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Progression from Nonalcoholic Fatty Liver to Nonalcoholic Steatohepatitis Is Marked by a Higher Frequency of Th17 Cells in the Liver and an Increased Th17/Resting Regulatory T Cell Ratio in Peripheral Blood and in the Liver

Monika Rau,* Anne-Kristin Schilling,* Jan Meertens,* Ilona Hering,* Johannes Weiss,* Christian Jurowich,[†] Theodor Kudlich,* Heike M. Hermanns,* Heike Bantel,[‡] Niklas Beyersdorf,[§] and Andreas Geier*

Nonalcoholic fatty liver disease is increasing in prevalence. It can be subdivided into nonalcoholic fatty liver (NAFL) and nonalcoholic steatohepatitis (NASH). Five to twenty percent of cases progress from NAFL to NASH. Increased hepatic Th17 cells and IL-17 expression were observed in NASH mice and patients, respectively. We analyzed CD4⁺ effector T cells and regulatory T cells (Tregs) from peripheral blood and livers of NAFL and NASH patients. A total of 51 NAFL patients, 30 NASH patients, 31 nonalcoholic fatty liver disease patients (without histology), and 43 healthy controls were included. FACS analysis was performed on PBMCs and intrahepatic lymphocytes. Compared with healthy controls, a lower frequency of resting Tregs (rTregs; CD4⁺ CD45RA⁺ CD25⁺) and higher frequencies of IFN- γ ⁺ and/or IL-4⁺ cells were detected among CD4⁺ T cells of peripheral blood in NASH, and to a lesser degree in NAFL. In hepatic tissue, NAFL to NASH progression was marked by an increase in IL-17⁺ cells among intrahepatic CD4⁺ T cells. To define immunological parameters in peripheral blood to distinguish NAFL from NASH, we calculated different ratios. Th17/rTreg and Th2/rTreg ratios were significantly increased in NASH versus NAFL. The relevance of our findings for NASH pathogenesis was highlighted by the normalization of all of the changes 1 y after bariatric surgery. In conclusion, our data indicate that NAFL patients show changes in their immune cell profile compared with healthy controls. NAFL to NASH progression is marked by an increased frequency of IL-17⁺ cells among intrahepatic CD4⁺ T cells and higher Th17/rTreg and Th2/rTreg ratios in peripheral blood. *The Journal of Immunology*, 2016, 196: 97–105.

Nonalcoholic fatty liver disease (NAFLD) is defined by an accumulation of liver fat exceeding 5% of its weight in the absence of significant alcoholic intake. The prevalence of NAFLD ranges from 20 and 35% in Europe and is up to 50% in the United States (1). In >50% of cases, patients with NAFLD are obese. Five to twenty percent of patients with nonalcoholic fatty liver (NAFL) progress to nonalcoholic steatohepatitis (NASH) (1). Until now, it is not well understood why only

some patients develop NASH. The pathogenesis of NAFLD is commonly explained by a “two-hit” hypothesis (2). The first hit leading to steatosis is favored by factors characterizing the metabolic syndrome (e.g., insulin resistance, obesity, hyperlipidemia). The second hit, initiated by bacterial translocation or elevated levels of pathogen-associated molecular patterns in the portal vein, leads to an inflammation in the fatty liver resulting in steatohepatitis (2).

Different T cell populations, such as regulatory T cells (Tregs), Th1 cells, and Th17 cells play a central role in the immunopathogenesis of fatty liver disease (3). Th17 cells are Th cells characterized by the secretion of various cytokines, such as IL-17 (IL-17A, IL-17F), IL-21, IL-22, and TNF- α (4). These cytokines lead to tissue inflammation and recruitment of leukocytes via the induction of neutrophil-attracting chemokines (CXCL1, CXCL2, or CXCL8) (5). For the differentiation of Th17 cells, the combined actions of TGF- β , IL-6, IL-21, IL-23, and IL-1 β are needed, whereas TGF- β alone induces Tregs instead of Th17 cells (6, 7). Tregs control effector T cell responses. Thus, Tregs and Th17 cells reflect two poles of the immune response (i.e., tolerance and inflammation) and, therefore, need to be balanced.

The inflammatory process underlying NASH is characterized by elevated expression of inflammatory cytokines, such as TNF- α and IL-6. A recent study showed hepatic Th17 cell infiltration in NASH patients (8). The investigators also assessed the influence of IL-17 in a mouse model in which IL-17 neutralization attenuated LPS-induced liver injury as a “second hit.” In vitro experiments using the HepG2 cell line indicated the induction of steatosis and IL-6 production by IL-17 in combination with free

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Abbreviations used in this article: aTreg, activated Treg; AUROC, area under ROC; CI, confidence interval; CK-18, cytokeratin-18 fragment M30; FU, follow-up; GGT, γ -glutamyl transferase; GOT, glutamic oxaloacetic transaminase; GPT, glutamic pyruvate transaminase; HC, healthy control; NAFL, nonalcoholic fatty liver; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; ROC, receiver operating characteristic; rTreg, resting Treg; Treg, regulatory T cell; ULN, upper limit of normal.

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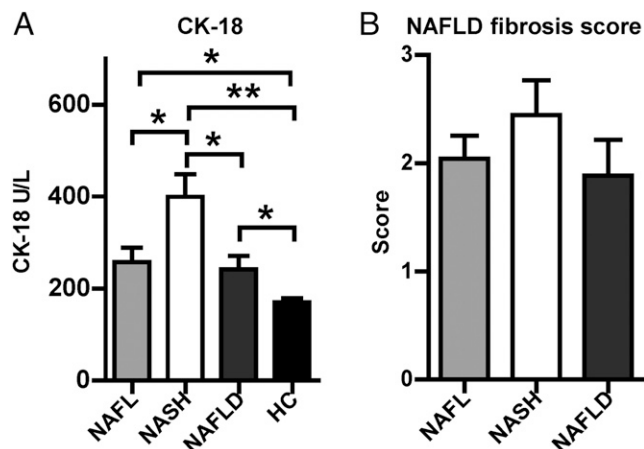


FIGURE 1. Parameters of noninvasive risk assessment. **(A)** CK-18 serum levels in patients with NAFL ($n = 49$), NASH ($n = 25$), or NAFLD (no available liver histology, $n = 28$) and in HCs ($n = 42$). Data are mean \pm SEM. **(B)** NAFLD fibrosis score in patients with NAFL ($n = 46$), NASH $n = 28$, or NAFLD $n = 29$; * $p < 0.05$, ** $p < 0.001$, t test.

fatty acids (8). IL-17A^{-/-} mice were resistant to the development of steatohepatitis, whereas wild-type mice showed progression from NAFL to NASH via induction of the IL-17 axis (9). Furthermore, IL-17 can induce the expression of neutrophil-attracting chemokines (e.g., CXCL1, CXCL2), and neutrophil and lymphocyte infiltration is characteristic of liver samples from NASH patients (10).

Taken together, the current data suggest that Th17 cells play an important role in inflammatory processes characterizing NASH. Therefore, it can be hypothesized that the progression from NAFL to NASH emerges on the background of a changed Treg/Th17 balance.

In this study, T cells in peripheral blood and hepatic tissue were characterized in patients with NAFLD. We focused on changes in effector T cells and Tregs to differentiate patients with NAFL and NASH.

Materials and Methods

Patient characteristics

In this prospective study, 112 patients with NAFLD were included after approval by the local ethics committee and informed consent. Eighty-one patients had histology-proven NAFL or NASH, and 31 patients were diagnosed with NAFLD by typical ultrasound findings, as described by sonographic NASH score (11). Patients without histology-proven NAFL or NASH were classified as having NAFLD throughout the study and were included in cytokeratin-18 fragment M30 (CK-18) serum analysis. A total of 54.8% of these NAFLD patients (without histology) had glutamic pyruvate transaminase (GPT) levels > 0.8 upper limit of normal (ULN), and 35.5% had glutamic oxaloacetic transaminase (GOT) > 0.8 ULN. Ninety-seven of all fatty liver patients ($n = 112$) were morbidly obese and had an evaluation of liver function before bariatric surgery. Fifteen obese patients were included without bariatric surgery. All patients were > 18 y old. Other

liver diseases were excluded by hepatitis B surface Ag, anti-hepatitis C virus, and antinuclear Ab. A questionnaire was answered upon enrollment in the study; patients with significant alcohol consumption (women > 20 g/d and men > 30 g/d) were excluded. A total of 78 patients had subcapsular liver biopsies during bariatric surgery, and 3 patients had percutaneous liver biopsy. Liver samples were diagnosed by an experienced pathologist and classified using the NASH activity score (12). In patients with available liver histology, 51 patients had NAFL, and 30 patients had NASH. A total of 43 healthy controls (HCs) was included. All control subjects had no evidence or history of liver pathology. Furthermore, liver ultrasound was without pathologies, and there was no elevated liver stiffness, as determined by transient elastography measurement (FibroScan). Laboratory analysis showed no elevation of GOT or GPT. NAFLD fibrosis risk score was calculated as a well-established noninvasive risk score according to Angulo et al. (13).

NASH patients were seen in a follow-up (FU) visit ≥ 12 mo after bariatric surgery to evaluate liver function by standard laboratory test and ultrasound and to analyze peripheral immune cells. Fourteen NASH patients were included in the FU study.

Cell isolation and preparation

Isolation of lymphocytes and PBMCs. Isolation of lymphocytes and PBMCs was performed by Lymphocyte Separation Medium (PAA) ex vivo on an individual basis. Cells were counted upon trypan blue staining and used in aliquots of 1×10^6 cells.

Isolation of intrahepatic mononuclear cells. Immediately after surgery and until processing on the same day, liver biopsy tissue was stored at room temperature in a centrifugation tube containing 10 ml RPMI 1640 medium. Liver tissue was cut in fragments of 1–2 mm length and gently passed through a cell strainer (70 μ m; BD). Intrahepatic mononuclear cells were separated using Lymphocyte Separation Medium (PAA). Cells were counted upon trypan blue staining and used in aliquots of $3\text{--}5 \times 10^4$ cells.

Cytokine stimulation and FACS analysis

Cytokine stimulation. A total of $3\text{--}5 \times 10^4$ intrahepatic mononuclear cells or 1×10^6 PBMCs was plated in a total volume of 1 ml RPMI 1640 + L-glutamine (PAA) (supplemented with 1 mM sodium pyruvate, $1 \times$ non-essential amino acids, 10 mM HEPES, $1 \times$ penicillin/streptomycin, 50 μ M 2-ME, and 10% human serum [clot type AB]) into separate wells of a 48-well plate. Three wells/sample were stimulated for 4 h at 37°C and 5% CO₂ with 10 ng/ml PMA (Calbiochem), 500 ng/ml ionomycin (Sigma-Aldrich), and $1 \times$ brefeldin A (BioLegend). After stimulation, 1 ml cell suspension was transferred to a 6-ml FACS tube and centrifuged at $500 \times g$ for 3 min. The sediment was resuspended in the remaining medium and transferred to wells of a 96-well V-bottom plate and centrifuged at $500 \times g$ for 3 min.

FACS analysis. After blocking with 20 μ g/ml normal mouse Ig (Sigma-Aldrich) for 15 min on ice, all cells underwent cell surface staining, followed by intracellular staining. The cells were treated with Foxp3/Transcription Factor Fixation/Permeabilization solution, according to the manufacturer's instructions (eBioscience), to stabilize the surface staining and permit intracellular staining through permeabilization of the cell membrane.

Anti-CD45RA-FITC, anti-HLA-DR-PE/Cy7, anti-CD4-Alexa Fluor 700, anti-CD8-allophycocyanin/Cy7, anti-CD25-BV421, anti-CD3-BV510, anti-Foxp3-Alexa Fluor 647, anti-CD4-allophycocyanin/Cy7, anti-Foxp3-Alexa Fluor 488, anti-IL-4-PE, anti-IFN- γ -PE/Cy7, anti-IL-17-BV421, isotype control MOPC-21-Alexa Fluor 647, MOPC-21-BV421, and MOPC-21-PE/Cy7 Abs were obtained from BioLegend. All Abs were used according to the manufacturer's instructions. FACS analyses were performed using an LSR II flow cytometer (Becton Dickinson,

Table I. Subject characteristics

	NAFL ($n = 51$)	NASH ($n = 30$)	NAFLD ($n = 31$) ^a	HC ($n = 43$)
Sex (male/female; n)	12/39	8/22	14/17	10/33
Age (y; mean \pm SD)	44.4 \pm 11.9	52.4 \pm 10.0	47.1 \pm 11.9	27.0 \pm 4.3
BMI (kg/m ² ; mean \pm SD)	51.7 \pm 9.0	49.8 \pm 8.7	46.3 \pm 11.6	22.1 \pm 4.5
Hypertension (% [n])	60.8 (31)	80.0 (24)	80.6 (25)	4.7 (2)
Diabetes mellitus (% [n])	31.4 (16)	56.7 (17)	29.0 (9)	0 (0)
Hyperlipidemia (% [n])	35.3 (18)	33.3 (10)	61.3 (19)	0 (0)

Characteristics of all patients and HCs in the cohort.

^a31 patients with NAFLD had no liver histology, but diagnosis was made by typical ultrasound findings according to a sonographic NASH score.

BMI, body mass index.

Table II. Laboratory findings in patients with NAFL or NASH

	NAFL (n = 51)	NASH (n = 30)
Elevated GPT (% [n])	27.5 (14)	73.3 (22)
Elevated GOT (% [n])	13.7 (7)	43.3 (13)
Elevated alkaline phosphatase (% [n])	9.8 (5)	23.3 (7)
Elevated GGT (% [n])	23.5 (12)	56.7 (17)
CK-18 (U/l; mean \pm SD)	257.8 \pm 226.0	399.3 \pm 244.1
Fibrosis stage (% [n])		
F0	52.1 (25)	27.6 (8)
F1	47.9 (23)	34.5 (10)
F2	0 (0)	17.2 (5)
F3	0 (0)	3.4 (1)
F4	0 (0)	17.2 (5)
Liver immune cells analysis	n = 35	n = 18

The data of fibrosis stage for NAFL and NASH total $n = 48$ and $n = 29$, respectively. In four patients, detailed fibrosis stage could not be sufficiently assessed due to subcapsular liver biopsies.

San Diego, CA), and the data were analyzed with FlowJo software (TreeStar, Ashland, OR).

CK-18 measurement

The apoptosis-associated neopeptide CK-18 was measured using the M30-Apoptosense ELISA, according to the manufacturer's instructions (Peviva, Bromma, Sweden) and as described (14). Patients were stratified into groups with low, moderately elevated, and high CK-18 serum levels (<157 U/l, 157–205 U/l, and >205 U/l, respectively). Cut-off levels for stratification of patients were chosen based on a recent publication that analyzed the diagnostic accuracy of CK-18 (15).

Statistical analyses were performed with SPSS (19.0, SPSS; Chicago, IL), and graphs were created with Prism5 (GraphPad, La Jolla, CA). Clinical parameters were compared using a χ^2 test. Differences in cell frequency were analyzed with an unpaired, two-sided t test, as appropriate. A paired t test was used for analysis of FU patients. The p values <0.05 were considered statistically significant. To evaluate the diagnostic accuracy of CK-18, Th17/resting Treg (rTreg) ratio and combined CK-18 immune score receiver operating characteristic (ROC) curve with area under ROC (AUROC) were calculated using SPSS.

Statistical analysis

Statistical analyses were performed with SPSS (19.0, SPSS; Chicago, IL), and graphs were created with Prism5 (GraphPad, La Jolla, CA). Clinical parameters were compared using a χ^2 test. Differences in cell frequency were analyzed with an unpaired, two-sided t test, as appropriate. A paired t test was used for analysis of FU patients. The p values <0.05 were considered statistically significant. To evaluate the diagnostic accuracy of CK-18, Th17/resting Treg (rTreg) ratio and combined CK-18 immune score receiver operating characteristic (ROC) curve with area under ROC (AUROC) were calculated using SPSS.

Results

Cohort characteristics

A total of 112 patients with NAFLD was included in this study, with characterization of the peripheral immune cell profile: 51 patients had histology-proven NAFL, and 30 patients had NASH. No liver histology was available for 31 patients; therefore, the samples were characterized as NAFLD, and the term NAFLD is used throughout the article. The latter patients were included in the CK-18 analysis and immune cell analysis, according to CK-18 levels (Fig. 1). In all groups, female patients exceeded the number of male patients. HCs were younger than NAFL patients, as seen also in other human studies (16, 17). In Germany, NAFLD has a high prevalence (~ 20 – 35%), with an increase in elderly people who also have a higher frequency of metabolic disorders (1). This difference in

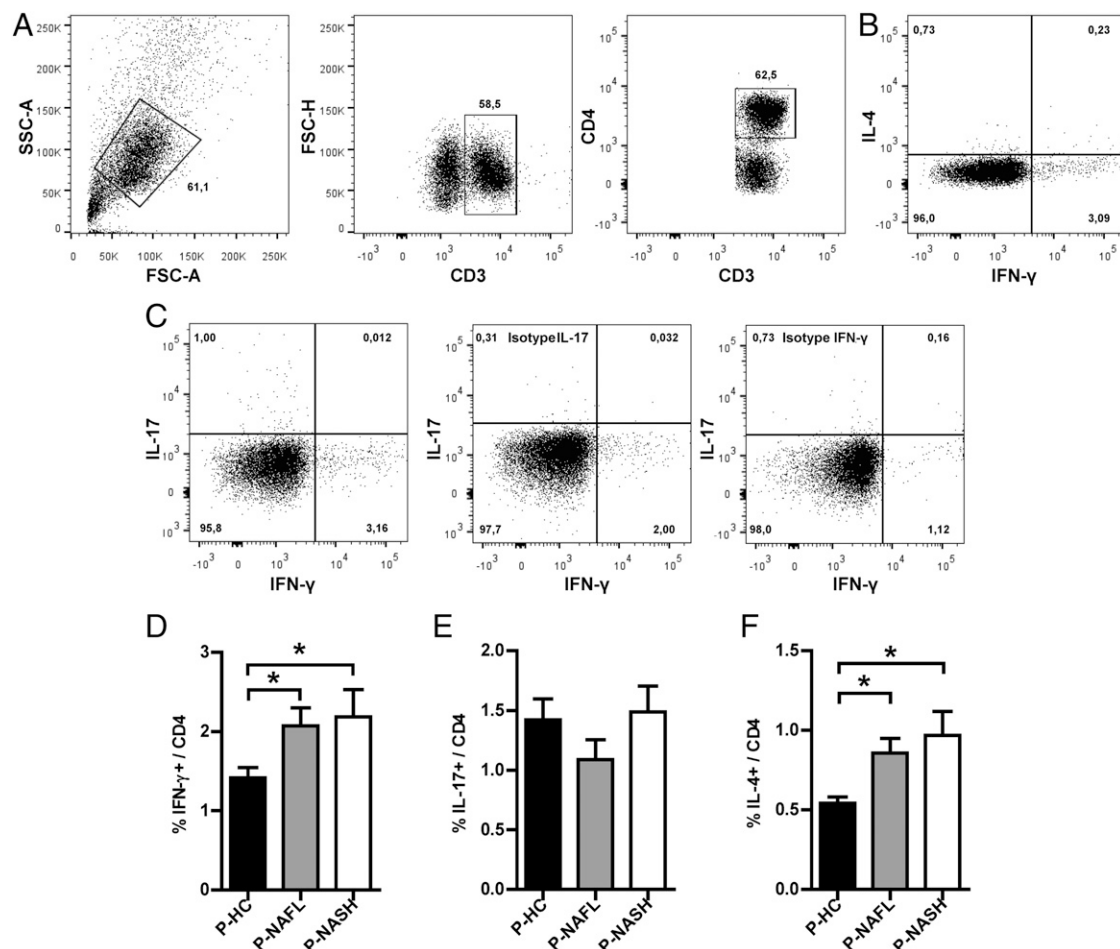


FIGURE 2. T effector cells in peripheral blood. (A) Representative gating scheme for analysis of T effector cells. (B) Representative staining of peripheral CD3 $^{+}$ /CD4 $^{+}$ cells for IL-4 and IFN- γ . (C) Representative staining of peripheral CD3 $^{+}$ /CD4 $^{+}$ cells for IL-17 and IFN- γ with isotype for IL-17 and isotype for IFN- γ . Frequency of IFN- γ -producing cells (D), IL-17-producing cells (E), and IL-4-producing cells (F) among CD4 $^{+}$ T cells of patients with NAFL ($n = 51$) or NASH ($n = 30$) and HCs ($n = 43$). Data are mean \pm SEM. * $p < 0.05$, t test. P-, peripheral blood.

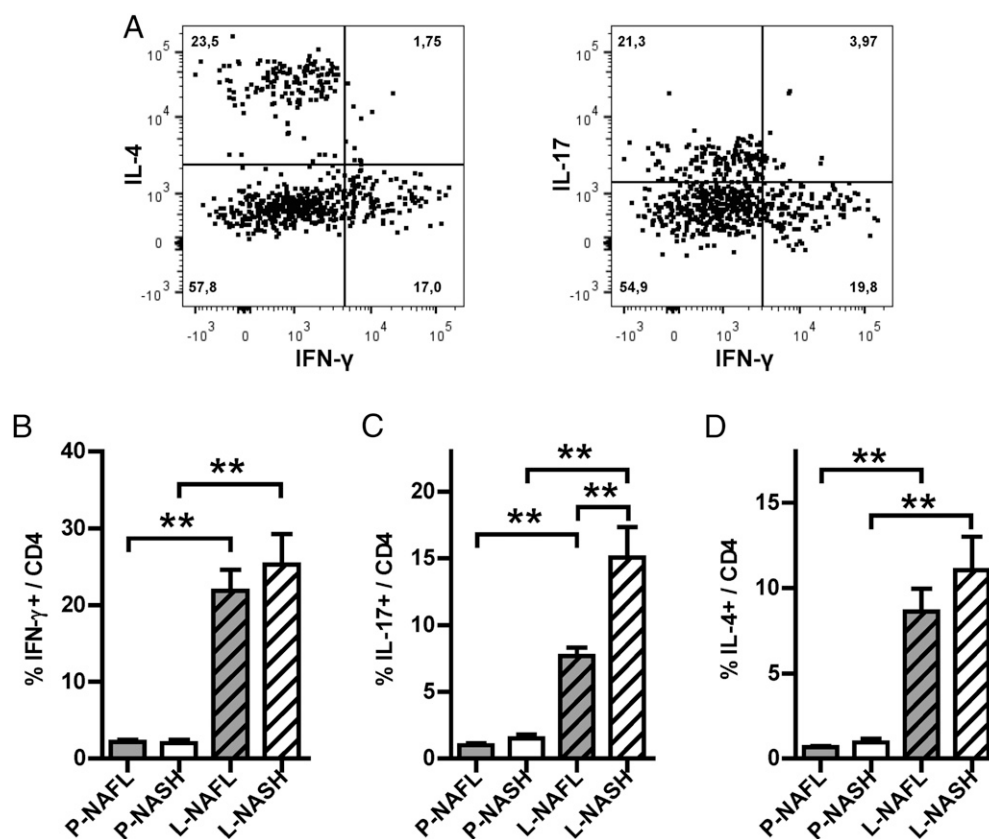


FIGURE 3. T effector cells in hepatic tissue compared with peripheral blood. (A) Representative IL-4, IL-17, and IFN- γ staining of hepatic CD3⁺/CD4⁺ cells. Frequencies of IFN- γ -producing cells (B), IL-17-producing cells (C), and IL-4-producing cells (D) among CD4⁺ T cells in peripheral blood and hepatic tissue in patients with NAFL ($n = 35$) or NASH ($n = 18$). Only patients with simultaneous quantification of peripheral and intrahepatic lymphocytes are included. Data are mean \pm SEM. ** $p < 0.001$, t test. L-, intrahepatic lymphocyte; P-, PBMC.

age was accepted because our study focused on differences between patients with NAFL and NASH. Patients had a significantly higher body mass index compared with HCs. In the NASH group, more patients had metabolic syndrome, with hypertension and diabetes mellitus, compared with the other patient groups (Table I). More patients with NASH had elevated laboratory parameters, such as GOT and GPT, as well as higher levels of alkaline phosphatase and γ -glutamyl transferase (GGT). Liver fibrosis stage was aggravated in the NASH group (Table II).

CK-18 was measured in the serum of patients as an established noninvasive marker for liver fibrosis and the presence of NASH in patients with fatty liver (15). Patients with histology-proven NAFL or NASH, as well as patients with NAFLD (without liver histology), had significantly higher CK-18 serum levels compared with HCs (Fig. 1A). NASH patients displayed the highest CK-18 serum levels, which were significantly higher than those of NAFL and NAFLD patients. Patients with a high histological NASH activity score (>4 points) had significantly higher CK-18 serum levels compared with patients whose NASH activity score was 1 or 2 points.

The NAFLD fibrosis score was used as another noninvasive tool to identify patients at risk. NASH patients had a trend toward higher NAFLD fibrosis scores compared with patients with NAFL and NAFLD, but the difference did not reach statistical significance (Fig. 1B).

Increased frequency of Th17 cells among intrahepatic CD4⁺ T cells in patients with NASH compared with NAFL

CD4⁺ effector T cells were more frequent in peripheral blood of patients. The frequencies of IL-4⁺ and/or IFN- γ ⁺ cells among

CD4⁺ T cells were significantly elevated in patients with NAFL and NASH in comparison with HCs (Fig. 2A–D, 2F). No significant change in IL-17-producing CD4⁺ T cells was observed in peripheral blood between HCs and patients (Fig. 2A, 2C, 2E).

Analysis of intrahepatic lymphocytes showed a pronounced increase in hepatic CD4⁺ effector T cells in comparison with peripheral blood. Liver specimens from HCs were not available in our study, and no in-depth FACS characterization of T cells from HCs has been carried out. Patients with NAFL and NASH had significantly higher frequencies of intrahepatic IL-17⁺, IL-4⁺, and IFN- γ -producing T cells compared with peripheral blood (Fig. 3). The greatest difference between intrahepatic and peripheral T cells was seen for the frequency of IFN- γ ⁺ cells among CD4⁺ T cells both for NAFL and NASH (Fig. 3B). However, IL-17⁺ cells were 1.5-fold more frequent in the liver of patients with NASH in comparison with hepatic tissue from patients with NAFL (Fig. 3C). Therefore, patients with NASH and NAFL could be differentiated in our cohort by the prevalence of IL-17⁺ cells among intrahepatic CD4⁺ T cells.

The ratio of Th17 cells/rTregs reflects disease progression in blood and liver

To characterize Tregs, PBMCs and intrahepatic lymphocytes were stained for CD3, CD4, CD45RA, and CD25 expression. Fig. 4A and 4B show typical staining profiles of CD45RA versus CD25 for CD3⁺CD4⁺ T cells of peripheral blood and liver, respectively. Activated Tregs (aTregs; CD4⁺CD45RA⁺CD25⁺) and rTregs (CD4⁺CD45RA⁺CD25⁺), two subsets of Tregs with suppressive function, were analyzed in more detail (18). In peripheral blood of

patients with NAFL and NASH, a significantly lower frequency of rTregs was observed among CD4⁺ T cells in comparison with HCs (Fig. 4C), suggesting increased turnover/consumption of Tregs in patients as a result of increased activation of the naive Tregs (18). NASH patients had an even lower frequency of rTregs than did NAFL patients ($p = 0.05$). aTregs (CD4⁺CD45RA⁻CD25⁺⁺⁺) were more frequent in patients with NAFL and, by trend, in patients with NASH, compared with HCs (Fig. 4D). Similarly, in hepatic tissue from NAFL and NASH patients, a lower frequency of rTregs and a trend toward a higher frequency of aTregs among intrahepatic CD4⁺ T cells were observed (Fig. 4E, 4F). Neither finding was statistically significant. The analysis of these two subsets of Tregs was restricted to patients from whom we could measure >500 intrahepatic CD4⁺ T cells.

Based on our study hypothesis that an imbalance between Th17 cells and Tregs drives liver pathology and in search of an immunological marker differentiating NAFL from NASH, the ratio of Th17/rTregs was calculated. Using this ratio, significant differences were observed in the peripheral blood and liver of patients with NAFL and NASH (Fig. 5A). To assess whether other ratios of T effector cells and rTregs reflected disease progression, we calculated the ratios of Th1/rTregs and Th2/rTregs. The Th2/rTreg ratio (IL-4⁺/rTregs) showed a significant difference in peripheral blood between patients with NAFL or NASH and HCs, but no significant difference was seen with regard to the liver. Using the Th1/rTreg ratio (IFN- γ /rTregs) in blood and liver, it was not possible to differentiate NAFL and NASH patients (Fig. 5B, 5C). Although the Th17/rTreg and Th2/rTreg ratios were increased in peripheral blood of NASH versus NAFL patients, this was only the case for the Th17/rTreg ratio in the liver. This supports our hypothesis that the balance between Th17 cells and rTregs plays a central role in NASH pathogenesis.

HLA-DR expression shows recent activation of intrahepatic CD4⁺ T cells

To determine whether the liver-infiltrating T cells are recently activated cells, we stained for HLA-DR expression (19). We detected a slightly higher frequency of HLA-DR⁺ cells among CD4⁺ T cells in peripheral blood of patients compared with HCs (Fig. 5D, 5E). In livers from both patient groups, up to 44% of CD4⁺ T cells expressed the activation marker HLA-DR (Fig. 5F). Therefore, the effector CD4⁺ T cells found in the livers of NAFL and NASH patients most likely contribute actively to pathogenesis in situ.

Immune cell changes according to CK-18 serum levels

To further analyze our findings with regard to peripheral immune cell changes for the entire study cohort (i.e., including the patients with NAFLD [without liver histology] and HCs), all subjects were stratified based on CK-18 serum levels. The cohort was subdivided into three groups with low, moderately elevated, and high CK-18 serum levels. In patients with moderately elevated (157–205 U/l) and high (>205 U/l) CK-18 serum levels reflecting increased necroinflammatory activity, a significantly reduced frequency of rTregs among CD4⁺ T cells was measured in peripheral blood (Fig. 6A), whereas a trend toward a higher frequency of IL-17⁺ cells among CD4⁺ T cells was observed (Fig. 6B). In addition, a higher Th17/rTreg ratio was associated with higher CK-18 serum levels (i.e., patients with the highest CK-18 levels had a significantly higher Th17/rTreg ratio in comparison with patients with low CK-18 levels) (Fig. 6C). No significant association between CK-18 levels and Th1/rTreg or Th2/rTreg ratio was observed (Supplemental Fig. 1). Therefore, in this analysis spanning the entire study cohort, the Th17/rTreg ratio also correlated positively with disease severity, as determined by CK-18 serum concentrations.

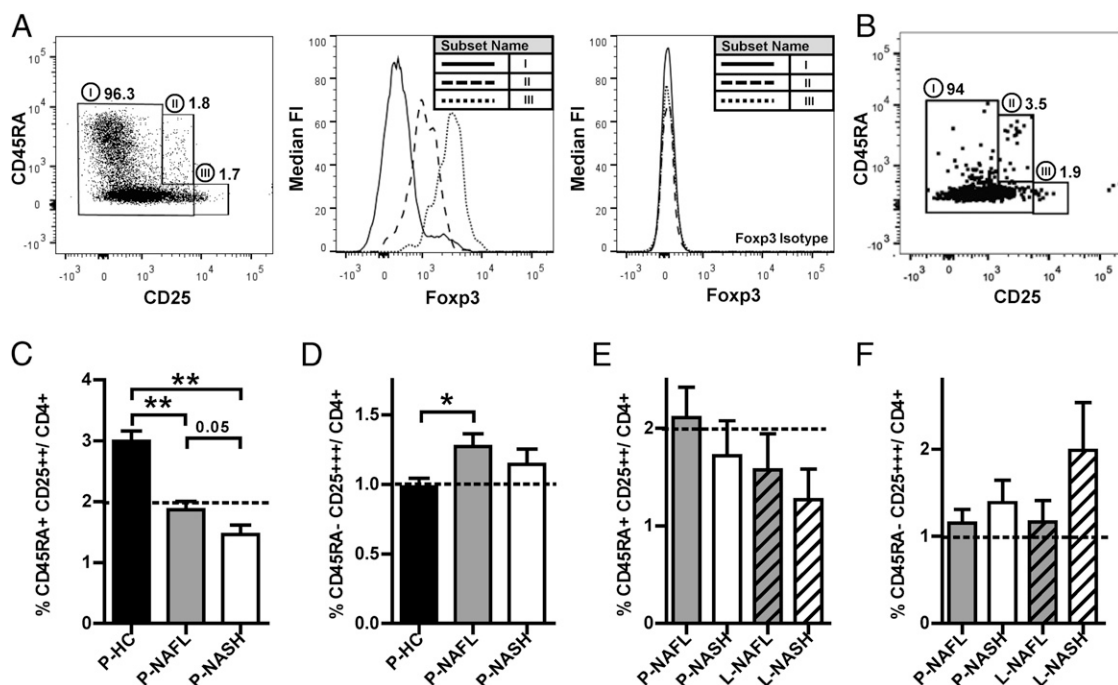


FIGURE 4. Resting and activated Tregs in peripheral blood and hepatic tissue. (A) FACS plot showing CD45RA and CD25 expression among CD3⁺CD4⁺ cells of peripheral blood. Expression of Foxp3 staining (middle panel) and isotype-control staining (right panel) for rTregs (II) and aTregs (III) and the remaining conventional CD4⁺ T cells (I). (B) CD45RA and CD25 expression profile of intrahepatic CD3⁺CD4⁺ cells. Summary of CD4⁺CD45RA⁺CD25⁺⁺ (rTregs) (C) and CD4⁺CD45RA⁻CD25⁺⁺⁺ (aTregs) (D) frequencies among CD4⁺ T cells from peripheral blood (P-NAFL, $n = 45$; P-NASH, $n = 29$; P-HC, $n = 42$). Frequencies of rTregs (E) and aTregs (F) in hepatic tissue (L-NAFL and L-NASH) compared with PBMCs. (C–F) Dashed lines were added to facilitate the comparison between peripheral blood and hepatic tissue [2% for (C) and (E) as well as 1% for (D) and (F)]. Only patients with simultaneous analysis of peripheral and intrahepatic lymphocytes were included (P-NAFL, $n = 15$; P-NASH, $n = 8$; L-NAFL, $n = 15$; L-NASH, $n = 8$). Data are mean \pm SEM. * $p < 0.05$, ** $p < 0.001$, t test. L-, hepatic tissue; P-, peripheral blood.

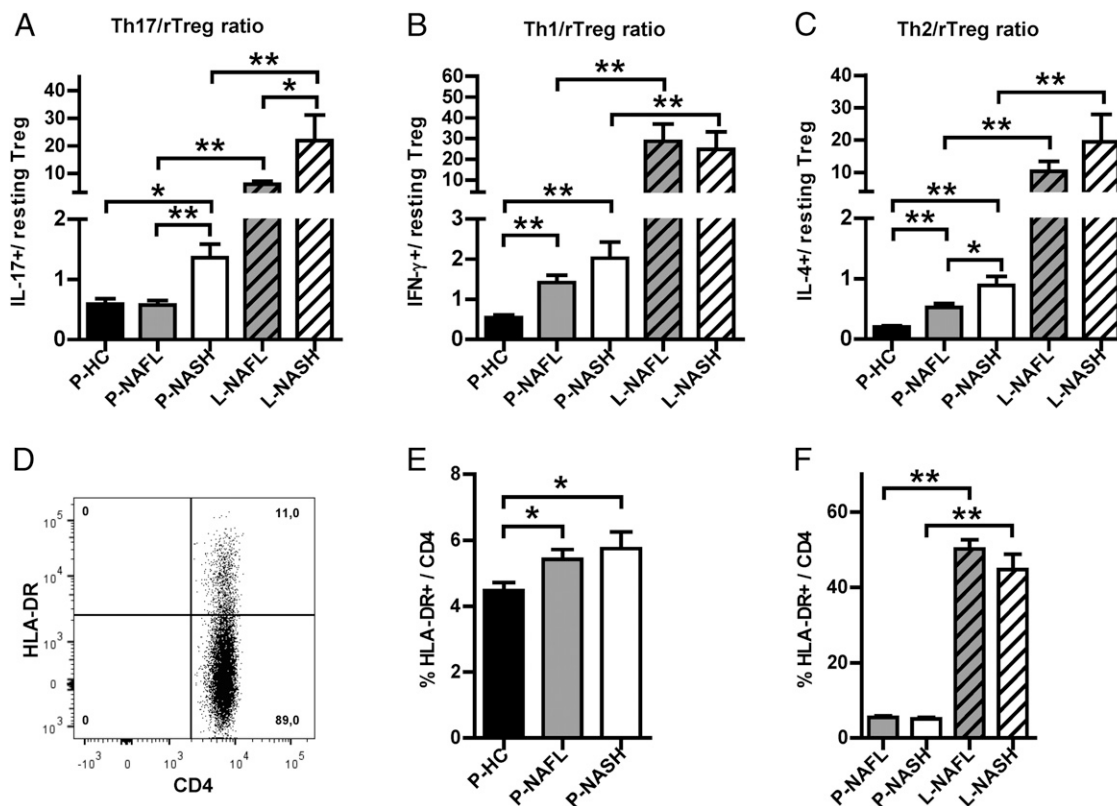


FIGURE 5. Th17/rTreg, Th1/rTreg, and Th2/rTreg ratios and HLA-DR expression on T cells. Th17/rTreg ratio (A), Th1/rTreg ratio (B), and Th2/rTreg ratio (C) in PBMCs and hepatic tissue of patients and HCs (P-HC, $n = 42$; P-NAFL, $n = 45$; P-NASH, $n = 29$; L-NAFL, $n = 15$; L-NASH, $n = 8$). (D) FACS plot of representative HLA-DR and CD4 staining of CD3⁺ cells in peripheral blood. (E) Frequency of CD4⁺HLA-DR⁺ T cells in peripheral blood of HCs and patients with NAFL or NASH. (F) CD4⁺HLA-DR⁺ T cells in PBMCs and hepatic tissue in patients with NAFL or NASH with simultaneous analysis of both lymphocyte compartments (P-NAFL, $n = 34$; P-NASH, $n = 18$; L-NAFL, $n = 34$; L-NASH, $n = 18$). Data are mean \pm SEM. * $p < 0.05$, ** $p < 0.001$, t test. L-, hepatic tissue; P-, peripheral blood/PBMC.

Diagnostic accuracy of CK-18 serum levels, Th17/rTreg, Th1/rTreg, and Th2/rTreg ratios, and a new combined CK-18 immune score

To evaluate the diagnostic accuracy of the identified parameters for NASH, ROC curve analyses were performed for Th17/rTreg, Th1/rTreg, and Th2/rTreg ratios in comparison with CK-18 serum levels. The Th17/rTreg ratio showed an AUROC of 0.68 (confidence interval [CI]: 0.58–0.78, $p < 0.01$) (Fig. 7A). A similar AUROC was observed for Th2/rTreg ratio (0.68 [CI: 0.57–0.78], $p < 0.01$), whereas the AUROC for Th1/rTreg ratio was 0.62 (CI: 0.5–0.7, $p = 0.05$) (Supplemental Fig. 2). The best AUROC for the diagnosis of NASH was seen for CK-18 serum levels (0.78 [CI:

0.68–0.88], $p < 0.001$) (Fig. 7B). To combine our findings of T effector ratios and CK-18 levels, a CK-18 immune score was calculated. The Th17/rTreg ratio was used for this score because it is the only one showing significant differences in both PBMCs and liver cells of patients with NAFL and NASH and a significant positive correlation with higher CK-18 serum levels. A CK-18 immune score was calculated as follows: $(\text{Th17/rTreg})^4 + \text{CK-18}$. This score provided an even better AUROC of 0.79 (CI: 0.68–0.89, $p < 0.001$) compared with CK-18 alone (Fig. 7C). To confirm these findings, the AUROC was calculated for NASH activity score > 4 points, with the highest result for CK-18 immune score (0.77 [CI: 0.64–0.90], $p < 0.01$) compared with CK-

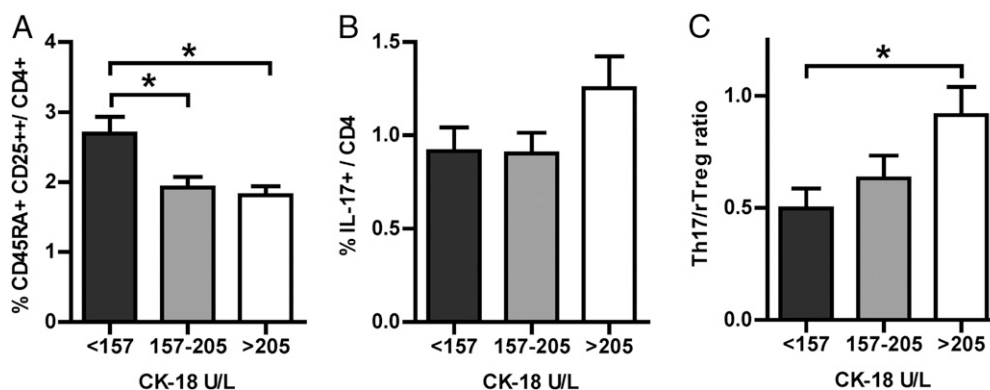


FIGURE 6. Immune cell profiles according to CK-18 serum level. The total cohort (NAFL, NASH, NAFLD without liver histology, and HC) was classified based on CK-18 serum levels. Frequencies of CD45RA⁺CD25⁺ (rTregs) (A) and IL17⁺ cells (B) among CD4⁺ T cells based on CK-18 serum levels. (C) Th17/rTreg ratio after CK-18 stratification (<157 U/L, $n = 34$; 157–205 U/L, $n = 43$; >205 U/L, $n = 67$). Data are mean \pm SEM. * $p < 0.05$, t test.

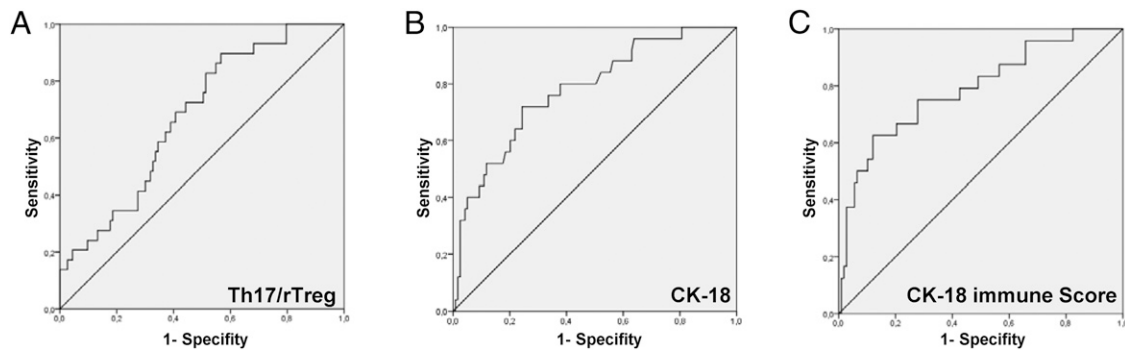


FIGURE 7. ROC curves for NASH diagnosis. ROC curve for Th17/rTreg ratio (A) and CK-18 (B). (C) The best AUROC was calculated for combined CK-18 immune score.

18 (0.75 [CI: 0.62–0.88], $p < 0.01$) and Th17/rTreg ratio (0.72 [CI: 0.60–0.84], $p < 0.05$) (Supplemental Fig. 3). AUROCs of the Th1/rTreg ratio and Th2/rTreg ratio were lower (0.51 [CI: 0.36–0.65], $p = 0.94$ and 0.60 [CI: 0.45–0.74], $p = 0.19$, respectively) (Supplemental Fig. 4).

CD4⁺ T cell subset composition in NASH FU patients after bariatric surgery and weight loss

Fourteen NASH patients undergoing bariatric surgery were seen in an FU visit ≥ 12 mo after surgery. All patients, with the exception of one, showed significant weight loss (mean \pm SD: 45.3 ± 19.4 kg). GPT and GGT serum levels were significantly lower at the time of the FU visit (GPT serum levels, mean \pm SD: 44.0 ± 17.6 U/l versus 25.8 ± 8.1 U/l, $p < 0.01$; GGT serum levels, mean \pm SD: 41.5 ± 22.8 U/l versus 21.0 ± 12.0 U/l, $p < 0.01$). A decrease was also seen in serum CK-18 levels (mean \pm SD: 342 ± 228 U/l versus 152 ± 63 U/l, $p = 0.07$). Analysis of lymphocytes in peripheral blood showed a significant increase in rTregs and a significant decrease in IL-17-producing T cells in comparison with preoperative levels (Fig. 8A, 8B). Consequently, the Th17/rTreg ratio was decreased significantly in all patients after surgery (Fig. 8C). Moreover, Th2/rTreg ratio and HLA-DR expression were significantly lower in FU patients compared with the levels seen in the same patients before bariatric surgery (Fig. 8D, 8E). Therefore, the FU of patients after bariatric surgery corroborated that the frequency of Th17 cells among CD4⁺ T cells and the Th17/rTreg ratio are markers of immunopathology in NASH patients.

Discussion

NAFLD has an increasing prevalence in the Western hemisphere, but it is unknown why some patients remain clinically stable with NAFL for years, whereas others develop progressive disease with

NASH and fibrosis. The inflammatory process and underlying immunological mechanisms in NASH are not well understood. Cells expressing several T cell markers were recently identified by immunostaining in NAFLD liver biopsy sections (20). In this study, we analyzed the peripheral and hepatic composition of CD4⁺ T cells in patients with NASH in comparison with NAFL in unprecedented detail, with ex vivo analysis of immune cells in the liver and peripheral blood. Furthermore, long-term changes in the immune cell profile after treatment of obesity by bariatric surgery were analyzed in 14 NASH patients.

Little data exist about the role of T effector cells in human NAFLD. In 20 patients with NASH, a Th1-dominant immune response was observed, with higher frequencies of IFN- γ -producing CD4⁺ and CD8⁺ cells in the peripheral blood; no change was observed for IL-4⁺CD4⁺ cells (21). Further data on the impact of intrahepatic Th1 and Th2 effector T cells in NASH are derived from mouse experiments in various models of NASH. Increased intrahepatic cytokine levels of TNF- α and IL-12 were induced by a choline-deficient diet alone, whereas Th1-dominant cytokines, such as TNF- α and IFN- γ , were elevated after concomitant ConA treatment (22). In ob/ob mice treated with endotoxin, higher IFN- γ mRNA expression was observed in hepatic tissue, again highlighting a Th1-dominant cell response (23). Moreover, our data showing increased frequency of Th1 cells among CD4⁺ T cells in peripheral blood of patients compared with HCs, as well as the increase in hepatic tissue in patients, are in line with a study of 50 obese children in which an increase in IFN- γ -producing cells was detected in peripheral blood compared with HCs (24). In morbidly obese adult patients, hepatic upregulation of various genes involved in immune regulation and T cell activation toward a Th1 phenotype were described, however, without characterizing the leukocytic infiltrate (25). Interestingly, an upregulation of these inflammatory genes was already observed in six patients with

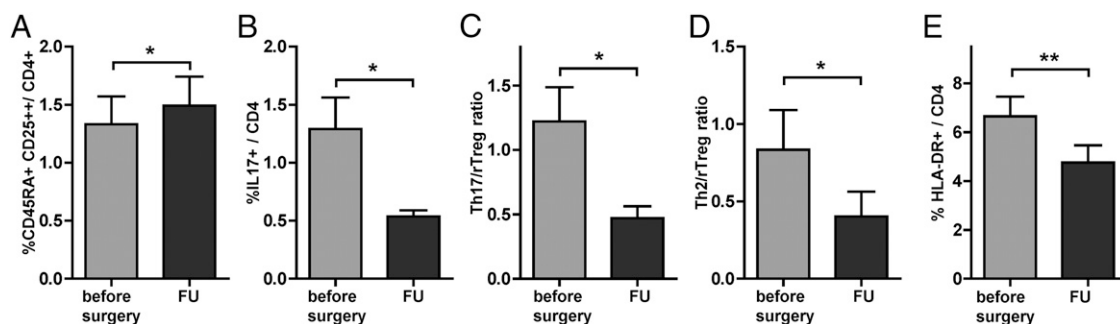


FIGURE 8. CD4⁺ T cell subset composition in NASH FU patients. Analysis of immune cells in peripheral blood in 14 NASH patients before bariatric surgery and at the FU visit ≥ 12 mo after surgery. Frequency of CD45RA⁺CD25⁺ (rTregs) (A) and IL17⁺ cells (B) among CD4⁺ T cells, Th17/rTreg ratio (C), Th2/rTreg ratio (D), and HLA-DR expression by CD4⁺ T cells (E) in peripheral blood. Data are mean \pm SEM. * $p < 0.05$, ** $p < 0.001$, paired t test.

simple steatosis (NAFL). Together with our findings of higher frequency of effector T cells in hepatic tissue of patients with NAFL, these two observations indicate that T cells are involved in this early disease stage.

Our human study demonstrates T cell activation in peripheral blood and hepatic tissue, with an increased frequency of HLA-DR⁺ cells among CD4⁺ T cells in NAFL and NASH. Higher frequencies of IFN- γ ⁺ and IL-4⁺ cells among CD4⁺ T cells could be detected in peripheral blood of NAFL and NASH patients in comparison with HCs. Importantly, patients with NASH could be differentiated from patients with NAFL by a significantly higher frequency of Th17 cells among hepatic CD4⁺ T cells. These results led us to conclude that patients with NAFL already show key pathological changes in their CD4⁺ T cell compartments in both peripheral blood and hepatic tissue. Progression from NAFL to NASH is marked by a more pronounced accumulation of Th17 cells in the liver. Intrahepatic Th17 cells are found in various liver diseases, such as viral hepatitis, alcoholic liver disease, hepatocellular carcinoma, and primary biliary cirrhosis (26–29). After a high-fat diet over 8 wk, a higher frequency of intrahepatic IL-17⁺IFN- γ ⁺CD4⁺ T cells was detected by FACS analysis in murine NAFLD. In liver specimens of NASH patients, higher numbers of IL-17⁺ cells were determined by immunohistochemistry, together with increased expression levels of Th17-related cytokines and transcription factors, such as IL-17, IL-21, IL-23, and ROR γ t (8). IL-17RA^{-/-} mice on a high-fat diet showed weight gain and hepatic triglyceride accumulation but no development of steatohepatitis (9).

Based on recent insights from mice and human studies, our focus was on the analysis of Th17 cells and Tregs (8). Two distinct subpopulations of Tregs expressing Foxp3 were described in humans (18). CD4⁺CD45RA⁺CD25⁺ cells (rTregs) convert into aTregs (CD4⁺CD45RA⁻CD25⁺ cells). aTregs are terminally differentiated and rapidly die after proliferation and exertion of suppression. The functional importance of a deficiency in the Treg compartment for the progression to steatohepatitis becomes evident from mice fed a high-fat diet and treated with endotoxin (30). Adoptive transfer of Tregs to these mice led to a significantly decreased liver injury, indicating that Tregs are capable of controlling the disease when present in sufficient numbers and fully active. In our study, rTregs and aTregs were analyzed separately for the first time, to our knowledge, in patients with NAFLD. A reduced frequency of rTregs among CD4⁺ T cells was observed in peripheral blood of patients with NAFL or NASH. NASH patients had an even more pronounced reduction in PBMCs compared with NAFL patients. Within the liver of histology-proven NASH patients, we detected a trend toward a lower frequency of rTregs and a higher frequency of aTregs. Analysis of patients stratified according to CK-18 serum levels as a biochemical marker showed the same changes. In the FU study carried out ≥ 12 mo after bariatric surgery, NASH patients showed a significantly reduced Th17/rTreg ratio in peripheral blood. Weight loss due to bariatric surgery seems to have a positive effect on liver function, with a reduction in serum GPT, GGT, and CK-18 levels in these patients. This effect is reflected by reversibility of the observed immune cell changes in peripheral blood, with a reduced Th17/rTreg ratio, lower numbers of IL-17-producing T cells, increased numbers of rTregs, lower Th2/rTreg ratio, and reduced HLA-DR expression.

Of major clinical importance, patients with NASH could be differentiated noninvasively from patients with bland NAFL by a significantly higher Th17/rTreg ratio in peripheral blood. The Th17/rTreg ratio may be used in combination with CK-18 to better identify patients at risk for developing NASH in future clinical practice. Th1/rTreg and Th2/rTreg ratios were not significantly different in both peripheral blood and hepatic tissue from NAFL

and NASH patients. Whether the key event for the observed increase in Th17 cell frequency in the liver is located within the liver or in distant sites of inflammation, such as adipose tissue or the intestine, needs further investigation. Weight loss through bariatric surgery as a mechanistic treatment of obesity seems to be an important factor and contributes to reduced inflammatory changes in peripheral blood of these patients.

To our knowledge, our study is the first to describe functional changes within CD4⁺ T cells in both peripheral blood and hepatic tissue for patients with different stages of fatty liver disease. Thus, it provides the basis for identifying novel biomarkers for patients at risk for disease progression and new therapeutic targets for future immune-based interventions. In future studies, additional immune cell compartments (e.g., NKT cells) need to be analyzed on the basis of our findings to further clarify the immunopathogenesis of NAFLD.

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Disclosures

The authors have no financial conflicts of interest.

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