^{-1}
HDL cholesterol (mg/dl)	52 (38–62)	35 (20–47)	6.63×10^{-2}
LDL cholesterol (mg/dl)	34 (25–120)	23 (17–112)	7.88×10^{-1}

Note: Values represent the median, range (minimum to maximum), and Student *t*-test was performed for statistical analysis.

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 without DTA (left panel) or with DTA (right panel). (h) Bar graphs of Foxp3 and (i) 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 Malle: 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; resources; writing – original draft. Reiner K. Mailer: conceptualization; data curation; formal analysis; funding acquisition; project administration; resources; supervision; writing – review and editing.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1: LDL-specific T cells are present in healthy donors. A) Representative histograms of $CD154^+$ enriched $CD4^+$ T cells following incubation with cytomegalovirus (CMV)-derived peptides in CMV-seronegative and CMV-seropositive donors (black lines and gray area, upper panel) and percentages of enriched CMV-specific T cells of the $CD4^+$ T-cell population (lower panel) displayed as median ± interquartile range and 5–95 percentile whiskers in CMV-seronegative ($n = 5$) and CMV-seropositive ($n = 9$) donors in comparison to samples without (w/o) CMV stimulation. **B)** Histogram (upper panel) and percentages of enriched LDL-specific T cells of the $CD4^+$ T-cell population (lower panel) displayed as median ± interquartile range and 5-95 percentile whiskers

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-γ+ (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. ■

2. Darstellung der Publikation

2.1 Einleitung

Herz-Kreislauf-Erkrankungen stellen weltweit die häufigste Todesursache dar (GBD 2017 Causes of Death Collaborators 2018). Sie sind durch eine chronische Entzündung der Arterienwand gekennzeichnet, die Atherosklerose. Sowohl Zellen der angeborenen als auch der adaptiven Immunabwehr sind an diesem komplexen Entzündungsgeschehen beteiligt.

Darüber hinaus spielen Lipoproteine, die im Körperkreislauf zirkulierenden heterogenen Transporteinheiten des Fettstoffwechsels, eine zentrale Rolle. Insbesondere die Lipoproteine geringer Dichte mit cholesterinesterreichem Inhalt (low density lipoproteins, kurz: LDL) durchqueren das geschädigte Endothel und interagieren mit subendothelialen Matrixproteinen. Es kommt zu einer Akkumulation von LDL in der Intima. Außerdem erfolgen Modifikationen des LDL durch reaktive Sauerstoffspezies, die eine Rekrutierung von Immunzellen und die Differenzierung von Monozyten zu Makrophagen und dendritischen Zellen herbeiführen. Vermittelt über Scavenger-Rezeptoren nehmen Makrophagen dieses oxidierte LDL auf, transformieren sich zu zytokinproduzierenden Schaumzellen und nekrotisieren schließlich. Eine Entzündungskaskade beginnt und es bildet sich eine atherosklerotische Plaque aus (Khatana et al. 2020, Ridker et al. 2017, Stemme et al. 1995, Tabas et al. 2007, Xu et al. 2019).

Als Vertreter der adaptiven Immunität machen T-Helferzellen einen Großteil der Immunzellen in atherosklerotischen Plaques aus. Ihr Phänotyp leistet einen entweder entzündungshemmenden oder entzündungsfördernden Beitrag zur Atherosklerose (Ait-Oufella et al. 2021, Geovanini et al. 2018, Ketelhuth et al. 2016, Saigusa et al. 2020). Die T-Zell-Aktivierung erfolgt durch Antigenpräsentierende Zellen (APC), die auf ihrer Oberfläche zuvor phagozytierte Peptide im Humanen Leukozyten Antigen der Klasse II (HLA II, engl. kurz: MHC II) anbieten. Zahlreiche Studien konnten in diesem Zusammenhang immunogenes Potential bei verschiedenen Peptiden des Apolipoprotein B100 (ApoB100) belegen (Marchini et al. 2021). Dieses lebereigene Protein, welches als Bestandteil von LDL die Cholesterinabgabe an das Gewebe reguliert, wird demnach von T-Zellrezeptoren erkannt und es kommt zu einer Proliferation mit anschließender Differenzierung in T-Zell-Subtypen. Den Hauptteil der in Plaques vorliegenden T-Zellen machen Interferon-γ-produzierende T-Helferzellen (Th1) aus. Während ihnen eine eindeutig proatherogene Wirkung nachgewiesen wurde, zeigen regulatorische T-Zellen (Treg) mit typischer Expression von FOXP3 (Forkhead-Box-Protein P3) anti-atherosklerotische Effekte. IL-17A-exprimierende T-Helferzellen 17 (Th17) haben hingegen gemischte

Eigenschaften, werden jedoch in erhöhter Zahl in der entzündeten Intima vorgefunden (Ait-Oufella et al. 2021, Erbel et al. 2011, Frostegård et al. 1999, Geovanini et al. 2018, Ketelhuth et al. 2016, Saigusa et al. 2020, Wolf und Ley 2019). Das Verhältnis von Treg- zu Th17-Zellen im Blut nimmt bei fortschreitender Atherosklerose ab (Cheng et al. 2007, Gisterå et al. 2013, Simon et al. 2013).

Als unabhängiger Risikofaktor der Atherosklerose gilt neben den Symptomen des metabolischen Syndroms mit Hypertonie, Adipositas, Diabetes mellitus Typ 2 und Dyslipidämie die Nicht-alkoholische Fettlebererkrankung (engl. kurz: NAFLD), welche unlängst als hepatische Komponente des Metabolischen Syndroms beschrieben wurde (Abdallah et al. 2020, Cai et al. 2020, Ference et al. 2017, Lee et al. 2018, Targher et al. 2015, Van Herck et al. 2019). Dabei kommt es durch chronische Energieüberladung zu einer Verfettung der Leber. Eine Lipotoxizität mit Hepatozytennekrose und der Einwanderung von Immunzellen ist die Folge. Auch hier ist das adaptive Immunsystem, insbesondere die T-Helferzellen, involviert (Kubes et al. 2018, Stols-Gonçalves et al. 2019). Wie bei der Atherosklerose sind proinflammatorische T-Zellsubtypen mit einem Fortschreiten der Erkrankung assoziiert, während regulatorische T-Zellen den chronischen Entzündungsprozess bremsen (Kremer et al. 2006). Das Treg- zu Th17-Zellen Verhältnis sinkt wie bei der Atherosklerose auch (Rau et al. 2016, Tang et al. 2011, Van Herck et al. 2019). Weitere entzündliche Lebererkrankungen wie etwa die toxische oder virale Hepatitis sind ebenfalls mit Atherosklerose assoziiert (Chan AT et al. 2006, Ostos et al. 2002, Petta et al. 2016, Rehm et al. 2016, Stoll et al. 2004) und eine Hemmung von intrahepatischen Entzündungsprozessen senkt die Atheroskleroselast (Luchtefeld et al. 2007). Folge der NAFLD ist die Ausbildung einer Dyslipidämie und Insulinresistenz mit proatherogenem Potential (DeFilippis et al. 2013, Siddiqui et al. 2015). Weiterhin konnten intrahepatisch differenzierte T-Zellen in der entzündeten Aorta im Atherosklerose-Mausmodells gefunden werden (Mailer et al. 2017a). Aufgrund dieser Verknüpfung von NAFLD und kardiovaskulären Erkrankungen ergibt sich folgende Arbeitshypothese:

Eine Leberschädigung im Rahmen einer NAFLD führt zur proatherogenen Differenzierung von ApoB100-spezifischen T-Zellen.

In dieser Arbeit wurden T-Zell-Restimulationen mit immunogenen Apolipoprotein-B100-Peptiden (Gisterå et al. 2017, Kimura et al. 2018) und einem Peptid des Proteins Gallensalzexportpumpe (Bile salt export pump, kurz: BSEP; Okushin et al. 2016, Stindt et

al. 2016) durchgeführt, um mithilfe magnetischer Separation spezifische T-Zellen über die Induktion des Aktivierungsmarkers CD154 zu detektieren und deren Treg- zu Th17-Zellen Verhältnis zu charakterisieren (Litjens et al. 2012, Frentsch et al. 2005). Dabei konnte die Immunantworten in gesunden Blutspendern mit denen in NAFLD- und Hypercholesterinämie-Patienten verglichen werden. In einem zweiten Teil erfolgte die Induktion eines vorrübergehenden Leberschadens im ApoB100 transgenen Mausmodell. Dessen Auswirkungen wurden anschließend histologisch, durchflusszytometrisch und in einem ähnlichen Restimulationsexperiment mit LDL bewertet.

2.2 Methoden

Die Beschaffung der humanen Proben erfolgte gänzlich über das Universitätsklinikum Hamburg-Eppendorf. Blut von gesunden Blutspendern konnte durch die Blutbank bezogen werden. Die ansässigen Ambulanzen für Hepatologie und Adipositas ermöglichten die Sammlung von Blutproben aus Patientenkollektiven mit apparativ nachgewiesener NAFLD oder kardiovaskulärem Risikoprofil (insbesondere Hypercholesterinämie und Adipositas; engl. kurz: CVD). Nach einer Aufklärung über die vollständige Anonymisierung der Daten, wurde im Rahmen der Routineuntersuchung Blut entnommen. Außerdem wurde ein Fragebogen gereicht, der Informationen über Alter, das Auftreten kardiovaskulärer Erkrankungen und die Einnahme von lipidsenkenden Medikamenten lieferte. Zwischen Oktober 2018 und April 2020 konnten so insgesamt 59 Blutproben gesammelt werden (Blutbank 12, NAFLD 23, CVD 24).

Die Anreicherung und immunphänotypische Analyse der antigenspezifischen T-Zellen erfolgte im direkten Anschluss und orientierte sich an der Anleitung des verwendeten CD154 MicroBead Kit der Firma Miltenyi. Hierzu wurden zunächst die mononukleären Zellen des peripheren Blutes mittels Dichtegradientenzentrifugation isoliert und anschließend über Nacht (für 14-16 Std.) mit Antigenen inkubiert. Zur Stimulation der Zellkultur dienten humanes LDL (MyBioSource) sowie synthetische Peptide des Zytomegalievirus (CMVpp65, Miltenyi), ApoB100 (P1: Aminosäuren 3961-3980; P2: Aminosäuren 4411-4430; P3: 3009-3023) und BSEP (Aminosäuren 534-554). Überstehendes Plasma wurde bei -20° C eingelagert und zu einem späteren Zeitpunkt die Parameter Cholesterin, Triglyzeride, LDL, HDL, AST, ALT, GGT und AP im hauseigenen Zentrallabor erhoben. Die aktivierungsbedingte Expression von CD154 an der Oberfläche der T-Zellen ermöglichte schließlich die selektive Anreicherung antigenspezifischer T-Zellen (Frentsch et al. 2005). Hierzu wurde das Oberflächenprotein CD154 durch Antikörper ferromagnetisch markiert

und anschließend im Magnetfeld von nicht markierten Zellen separiert. Die darauffolgende Bindung von fluoreszenzmarkierten Antikörpern an CD4 sowie intrazellulär Interferon-γ, IL-17A und FOXP3 (alle Isoformen; Mailer et al. 2018) ermöglichte die immunologische Charakterisierung der Zellen in der Durchflusszytometrie. Außerdem wurde LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (ThermoFisher) und Fc Block (BD Bioscience) hinzugefügt, um tote Zellen und unspezifische Antikörperfärbungen von der Analyse auszuschließen.

Für das Mausmodell eines vorübergehenden Leberschadens wurden transgene Mäuse des Stammes B6.Cf-Ldlr<tm1Her> Tg(APOB100)/Kctt (European Mouse Mutant Archive, #09689) verwendet. Diese Mäuse zeigen einen normalen LDL-Rezeptor-Phänotyp bei heterozygoter Expression von humanem ApoB100. Im Alter von 10-12 Wochen erfolgte eine hydrodynamische Schwanzveneninjektion (HDI) mit oder ohne Diphterietoxin-A(DTA)-kodierendem Plasmid (pLive, Mirusbio). Vier oder 14 Tagen nach Schwanzveneninjektion wurden die Mäuse getötet, Blut mittels Herzpunktion gesammelt und Leber und Milz nach PBS-Perfusion entnommen. Fluoreszierende Anti-CD4-, Anti-CD11b-, Anti-CD154- und Anti-F4/80-Antikörper dienten der durchflusszytometrischen Analyse hepatischer oder splenischer Zellen. Blutwerte (z.B. ALT, AST) wurden erhoben. Außerdem wurden histologische Schnitte der Leber mit Hämatoxilin- und Eosinfärbung angefertigt. Peripher mononukleäre Zellen der Leber und Milz von nach 14 Tagen getöteten Mäusen wurden ähnlich dem Verfahren bei humanen Proben mit oder ohne LDL stimuliert, magnetisch separiert (CD154 Enrichment and Detection Kit mouse, Miltenyi) und nach fluoreszierender Antikörpermarkierung durchflusszytometrisch ausgewertet (CD4, CD154, Foxp3, IL-17a).

Weitere Angaben zu Antikörpern, Assays, Reagenzien sowie deren verwendeten Konzentrationen und statistischen Methoden sind der beiliegenden Publikation zu entnehmen. Die Versuche wurde durch örtliche Behörden sowie die Ethikkommission genehmigt.

2.3 Resultate

Zunächst gelang es, den Versuchsaufbau für humane Proben zu etablieren. Dank großer Verbreitung des Humanen Herpesvirus 5 (Zytomegalievirus, kurz: CMV) und der routinemäßigen Erhebung des CMV-Serostatus in der Blutbank bot sich die Exposition mononukleärer Zellen mit Zytomegalieviruspeptiden an. Abhängig vom Zytomegalie-Serostatus gesunder Blutspender konnte die spezifische T-Zell-Antwort eines noch naiven

Immunsystems von der Gedächtnis-T-Zell-Antwort unterschieden werden. Nach CMV-Inkubation zeigten sich also größere Anteile aktivierter T-Zellen ($CD154^+CD4^+$) in seropositiven Blutproben im Vergleich zu gleichbehandelten seronegativen Blutproben (37,6%± 5,3% gegenüber 9,5%± 2,0%). Diese Populationen von aktivierten Gedächtnis-T-Zellen boten darüber hinaus ein signifikant höheres Maß an Differenzierung durch Interferon-γ- bzw. FOXP3-Expression.

Im weiteren Verlauf erfolgte die Analyse der antigenspezifischen T-Zell-Antwort auf weitere definierte Peptide in gesunden Blutspendern. ApoB100-Peptide, die bereits in vorhergehenden Publikationen als T-Zellepitope identifiziert werden konnten (P1, P2, P3: Gisterå et al. 2017, Kimura et al. 2018), und ein Peptid aus dem Gallensalzexportpumpen-Protein BSEP, welches als B-Zellepitop in NAFLD und Lebertransplantation bekannt ist (Okushin et al. 2016, Stindt et al. 2016), konnten synthetisch hergestellt und mit den mononukleären Zellen der Blutproben inkubiert werden. Hierbei konnten erhöhte Anteile von antigenspezifischen T-Zellen (also $CD154^+$ $CD4^+$ Zellen unter den $CD4^+$ Zellen) nach Stimulation mit jedem der Peptide festgestellt werden, wohingegen nur die Stimulation durch P2, P3 und BSEP im Vergleich zur Negativkontrolle die Signifikanz erreichte.

Die anschließende Analyse von Blutproben aus Patientengruppen mit erhöhtem kardiovaskulärem Risikoprofil bzw. nachgewiesener NAFLD zeigten ebenfalls signifikant erhöhte Anteile von $CD154^+CD4^+$ Zellen nach Stimulation durch die ApoB100-Peptide P2 und P3 im Vergleich zur Negativkontrolle. Die Relation der T-Zell-Antwort zu der gesamten vitalen T-Zellpopulation in der separierten Probe überstieg sogar jene aus der Gruppe der Gesunden signifikant. Es konnte also eine stärkere ApoB100-spezifische T-Zellantwort in Hypercholesterinämie- und NAFLD-Patienten nachgewiesen werden als in gesunden Blutspendern. Zusätzlich zeigte sich ein signifikant verändertes Expressionsmuster dieser Zellpopulation. Im Vergleich zu gesunden Blutspendern exprimierten die ApoB100-spezifischen T-Zellen häufiger IL-17A und seltener FOXP3. Veranschaulicht wird diese Verlagerung zusätzlich durch den Quotienten der $IL\text{-}17A^+$ zu $FOXP3^+$ Zellen, der um das 7,9-fache in NAFLD- und sogar das 8,7-fache in CVD-Patienten erhöht war. Eine gleichgerichtete Änderung der Expressionsverhältnisse von FOXP3 und IL-17A konnte auch in der Population unspezifischer T-Zellen nachgewiesen werden, allerdings in geringerem Ausmaß. Interessant ist, dass die Anzahl doppeltpositiver T-Zellen, also $IL\text{-}17A^+$ und $FOXP3^+$ Zellen, unter den spezifischen T-Zellen nicht erhöht ist.

Betrachtet man nun diese spezifischen T-Zellantworten in NAFLD- oder Hypercholesterinämie-Patienten im Vergleich zu denen in Gesunden gesondert zeigt sich,

dass P2-spezifische Zellen eine Zunahme der IL-17A-Expression aufweisen, während die P3-spezifischen Zellen zusätzlich eine Abnahme der FOXP3-Expression zeigen. Das Differenzierungsprofil der ApoB100-spezifischen T-Zellen ist also abhängig von Leberstörung durch NAFLD, CVD bzw. Plasmacholesterinniveau und peptidspezifisch.

Diese Erkenntnisse spiegeln sich in den Messungen der Blutplasmaparameter von Hypercholesterinämie- und NAFLD-Patienten wider. So sind leichte Korrelationen erhöhter Werte von ALP (Alkalische Phosphatase) und ALT (Alanin-Aminotransferase), als Korrelat des Leberschadens, mit erhöhtem IL-17A-Vorkommen in der Population ApoB100-spezifischer T-Zellen auszumachen. Zusätzlich ergibt sich eine negative Korrelation zwischen der FOXP3-Expression und steigenden LDL- und Gesamtcholesterinwerten in derselben Zellpopulation. Dyslipidämie und Leberschaden stehen also in Verbindung mit einer proinflammatorischen T-Zell-Antwort auf ApoB100-Peptide.

Weitere interessante Resultate lieferte die Stimulation mit dem BSEP-Peptid. In der Gruppe der Gesunden konnten signifikant erhöhte Anteile von spezifischen T-Zellen nachgewiesen werden. Diese BSEP-spezifischen Zellen in Patienten mit NAFLD exprimierten signifikant vermehrt IL-17A. Außerdem ergab sich eine positive bzw. negative Korrelation dieser Zellpopulationsgröße mit jener der ApoB100-spezifischen IL-17A$^+$ bzw. FOXP3$^+$ Zellen.

Das bereits beschriebene Mausmodell eines vorübergehenden Leberschadens in Mäusen, die humanes Apolipoprotein B100 exprimieren, konnte auf histologischer und molekularer Ebene verifiziert werden. Vier Tage nach Transfektion des Diphterietoxins wurden erhöhte Leberparameter im Blut nachgewiesen und die Histologie zeigte eine massive hepatische Immunzellinfiltration. Durchflusszytometrische Analysen der Leber deuteten zudem auf einen Anstieg CD11b$^+$/F4/80$^+$ und CD154$^+$ Zellzahlen bei unverändertem CD4$^+$ Zellzahlniveau hin. Zehn Tage später normalisierten sich Blutparameter und Histologie. Hepatische CD11b$^+$/F4/80$^+$ und CD154$^+$ Zellen glichen sich zwischen transfizierten und nicht-transfizierten Mäusen wieder an. Eine ebenfalls nach 14 Tagen durchgeführte LDL-Stimulation und Anreicherung von splenischen und hepatischen aktivierten T-Zellen wies signifikant erhöhte Anteile LDL-spezifischer T-Zellen in der Milz, nicht aber in der Leber nach. Diese Zellpopulation zeichnete sich darüber hinaus durch nicht-signifikant verstärkte Foxp3- und signifikant verstärkte IL-17a-Exprimierung aus. Außerdem konnte im Gegensatz zu den Resultaten der Untersuchungen mit menschlichen Proben eine gesteigerte spezifische Foxp3$^+$IL-17a$^+$ T-Zellpopulation in transfizierten Mäusen nachgewiesen werden.

2.4 Diskussion

Unsere Ergebnisse zeigen anschaulich, dass es im Rahmen der NAFLD zur Ausbildung einer ApoB100-spezifischen $CD4^+$ T-Zellreaktion kommt. Diese adaptive Immunantwort, vermittelt durch autoreaktive T-Zellen, ist von proinflammatorischer Natur. Während in gesunden Blutspendern ApoB100-Peptide eine leichtgradige Aktivierung über dem Hintergrundniveau von vorwiegend regulatorischen T-Zellen hervorrufen, ist in Patienten mit NAFLD unter gleichen Bedingungen eine vergrößerte Population proinflammatorischer $IL\text{-}17A^+$ Zellen zu verzeichnen. Genauso verhält es sich im Blut von Probanden mit Hypercholesterinämie. Diese Verschiebung der T-Zellantwort korreliert darüber hinaus mit den Blutwerten für Leberschaden und Dyslipidämie. Weiterhin konnte durch eine vorübergehende Leberschädigung im Mausmodell mit humanem ApoB100-Gen eine LDL-spezifische Th17-Zellantwort in der Peripherie induziert werden. Damit verknüpft die Arbeit erstmals den Leberschaden direkt mit einer proatherogenen ApoB100-spezifische T-Zellantwort.

Um diese Ergebnisse einordnen zu können, ist ein Verständnis über die besondere immunologische Stellung der Leber notwendig. Sie fungiert als Brandmauer zwischen Gastrointestinaltrakt und systemischen Kreislauf. Einerseits müssen gefährliche Erreger erkannt und bekämpft werden, andererseits eine Vielzahl von harmlosen Antigenen toleriert werden. Dieser Balanceakt wird durch einen hepatischen Toleranzmechanismus realisiert, an dem neben Kupfferzellen, dendritischen Zellen und Hepatozyten vor allem regulatorische T-Zellen und dessen Zytokin IL-10 beteiligt sind (Cai et al. 2018, Crispe et al. 2003, Osei-Bordom et al. 2020). So konnte zum Beispiel die Krankheitslast im Mausmodell der Multiplen Sklerose durch die hepatische Expression eines krankheitsassoziierten Autoantigens Treg-vermittelt reduziert werden (Lüth et al. 2008). Außerdem ist im Mausmodell erwiesen worden, dass intrahepatisch differenzierte T-Zellen am Entzündungsprozess im Gefäß teilnehmen können (Mailer et al. 2017a). Die bereits benannten intrahepatischen Vorgänge bei NAFLD könnten diese Toleranzmechanismen empfindlich stören und systemische Auswirkungen zur Folge haben. Demzufolge bilden hepatische Makrophagen im Rahmen der NAFLD verstärkt Zytokine wie TGF-β und IL-6, welche die Th17-Differenzierung fördern (Van Herck et al. 2019). Zudem konnte eine veränderte intrahepatische Antigenpräsentation durch APC im Rahmen von Leberfibrose belegt werden und somit Einfluss auf die T-Zelldifferenzierung nehmen (Ramachandran et al. 2019, Cai et al. 2018). Unsere Ergebnisse bestätigen die Auffassung der Leber als Immunorgan der Toleranz.

Die hier ermittelte Autoreaktivität der T-Zellen gegen ApoB100-Peptide in gesunden Blutspendern ist nicht verwunderlich und steht im Einklang mit aktuellen Studienergebnissen. Das Vorherrschen der Toleranz bzw. die Abwesenheit eines chronischen Entzündungsprozesses in der Gefäßwand ist durch die Dominanz der spezifischen regulatorischen T-Zellpopulation begründet (Marchini et al. 2021). Ihre immunsuppressive Funktion bildet den Toleranzmechanismus. Dementsprechend zeigten sich in gesunden menschlichen Proben und Mausexperimenten ApoB100-spezifische T-Zellpopulationen, die vor allem dem regulatorischen T-Zellsubtyp entsprechen (Kimura et al. 2018). Im Verlauf der Atherosklerose veränderte sich das Expressionsmuster dieser spezifischen Zellen an einem bislang nicht erfassbaren Kipppunkt, der zu einem Verlust der FOXP3-Expression führt. Gleichzeitig liegen vermehrt Th1- und Th17-Subtypen vor (Wolf et al. 2020). Folglich sinkt das Verhältnis von Treg- zu Th17-Zellen. Gleiches geschieht in der Population unspezifischer T-Helferzellen (Cheng et al. 2008, Gisterå et al. 2013). Die proinflammatorische Population der spezifischen T-Helferzellen überwiegt und dominiert mit seinen proatherogene Eigenschaften (Kimura et al. 2018, Wolf et al. 2020). In der Literatur wird außerdem die Umwandlung von Treg-Zellen zu sogenannten ex-Treg-Zellen mit starker Produktion von proinflammatorischen Zytokinen beschrieben (Ali et al. 2020, Saigusa et al. 2020). Wie diese Differenzierungsverschiebung verursacht wird, ist hingegen bisher nicht geklärt. Werden ApoB100-spezifische Treg-Zellen in atherosklerotische Mäuse transferiert, ändern sie ihren Phänotyp zu großen Teilen. Demzufolge verfügen regulatorische T-Zellen über eine erhöhte Plastizität (Tse et al. 2013, Wolf et al. 2020). Diese kann jedoch nicht die gesamte Population proinflammtorischer T-Helferzellen erklären, worauf Abstammungsuntersuchungen von $FOXP3^+$ T-Zellen hindeuten (Wolf und Ley 2019). Folglich muss zusätzlich ein T-Zellpriming, also die erstmalige Differenzierung von naiven T-Zellen zu proinflammatorischen Subtypen existieren. Neben der Ausbildung einer sogenannten immunologischen Synapse zwischen T-Zelle und APC mit kostimulatorischen Signalen ist dabei vor allem das umgebende Zytokinmilieu von entscheidender Bedeutung (Curtsinger et al. 1999).

Während unsere Ergebnisse den Verlust der Treg-Zellantwort untermauern, gelang es nicht Veränderungen der Interferon-γ-Expression, eines der typischen Zytokine der Th1-Zellen, nachzuweisen. Außerdem zeigen sich kaum plastische ApoB100-spezifische $CD4^+$ Zellen im Menschen, die Kennzeichen unterschiedlicher T-Helferzellsubtypen gleichzeitig ausprägen, obwohl zuvor publizierte Daten die Population $FOXP3^+$IL-$17A^+$ bzw. $FOXP3^+$ Ror-γ-T^+ Zellen und deren Expansion in der Atherosklerose hervorheben (Kimura et al.

2018, Mailer et al. 2015, Mailer et al. 2017a, Wolf et al. 2020). In unserem Modell des transienten Leberschadens der Maus ist dieser T-Helferzellphänotyp jedoch nachweisbar.

Damit liefern die Ergebnisse dieser Arbeit eine mögliche Ursache für das Kippen der ApoB100-spezifischen T-Zellantwort in Richtung Inflammation und Atherosklerose und zugleich einen immunologischen Zusammenhang von NAFLD und CVD. Die Leber gewinnt damit zunehmend an Bedeutung als Ort der systemischen Toleranzinduktion bzw. des Toleranzverlusts im Kontext der Atherosklerose.

Das intrahepatische BSEP-Vorkommen nimmt mit zunehmender Leberschädigung, gemessen am NAFLD-Aktivitätsscore, ab (Stindt et al. 2016, Okushin et al. 2016). Dies könnte möglicherweise durch die erhöhte Anzahl BSEP-spezifischer IL-17A^{+} T-Zellen in NAFLD-Patienten erklärt werden. Eine autoreaktive IL17-A-gesteuerte Entzündungsreaktionen mit Zelluntergang von BSEP-exprimierender Leberzellen ist also denkbar. Dieser Schluss erweitert die Vorstellung der hepatischen Toleranz und dessen Verlust in der Leberverfettungssituation gegenüber weiteren körpereigenen Peptiden. Außerdem unterstreicht der Zusammenhang potentiell proinflammatorischer BSEP-spezifischer T-Zellen mit proinflammatorischen ApoB100-spezifischen T-Zellen den Einfluss des Leberschadens auf die proatherogene T-Zelldifferenzierung.

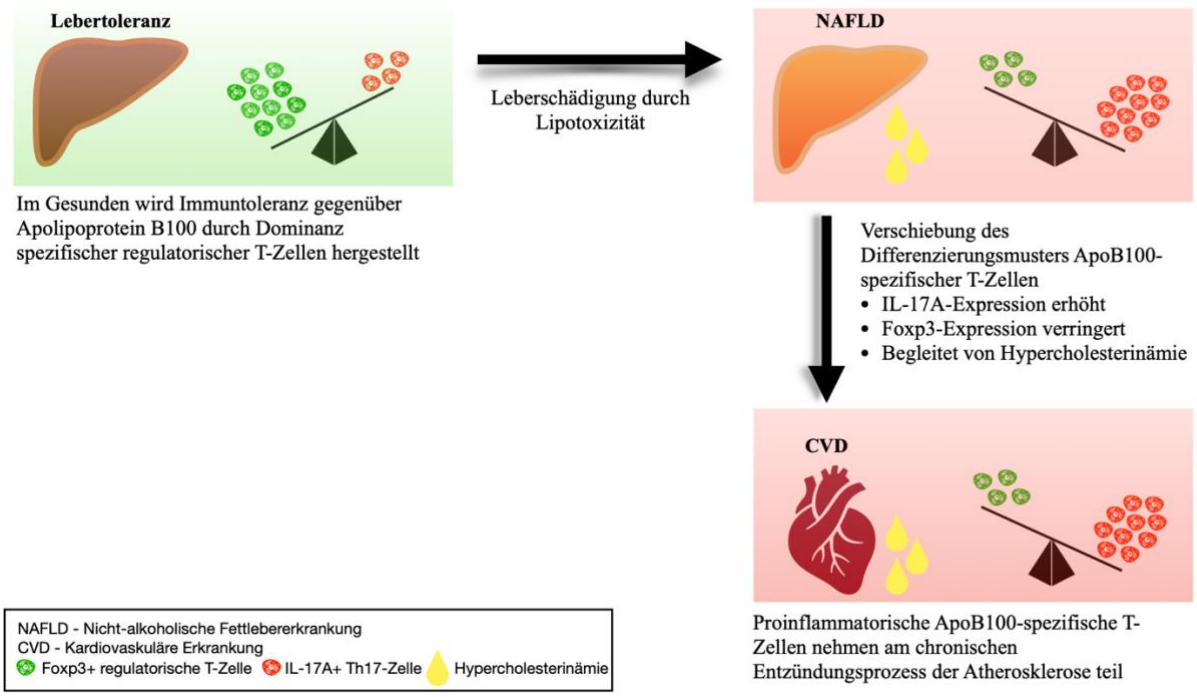
Die in unseren Patienten nachgewiesene Hypercholesterinämie liefert eine mögliche Erklärung für die erhöhte Anzahl ApoB100-spezifischer T-Zellen. Einerseits liegt mehr Autoantigen, also ApoB100, vor. Andererseits ist Cholesterin in der Lage die T-Zellrezeptorfunktion direkt positiv zu beeinflussen, wie vorausgegangene Arbeiten zeigen konnten (Chan DC et al. 2010, Guasti et al. 2016, Mailer et al. 2017b, Lundberg et al. 2017, Proto et al. 2018). Allerdings erfasst das Studiendesign schlussendlich nicht, ob die verstärkte und verschobene ApoB100-spezifische T-Zellantwort ursächlich für NAFLD bzw. CVD ist oder ob nur deren gemeinsame Endstrecke, die Hypercholesterinämie, abgebildet wird (Ketelhuth et al. 2022, Li et al. 2022). Darüber hinaus ist sehr interessant, dass regulatorische T-Zellen selbst Einfluss auf den Lipidstoffwechsel nehmen können (Getz et al. 2014). Die starke Verknüpfung von NAFLD und Herz-Kreislauf-Erkrankungen, insbesondere die Funktionen der Leber als Lipidstoffwechsel- und Immunorgan, machen einen ätiologischen Zusammenhang wahrscheinlich.

Eine Limitation bei dieser Studie ist die Erfassung der T-Zellantwort von lediglich drei ApoB100-Epitopen, die nur eine beschränkte Aussage über die T-Zellantwort aller ApoB100-spezifischen Zellen liefern können. Außerdem zeigt die Restimulation mit Peptid P1 keine T-Zellaktivierung über die Negativkontrolle hinaus, was auf eine reduzierte HLA

II Bindungsaffinität zurückzuführen sein könnte. Weiterhin sind neben ApoB100 weitere Autoantigene im Rahmen der Atherosklerose beteiligt wie etwa Hitzeschockproteine oder β2-Glykoprotein1, die hier keine Beachtung bekommen (Marchini et al. 2021, Rahman et al. 2017).

Nichtsdestotrotz liefert diese Arbeit einen detaillierten Einblick in die spezifische T-Zellantwort auf körpereigenes ApoB100 und unterstreicht die Relevanz weiterer Forschung. Der immunologische Zusammenhang zwischen der häufigsten chronischen Lebererkrankung NAFLD (Lonardo et al. 2016, Targer et al. 2015) und den kardiovaskulären Erkrankungen muss in Zukunft weiter beleuchtet werden, um die Impfstoffentwicklung gegen Atherosklerose oder neue Therapieansätze der NAFLD voranzutreiben (Nilsson et al. 2020 und 2021).

2.5 Schemazeichnung



2.6 Literaturverzeichnis

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3. Zusammenfassung

Das lebereigene Apolipoprotein B100 (ApoB100) ist ein Autoantigen atherogener $CD4^+$ T-Zellen bei kardiovaskulären Erkrankungen. Welche Auswirkungen Leberschäden auf die ApoB100-spezifische T-Zellantwort haben ist jedoch unbekannt. In einem Restimulationsexperiment mit immunogenen ApoB100-Peptiden konnte mithilfe des aktivierungsinduzierten Oberflächenproteins CD154 die Immunantwort des T-Zellrepertoires erfasst und analysiert werden. Dabei wurden die Immunreaktionen von Patienten mit Nicht-alkoholischer Fettlebererkrankung oder kardiovaskulärem Risikoprofil mit Gesunden verglichen. Darüber hinaus wurde ein ApoB100-humanisiertes Mausmodell des transienten Leberschadens auf ApoB100-spezifischer T-Zellreaktionen untersucht. Während wir in Gesunden eine kleine Population ApoB100-spezifischer T-Helferzellen mit überwiegender FOXP3-Expression fanden, zeigten beide Patientenkohorten hingegen eine vergrößerte Population ApoB100-spezifischer T-Zellen mit gesteigerter IL-17A- und verringerter FOXP3-Expression. Diese proinflammatorische Verlagerung des Expressionsmusters korrelierte mit im Blutplasma erhobenem Leberschaden und Dyslipidämie. Der transiente Leberschaden im Mausmodell resultierte in erhöhter IL-17A-Expression der splenischen T-Zellpopulation. Damit liefern unsere Ergebnisse eine immunologische Ursache für den Zusammenhang von Leberschädigung und Herz-Kreislauf-Erkrankungen.

Liver-derived apolipoprotein B100 (ApoB100) is an autoantigen of atherogenic $CD4^+$ T cells in cardiovascular disease. However, the effects of liver injury on the ApoB100-specific T-cell response is still unknown. In a restimulation experiment with immunogenic ApoB100 peptides, the activation-induced surface protein CD154 was used to analyze the immune response of the T cell repertoire. The immune responses of patients with non-alcoholic fatty liver disease or cardiovascular risk profile were compared with healthy individuals. In addition, an ApoB100-humanized mouse model of transient liver injury was examined for ApoB100-specific T cell responses. Whereas in healthy individuals we found a small population of ApoB100-specific T helper cells with predominant FOXP3 expression, both patient cohorts, in contrast, showed an enlarged population of ApoB100-specific T cells with increased IL-17A and decreased FOXP3 expression. This proinflammatory shift in expression pattern correlated with liver damage and dyslipidemia elevated in blood plasma. Transient liver damage in the mouse model resulted in increased IL-17A expression by the splenic T-cell population. Thus, our results provide an immunological cause for the association of liver damage and cardiovascular disease.

4. Erklärung des Eigenanteils

1. Gesamter Prozess der Datenerhebung aller humanen Proben
    a. Koordination und Organisation der Blutentnahme
        i. Akquirierung von Patienten
        ii. Durchführung von Befragung
    b. Durchführung experimenteller Arbeiten
    c. Durchflusszytometrische Analyse
    d. Organisation der Messungen im Zentrallabor
2. Mitwirkung an Analyse der Ergebnisse
3. Mitwirkung an schriftlicher Fassung des Manuskripts

5. Danksagung

Ohne tatkräftige Unterstützung von vielen Seiten hätte die Arbeit in dieser Form nicht gelingen können. Daher möchte ich im Folgenden großen Dank aussprechen.

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6. Lebenslauf

Persönliche Daten

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Humanmedizinstudium — Oktober 2014 - Dezember 2021

am Universitätsklinikum Hamburg-Eppendorf, Abschluss mit der Note „sehr gut“

7. Eidesstattliche Versicherung

Ich versichere ausdrücklich, dass ich die Arbeit selbständig und ohne fremde Hilfe verfasst, andere als die von mir angegebenen Quellen und Hilfsmittel nicht benutzt und die aus den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen einzeln nach Ausgabe (Auflage und Jahr des Erscheinens), Band und Seite des benutzten Werkes kenntlich gemacht habe.

Ferner versichere ich, dass ich die Dissertation bisher nicht einem Fachvertreter an einer anderen Hochschule zur Überprüfung vorgelegt oder mich anderweitig um Zulassung zur Promotion beworben habe.

Ich erkläre mich einverstanden, dass meine Dissertation vom Dekanat der Medizinischen Fakultät mit einer gängigen Software zur Erkennung von Plagiaten überprüft werden kann.

Unterschrift: .....