Monocytes as Potential Mediators of Pathogen-Induced T-Helper 17 Differentiation in Patients With Primary Sclerosing Cholangitis (PSC)

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BACKGROUND AND AIMS: T cells from patients with primary sclerosing cholangitis (PSC) show a prominent interleukin (IL)-17 response upon stimulation with bacteria or fungi, yet the reasons for this dominant T-helper 17 (Th17) response in PSC are not clear. Here, we analyzed the potential role of monocytes in microbial recognition and in skewing the T-cell response toward Th17.

APPROACH AND RESULTS: Monocytes and T cells from blood and livers of PSC patients and controls were analyzed ex vivo and in vitro using transwell experiments with cholangiocytes. Cytokine production was measured using flow cytometry, enzyme-linked immunosorbent assay, RNA in situ hybridization, and quantitative real-time PCR. Genetic polymorphisms were obtained from ImmunoChip analysis. Following ex vivo stimulation with phorbol myristate acetate/ionomycin, PSC patients showed significantly increased numbers of IL-17A-producing peripheral blood CD4+ T cells compared to PBC patients and healthy controls, indicating increased Th17 differentiation in vivo. Upon stimulation with microbes, monocytes from PSC patients produced significantly more IL-1β and IL-6, cytokines known to drive Th17 cell differentiation. Moreover, microbe-activated monocytes induced the secretion of Th17 and monocyte-recruiting chemokines chemokine (C-C motif) ligand (CCL)-20 and CCL-2 in human primary cholangiocytes. In livers of patients with PSC cirrhosis, CD14hiCD16int and CD14hiCD16hi monocytes/macrophages were increased compared to alcoholic cirrhosis, and monocytes were found to be located around bile ducts.

CONCLUSIONS: PSC patients show increased Th17 differentiation already in vivo. Microbe-stimulated monocytes drive Th17 differentiation in vitro and induce cholangiocytes to produce chemokines mediating recruitment of Th17 cells and more monocytes into portal tracts. Taken together, these results point to a pathogenic role of monocytes in patients with PSC. (Hepatology 2020;72:1310-1326).

Primary sclerosing cholangitis (PSC) is a chronic liver disease characterized by inflammation and fibrosis of the intra- and extrahepatic bile ducts,
leading to liver cirrhosis and hepatobiliary malignancy.\(^{1}\) PSC is strongly associated with inflammatory bowel disease (IBD), particularly a unique phenotype of ulcerative colitis (UC).\(^{2,3}\) There is currently no medical treatment available with a proven effect on disease progression, rendering PSC a major indication for liver transplantation.\(^{4,5}\) Several mechanisms have been considered to be part of a complex and multifactorial disease pathogenesis, including dysregulated adaptive immune responses, autoimmunity, and defects in mechanisms protecting against bile acid toxicity.\(^{1,6}\) The strong association with colitis and characteristic fecal microbiota alterations support the notion that responses to bacteria or their products at mucosal surfaces play a role in disease pathogenesis.\(^{7,8}\) In line with this, our group recently demonstrated a significantly altered microbial composition in ductal bile of patients with PSC.\(^{9}\) In addition, genetic studies identified a number of PSC risk loci that encode for key components of the innate and adaptive immune system.\(^{10}\) Some of them are involved in regulating microbiota diversity, pathogen defense, as well as Th17 differentiation and—maintenance.\(^{11-13}\)

We have previously shown an increased production of interleukin (IL)-17A by CD4\(^+\) T cells upon pathogen stimulation in vitro and IL-17–producing T cells around bile ducts within livers of PSC patients.\(^{14}\) Increased production of the chemokine, chemokine (C-C motif) (CCL)-20, by cholangiocytes was shown to induce recruitment of Th17 cells to bile ducts.\(^{15}\) Th17 cells contribute to protection against extracellular bacterial and fungal pathogens at mucosal surfaces in the gastrointestinal tract, airway, lungs, and skin.\(^{16,17}\) However, Th17 cells are also described to be critically involved in the pathogenesis of several human autoimmune and inflammatory diseases\(^{18,19}\) such as multiple sclerosis,\(^{20}\) rheumatoid arthritis,\(^{21}\) or Crohn's disease.\(^{22,23}\) Of note, bacterial species with the ability to penetrate intestinal mucosa have recently been shown to induce IL-17A–producing T cells and aggravate mouse models of cholangitis.\(^{24}\) Monocytes within blood and liver significantly contribute to pathogen surveillance and are efficient professional antigen-presenting cells with the ability to induce effector T–cell function as well as stimulation of epithelial cells.\(^{25,26}\) It was recently shown that monocytes contribute to disease pathogenesis in mouse models of sclerosing cholangitis, and that depletion of monocytes improved disease severity.\(^{27}\)

We here aimed to investigate the pathogenic role of monocytes in patients with PSC by analyzing their capacity to induce Th17 differentiation and activation of cholangiocytes.

**Patients and Methods**

**PATIENTS**

A total of 135 patients with PSC, 99 with primary biliary cholangitis (PBC), 29 with autoimmune hepatitis (AIH), 19 with nonalcoholic fatty liver disease (NAFLD), 8 with inflammatory bowel disease...
(IBD) only, and 8 with alcohol-induced liver disease (ALD) who attended the outpatient service of the I. Department of Medicine, University Medical Centre Hamburg-Eppendorf (UKE; Hamburg, Germany) and 97 healthy controls were included in the study. PSC, PBC, and AIH were diagnosed by generally accepted criteria according to the European Association for the Study of the Liver guidelines. Exclusion criteria for this study were acute deterioration/flares of disease or an immunosuppressive therapy with >10 mg/d prednisolone or >1.5 mg/kg body weight azathioprine per day. For patients with IBD, exclusion criteria were no intense immunosuppressive therapy and no anti–tumor necrosis factor treatment.

Clinical characteristics of all included patients are shown in Table 1. For flow cytometric ex vivo analysis of Th17 and T-helper 1 (Th1) frequencies, blood samples of 62 PSC patients, 56 PBC patients, 29 AIH patients, 19 patients with NAFLD, 8 patients with IBD only, and 34 healthy controls were used (Table 2). Eight PSC patients and 4 PBC patients from ex vivo analysis were categorized as advanced disease according to imaging signs compatible with liver cirrhosis (ultrasound/magnetic resonance imaging/computed tomography) or advanced fibrosis using transient elastography/fibroscan (≥9.6 kPa), splenomegaly (≥12.0 cm), esophageal/gastric varices, ascites, or hepatic encephalopathy. IL-17 and interferon-gamma (IFNγ) cytokine secretion of peripheral blood mononuclear cells (PBMCs) was analyzed in blood samples of 30 patients with PSC, 19 patients with PBC, and 16 healthy donors. Single-nucleotide polymorphism (SNP) analysis was performed for 31 PSC patients and 29 healthy controls. Microbiologically induced Th17 differentiation in PBMCs was performed with PBMCs derived from 32 patients with PSC, 14 patients with PBC, and 17 healthy donors. Cytokine-induced Th17 differentiation was investigated in PBMCs derived from 24 patients with PSC, 15 patients with PBC, and 12 healthy donors. Cytokine production of PBMCs stimulated with heat-inactivated pathogen was analyzed from 28 patients with PSC, 20 PBC patients, and 26 healthy donors. Cytokine production of monocytes was analyzed in blood samples of 25 PSC patients and 21 healthy controls.

Monocyte populations were analyzed from blood and explanted livers of 10 patients with PSC and 8 patients with ALD undergoing liver transplantation.
Cholangiocytes were isolated from liver tissue of 2 explant patients with PSC. No donor organs were obtained from executed prisoners or other institutionalized persons. This study was approved by the Ethics Committee of Hamburg, and written informed consent was obtained from all patients and healthy controls (PV4081).

**ISOLATION OF PBMCs, NAÏVE CD4+ T CELLS, AND MONOCYTES FROM PERIPHERAL BLOOD**

Peripheral blood mononuclear cells (PBMCs) were isolated as described. Monocytes and naïve CD4+ T cells were freshly isolated from PBMCs using the “human Pan Monocytes Isolation Kit” and “naive CD4+ T cell Isolation Kit” (both Miltenyi Biotech, Germany), according to the manufacturer’s instructions. Purity of isolated cells was analyzed by flow cytometry and confirmed to be 80%-90% for monocytes and 90%-95% for naïve CD4+ T cells.

**ISOLATION OF LIVER-INFILTRATING LEUKOCYTES**

Liver tissue was processed immediately after surgery. Tissue was cut into small pieces and transferred into gentleMACS C Tubes (Miltenyi Biotech, Germany) slightly covered with RPMI1640+Glutamax medium (Gibco, Life Technologies, Darmstadt, Germany), supplemented with 10% fetal calf serum (FCS; PAA Laboratories, Pasching, Austria) and 1% penicillin/streptomycin (P/S). Pieces were hashed using the gentleMACS Octo Dissociator (Miltenyi Biotech, Germany), and suspension was successively filtered using by cell-strainer separation with mesh sizes from 300 to 40 µm. Purified cells were frozen in FCS containing 10% dimethyl sulfoxide.

**ISOLATION OF PRIMARY CHOLANGIOCYTES**

Cholangiocytes were isolated from human explant tissue with a collagenase digestion followed by cell-strainer separation (100 µm). Hepatocytes were removed by centrifugation (40g) to further enrich the cholangiocyte population. DynaBead separation with an epithelial cell adhesion molecule HEA124 antibody (Progen, Heidelberg, Germany) and goat anti-mouse immunoglobulin G (IgG) DynaBeads
(Invitrogen, Carlsbad, CA) was performed. Cells were grown in cholangiocyte medium as described.\(^{30}\)

**FLOW CYTOMETRY**

Immunofluorescence staining of cells was performed with antibodies to CD3, CD4, CD14, CD16, CD20, CD45, CD45RA, CD56, human leukocyte antigen-DR, IL-17A, and IFN\(\gamma\) (all Biolegend, Koblenz, Germany). Dead cells were stained with Pacific Orange-NHS (Life technologies, Carlsbad, CA) and excluded from further analysis. Flow cytometry data were analyzed with fluorescence-activated cell sorting (FACS) Diva Software (BD Biosciences, Heidelberg, Germany).

**CELL RESTIMULATION AND CYTOKINE MEASUREMENT**

For intracellular cytokine staining, peripheral whole blood was restimulated with phorbol myristate acetate (PMA; 25 ng/mL) and ionomycin (5 \(\mu\)g/mL; both Sigma-Aldrich, Darmstadt, Germany) in the presence of GolgiPlug (1 \(\mu\)g/mL; BD Biosciences, Germany) for 4 hours. Surface staining was performed with FACS Lysing Solution and Cytofix/Cytoperm (both BD Biosciences, Germany). Cells were stained for IFN\(\gamma\) and IL-17A. For enzyme-linked immunosorbent assay (ELISA) measurement, isolated PBMCs (1 \(\times\) \(10^6\) cells/mL) were restimulated for 86 hours with 1 \(\mu\)g/mL of anti-CD3 (clone OKT3) and 1 \(\mu\)g/mL of anti-CD28 antibodies (both BioLegend, Germany) in RPMI1640+Glutamax medium (5% FCS and 1% P/S). Supernatants were analyzed for IFN\(\gamma\) and IL-17A by ELISA (both Peprotech, Hamburg, Germany), according to the manufacturer’s protocol.

**STIMULATION OF PBMCs/MONOCYTES WITH HEAT-INACTIVATED MICROBES**

PBMCs or monocytes (3 \(\times\) \(10^6\) cells/mL) were stimulated with heat-inactivated microbes (10^9 U/mL) for 24 hours in RPMI1640+Glutamax medium (2% FCS and 1% P/S). Supernatants were analyzed by ELISA using the human IL-1\(\beta\) DuoSet ELISA kit (R&D Systems, Germany) and the human IL-6 ELISA Kit (Peprotech, Germany), according to the manufacturer’s protocol.

**CHOLANGIOCYTES AND MONOCYTE COCULTURE**

For stimulation, 3 \(\times\) \(10^5\) cells were cultured in cholangiocyte medium\(^{26}\) overnight. Epidermal growth factor and FCS were withdrawn and cells were incubated overnight again, reaching approximately 90% confluence. Human IL-1\(\beta\) (1 ng/mL) or 30 ng/mL of human IL-6 (both BioLegend, Germany) was added to the culture for 6 hours. For transwell experiments, 5 \(\times\) \(10^5\) cholangiocytes were cultured in cholangiocyte medium to reach 80% confluence the next day. Medium was changed to RPMI1640+Glutamax medium (2% FCS and 1% P/S). Freshly isolated monocytes (5 \(\times\) \(10^5\)/well) and heat-inactivated \(C.\) albicans were added into the tissue culture inserts (Sarstedt, Nümbrecht, Germany) for 24 hours. For IL-1\(\beta\) neutralization, 5 ng/mL of human anti-IL-1\(\beta\)/IL-1F2 antibody (R&D Systems, Germany) was supplemented. Supernatants were analyzed by ELISA for IL-6, IL-8, CCL-2 (all Peprotech, Germany), and (2 \(\mu\)g/mL, clone CD28.2; BioLegend, Germany) in RPMI1640+Glutamax medium, supplemented with 10% FCS and 1% P/S. For Th17 differentiation, IL-6 (30 ng/mL), IL-1\(\beta\) (30 ng/mL), IL-23 (30 ng/mL; all Miltenyi Biotec, Germany), and transforming growth factor beta 1 (TGF\(\beta\)1; 2.25 ng/mL; R&D Systems, Wiesbaden, Germany) were supplemented. T-helper 1 (Th1) and T-helper 2 differentiation was suppressed by addition of 1 \(\mu\)g/mL of anti-IL-12 (PeproTech, Germany), 1 \(\mu\)g/mL of anti-IFN\(\gamma\), and 2.5 \(\mu\)g/mL of anti-IL-4 antibodies (both Miltenyi Biotec, Germany).

**IN VITRO Th17 DIFFERENTIATION**

PBMCs (1 \(\times\) \(10^6\) cells/mL) or naive CD4\(^+\) T cells were cultured with anti-CD3 (2 \(\mu\)g/mL, clone UCHT1; BD Biosciences, Germany) and anti-CD28 antibodies (2 \(\mu\)g/mL, clone CD28.2; BioLegend, Germany) in RPMI1640+Glutamax medium, supplemented with 10% FCS and 1% P/S. For Th17 differentiation, IL-6 (30 ng/mL), IL-1\(\beta\) (30 ng/mL), IL-23 (30 ng/mL; all Miltenyi Biotec, Germany), and transforming growth factor beta 1 (TGF\(\beta\)1; 2.25 ng/mL; R&D Systems, Wiesbaden, Germany) were supplemented. T-helper 1 (Th1) and T-helper 2 differentiation was suppressed by addition of 1 \(\mu\)g/mL of anti-IL-12 (PeproTech, Germany), 1 \(\mu\)g/mL of anti-IFN\(\gamma\), and 2.5 \(\mu\)g/mL of anti-IL-4 antibodies (both Miltenyi Biotec, Germany). On days 5 and 8, medium and cytokine mix were substituted. Th17 differentiation with PBMCs and heat-inactivated \(C.\) albicans was performed as described.\(^{14}\) Th17 levels were analyzed on day 12 by flow cytometry.
CCL-20 (BioLegend, Germany), and cells were harvested for RNA isolation and qPCR analysis.

**REAL-TIME qPCR**

Total RNA was extracted from cholangiocytes using the Nucleospin Kit (Macherey Nagel, Düren, Germany), and complementary DNA (cDNA) was reverse-transcribed from total RNA (High Capacity cDNA Reverse Transcription Kit; Applied Biosystems, Germany). Expression of IL-6 (Hs00174131_m1), IL-8 (Hs00174103_m1), IL-23 (Hs00372324_m1), CCL-20 (Hs00355476_m1), CCL-2 (Hs00172878_m1), and TGF\(\beta\)1 (Hs00998133_m1) was measured using the Taqman Universal PCR master mix. Taqman gene expression kits (all Applied Biosystems, Weiterstadt, Germany) were used for amplification, and target gene expression was normalized to hypoxanthine-guanine phosphoribosyltransferase (Hs02800695_m1) housekeeper levels by using the delta delta threshold cycle method.

**HISTOLOGY**

IL-17A, CD3, CD14, and RNAscope staining was carried out on paraffin-embedded human PSC explant liver tissue. RNA in situ hybridization (RISH) was performed using the RNAscope 2.5 HD Detection Reagent Kit (Advanced Cell Diagnostic, Milan, Italy), according to manufacturer’s instruction. Briefly, formalin-fixed, paraffin-embedded human liver sections were dehydrated, and endogenous peroxidase activity was blocked and followed by antigen retrieval. Protease Plus (Vector Laboratories, Dossenheim, Germany) treatment was performed for 30 minutes at 40°C. Slides were incubated for 2 hours at 40°C with target probes specific for IL-1\(\beta\) (HS-IL1B). The signal was amplified and developed with alkaline phosphatase (AP)-based Fast Red chromogens. CD3 was stained using anti-human CD3 (Invitrogen) and goat anti-mouse horseradish peroxidase (HRP; DAKO, Hamburg, Germany), and CD14 was stained using anti-human CD14 (clone CL1638; Atlas Antibodies, Germany) and polyclonal rabbit anti-mouse IgG HRP (DAKO, Germany). IL-17A was stained using anti-human IL-17A (R&D Systems, Germany) polyclonal rabbit anti-goat IgG (DAKO, Germany) and anti-rabbit AP-complex (Polap kit; Zytoxed, Germany). For CD3 and CD14, peroxidase activity was visualized using 3.3′-diaminobenzidine, and AP was used to visualize IL-17 using the POLAP-Kit (Zytoxed, Bargteheide, Germany). Sections were counterstained with hematoxylin (both Roth, Karlsruhe, Germany). All pictures were taken with Biorevo BZ-9000 and analyzed using BZ-II Analyzer software (both Keyence, Osaka, Japan).

**GENOTYPING**

SNP analysis of IL-21 (rs11936230), REL (rs13017599 and rs6706689), and caspase recruitment domain-containing protein 9 (CARD9; rs4077515) gene loci were conducted in PSC patients and healthy controls. Data were derived from ImmunoChip genotyping (31) and Sanger sequencing at the Institute of Clinical Molecular Biology, Christian-Albrechts-University of Kiel, Germany.

**STATISTICAL ANALYSIS**

Data are presented as mean ± SEM. Differences between two groups were assessed for statistical significance using the Mann-Whitney U test. Differences between three or more groups were assessed using the nonparametric Kruskal-Wallis test, followed by Dunn’s multiple comparison or one-way ANOVA (analysis of variance) test and Tukey’s post-hoc test (\(P\) value <0.05 was considered as significant and \(P\) values are as indicated: \(*P\) ≤ 0.05, \(**P\) ≤ 0.01, \(***P\) ≤ 0.001, and \(****P\) ≤ 0.0001).

**Results**

**INCREASED IN VIVO Th17 DIFFERENTIATION IN PATIENTS WITH PSC**

CD4+ T cells from PSC patients showed increased IL-17A production in response to in vitro stimulation with microbes derived from bile. In order to explore whether Th17 differentiation already occurs in vivo in patients with PSC, we investigated Th17 frequencies in peripheral blood of PSC patients and control groups ex vivo upon stimulation with PMA/ionomycin (Table 2). PSC patients showed the highest frequencies of IL-17A–expressing CD4+ T cells in peripheral blood compared to other inflammatory liver diseases with similar liver stiffness and bilirubin levels and to IBD-only patients (Fig. 1A and Supporting Fig. S1). Patients with PSC showed significantly higher frequencies of Th17 cells in peripheral...
FIG. 1. Increased frequency of Th17 cells in peripheral blood of patients with PSC. Peripheral blood of patients with PSC, PBC, AIH, NAFLD, IBD only, and healthy donors was stimulated with PMA/ionomycin for 4 hours and analyzed for cytokine production using flow cytometry. (A) PSC patients showed significantly increased frequencies of IL-17A–producing CD4+ T cells as compared to healthy donors and PBC patients. (B) Expression of IFNγ was decreased in patients with AIH, but similar between CD4+ T cells of PSC, PBC, AIH, NAFLD, IBD-only patients, and healthy donors. Isolated PBMCs were stimulated with anti-CD3 and anti-CD28 antibodies for 96 hours, and (C) IL-17 and (D) IFNγ levels were measured in supernatants using ELISA. (E) Th17 frequencies were increased already in early-stage PSC patients. Data were analyzed using the Kruskal-Wallis test, followed by Dunn's multiple comparison: *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001.
blood compared to healthy controls (PSC, 2.5% ± 0.2 vs. healthy, 1.75% ± 0.1; \( P = 0.0012 \)) and to a group of patients with PBC, another autoimmune cholestatic liver disease (PSC, 2.5% ± 0.2 vs. PBC, 1.6% ± 0.1; \( P = 0.0037 \)). In order to demonstrate that sex and age did not have significant effects on the results obtained, we analyzed the frequencies of IL-17–producing CD4+ T cells in PSC patients and matched healthy controls (Supporting Fig. S2). Compared to healthy controls or patients with PBC, PSC patients showed significantly increased frequencies of Th17 cells in blood already at early disease stages (nonadvanced PSC, 2.6% ± 0.2 vs. healthy, 1.6% ± 0.2 vs. nonadvanced PBC, 1.9% ± 0.2; **\( P = 0.0017 \) and *\( P = 0.0382 \); Fig. 1E). Given that Th1 cells have been implicated in autoimmunity and were reported to be involved in the development of cholangitis,\(^{(32-34)}\) we further analyzed the ex vivo frequency of Th1 cells in peripheral blood. We found that Th1 cell frequencies in PSC patients were not significantly different compared to healthy controls and PBC, NAFLD, or IBD patients (PSC, 19.74% ± 1.2; healthy, 15.69% ± 2.1; PBC, 20.68% ± 1.6; NAFLD, 14.95% ± 2.8; IBD, 16.46% ± 3.4; Fig. 1B).

In order to support the flow-cytometry–based finding of increased Th17 frequencies in blood of patients with PSC compared to healthy controls, we determined IL-17A levels in supernatants of ex vivo restimulated PBMCs. To exclude an effect of ursodeoxycholic acid (UDCA) treatment or cholestasis per se, we analyzed IL-17 levels in patients with PBC who had similar treatment regimens and serum bilirubin levels (Table 1). We confirmed an increased Th17 differentiation in PSC patients by showing significantly increased production of IL-17A by PBMCs from PSC patients in comparison to healthy controls and PBC patients (PSC, 1,639 pg/mL ± 131 vs. healthy, 939 pg/mL ± 120 vs. PBC, 1,132 pg/mL ± 141; \( P = 0.0014 \); Fig. 1C). In contrast, there was no difference in IFN\(\gamma\) production between the groups (Fig. 1D).

Th17 FREQUENCIES IN PERIPHERAL BLOOD WERE NOT ASSOCIATED WITH THE PRESENCE OF ASSOCIATED COLITIS OR POLYMORPHISMS IN IL-21, REL, AND CARD9

Associated IBD is the most common inflammatory comorbidity in PSC and is thought to be a central characteristic of the disease.\(^{(2,3)}\) Given that IL-17A is also involved in the pathogenesis of IBD,\(^{(23)}\) we next investigated whether the increased frequencies of IL-17–producing CD4+ T cells in peripheral blood of PSC patients were associated with the presence of colitis. Th17 cell frequencies and IL-17A production were similar between PSC patients with or without associated colitis (Supporting Fig. S3A,B).

Moreover, we analyzed polymorphisms in genetic loci of IL-21, REL, and CARD9, which has been shown to be associated with PSC and could potentially be involved in Th17 differentiation.\(^{(11,12,35)}\) In a limited number of patients, the analyzed genotypes were not associated with frequencies of Th17 cells in PSC patients or healthy controls (Supporting Fig. S4).

INCREASED SECRETION OF TH17-INDUCING CYTOKINES IL-1\(\beta\) AND IL-6 BY MONOCYTES FROM PATIENTS WITH PSC AFTER MICROBIAL STIMULATION

Differentiation of naïve CD4+ T cells into Th17 cells depends, to a large extent, on action of IL-1\(\beta\) together with IL-6.\(^{(36)}\) It has been well described that circulating monocytes could trigger and polarize CD4+ T-cell responses.\(^{(36-38)}\) Given that monocytes are a major source of IL-1\(\beta\) and IL-6, we hypothesized that in vivo monocytes may represent the functional link between microbial recognition and induction of IL-17 production in T cells. Upon stimulation with \(\text{C. albicans} \) (Fig. 2A) or \(\text{E. faecalis} \) (Fig. 2B), PBMCs from patients with PSC produced significantly higher amounts of IL-1\(\beta\) and IL-6 compared to PBMCs derived from controls. Interestingly, we could not detect differences in secretion of IL-1\(\beta\) or IL-6 upon stimulation with \(\text{E. coli} \) (Fig. 2C). This finding was independent of the presence or absence of PSC-associated colitis (Supporting Fig. S5).

We next aimed to investigate whether monocytes contribute to the observed production of IL-1\(\beta\) and IL-6 after pathogen stimulation in peripheral blood of PSC patients. Thus, we stimulated PBMCs, blood-derived monocytes, or residual PBMCs without monocytes from the same individuals with \(\text{C. albicans} \). In our previous studies, stimulation
with *C. albicans* induced the strongest Th17 differentiation among tested pathogens, and *C. albicans* has recently been identified as the major fungal inducer of human Th17 responses.\(^{14,39}\) Monocytes obtained from healthy donors as well as from PSC patients showed increased IL-1β secretion compared...
to total PBMCs or PBMCs without monocytes (Fig. 3A,B). Interestingly, monocytes from PSC patients produced significantly higher amounts of IL-1β compared to healthy controls (PSC, 18,901 pg/mL ± 1,068 vs. healthy, 14,698 pg/mL ± 1,585; \( P = 0.046 \); Fig. 3C). Similar results were obtained for production of IL-6. We found that monocytes obtained from healthy donors as well as from PSC patients were the main producers of IL-6 upon pathogen stimulation (Fig. 3D,E), and monocytes from PSC patients produced significantly higher amounts of IL-6 compared to healthy controls (PSC, 46,603 pg/mL ± 3,130 vs. healthy, 35,903 pg/mL ± 3,375; \( P = 0.037 \); Fig. 3F).

**INCREASED Th17 DIFFERENTIATION OF NAÏVE CD4⁺ T CELLS FROM PSC PATIENTS UPON IN VITRO STIMULATION WITH IL-1β AND IL-6**

After having shown that monocytes from patients with PSC produce increased amounts of Th17-differentiating cytokines IL-1β and IL-6, we next investigated whether these cytokines could induce IL-17A production in T cells also in the absence of microbial stimulation. PBMCs were isolated from blood of PSC and PBC patients or healthy controls and differentiated in vitro in a cytokine-dependent manner in comparison

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**FIG. 3.** Monocytes from patients with PSC secreted increased amounts of IL-6 and IL-1β upon pathogen stimulation. Whole PBMCs, monocytes, and PBMCs without monocytes of (A,D) healthy controls and (B,E) patients with PSC were stimulated for 24 hours with heat-inactivated *C. albicans*. (A–C) IL-1β or (D–F) IL-6 cytokine secretion was analyzed in supernatants after stimulation with *C. albicans* for 24 hours by ELISA. Data were analyzed using the Kruskal-Wallis test, followed by Dunn’s multiple comparison: *\( P \leq 0.05 \); **\( P \leq 0.01 \); ***\( P \leq 0.001 \); ****\( P \leq 0.0001 \). (C) IL-1β or (F) IL-6 concentrations were increased in supernatants of stimulated monocytes isolated from PSC patients compared to healthy donors. Data were analyzed using one-way ANOVA and Tukey’s post-test: *\( P < 0.01 \).
FIG. 4. Increased in vitro Th17 differentiation in patients with PSC. PBMCs and naïve CD4+ T cells of patients with PSC or PBC and healthy donors were isolated and cultured in vitro under Th17 differentiation conditions for 12 days and analyzed for cytokine production using flow cytometry. (A) Gating strategy for the analysis of CD4+ T cells according to IL-17 and IFN-γ expression following Th17 differentiation. IL-17 and IFN-γ expression of (B) pathogen-stimulated PBMC with heat-inactivated C. albicans, (C) cytokine-stimulated PMBCs, and (D) cytokine-stimulated naïve CD4+ T cells from patients with PSC compared to PBC and healthy donors. Data were analyzed using the Kruskal-Wallis test, followed by Dunn's multiple comparison: *P ≤ 0.05; **P ≤ 0.01.
to stimulation with \textit{C. albicans} (Fig. 4A-C). Cytokine-induced Th17 differentiation was significantly increased in PSC patients compared to healthy controls and showed a tendency toward higher differentiation compared to PBMCs derived from PBC patients (PSC, 4.3% ± 0.8 vs. healthy, 2.1 ± 0.3 vs. PBC, 3.6 ± 0.7; \( P = 0.034 \); Fig. 4C). These findings were confirmed using isolated naïve CD4+ T cells, which showed a significantly higher capacity to convert into Th17 cells in PSC patients compared to CD4+ T cells derived from PBC patients or healthy controls (PSC, 1.36% ± 0.24 vs. healthy, 0.41% ± 0.1 vs. PBC, 0.31% ± 0.1; \( P = 0.0002 \); Fig. 4D).

INCREASED FREQUENCIES OF CD14\textsuperscript{hi}CD16\textsuperscript{int} AND CD14\textsuperscript{lo}CD16\textsuperscript{hi} MONOCYTES IN THE LIVERS OF PATIENTS WITH PSC COMPARED TO ALCOHOLIC LIVER CIRRHOSIS

After finding that monocytes from peripheral blood potentially contribute to Th17 differentiation in PSC, we were interested to characterize monocyte-derived macrophage populations within PSC livers. To that end, monocytes/macrophages were analyzed in explant livers of patients with PSC. Explant tissue from ALD patients was used as controls. CD14 and CD16 expression was determined using flow cytometry.\(^{(40)}\) Thereby, the classical (CD14\textsuperscript{hi}CD16\textsuperscript{lo}) and the more proinflammatory intermediate (CD14\textsuperscript{hi}CD16\textsuperscript{int}) and nonclassical (CD14\textsuperscript{lo}CD16\textsuperscript{hi}) monocyte/macrophage populations were identified.\(^{(41)}\) We observed a significant increase in CD14\textsuperscript{hi}CD16\textsuperscript{int} and CD14\textsuperscript{lo}CD16\textsuperscript{hi} monocyte/macrophage subpopulations in livers of PSC patients compared to ALD (Fig. 5A), but not in peripheral blood. This is of interest given that increased hepatic infiltration with monocyte-derived CD14\textsuperscript{+}CD16\textsuperscript{+} macrophages in chronic liver diseases has been described, and a peribiliary infiltration of monocyte-derived macrophages with a proinflammatory phenotype has recently been shown in liver parenchyma of patients with advanced PSC.\(^{(27,42-44)}\)

IL-17A–PRODUCING T CELLS AND MONOCYTES ARE LOCALIZED AROUND BILE DUCTS OF PSC PATIENTS

To clarify the hepatic localization of Th17 cells and macrophages, we performed immunohistochemistry (IHC) staining of explanted PSC livers. We confirmed that IL-17A\textsuperscript{+} cells and CD3+IL-17\textsuperscript{+} cells are found in portal infiltrates with preferential localization around bile ducts (Fig. 5B,C) and observed CD14-positive monocytes/macrophages close to bile ducts within portal tracts of PSC patients (Fig. 5D). Moreover, IHC RNAscope staining of liver tissue from PSC patients revealed mRNA expression of IL-1β in CD14-positive monocytes/macrophages within inflamed portal tracts and a strong IL-1β expression in PSC cholangiocytes (Fig. 5E).

MONOCYTE-DERIVED IL-1β CONTRIBUTES TO PATHOGEN-INDUCED ACTIVATION OF HUMAN CHOLANGIOCYTES

Circulating blood cells are recruited into injured tissue by the action of chemokines. It has been previously described that localization of Th17 cells around human bile ducts was related to CCL-20 secretion by cholangiocytes.\(^{(15)}\) Localization of monocyte-derived cells close to bile ducts and their ability to secrete IL-1β suggested that monocyte-derived cytokines could be potential activators of cholangiocytes. To analyze whether cholangiocytes can be activated by cytokines released from monocytes, we performed \textit{in vitro} stimulation assays of the human H69 cholangiocyte cell line as well as primary cholangiocytes isolated from PSC patients. We observed that CCL-20, IL-6, IL-8, and monocyte-recruiting chemokine CCL-2 mRNA and protein expression in H69 cells was markedly increased in response to activation with IL-1β (Supporting Fig. S6). Additional supplementation with IL-6 did not further increase activation status, implicating that IL-1β is the main driver for cholangiocyte activation (Supporting Fig. S7). To confirm that pathogen-stimulated monocytes have the ability to activate cholangiocytes through secretion of cytokines, we performed transwell experiments in which freshly isolated monocytes and heat-inactivated \textit{C. albicans} were added to the culture of H69 cells. Pathogen-stimulated monocytes induced by soluble factors the secretion of chemokines and proinflammatory cytokines, such as CCL-2, CCL-20, IL-6, and IL-8, in H69 cells (Fig. 6A,B). Importantly, we could confirm these results in primary cholangiocytes derived from PSC patients (Fig. 6C,D). Administration of an anti-IL-1β blocking antibody significantly decreased
FIG. 5. Increased frequencies of intermediate and nonclassical monocyte subpopulations and presence of IL-17A–expressing T cells in livers of PSC patients. (A) Flow cytometry analysis of CD14 and CD16 expression on monocytes/macrophages in peripheral blood and liver tissue from PSC patients compared to patients with alcoholic cirrhosis. Data were analyzed using the Kruskal-Wallis test, followed by Dunn's multiple comparison: *P ≤ 0.05. (B) IL-17 (red), (C) CD3 (brown), and IL-17 (red) double staining and (D) CD14 IHC staining of explant liver tissue from a PSC patient. (E) CD14 IHC staining combined with RISH (RNAscope) of PSC-liver explant sections shows the expression of IL-1β (red) in CD14-positive macrophages (brown).
FIG. 6. Activation of cholangiocytes by monocyte-derived IL-1β. (A,B) H69 cells or (C,D) isolated primary cholangiocytes of PSC patients were cocultured with freshly isolated monocytes in the presence or absence of heat-inactivated C. albicans for 24 hours in transwell experiments. Cytokine expression was analyzed (A,C) in supernatants using ELISA or (B,D) in harvested cells performing qPCR analysis. (E) ELISA of supernatants of H69 cells cocultured with monocytes and C. albicans in transwells in the presence or absence of neutralizing IL-1β antibody. Data were analyzed using one-way ANOVA and Tukey’s post-test: **P < 0.01; ***P < 0.001; ****P < 0.0001. Abbreviation: BEC, biliary epithelial cells.
CCL-20 production by cholangiocytes (Fig. 6E,F), demonstrating that monocyte-derived IL-1β contributes to pathogen-induced activation of cholangiocytes.

Thus, our data indicate a positive feedback loop through which activated monocytes, in addition to inducing differentiation of Th17 cells, could amplify their own recruitment and recruitment of Th17 cells into the periductular area of the liver.

Discussion

The pathogenesis of PSC remains largely unknown, and this contributes to the fact that effective treatment options for this progressive disease are lacking. Intestinal bacteria inducing IL-17A production in T cells as well as monocytes have recently been implicated in the pathogenesis of PSC and mouse models of sclerosing cholangitis. We have previously shown that in patients with PSC, in vitro stimulation of PBMCs with microbes resulted in increased IL-17A production. In this study, we show that increased Th17 differentiation previously observed in vitro seems to be the result of increased in vivo Th17 differentiation in PSC patients.

We analyzed whether increased Th17 frequencies in peripheral blood of PSC patients were associated to genetic susceptibility loci known to be associated with PSC and involved in pathogen defense or Th17 differentiation. However, acknowledging the limited number of patients included, our data suggest that genetic associations within the loci of REL (rs13017599 and rs6706689), CARD9 (rs4077515), or IL-21 (rs11936230) did not account for the increased Th17 frequencies observed and rather point toward environmental triggers. Presence of intestinal inflammation could represent one extrahepatic trigger, but we here did not detect an association of PSC-associated colitis with changes in frequencies of Th17 cells. However, intestinal microbiota diversity was shown to be reduced in patients with PSC independently from the presence of associated colitis, and the abundance of potentially proinflammatory bacterial species such as Veillonella sp. was found to be increased. Recently, we have described microbial alterations in the ductal bile of patients with PSC. It therefore seems conceivable that microbiota shape T-cell differentiation in PSC. Interestingly, in vivo differentiation of Th17 cells in PSC may be induced by specific microbiota translocating through the intestinal barrier, as has been recently shown, for example, in Klebsiella pneumoniae. For Th17 differentiation and maintenance, T cells require the action of IL-1β, IL-6, and other cytokines typically produced by antigen-presenting cells. Given that monocytes have recently been implicated in the pathogenesis of sclerosing cholangitis in mouse models of PSC, we hypothesized that monocytes may represent the link between microbial stimulation and T-cell differentiation into Th17 cells. We observed that after stimulation with C. albicans and E. faecalis, two pathogens previously associated with PSC and progression of disease, PBMCs from patients with PSC produced increased amounts of Th17-inducing cytokines IL-1β and IL-6 and identified monocytes as the main producers of these cytokines in blood. CD14^highCD16^ monocytes have been previously described to promote Th17 expansion and maintenance in rheumatoid arthritis and chronic viral hepatitis B infection. In PSC livers, the numbers of perisinusoidal macrophages and recently also of periductular monocyte-derived macrophages were found to be increased. We here detected increased frequencies of CD14^highCD16^ monocyte-derived macrophages in explanted PSC livers compared to ALD. These markers have been associated to a more proinflammatory phenotype. However, the use of CD14 and CD16 alone is not sufficient for the functional characterization of monocyte subsets, and further analyses have to be performed to clarify their phenotypic differentiation. Moreover, we could show that monocytes/macrophages expressing IL-1β located around bile duct epithelial cells in patients with PSC, and that human cholangiocytes responded to monocytes by secreting high levels of the Th17-attracting chemokine, CCL-20, and the monocyte-recruiting chemokine, CCL-2, in addition to the proinflammatory cytokines, IL-6 and IL-23, again both involved in Th17 differentiation and maintenance. Thus, activation of cholangiocytes by monocyte-released IL-1β could create a feedback loop through which recruitment of more IL-17-secreting effector cells and monocytes/macrophages to bile ducts could be amplified.

It is tempting to speculate that Th17 cells as well as monocytes/macrophages could serve as therapeutic targets in PSC. Our ex vivo analyses of peripheral blood demonstrated increased Th17 frequencies already at early stages of PSC. Various blockers of the
IL-17 axis have been developed and shown to effectively treat autoimmune diseases, such as psoriasis, whereas they failed to improve Crohn’s disease. IL-17A has a proinflammatory function, but also aids to combat microbes, for example, by recruiting neutrophils to the site of infection. Therefore, a better understanding of the function of IL-17, including its isoforms, seems fundamental before therapeutic targeting of IL-17 can be considered in PSC. Inhibition of monocytes/macrophage recruitment by cenicriviroc, an oral, dual antagonist of CCR2/CCR5, has been recently shown to ameliorate liver fibrosis in mouse models of sclerosing cholangitis and achieved improvement in fibrosis and no worsening of steatohepatitis in a randomized, placebo-controlled trial. Blocking monocyte/macrophage recruitment to the liver could therefore be an interesting treatment approach in PSC.

Limitations of our study include the single-center design and inclusion of late-stage liver tissue only for assessment of monocyte subsets. Analysis of genotype-phenotype correlations was clearly limited by patient numbers.

In conclusion, we here show that an increased Th17 differentiation occurred in PSC patients already in vivo, and that monocytes may provide the functional link between microbiota and T cells in PSC. These results should stimulate further research into the role of mononcytes as therapeutic targets in PSC.

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