Follicular T Helper Cell Signatures in Primary Biliary Cholangitis and Primary Sclerosing Cholangitis

Leonie Adam,[1] Katharina Zoldan,[1] Maike Hofmann,[1] Michael Schultheiss,[1] Dominik Bettinger [ID] ,[1,2] Christoph Neumann-Haefelin,[1] Robert Thimme,[1] and Tobias Boettler [ID] ,[1]

Primary sclerosing cholangitis (PSC) and primary biliary cholangitis (PBC) are the most common cholestatic liver diseases. While PBC is generally accepted to be an autoimmune disorder characterized by pathognomonic autoantibodies against mitochondrial antigens, the pathogenesis of PSC is less precisely defined; however, some degree of altered immunity toward autoantigens has been suggested. Follicular T helper (Tfh) cells, a distinct clusters of differentiation (CD)4 T-cell subset specialized in facilitating antibody responses, have been shown to contribute to humoral autoimmunity in various disorders; yet, there is only limited information on possible alterations of Tfh cells in the context of cholestatic liver diseases. Thus, we addressed this important question by analyzing the frequency, activation status, and function of Tfh cells and frequencies of regulatory follicular T helper (Tfr) cells in well-defined cohorts of patients with PBC and patients with PSC. Interestingly, we observed a significant increase in circulating chemokine (C-X-C motif) receptor 5 (CXCR5)+programmed death 1 (PD-1) +CD4+ Tfh cells in patients with PBC but not in those with PSC. Although the frequency of potentially pathogenic chemokine (C-C motif) receptor 7 (CCR7)lowCXCR5+PD-1+CD4+ Tfh cells was increased in both disorders compared to healthy donors, the increase was significantly more pronounced in PBC. Furthermore, in patients with PBC, Tfh cells displayed stronger expression of the activation markers OX40 and inducible costimulator of T cells, correlated with anti-anti-mitochondrial antibody M2 and immunoglobulin M titers, and were most significantly increased in patients with cirrhosis. Tfr cell numbers were similarly increased; however, Tfh/Tfr ratios were unaltered in PSC and PBC. These alterations did not correlate with increased secretion of the Tfh signature cytokine interleukin-21 in sorted CD4 T cells. *Conclusion:* Significant alterations occur in the Tfh cell compartment in cholestatic liver diseases, suggesting that Tfh cells influence the pathogenesis of PBC and to a lesser extend PSC. (*Hepatology Communications* 2018;2:1051-1063)

Cholestatic liver diseases (CLDs) are defined as disorders that target the intrahepatic or extra-hepatic bile ducts and subsequently lead to impaired bile acid secretion into the duodenum, intra-hepatic cholestasis, and inflammation. While the term CLD comprises a variety of disorders, primary sclerosing cholangitis (PSC) and primary biliary cholangitis (PBC) are the most common CLDs, both of which can result in liver cirrhosis in the majority of untreated patients.[1,2] PBC is more prevalent in female individuals and is associated with other autoimmune diseases, such as autoimmune thyroiditis and Sjögren's syndrome.[1] In contrast, PSC typically affects male individuals and is strongly associated with the presence of inflammatory

bowel disease, ulcerative colitis in particular.[2] Both diseases are histologically characterized by lymphocytic infiltrates that surround the bile ducts and that are comprised mostly of T lymphocytes.[3] However, the immunopathogenesis of both disorders differs. PBC is generally accepted to be an autoimmune disorder with precisely defined autoantigens that are targeted by both humoral and cellular immune components.[4] In PSC, the pathogenesis is less precisely defined. While the majority of patients present with autoantibodies in the serum (mostly perinuclear anti-neutrophil cytoplasmic antibodies), several features of the disease do not support the hypothesis that PSC is an autoimmune disorder; these include male predominance, the lack of defined autoantigens, and the failure of immunosuppressive agents to influence the course of the disease, although the latter also holds true for PBC.[1]

Follicular T helper (Tfh) cells are a distinct subset of clusters of differentiation (CD)4 T cells that are central facilitators of humoral immunity.[5] While their key task is the establishment of germinal center reactions with B cells in lymphoid tissues, they can also be found in the periphery and are characterized by the expression on chemokine (C-X-C motif) receptor type 5 (CXCR5) and the inhibitory receptor programmed death 1 (PD-1).[6,7] In recent years, a variety of studies have demonstrated that circulating Tfh cells contribute to humoral immunity, that they display distinct phenotypes based on their activation status, and that they can be altered in patients with autoimmune disorders. While it has been shown that recently activated and pathogen-specific Tfh cells up-regulate the chemokine receptor CXCR3,[8,9] highly functional, resting, or memory Tfh cells lack CXCR3 expression.[6] Moreover, it has been shown that circulating Tfh cells

lacking chemokine (C-C motif) receptor 7 (CCR7) closely resemble lymphoid tissue-derived Tfh cells that are highly functional but are also pathogenic in the context of autoimmunity.[10] The development of pathogenic Tfh cells has recently been associated with changes in the ratio between Tfh cells and regulatory Tfh (Tfr) cells[11] as Tfr cells regulate the magnitude and specificity of germinal center reactions.[12] More specifically, it has been shown that Tfr cells prevent the generation of autoantibody-producing B cells.[13]

Importantly, Tfh cells have been suggested to contribute to the pathogenesis of PBC as they have been found in liver samples of patients with PBC and their frequency in the peripheral blood of patients with PBC has been shown to be increased compared to healthy donors and patients with autoimmune hepatitis.[14] However, a precise characterization of potentially pathogenic Tfh subsets has not been performed in PBC and a possible role of Tfh cells in patients with PSC has not been analyzed. Thus, in this study, we aimed to identify the precise phenotype and functional aspects of Tfh and Tfr cells in both PBC and PSC in order to determine a role for Tfh cells in the pathogenesis of CLDs.

Patients and Methods

STUDY SUBJECTS

In this cross-sectional study, 18 patients with PBC and 20 patients with PSC, who were treated at the Gerok-Liver Center of the University Hospital Freiburg, Freiburg, Germany, were included in the analysis and compared to a group of 23 healthy donors and 14 patients with compensated liver cirrhosis of other etiology (alcoholic cirrhosis, n = 8; nonalcoholic

ARTICLE INFORMATION:

From the [1]Department of Medicine II, University Medical Center, Faculty of Medicine, University of Freiburg, Freiburg, Germany; [2]Berta-Ottenstein Program, Faculty of Medicine, University of Freiburg, Freiburg, Germany

ADDRESS CORRESPONDENCE AND REPRINT REQUESTS TO:

Tobias Böettler, M.D.
Department of Medicine II, University Medical Center
Hugstetter Str. 55

D-79106 Freiburg, Germany
E-mail: tobias.boettler@uniklinik-freiburg.de
Tel: +49 761 270-34010

TABLE 1. CHARACTERISTICS OF PATIENTS INCLUDED IN THE STUDY

	PBC	PSC	Cirrhosis*
Number (n)	18	20	14
Sex (female/male)	female n = 18 (100%)	female n = 7 (35%)	female n = 4 (28.5%)
	male n = 0 (0%)	male n = 13 (65%)	male n = 10 (71.5%)
Age (mean ± SD)	52.4 ± 9.5	44.6 ± 15.1	54.6 ± 8.2
Concurrent IBD	n = 1 (5.5%)	n = 16 (80%)	n = 0 (0%)
Medication			
UDCA	n = 18 (100%)	n = 19 (95%)	n = 0 (0%)
No immunosuppressive therapy	n = 15 (83.3%)	n = 10 (50%)	n = 14 (100%)
Steroids	n = 3 (16.7%)	n = 0 (0%)	n = 0 (0%)
Steroids + IS	n = 0 (0%)	n = 10 (50%)	n = 0 (0%)

Abbreviations: IBD, inflammatory bowel disease; IS, immunosuppressive drug; UDCA, ursodeoxycholic acid.
*14 patients with compensated liver cirrhosis comprising 8 patients with alcoholic cirrhosis, 4 patients with nonalcoholic steatohepatitis cirrhosis, 2 patients with hepatitis C virus cirrhosis.

steatohepatitis cirrhosis, n = 4; hepatitis C virus cirrhosis, n = 2; all Child-Pugh class A). Clinical details of the cohorts are presented in Table 1. Each patient gave written informed consent before ethylene diamine tetraacetic acid-anticoagulated blood samples were collected for flow cytometric analysis. The Ethics Committee of the Albert-Ludwigs-University Freiburg approved the study (EK-Freiburg 383/14).

PREPARATION OF PERIPHERAL BLOOD MONONUCLEAR CELLS AND FLOW CYTOMETRIC ANALYSES

Peripheral blood mononuclear cells were isolated from blood samples by density gradient centrifugation using Ficoll Histopaque (PAA, Vienna, Austria). The cells were washed with phosphate-buffered saline before being cryopreserved in dimethyl sulfoxide freezing medium at −80°C. Roswell Park Memorial Institute 1640 cell culture medium supplemented with 10% fetal bovine serum was used to thaw the cells for analysis.

Polychromatic staining with fluorochrome-labeled antibodies was performed with the following reagents: anti-CD3-PacBlue, anti-CD3-allophycocyanin (APC)-H7, anti-CD4-APC-H7, anti-CD8-phycoerythrin (PE), anti-CD25-peridinin chlorophyll protein (PerCP), anti-CCR6-fluorescein isothiocyanate (FITC), anti-CXCR5-Brilliant Violet (BV)421, anti-interferon (IFN)-γ-APC, anti-interleukin (IL)-2-BV421, anti-IL-21-PE, anti-Ox40-PE, anti-tumor necrosis factor α-FITC (all from BD Biosciences, San Jose, CA), anti-CCR7-FITC, anti-CXCR3-APC, anti-PD-1-PE-cyanine 7 (Cy7) (all from BioLegend, San Diego, CA), anti-forkhead box P3 (FoxP3)-FITC, anti-FoxP3-fluorophore (eFluor)450, anti-inducible costimulator of T cells (ICOS)-APC, and anti-IL-17-PE-Cy7 (all from eBioscience, Frankfurt am Main, Germany). To exclude dead cells Viaprobe-V500 (BD Biosciences) was used. Prior to staining with the listed antibodies, Fc receptors were blocked using a purified immunoglobuIin (Ig)G1 whole molecule. Subsequently, cells were stained, washed with phosphate-buffered saline, and analyzed by flow cytometry on a FACS Canto II machine using DIVA software (BD Biosciences). Postacquisition analysis was performed with FlowJo v10.0.8 software (Tree Star Inc., Ashland, OR).

INTRACELLULAR CYTOKINE STAINING

CD4 T cells were sorted by negative magnetic selection using a CD4+ T Cell Isolation Kit (MACS; Miltenyi Biotec). The purity of the sorted cell population was higher than 90%. To investigate the cytokine production of the sorted CD4 T cells, 1×10^6 cells were stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin (Sigma Aldrich, Steinheim, Germany) and incubated for 5 hours at 37°C in the presence of Golgi Plug/Golgi Stop (BD Biosciences) in 96-well plates. Following surface staining, permeabilization was performed by adding Cytofix/Cytoperm (BD Biosciences) followed by intracellular staining with antibodies against IFN-γ, IL-2, IL-21, tumor necrosis factor α (all BD Biosciences), and IL-17 (eBioscience). The Simulation Program With

Integrated Circuit Emphasis (SPICE)[15] was used to analyze polyfunctionality.

ENZYME-LINKED IMMUNOSORBENT ASSAY

Analyses of anti-mitochondrial antibodies (AMAs) were performed by enzyme-linked immunosorbent assay (ELISA) (Anti-M2-3E-ELISA; Euroimmun, Luebeck, Germany). The antigens included in this assay are the E2 subunits of pyruvate dehydrogenase, branched-chain 2-oxoacid dehydrogenase, and 2-oxoglutarate dehydrogenase. Plasma samples from patients and healthy controls were diluted (1:101) and ELISA was performed according to the manufacturer's instructions. IgG and IgM in the plasma were quantified by ELISA using specific antibodies (Jackson ImmunoResearch, Dianova, Hamburg, Germany). Plasma samples were diluted 1:100,000 and 1:10,000,000. Plates (Nunc MaxiSorp; VWR, Bruchsal, Germany) were coated with anti-human IgG (heavy chain + light chain) antibody. Human IgG and IgM (whole molecule) were used for calibration. For detection, peroxidase-conjugated anti-human F(ab')2 fragments specific for Fc5μ or gamma fragments were applied. Plates were measured with a Spark plate reader (Tecan, Crailsheim, Germany).

STATISTICAL ANALYSIS

GraphPad Prism 6 software (GraphPad Prism Software, Inc.) was used to perform statistical analyses. Differences between multiple groups were analyzed by the Kruskal-Wallis H nonparametric test and Dunn's multiple comparison test. The Mann-Whitney U test was used when comparing only two groups. Linear regression was performed to investigate the potential relationship between two variables. Data are presented
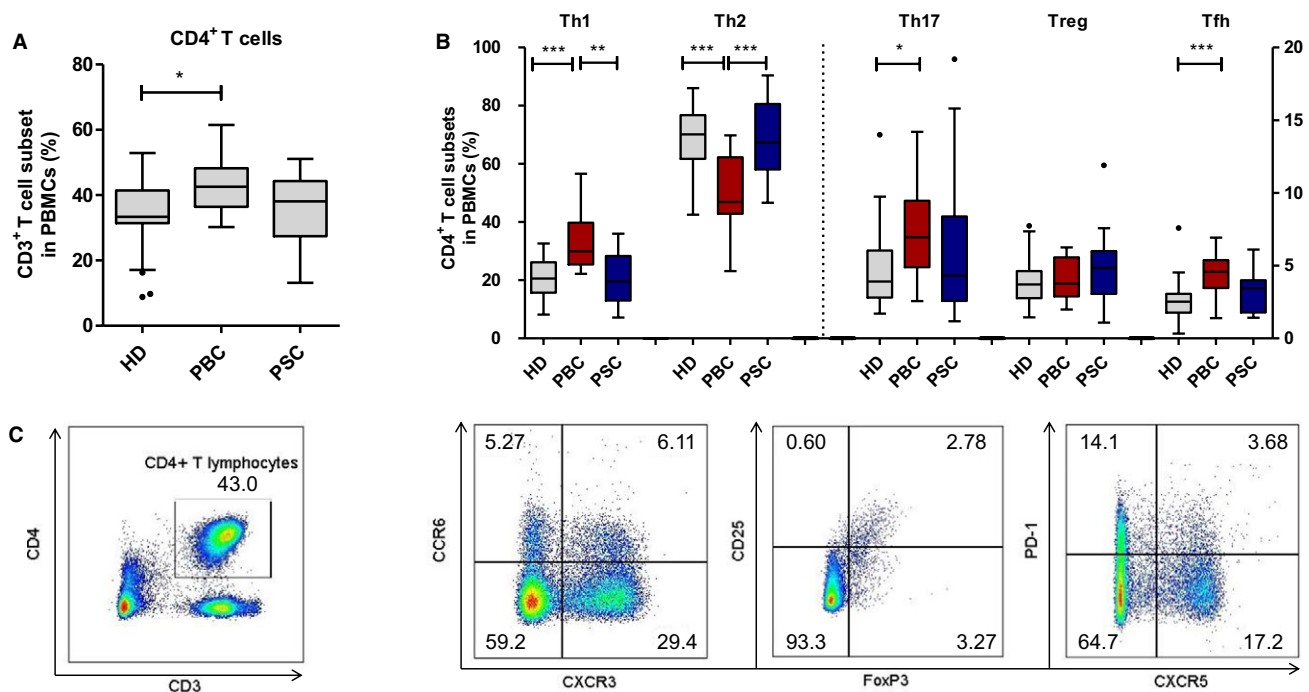

FIG. 1. CD4+ helper T-cell subsets in patients with PBC and PSC. Frequencies of the indicated subsets are shown. (A) Bulk CD4 T cells, characterized as CD4+ cells of CD3+ T lymphocytes in PBMCs, are shown in patients with PBC, PSC and healthy donors. (B) CD4 T cell subsets. CXCR3+ CCR6- cells of CD4 T cells represent Th1 cells, CXCR3- CCR6- cells of CD4 T cells represent Th2 cells, whereas CXCR3- CCR6+ cells of CD4 T cells represent Th17 cells. Regulatory T cells are characterized as CD25+ FoxP3+ cells of CD4 T cells. Circulating Tfh cells are identified by coexpression of CXCR5 and PD-1 by CD4 T cells. Data are presented as box-and-whisker plots with upper and lower quartile. The horizontal line inside the box represents the median and whiskers show the maximum and minimum values. Outliers are shown as dots representing data points which exceed 1.5 X the upper or lower quartile. The dotted vertical line separates the graphs according to the two different y-intercepts. (C) Gating strategies. Plot on the left was gated on live single cells within the lymphocyte gate. Plots on the right are gated on CD4+ T cells. *$P \leq 0.05$, **$P \leq 0.01$ and ***$P \leq 0.001$.

as box-and-whisker plots or as median. For all analyses, a two-sided *P* value of <0.05 was determined to be statistically significant.

Results

CD4 T-CELL SUBSETS IN CLDS

CD4 T cells can be divided into several subsets that display different functional properties. Although there is considerable overlap and plasticity between the distinct subsets, the analysis of the composition of the entire CD4 T-cell population can be helpful to determine subset variations in immune-mediated disorders. Thus, we analyzed CD4 T cells in patients with PBC, those with PSC, and healthy volunteers in order to analyze their frequency and their distinct subset composition (patient characteristics are detailed in the methods section). When analyzing CD4 T-cell frequencies, we observed higher percentages of CD4 T cells among all lymphocytes in patients with PBC (Fig. 1A), supporting a special role for CD4 T cells in PBC.[16] To analyze the CD4 T-cell subset distribution, we stained for CCR6 and CXCR3 in order to separate T helper (Th)1, Th2, and Th17 cells as has been described by several groups.[7,17-19]
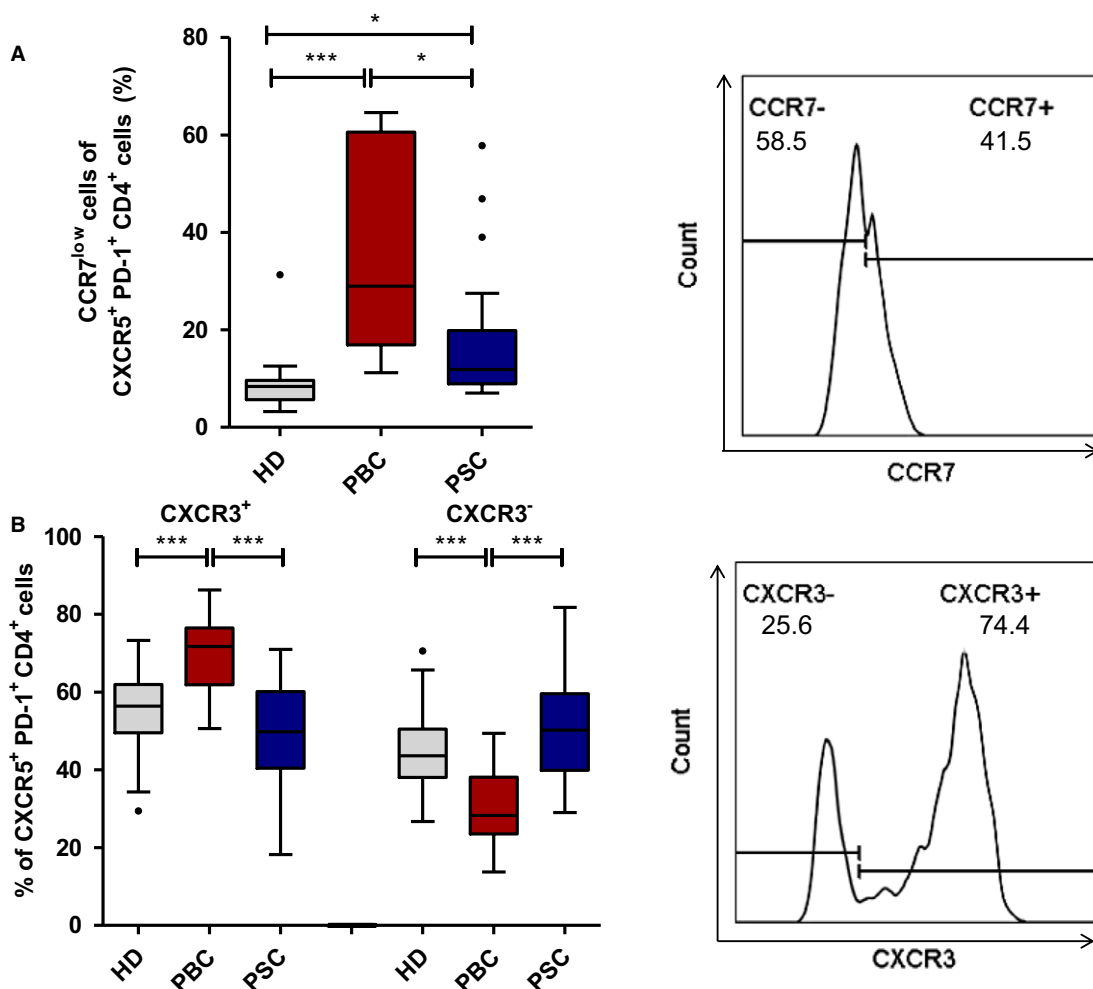
FIG. 2. Altered Tfh cell subsets in patients with PBC or PSC. Frequencies of the indicated subsets are shown as % of CD4 T cells. (A) CCR7low cells of circulating Tfh cells, characterized as CXCR5+ PD-1+ CD4 T cells, are illustrated. (B) CXCR3+ (left) and CXCR3- (right) of CXCR5+ PD-1+ CD4 T cells are depicted. Data on the left graphs are presented as box-and-whisker plots with upper and lower quartile. The horizontal line inside the box represents the median and whiskers show the maximum and minimum values. Outliers are shown as dots representing data points which exceed 1.5 X the upper or lower quartile. The representative histograms on the right side of the figure are gated on CXCR5+ PD-1+ CD4 T cells. *P≤0.05, **P≤0.01 and ***P≤0.001.

Interestingly, we observed increased frequencies of Th1 and Th17 cells in patients with PBC but not PSC while Th2 cells were decreased in patients with PBC (Fig. 1B,C). Regulatory T cells, defined by co-expression of CD25 and FoxP3, were unaltered in our cohort of patients with CLDs compared to healthy volunteers. Most importantly, however, we observed an increase of Tfh cells as characterized by the expression of CXCR5 and PD-1 in patients with PBC and a tendency toward higher frequencies in patients with PSC (Fig. 1B). These observations suggest that cells with a Tfh phenotype are altered in PBC and to a lesser extent in PSC, warranting a more detailed analysis of circulating Tfh cells in both conditions.

INCREASE OF POTENTIALLY PATHOGENIC Tfh CELLS IN PATIENTS WITH PBC OR PSC

The co-expression of CXCR5 and PD-1 identifies cells with a Tfh phenotype, and from here on we refer to CD4+CXCR5+PD-1+ cells as Tfh cells.[5] However, only a subset of cells with this phenotype displays functional properties of Tfh cells.[6,7,10] It has been shown that circulating Tfh cells in humans that lack CCR7 expression closely resemble functional Tfh cells from lymphoid tissues. Moreover, it could be shown that these cells can be pathogenic and contribute to autoimmunity.[10] To date, these highly active Tfh cells have not been analyzed in
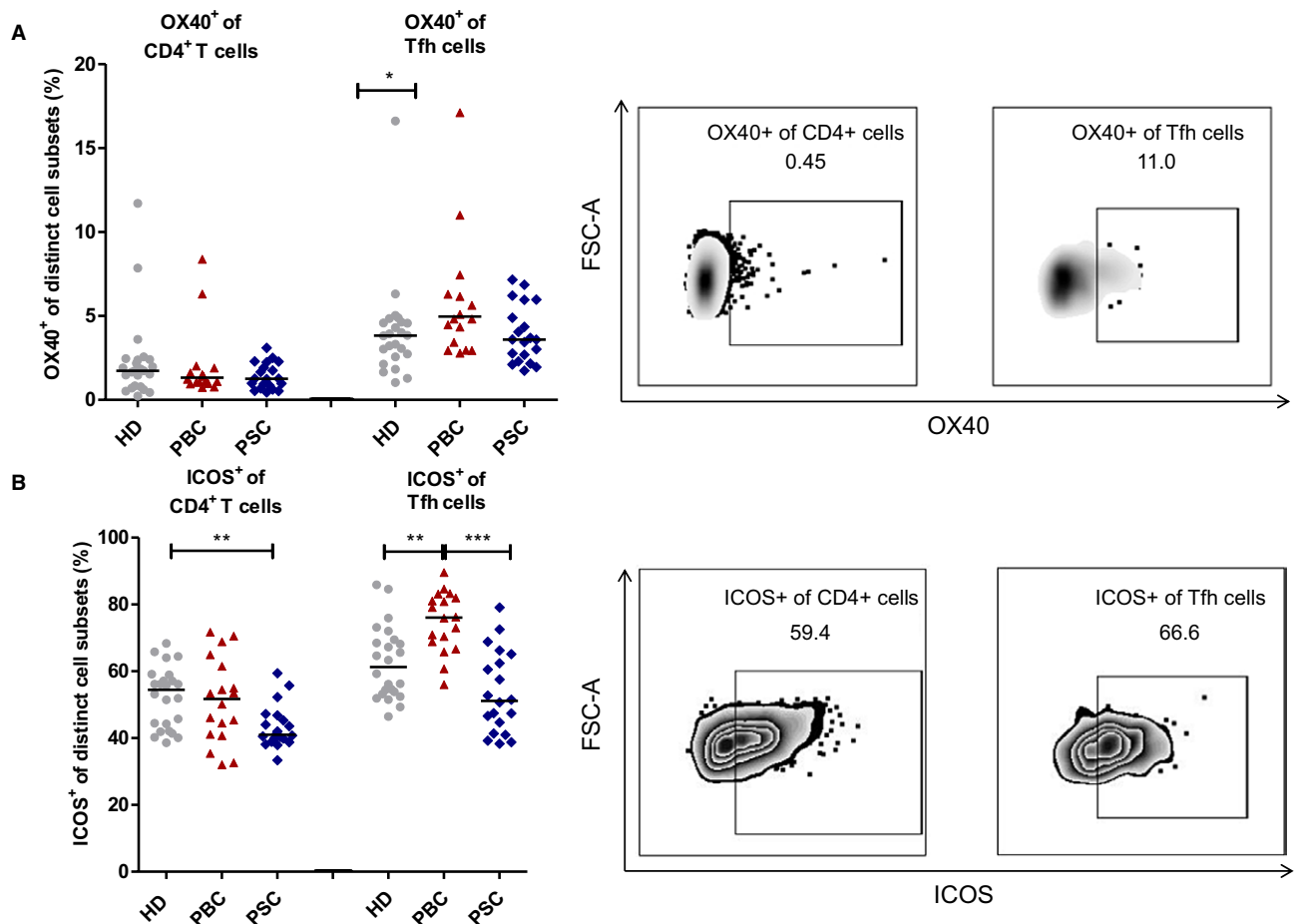
FIG. 3. Tfh cell activation in patients with PBC or PSC. Frequencies of the indicated subsets are shown. (A) OX40 expression on CD4 T cells and Tfh cells (CXCR5+ PD-1+ CD4 T cells) is illustrated as a marker for cell activation. (B) ICOS expression on CD4 T cells and Tfh cells. Data on the left graphs are presented as scatter dot plots. The horizontal lines represent the median. The representative density plots (A) and zebra plots (B) on the right side of the figure are gated on CD4 T cells and Tfh cells (CXCR5+ PD-1+ CD4 T cells) as indicated in the plots. The gating rectangle is set on OX40 (A) and ICOS (B) expressing cells. *$P \leq 0.05$, **$P \leq 0.01$ and ***$P \leq 0.001$.

the context of CLDs. Interestingly, we observed that CCR7lowCXCR5+PD-1+ Tfh cells were dramatically increased in patients with PBC compared to healthy volunteers and patients with PSC, further supporting a special role for Tfh cells in PBC pathogenesis (Fig. 2A); however, compared to healthy donors, CCR7lowCXCR5+PD-1+ Tfh cells were also increased in patients with PSC (Fig. 2A), although to a much lesser extent than in PBC. Next, we analyzed CXCR3 expression on Tfh cells as this chemokine receptor has been shown to be expressed on activated and pathogen-specific Tfh cells[8,9] while resting or memory Tfh cells lack CXCR3 expression.[6] We observed significant increases of CXCR3 expression on circulating Tfh cells in patients with PBC compared to healthy volunteers and PSC while no significant changes were observed in patients with PSC compared to healthy volunteers (Fig. 2B). These observations indicate that potentially pathogenic alterations of

circulating Tfh cells are present in patients with PSC and those with PBC. However, these alterations are significantly more pronounced in PBC compared to PSC.

Tfh CELLS SHOW ACTIVATED PHENOTYPE IN PATIENTS WITH PBC

Upon activation, Tfh cells up-regulate the ICOS receptor as well as the costimulatory molecule OX40 (CD134), a tumor necrosis factor superfamily receptor.[8,20] Both receptors are important mediators of intracellular signaling pathways that are closely related to Tfh differentiation and activation. Indeed, ICOS expression is required for Tfh differentiation and following antigen re-exposure.[8,21] OX40 signaling has been implicated in the induction and maintenance
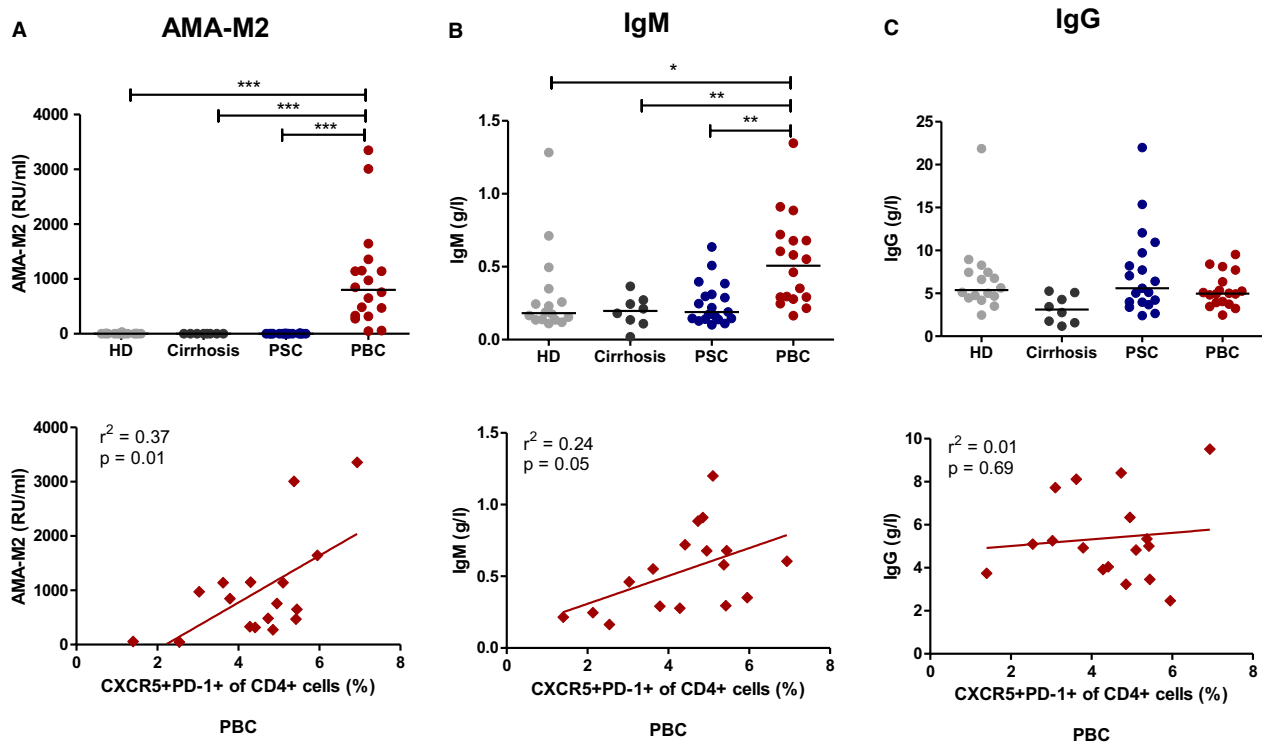

FIG. 4. Autoantibodies and immunoglobulins and their correlation to Tfh cells in PBC. Analyses of antimitochondrial antibodies (AMA-M2), IgM and IgG performed by ELISA in patients with PBC as well as in patients with PSC, cirrhosis and in healthy volunteers and their correlation with the frequency of Tfh cells in patients with PBC are shown. (A) The levels of AMA-M2 antibodies are shown in the upper panel. The figure below shows the correlation between the AMA-M2 titer and the frequency of Tfh cells (% CXCR5+ PD-1+ of CD4 T cells) in patients with PBC. (B + C) The levels of IgM and IgG in the plasma of the four cohorts is displayed in the upper figures. In patients with PBC, the levels of IgM and IgG are correlated with the frequency of Tfh cells. Data is presented as scatter dot plots (upper panels). The horizontal lines represent the median. In the lower panels, linear regression analyses are shown. r2 is shown as quality criterion for linear regression. *$P \leq 0.05$, **$P \leq 0.01$ and ***$P \leq 0.001$.
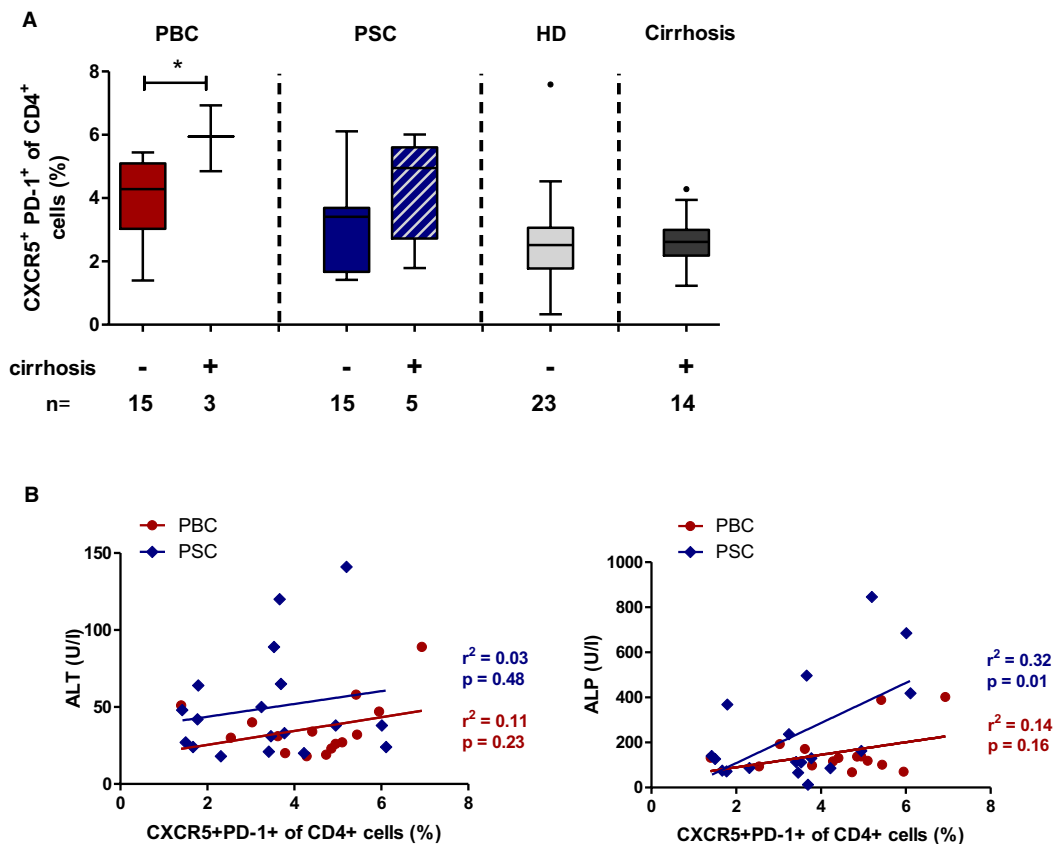
FIG. 5. Correlation of Tfh cells with disease activity and stage. (A) The frequency of Tfh cells (% CXCR5+ PD-1+ of CD4 T cells) in patients with PBC, PSC, healthy donors and in patients with liver cirrhosis of other etiologies (alcohol, NASH and HCV, all compensated) is shown. Data are presented as box-and-whisker plots with upper and lower quartile. The horizontal line inside the box represents the median and whiskers show the maximum and minimum values. Outliers are shown as dots representing data points which exceed 1.5 X the upper or lower quartile. The dotted vertical line separates the graphs between disease etiologies. (B) Correlation between the frequency of Tfh cells and the levels of laboratory markers of disease activity such as ALT and ALP are presented in patients with PBC and PSC. Linear regression analyses are shown. r2 is shown as quality criterion for linear regression. *$P \leq 0.05$, **$P \leq 0.01$ and ***$P \leq 0.001$.

of Tfh-mediated germinal center formation.[20,22–24] Thus, we stained Tfh cells for co-expression of OX40 and ICOS to analyze the activation state of circulating Tfh cells in PBC and PSC. Both ICOS and OX40 were significantly up-regulated on Tfh cells in patients with PBC but not in those with PSC, further pointing toward an active role of Tfh cells in PBC (Fig. 3).

LEVELS OF AMAs CORRELATE WITH FREQUENCY OF Tfh CELLS

PBC is characterized by autoreactive immune responses targeting mitochondrial antigens, particularly E2 units of enzymes within the respiratory chain of the mitochondria. In order to correlate the frequency of circulating Tfh cells with disease-specific anti-AMA-M2 antibodies, we quantified anti-AMA-M2 levels in patients with PBC as well as in patients with PSC, those with cirrhosis, and in healthy volunteers from date-matched plasma samples. As expected, only patients with PBC displayed AMA-M2 antibodies. Interestingly, anti-AMA-M2 levels were significantly correlated with circulating Tfh frequencies (Fig. 4A). Similarly, PBC is characterized by an increase of IgM levels in the sera of patients. We observed that higher IgM levels also appear to be associated with increased Tfh frequencies (Fig. 4B), although this correlation did not reach statistical significance (P = 0.05). No correlation was observed between IgG levels and circulating Tfh frequencies in patients with PBC (Fig. 4C).
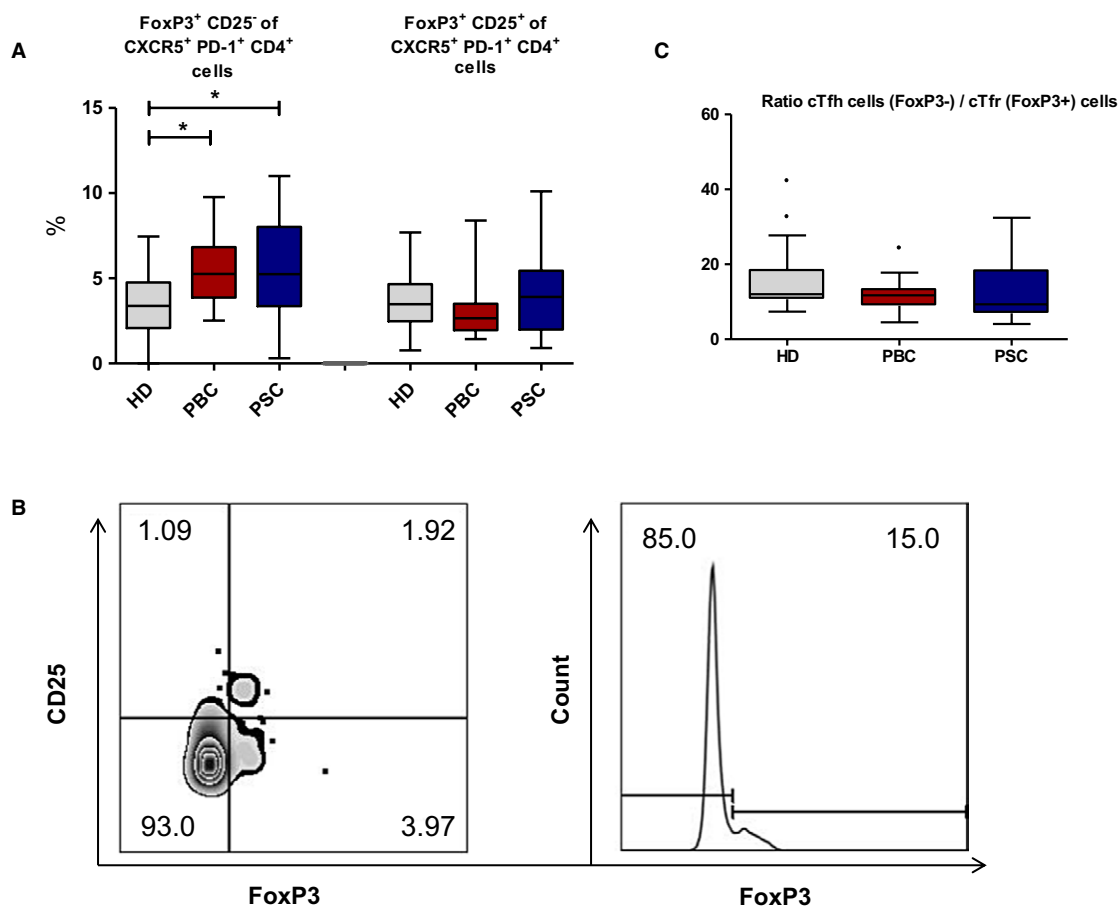
A

FoxP3+ CD25- of CXCR5+ PD-1+ CD4+ cells

FoxP3+ CD25+ of CXCR5+ PD-1+ CD4+ cells

C

Ratio cTfh cells (FoxP3-) / cTfr (FoxP3+) cells

B

FIG. 6. Frequencies of regulatory follicular T-helper cells. (A) Frequencies of the indicated subsets are shown as % of CXCR5+PD-1+CD4 T cells. cTfr cells, characterized as FoxP3+ of CXCR5+ PD-1+ CD4 T cells, are depicted focusing on CD25- cTfr (left) and CD25+ cTfr subsets (right). (B) Representative density plot and histogram are shown gated on CXCR5+ PD-1+ CD4 T cells. (C) Ratio of cTfh (FoxP3- cells of CXCR5+ PD-1+ CD4 T cells) and cTfr cells (FoxP3+ cells of CXCR5+ PD-1+ CD4 T cells) is shown. Data in (A) and (C) are presented as box-and-whisker plots with upper and lower quartile. The horizontal line inside the box represents the median and whiskers show the maximum and minimum values. Outliers are shown as dots representing data points which exceed 1.5 X the upper or lower quartile. *$P \leq 0.05$, **$P \leq 0.01$ and ***$P \leq 0.001$.

Tfh CELLS CORRELATE WITH DISEASE ACTIVITY AND STAGE

The observation that disease-specific antibody levels are positively correlated with circulating Tfh frequencies in patients with PBC raises the question whether Tfh cells are also associated with clinical markers of disease stage and progression. Patients with cirrhosis (all compensated, Child A) due to CLDs tended to have higher frequencies of Tfh cells compared to healthy volunteers and patients with cirrhosis due to other etiologies (alcoholic cirrhosis, n = 8; nonalcoholic steatohepatitis cirrhosis, n = 4; hepatitis C virus cirrhosis; n = 2; all compensated, Child A) (Fig. 5A). In addition, we sought to analyze whether biochemical

markers of disease activity, such as alkaline phosphatase (ALP) or alanine aminotransferase, were positively correlated with circulating Tfh cells. At the time of analysis, however, there was no clear correlation between alanine aminotransferase or ALP levels and circulating Tfh cells in patients with PBC. In patients with PSC, however, the ALP level positively correlated with the circulating Tfh frequency (Fig. 5B).

REGULATION OF Tfh RESPONSES BY Tfr CELLS

Tfr cells share several phenotypic features with Tfh cells, most importantly the expression of CXCR5 to
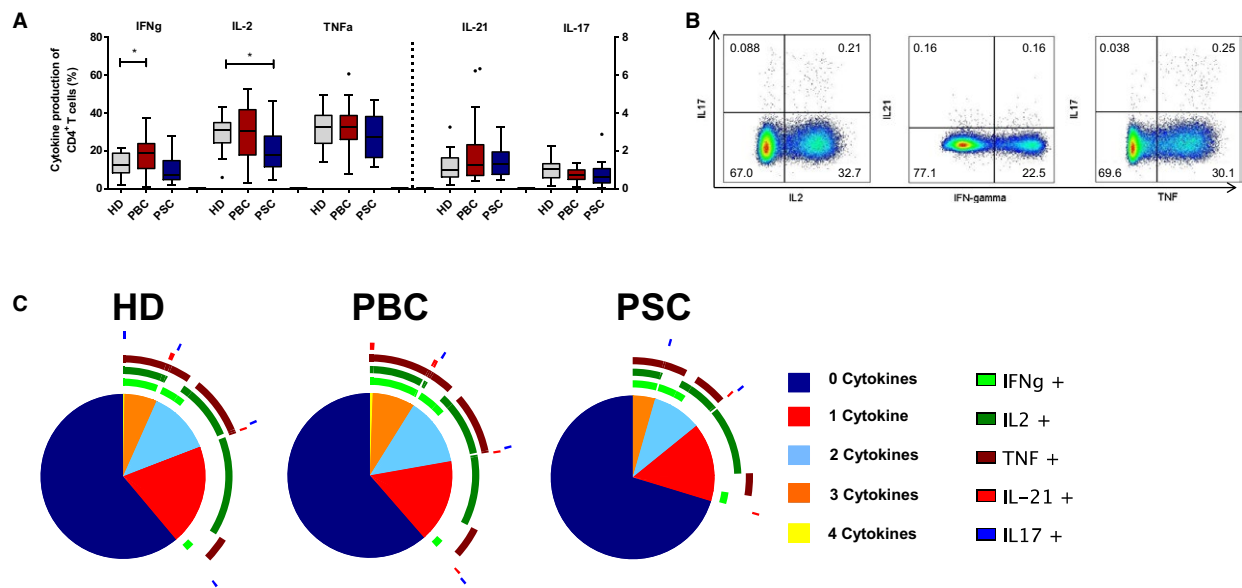
FIG. 7. Cytokine expression patterns of sorted CD4 T cells in patients with PBC and those with PSC. CD4 T cells were sorted by magnetic bead separation. Short-term stimulation over five hours with PMA / ionomycin and subsequent intracellular cytokine staining was performed. (A) Frequencies of cytokine producing cells of CD4 T cells. Secretion of IFN-γ, IL-2, TNFα, IL-21 and IL-17 secretion in patients with PBC, PSC and healthy donors is presented. Data are presented as box-and-whisker plots with upper and lower quartile. The horizontal line inside the box represents the median and whiskers show the maximum and minimum values. Outliers are shown as dots representing data points which exceed 1.5 X the upper or lower quartile. (B) Representative density plots gated on CD4 T cells are displayed. (C) Boolean-Gating and SPICE polyfunctionality analysis of the cytokine production revealed largely similar cytokine expression patterns for HD and patients with PBC and PSC. Pie charts represent the entire CD4 T cell population secreting either no cytokines (dark blue), one cytokines (red), 2 cytokines (light blue), 3 cytokines (orange) or 4 cytokines (yellow). The distribution of the individual cytokines is displayed outside the pie charts. *P≤0.05, **P≤0.01 and ***P≤0.001.

enable migration to germinal centers. Like Tfh cells, however, they can also be detected in the peripheral blood.[25] Recently, it has been shown that in contrast to conventional regulatory cells, human Tfr cells express significantly reduced amounts of the IL-2 receptor alpha chain CD25.[26] Interestingly, the highly functional Tfr population completely lacking CD25 expression was predominantly found to be located within lymphoid tissues while circulating Tfr (cTfr) cells retained some degree of CD25 expression.[26] Thus, in order to identify whether the increase in circulating Tfh cells found in patients with PBC and to a lesser extent those with PSC (Fig. 2A) is reflected in a decreased frequency of cTfr cells, we analyzed cTfr cells in healthy donors, patients with PBC, and those with PSC. Surprisingly, we observed increased frequencies of FoxP3+CD25– cells within circulating Tfh cells of patients with PBC and those with PSC compared to healthy donors (Fig. 6A,B). In contrast, FoxP3+ cells expressing CD25 were not increased in the patients, and due to the increased frequencies of

circulating Tfh cells, the ratio of Tfh and Tfr cells was comparable in all three groups (Fig. 6C), suggesting that the increased frequency of Tfh cells may not be a direct consequence of impaired regulation by Tfr cells.

CYTOKINE EXPRESSION PATTERNS OF CD4 T CELLS IN PBC AND PSC

IL-21 is the signature cytokine of Tfh cells and is crucial for their ability to provide B-cell help and for self-maintenance of Tfh cells by autocrine signaling. However, circulating Tfh cells have been shown to also express other cytokines, such as IFN-. We aimed to assess whether IL-21 production by CD4 T cells is more pronounced in patients with CLDs. Therefore, we sorted CD4 T cells by magnetic bead separation and performed short-term stimulation with PMA/ ionomycin. CD4-negative selection was performed prior to stimulation as stimulation with PMA/iono-mycin results in down-regulation of CD4 on T cells

that negatively affects the precise analysis of CD4-expressing cells. We also observed relevant alterations of CXCR5 expression following PMA/ionomycin stimulation. Thus, we did not assess cytokine secretion patterns specifically in CXCR5-expressing cells but rather analyzed cytokine secretion within the entire CD4 T-cell population. Interestingly, we observed that IL-21 secretion was slightly increased in patients with PBC; however, this trend did not reach statistical significance. In agreement with our observation of increased Th1 frequencies in patients with PBC, we observed increased frequencies of IFN--secreting CD4 T cells in patients with PBC (Fig. 7A,B). Boolean-Gating and SPICE analysis[15] revealed that cytokine secretion patterns of CD4 T cells were largely similar between healthy controls and patients with PBC or PSC (Fig. 7C), although patients with PSC tended to have lower frequencies of cytokine-secreting cells compared to healthy donors and patients with PBC.

Discussion

PSC and PBC are CLDs that can cause progressive liver damage leading to cirrhosis and its complications, such as hydropic decompensation, variceal bleeding, and liver cancer. The pathogenesis of both disease entities is closely linked to T cells, CD4 T cells in particular. Indeed, CD4 T cells are present in the inflamed areas surrounding the bile ducts.[27,28] Moreover, genome-wide association studies have identified several major histocompatibility complex class II genes that are associated with an increased risk of developing PBC and PSC.[29-31] Furthermore, pyruvate dehydrogenase E2 has been identified as an autoantigen, targeted by autoreactive CD4 T cells in patients with PBC.[32,33] Thus, PBC and PSC display features of cellular autoimmunity. PBC, however, is also characterized by development of humoral autoimmunity with the presence of AMAs that also target pyruvate dehydrogenase E2 and that serve as a diagnostic marker that can establish the clinical diagnosis of PBC in around 90% of affected patients.[1] Perinuclear anti-neutrophil cytoplasmic antibodies are present in the majority of patients with PSC; however, they neither establish the clinical diagnosis nor has their functional role in the pathogenesis of PSC been demonstrated.[2] Thus, it remains a matter of debate whether PSC can be considered a genuine autoimmune disease. In this study, we aimed to gain more detailed insights into the composition of the T-cell response in patients with PBC or PSC, specifically focusing on Tfh cells because alterations in this T-cell subset have been shown to be associated with autoimmunity.[7,10] Importantly, our data reveal an increased frequency of CD4+CXCR5+PD-1+ T cells in patients with PBC (Fig. 1B), extending previous observations by Wang et al.[14] who demonstrated that CD4+CXCR5+ T cells are enriched in patients with PBC. However, it is well accepted that circulating Tfh cells are comprised of different subsets with different abilities to stimulate B cells.[6,7,10] Indeed, it has been shown that peripheral Tfh cells lacking CCR7 expression can mirror germinal center activity and that this specific Tfh cell subset is up-regulated in patients with autoimmune diseases, such as rheumatoid arthritis and systemic lupus erythematosus.[10] Thus, this highly functional Tfh cell subset has been shown to serve as a biomarker for diseases involving humoral autoimmunity. Importantly, CCR7-negative Tfh cells were strongly enriched in patients with PBC but were also increased in patients with PSC compared to healthy donors (Fig. 2A), although frequencies of total CD4+CXCR5+PD-1+ T cells were not found to be increased in the peripheral blood of patients with PSC (Fig. 1B). The lack of CXCR3 expression on circulating Tfh cells has been suggested to serve as another marker of functional Tfh cells. It has been shown that Tfh cells expressing CXCR3 failed to provide help to B cells.[6] This observation, however, most likely reflects the phenotype of resting memory Tfh cells as these analyses have been performed on bulk CD4 T cells in healthy volunteers and it has been shown that circulating Tfh cells up-regulate CXCR3 in the context of recent antigen exposure.[8,9] Thus, CXCR3 expression might also serve as a marker of recent antigen-specific stimulation or Tfh cell activation. The observation that CXCR3-expressing Tfh cells are more abundant in patients with PBC (Fig. 2B) might therefore indicate activation of Tfh cells. Interestingly, this assumption is supported by the up-regulation of the activation markers ICOS and OX40 on Tfh cells in patients with PBC (Fig. 3). Collectively, our data indicate that Tfh cell immunity is significantly altered in patients with PBC characterized by up-regulation of highly functional CCR7low circulating Tfh cells and up-regulation of activation markers, such as CXCR3, OX40, and ICOS. Moreover, our in-depth analyses of predefined circulating Tfh cell subsets also revealed an increase of CCR7low circulating Tfh cells in patients with PSC, although to a lesser extent compared

to PBC, including reduced expression of the described activation markers.

The significant alterations of circulating Tfh cells in PBC suggest an association with disease-specific alterations of the humoral immune system, such as AMAs and elevated levels of IgMs. Indeed, AMA-M2 levels directly correlated with the frequency of circulating Tfh cells, as shown in Fig. 4. This trend was also observed for IgM levels but fell just short of reaching statistical significance. These observations extend previous findings by Wang et al.[14] who observed an association with anti-AMA positivity and the frequency of CXCR5+CD4+ T cells. In contrast to their observations, we quantified AMA levels by using a highly specific AMA-M2 ELISA and used date-matched samples for our correlations between the humoral and cellular features of autoimmunity. Furthermore, we specifically analyzed circulating Tfh cells (CXCR5+PD-1+) instead of analyzing the entire CXCR5+ CD4 cell population. It is intriguing to speculate whether the circulating T cells with a Tfh cell phenotype are also specific for mitochondrial antigens. However, due to several challenges in detecting antigen specificity of Tfh cells, including the unstable expression of Tfh markers (predominantly CXCR5) in *in vitro* cultures and the large diversity of human leukocyte antigen class II molecules, we were unable to show antigen specificity for the circulating Tfh cells in PBC. Another interesting finding is the increase of Tfh cell frequencies in patients with cirrhosis due to PBC and to a lesser extent also due to PSC while compensated cirrhosis due to other etiologies did not alter the frequency of circulating Tfh cells (Fig. 5). Although the number of patients with cirrhosis is small in our cohort, a similar observation was shown by Wang et al.[14] who showed a gradual increase of CXCR5+CD4+ T cells with increasing fibrosis levels. Of note, some patients were treated with corticosteroids and/or immunosuppressive drugs; however, there was no correlation between the intake of steroids and or immunosuppressive drugs and the frequency of circulating Tfh cells (data not shown).

We hypothesized that the increased frequency of highly functional Tfh cells and their activated state might be linked to decreased frequencies of Tfr cells in PBC and PSC. While we observed that the ratio of circulating Tfr and Tfh cells was comparable between healthy donors and patients with PSC or PBC (Fig. 6), we cannot rule out the possibility that the balance of Tfh and Tfr cells might be altered in favor of Tfh cells within germinal centers. However, germinal center-derived Tfr cells lack expression of CD25, and

CD25-negative Tfr cells were increased in patients with PSC and patients with PBC (Fig. 6A), suggesting that other mechanisms, such as stimulation by bacterial antigens, as suggested recently,[34] might be driving the expansion of potentially pathogenic Tfh cells in the context of CLDs.

Collectively, our results suggest that Tfh cells have a role in the pathogenesis of PBC and to a lesser extent of PSC. Although this study is descriptive and future studies will have to focus on the functionality of the altered Tfh cells in CLDs, our findings support the notion that while PSC displays features of autoreactivity, it lacks typical characteristics of a genuine autoimmune disorder.

REFERENCES

1) Carey EJ, Ali AH, Lindor KD. Primary biliary cirrhosis. Lancet 2015;386:1565-1575.

2) Lazaridis KN, LaRusso NF. Primary sclerosing cholangitis. N Engl J Med 2016;375:1161-1170.

3) Pollheimer MJ, Fickert P, Stieger B. Chronic cholestatic liver diseases: clues from histopathology for pathogenesis. Mol Aspects Med 2014;37:35-56.

4) Gershwin ME, Ansari AA, Mackay IR, Nakanuma Y, Nishio A, Rowley MJ, et al. Primary biliary cirrhosis: an orchestrated immune response against epithelial cells. Immunol Rev 2000;174:210-225.

5) Vinuesa CG, Linterman MA, Yu D, MacLennan IC. Follicular helper T cells. Annu Rev Immunol 2016;34:335-368.

6) Locci M, Havenar-Daughton C, Landais E, Wu J, Kroenke MA, Arlehamn CL, et al. International AIDS Vaccine Initiative Protocol C Principal Investigators. Human circulating PD-1CXCR6CXCR6 memory Tfh cells are highly functional and correlate with broadly neutralizing HIV antibody responses. Immunity 2013;39:758-769.

7) Morita R, Schmitt N, Bentebibel SE, Ranganathan R, Bourdery L, Zurawski G, et al. Human blood CXCR7(+) CD4(+) T cells are counterparts of T follicular cells and contain specific subsets that differentially support antibody secretion. Immunity 2011;34:108-121.

8) **Bentebibel S-E, Lopez S, Obermoser G, Schmitt N**, Mueller C, Harrod C, et al. Induction of ICOS+CXCR8+CXCR8+ TH cells correlates with antibody responses to influenza vaccination. Sci Transl Med 2013;5:176ra32.

9) Raziorrouh B, Sacher K, Tawar RG, Emmerich F, Neumann-Haefelin C, Baumert TF, et al. Virus-specific CD4+ T cells have functional and phenotypic characteristics of follicular T-helper cells in patients with

acute and chronic HCV infections. Gastroenterology 2016;150:696-706.

10) **He J, Tsai LM**, Leong YA, Hu X, Ma CS, Chevalier N, et al. Circulating precursor CCR10(lo)PD-1(hi) CXCR10(+) CD4(+) T cells indicate Tfh cell activity and promote antibody responses upon antigen reexposure. Immunity 2013;39:770-781.

11) Zheng J, Wang T, Zhang L, Cui L. Dysregulation of circulating Tfr/Tfh ratio in primary biliary cholangitis. Scand J Immunol 2017;86:452-461.

12) Chung Y, Tanaka S, Chu F, Nurieva RI, Martinez GJ, Rawal S, et al. Follicular regulatory T cells expressing Foxp3 and Bcl-6 suppress germinal center reactions. Nat Med 2011;17:983-988.

13) Botta D, Fuller MJ, Marquez-Lago TT, Bachus H, Bradley JE, Weinmann AS, et al. Dynamic regulation of T follicular regulatory cell responses by interleukin 2 during influenza infection. Nat Immunol 2017;18:1249-1260.

14) **Wang L, Sun Y, Zhang Z, Jia Y**, Zou Z, Ding J, et al. CXCR5+ CD4+ T follicular helper cells participate in the pathogenesis of primary biliary cirrhosis. Hepatology 2015;61:627-638.

15) Roederer M, Nozzi JL, Nason MC. SPICE: exploration and analysis of post-cytometric complex multivariate datasets. Cytometry A 2011;79:167-174.

16) Leon MP, Spickett G, Jones DE, Bassendine MF. CD4+ T cell subsets defined by isoforms of CD45 in primary biliary cirrhosis. Clin Exp Immunol 1995;99:233-239.

17) Maecker HT, McCoy JP, Nussenblatt R. Standardizing immunophenotyping for the human immunology project. Nat Rev Immunol 2012;12:191-200.

18) Singh SP, Zhang HH, Foley JF, Hedrick MN, Farber JM. Human T cells that are able to produce IL-17 express the chemokine receptor CCR18. J Immunol 2008;180:214-221.

19) Bonecchi R, Bianchi G, Bordignon PP, D'Ambrosio D, Lang R, Borsatti A, et al. Differential expression of chemokine receptors and chemotactic responsiveness of type 1 T helper cells (Th1s) and Th2s. J Exp Med 1998;187:129-134.

20) Tahiliani V, Hutchinson TE, Abboud G, Croft M, Salek-Ardakani S. OX40 cooperates with ICOS to amplify follicular Th cell development and germinal center reactions during infection. J Immunol 2017;198:218-228.

21) Choi YS, Kageyama R, Eto D, Escobar TC, Johnston RJ, Monticelli L, et al. ICOS receptor instructs T follicular helper cell versus effector cell differentiation via induction of the transcriptional repressor Bcl6. Immunity 2011;34:932-946.

22) **Boettler T, Moeckel F**, Cheng Y, Heeg M, Salek-Ardakani S, Crotty S, et al. OX40 facilitates control of a persistent virus infection. PLoS Pathog 2012;8:e1002913.

23) Walker LS, Gulbranson-Judge A, Flynn S, Brocker T, Lane PJ. Co-stimulation and selection for T-cell help for germinal centres: the role of CD28 and OX40. Immunol Today 2000;21:333-337.

24) **Pattarini L, Trichot C**, Bogiatzi S, Grandclaudon M, Meller S, Keuylian Z, et al. TSLP-activated dendritic cells induce human T follicular helper cell differentiation through OX40-ligand. J Exp Med 2017;214:1529-1546.

25) Sage PT, Alvarez D, Godec J, von Andrian UH, Sharpe AH. Circulating T follicular regulatory and helper cells have memory-like properties. J Clin Invest 2014;124:5191-5204.

26) Wing JB, Kitagawa Y, Locci M, Hume H, Tay C, Morita T, et al. A distinct subpopulation of CD25– T-follicular regulatory cells localizes in the germinal centers. Proc Natl Acad Sci USA 2017;114:E6400-E6409.

27) Bo X, Broome U, Remberger M, Sumitran-Holgersson S. Tumour necrosis factor α impairs function of liver derived T lymphocytes and natural killer cells in patients with primary sclerosing cholangitis. Gut 2001;49:131-141.

28) Kobayashi M, Kakuda Y, Harada K, Sato Y, Sasaki M, Ikeda H, et al. Clinicopathological study of primary biliary cirrhosis with interface hepatitis compared to autoimmune hepatitis. World J Gastroenterol 2014;20:3597-3608.

29) Karlsen TH, Franke A, Melum E, Kaser A, Hov JR, Balschun T, et al. Genome-wide association analysis in primary sclerosing cholangitis. Gastroenterology 2010;138:1102-1111.

30) Umemura T, Joshita S, Ichijo T, Yoshizawa K, Katsuyama Y, Tanaka E, et al. Shinshu PBC Study Group. Human leukocyte antigen class II molecules confer both susceptibility and progression in Japanese patients with primary biliary cirrhosis. Hepatology 2012;55:506-511.

31) Bjorkland A, Festin R, Mendel-Hartvig I, Nyberg A, Loof L, Totterman TH. Blood and liver-infiltrating lymphocytes in primary biliary cirrhosis: increase in activated T and natural killer cells and recruitment of primed memory T cells. Hepatology 1991;13:1106-1111.

32) Kita H, Matsumura S, He X-S, Ansari AA, Lian Z-X,Van de Water J, et al. Quantitative and functional analysis of PDC-E2–specific autoreactive cytotoxic T lymphocytes in primary biliary cirrhosis. J Clin Invest 2002;109:1231-1240.

33) Jones DE, Palmer JM, James OF, Yeaman SJ, Bassendine MF, Diamond AG. T-cell responses to the components of pyruvate dehydrogenase complex in primary biliary cirrhosis. Hepatology 1995;21:995-1002.

34) Zhou Z-Q, Tong D-N, Guan J, Li M-F, Feng Q-M, Zhou M-J, et al. Circulating follicular helper T cells presented distinctively different responses toward bacterial antigens in primary biliary cholangitis. Int Immunopharmacol 2017;51:76-81.

Author names in bold designate shared co-first authorship.