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Increased type-I interferon level is associated with liver damage and fibrosis in primary sclerosing cholangitis

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Abstract

Background: The level of type-I interferons (IFNs) in primary sclerosing cholangitis (PSC) was investigated to evaluate its association with disease activity and progression.

Methods: Bioactive type-I IFNs were evaluated in a murine model of PSC

Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferase; FIB-4, fibrosis 4 score; FVB/N, Friend Virus B NIH Jackson; GGT, gamma-glutamyl transpeptidase; IBD, inflammatory bowel disease; IFN: interferon; IFNAR, type-I interferon receptor; KO, knockout; LSM, liver stiffness measurement, Mdr2, multidrug resistance protein 2; OD, optical density; PAMP, pathogen-associated molecular pattern; PSC, primary sclerosing cholangitis; PBC, primary biliary cholangitis; TLR4/9, toll-like receptor 4/9.

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and human patients' sera using a cell-based reporter assay and ELISA techniques. In total, 57 healthy participants, 71 PSC, and 38 patients with primary biliary cholangitis were enrolled in this study.

Results: Bioactive type-I IFNs were elevated in the liver and serum of multidrug resistance protein 2–deficient animals and showed a correlation with the presence of $CD45^+$ immune cells and serum alanine transaminase levels. Concordantly, bioactive type-I IFNs were elevated in the sera of patients with PSC as compared to healthy controls (sensitivity of 84.51%, specificity of 63.16%, and AUROC value of 0.8267). Bioactive IFNs highly correlated with alkaline phosphatase ($r = 0.4179$, $p < 0.001$), alanine transaminase ($r = 0.4704$, $p < 0.0001$), and gamma-glutamyl transpeptidase activities ($r = 0.6629$, $p < 0.0001$) but not with serum bilirubin. In addition, patients with PSC with advanced fibrosis demonstrated significantly higher type-I IFN values. Among the type-I IFN subtypes IFNα, β and IFNω could be detected in patients with PSC with IFNω showing the highest concentration among the subtypes and being the most abundant among patients with PSC.

Conclusions: The selectively elevated bioactive type-I IFNs specifically the dominating IFNω could suggest a novel inflammatory pathway that might also have a hitherto unrecognized role in the pathomechanism of PSC.

INTRODUCTION

Primary sclerosing cholangitis (PSC) is a highly heterogeneous, idiopathic, immune-mediated cholestatic disease of the liver and is characterized by biliary inflammation and the destruction of the intrahepatic and extrahepatic bile ducts.[1,2] Since the tissue damage is progressive, it will ultimately result in end-stage liver disease or cholangiocarcinoma.[1,3] More than 50% of patients need liver transplantation within 10–15 years of symptom development, due to biliary cirrhosis and hepatobiliary malignancy.[1,4] Besides liver transplantation, definitive therapeutic interventions are missing. Together with the rising PSC cases worldwide, this urges more effective and novel therapeutic solutions. The pathomechanism of the disease is complex and likely involves multiple genetic and environmental factors that trigger an inflammatory reaction which results in a persistent injury of the liver. Human Leukocyte Antigen locus and interleukin 2 receptor association with disease susceptibility, aberrant T-cell homing, and recent evidence indicating the importance of lymphocyte- and natural killer cells cell–derived IFNγ point to close cooperation between innate and adaptive immunity as drivers of the disease.[4–7] PSC is associated with inflammatory bowel disease (IBD) in up to 80% of patients, suggesting a close correlation with microbial dysbiosis and aberrant response to pathogen-associated molecular patterns (PAMPs).[2] PAMPs activation is closely linked with type-I IFN production that can ultimately affect adaptive and innate immune mechanisms.[8]

Type-I interferons (type-I IFNs) are secreted polypeptides that are involved in antiviral response and modulate innate and adaptive immunity.[8] Type-I IFNs include 13 subtypes of IFNs such as IFNα, IFNβ, as well as IFNε, IFNκ, and IFNω. They all bind to the type-I IFN receptor (IFNAR), consisting of 2 subunits IFNAR1 and IFNAR2, which leads to the transcription of interferon-stimulated genes.[8] IFNAR signaling is linked to dendritic cell maturation and affects T-cell responses.[9] They can influence CD4 follicular T-helper cell differentiation and can directly act on the proliferation and survival of activated CD4 and CD8 T cells.[9] Despite their elaborate function in linking innate and adaptive immunity, the role of type-I IFNs in liver diseases is not fully understood. Type-I IFNs show protective capacity in toll-like receptor 9 (TLR9)-induced liver injury and trigger interleukin 1 receptor antagonist production that antagonizes the proinflammatory cytokine IL-1β.[10] Opposing to this, during ischemic stress, type-I IFNs aggravate the TLR4 response of liver macrophages and thereby promote liver injury.[11] IFNs also prompt the accumulation and activation of pathogenic CD8 T cells in metabolic dysfunction–associated steatotic liver disease, thereby contributing to disease progression.[12] Enhanced expression of type-I IFNs has been identified in immune cells within the portal tract in primary biliary cholangitis (PBC), another major immune-mediated cholestatic

disease. Here they play a crucial role in the aberrant B-cell activation during disease development.[13,14]

Despite the increasing interest in the PSC immune milieu, there are no studies that researched the potential role of type-I IFNs in the pathomechanism of PSC. Sensing of PAMPs (derived from pathogens or damaged tissues), in both immune and nonimmune cells, could result in type-I IFN release paralleling the TNF and proinflammatory cytokine production due to interferon regulatory factor 3 or interferon regulatory factor 7.[15] Therefore, we hypothesized that the abundant chronic inflammatory process and the presence of bacterial leakage in PSC could affect type-I IFN production in the liver microenvironment that could be detected in patient sera.

In accordance with this, we have identified that type-I IFNs are upregulated in cholestatic liver tissue in mice, and bioactive type-I IFNs can be readily detected in the serum of both mice and patients with PSC. Moreover, serum IFN levels in patients with PSC were correlated with serological and clinical parameters and indicated increased liver damage. Multiple subtypes of type-I IFNs could be identified but the dominating subtype was IFNω. Our data indicate that bioactive serum type-I IFNs might play a significant role in the pathomechanism of PSC. Further understanding of type-IFN–related signaling and the role of IFN subtypes in PSC pathogenesis could lead to novel therapeutic targets in the future.

METHODS

Human study cohort

The current study was approved by the appropriate regional ethics committees (Poland: reference number KB/58/A/2016, Romania: reference number 3043/07.03.2018, Bonn: State Chambers of Medicine in Nordrhein-Westfalen, approval number: 003(2020) and Hildesheim: State Chambers of Medicine in Niedersachsen, approval number: 118-2019). All study participants provided written informed consent. Patients with associated viral infections were excluded from the study. The characteristics of patients are summarized in Table 1. Standard biochemical analyses were retrieved from clinical laboratory databases including C-reactive protein, white blood cell count, platelet count, total bilirubin, aspartate aminotransferase, alanine aminotransferase (ALT), alkaline phosphatase (ALP), gamma-glutamyltransferase (GGT), and antibody profiles (including anti-mitochondrial antibody and Perinuclear Anti-Neutrophil). PSC was diagnosed based on cholangiographic findings according to accepted criteria,[16,17] and secondary sclerosing cholangitis, IgG4-associated cholangitis, small-duct PSC, and autoimmune hepatitis-PSC overlap syndromes were excluded. Patients with active immunosuppressive therapy were excluded from the study. Diagnosis of Crohn disease and ulcerative colitis were based on accepted criteria. The healthy cohort represented 60% males with a median age of 25.5 years (19.5–32) and were not under any medication.

Human and murine serum preparation

Blood was collected in standard S-Monovette 7.5 mL, Serum Gel with Clotting Activator (Sarstedt AG). Blood from mice was drawn using cardiac puncture and was collected into 1.5 mL reaction tubes. Blood in both cases was left for 30 minutes at room temperature to allow clot formation. Samples were centrifuged at 1500*g* for 20 minutes at 4°C. Supernatant was collected, aliquoted to avoid multiple freeze and melt cycles, and stored at −80°C until further analyses.

Detection of bioactive type-I IFNs in human and murine serum

Reporter HEK-Blue IFNα/β cells from Invivogen were used for semiquantitative detection of bioactive human type-I IFNs according to the manufacturer's instructions. Briefly, reporter HEK-Blue IFNα/β cells were grown in DMEM (Life Technologies) containing 10% (v/v) fetal bovine serum (Life Technologies), 1% (v/v) penicillin-streptomycin (10,000 U/mL, Life Technologies), 100 μg/mL normocin, 100 μg/mL zeocin, and 30 μg/mL blasticidin (Invivogen).

B16-Blue IFNα/β cells (Invivogen) were used for the detection of mouse bioactive type-I IFNs. Cells were grown in Roswell Park Memorial Institue Medium 1640 medium (Life Technologies) containing 10% (v/v) fetal bovine serum (Life Technologies), 1% (v/v) penicillin-streptomycin (10,000 U/mL, Life Technologies), 100 μg/mL normocin, and 100 μg/mL zeocin (Invivogen).

For both human and murine type-I IFN detection, the following protocol was used: Cells were harvested and suspended in a HEK-Blue Detection medium. 180 μL cell suspension containing 5×10^4 HEK-Blue IFNα/β cells (for humans) or 7.5×10^4 B16-Blue IFNα/β cells (for mice) were seeded into a 96-well plate with complete growth medium without selective antibiotics (zeocin and blasticidin). 20 μL human or murine undiluted serum was added and incubated with the cells for 24 hours. 20 μL supernatant containing secreted embryonic ALP was collected after 24 hours and incubated with 180 μL QUANTI-Blue reagent (Invivogen) using a 96-well plate (clear microplate, Biotechne). The OD (optical density) of the samples was measured at 620 nm using the Tecan Sunrise Microplate reader (Tecan Group Ltd). As a positive control, recombinant IFNα was added to the assay

TABLE 1 Characteristics of PSC and PBC patient cohort

	PSC	PBC
N	71	38
Males, n (%)	45.1	16.7
Age, median (range)	44 (1–76)	62 (44–81)
PSC duration, y, median (range)	5 (0–39)	
IBD, n (%)	49.3	
Ulcerative colitis, n (% of IBD)	42.9	
Crohn disease, n (% of IBD)	5.7	
Laboratory data		
CRP, mg/L, median (range)	1.7 (0.34–77.4)	0.52 (0.03–7.29)
White blood cells, $\times 10^3/\mu L$, median (range)	6.255 (0–14.19)	5.935 (3.26–9.72)
Platelets, $\times 10^3/\mu L$, median (range)	216 (64–468)	188.5 (46–336)
Bilirubin, mg/dL, median (range)	0.585 (0.16–2.29)	1.03 (0.5–10.7)
AST, U/L, median (range)	29 (8–95)	46.8 (18.6–271)
ALT, U/L, median (range)	27 (6–172)	36.35 (15.4–326)
ALP, U/L, median (range)	171 (48–868)	154.5 (58.8–1489)
GGT, U/L, median (range)	43 (10–956)	55.05 (11.4–632)

Abbreviations: AST, aspartate aminotransaminase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; GGT, gamma-glutamyl transpeptidase; CRP, C-reactive protein; IBD, inflammatory bowel disease; PBC, primary biliary cholangitis; PSC, primary sclerosing cholangitis.

and as a negative control, IFNγ was added to the assay.

ELISA

Serum concentrations of IFNα, IFNβ, and IFNω were quantified by using Human IFNα ELISA Kit (41135-1, pbl Assay Science), VeriKine Human IFN Beta ELISA Kit (41410, pbl Assay Science), Human IFN omega ELISA Kit (BMS233, Invitrogen) according to the manufacturer's recommendations. For the detection of IL-22, the Duo-Set ELISA kit (Biotechne) was used according to the manufacturer's instructions. The OD of the samples was measured using the Tecan Sunrise Microplate reader (Tecan Group Ltd).

Murine model of sclerosing cholangitis

Multidrug resistance protein 2 (Mdr2)-deficient and Friend Virus B NIH Jackson (FVB/N) control mice were obtained from Jackson Laboratories. Mice were kept in an assigned mouse cabinet (Bioscape) at the Department for Internal Medicine II (Saarland University Medical Centre) (AZ:C1-24.1.1). All experiments were conducted in accordance with the animal approval of the ethics and animal care committees of Saarland University Medical Centre. Only male mice were utilized for the experiments.

Preparation of liver single-cell suspension

Livers were isolated, cut into small pieces, and digested in a 37°C water bath for 60–80 minutes as described.[18,19] Briefly, Roswell Park Memorial Institue Medium, 1% fetal bovine serum with 0.1 mg/mL DNase-I (Life Technologies), 0.2 mg/mL Collagenase P (Roche), and 0.8 mg/mL Dispase (Roche) was used as digestion media. The supernatant was removed regularly and replaced with fresh digestion buffer. Cells were collected by centrifugation for 8 minutes at 180*g*, and erythrocytes were lysed with the ACK-Lysis buffer (Life Technologies) according to the manufacturer's instructions. Cells were washed and resuspended in MACSQuant Running Buffer and kept on ice until further analyses (Miltenyi Biotec).

Flow cytometry analysis

From the liver cell suspension, 1.25×10^5 cells were stained as described.[18–20] First, unspecific binding of antibody was blocked using anti-CD64 (clone: X57-5/7.1, 1:100) (BioLegend) and FcR Blocking reagent (10 μL/staining) (Miltenyi Biotec) for 5 minutes on ice. Then cells were stained with a 50 μL staining mix containing CD45 (30F11) (BioLegend) for 15 minutes on ice. After washing, cells were measured on MACSQuant Analyzer 10. Cell debris (FSC-A vs. SSC-A gate) and cell doublets (FSC-A vs. FSC-H) were excluded and living (negative for propidium iodide, Miltenyi Biotec) cells were analyzed using FlowJo 10.8.1 (FlowJo LLC).

Quantitative PCR analyses

Liver tissue samples from Mdr2 knockout (KO) and FVB/N control mice were homogenized in RLT plus buffer (Qiagen) using Tissuelyser LT (Qiagen). RNA extraction of liver samples was performed using RNeasy plus mini kit (Qiagen) followed by RT reaction using the Quantinova RT system (Qiagen) according to the manufacturer's guidelines. qPCR was carried out with validated primers using the Quantinova SYBR Green qPCR reagent (Qiagen) using the following program: 95°C for 2 minutes, and 40 cycles of amplification at 95°C for 5 seconds and 60°C for 30 seconds. Melt curve analysis was also included. Plates were measured using Applied Biosystem 7500 fast real-

time PCR system. Relative levels of target mRNA were compared with beta-actin using the 2-delta delta Ct method.[21] Primers were purchased from Qiagen: Actb (QT00095242), Ifna4 (QT01774353), Ifnb1 (QT00249662), and Ifng (QT01038821).

Statistical analyses

Data were compared using an unpaired two-tailed *T* test or Mann-Whitney test. Significance is reported as $*p < 0.05$, $**p < 0.001$, $***p < 0.0001$, and $****p \leq 0.0001$. We calculated Pearson correlation, linear regression, sensitivity, specificity, positive predictive value, negative predictive value, and AUROC values using Prism9 (Graphpad Software). Figures were created with Prism9 (Graphpad Software), the G*Power program (version 3.1.9.2.), or with BioRender.com.

RESULTS

Type-I IFNs are elevated in the murine model of sclerosing cholangitis

Mdr2 (Abcb4)-deficient mice provide a robust and reproducible model of spontaneously progressive chronic biliary disease resembling human PSC.[22] Among the PSC animal models, Mdr2−/− mice have been recognized to share several important morphologic and pathogenetic characteristics with human PSC.[22,23] Using this animal model on the FVB/N background, we have analyzed the expression of type-I IFNs.

Indeed, IFNα and IFNβ showed an up to 3-fold increase in 7-week-old Mdr2 KO animals compared to FVB/N controls (Figure 1A). In addition, IFNγ, previously described as an early inflammatory signal,[23] showed elevated expression in diseased animals (Figure 1A).

To validate whether type-I IFNs released within the liver microenvironment could be detected also in serum samples, we have utilized a reporter cell line, the B16-Blue IFNα/β cells, which specifically detects bioactive murine type-I IFNs by monitoring the activation of the JAK/STAT/ISGF3 and/or interferon regulatory factor 3 pathway.[24] These cells do not respond to IFNγ due to the absence of the interferon-gamma receptor but respond to any IFN subtype acting through the IFNAR1/2. Accordingly, elevated OD values were measured in Mdr2 KO animals reflecting the presence of bioactive type-I IFNs in murine serum (Figure 1B). It has been reported previously that Mdr2 KO animals show increased inflammatory infiltrate during liver damage.[23] Accordingly, the abundance of $CD45^+$ immune cells was elevated in the liver of 7-week-old animals, and immune cells further accumulated by 15 weeks of age as disease progressed (Figure 1C). Importantly, the measured OD values indicating bioactive type-IFNs in murine serum correlated well with the increased presence of $CD45^+$ cells in the liver (Figure 1C) ($r = 0.8396$, $p = 0.0091$). In addition, the elevated serum ALT values reflective of liver damage in Mdr2 KO animals also exhibited a good correlation with bioactive type-IFNs in the serum ($r = 0.837$, $p = 0.0013$) (Figure 1D).

Bioactive type-I IFNs are elevated in the serum of patients with PSC

To investigate whether our findings in the animal model would also be reflected in human samples, we have measured bioactive type-I IFNs using the reporter cell line HEK-Blue IFNα/β (Figure 2A) in sera of patients with PSC. Stimulation of HEK-Blue IFNα/β cells with type-I IFNs activates the JAK/STAT/ISGF3 pathway and subsequently induces the production of secreted embryonic alkaline phosphatase that is measured in the culture supernatant (Figure 2A). The cell model was capable of identifying any type-I IFN subtypes (among the 13 subtypes) present that use the IFNAR1/2 complex. In addition, we added 2 independent cohorts: healthy individuals and patients with PBC (Figure 2B). As a comparison, PBC samples were used, since it represent an immune-mediated cholestatic disease characterized by pathologic activation of CD4 and CD8 T cells, leading to the destruction of intrahepatic bile ducts and the wide range of autoantibody production.[25–27] Notably, the diagnostic criteria are clearly separated for both diseases and the addition of this cohort was not indicative of any diagnostic marker comparison. Importantly, type-I IFNs have been implicated in disease development in PBC within the liver environment[13,28] and therefore we analyzed whether liver microenvironmental changes in PBC liver would be detectable systemically in patients' sera in comparison with PSC.

As expected from murine data (Figure 1), bioactive type-I IFNs were detected in the majority of PSC sera and the level of IFNs was elevated compared to healthy individuals (Figure 2B). Cutoff value for PSC compared to healthy individuals was set at 0.4644 and accordingly, data reached a sensitivity of 84.51% and a specificity of 63.16% (Figure 2C). The index of accuracy (AUROC value) is the area under the receiver operating characteristic curve curve (sensitivity vs. 1−specificity). Indeed, 0.8267 AUROC value for PSC samples reflected high distinguishing precision of serum bioactive type-I IFNs (Figures 2B, C). Healthy individuals without ongoing viral infection or disease are expected to be low in IFNs in the peripheral blood. Thus, the pathological elevation of type-I IFNs due to disease conditions could be readily detected in our assay and distinguished from healthy individuals. It is important to note that patients with glucocorticoid-

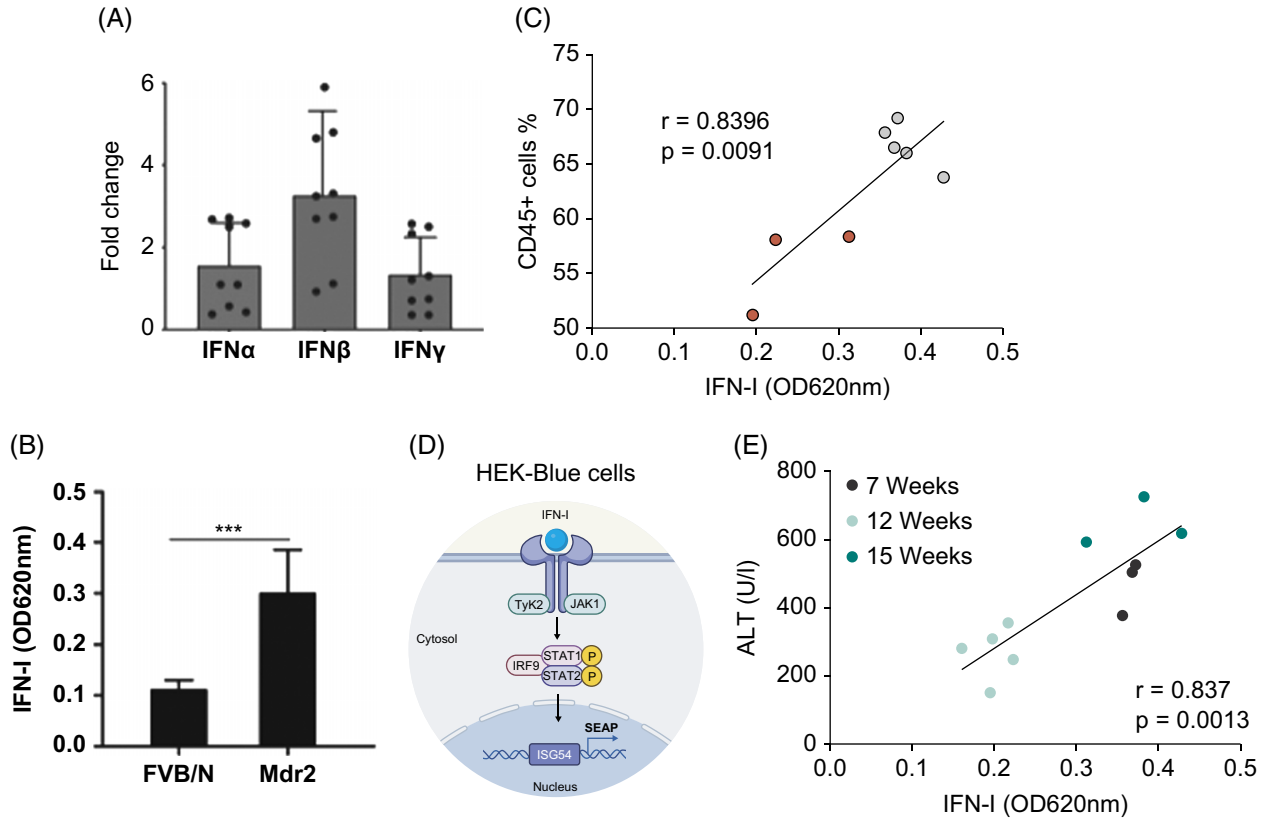

FIGURE 1 Bioactive type-I IFNs in murine model of PSC. Gene expression changes of IFNα, β, and γ (A) in 7-week-old Mdr2-deficient animals (n = 9) compared to wild type (n = 5). (B) Bioactive type-I IFNs in murine serum (n = 5–7/group). Unpaired *t* test mean ± SEM. ***$p < 0.001$. (C) The frequency of CD45$^+$ immune cells among live cells in the liver and its correlation with serum type-I IFNs levels (Pearson correlation) in 7-week (red) (n = 3) and 15-week (gray)-old animals (n = 5). (D) HEK-Blue reporter cell line capable of detecting bioactive type-I IFNs through IFNAR. (E) Serum ALT levels of Mdr2-deficient animals correlated with serum type-I IFN levels (Pearson correlation). Abbreviations: ALT, alanine aminotransferase; FVB/N, Friend Virus B NIH Jackson; IFN, interferons; IFNAR, type-I interferon receptor; Mdr2, multidrug resistance protein 2; OD, optical density; PSC, primary sclerosing cholangitis.

based or azathioprine drugs as part of systemic immunosuppressive therapy (both patients with PSC and patients with PBC) were excluded from our study.

Among patients with PBC, significantly less patients showed increased bioactive type-I IFNs (Figure 2B) and mostly remained similar to the level of the healthy cohort despite the previously described presence of type-I IFN–positive portal cellular infiltrate.[13,28] This could indicate a higher abundance and possibly a different immune pathogenic role of type-I IFNs in the development of PSC compared to PBC.

In addition, among patients with PSC, there was a gender bias in type-I IFN levels (Figures 2D–F). This meant that more male individuals with PSC showed higher OD levels (indicating higher type-I IFN levels) measured at 620 nm for the presence of bioactive type-I IFNs than female patients with the disease (Figures 2D–F). The mean OD values for male patients with PSC were 1.242 ± 0.1517 (SEM), and for female patients with PSC were 0.7362 ± 0.0434 (SEM) (Figures 2E, F). The OD values showed similarly higher IFN levels in male healthy subjects than female subjects (male mean OD: 0.2737 ± 0.0245 [SEM] and female mean OD: 0.2353 ± 0.0179 [SEM]) (Figures 2E, F). This indicates a gender bias in type-I IFN levels in patients with PSC but it seems to exist also among healthy individuals (with lower IFN levels) without the disease.

Correlation of clinical serum parameters with bioactive type-I IFNs in patients with PSC

To understand better how the measured IFNs relate to disease parameters, we have set various correlations with standard laboratory values using Pearson correlations (Figure 3). ALP, frequently elevated in PSC[29] demonstrated a modest correlation with bioactive type-I IFN levels (Figures 3A, B) (ALP: $r = 0.41779$, $p = 0.0003$). Enzymes associated with liver function such as ALT ($r = 0.4704$, $p = 0.0001$), aspartate

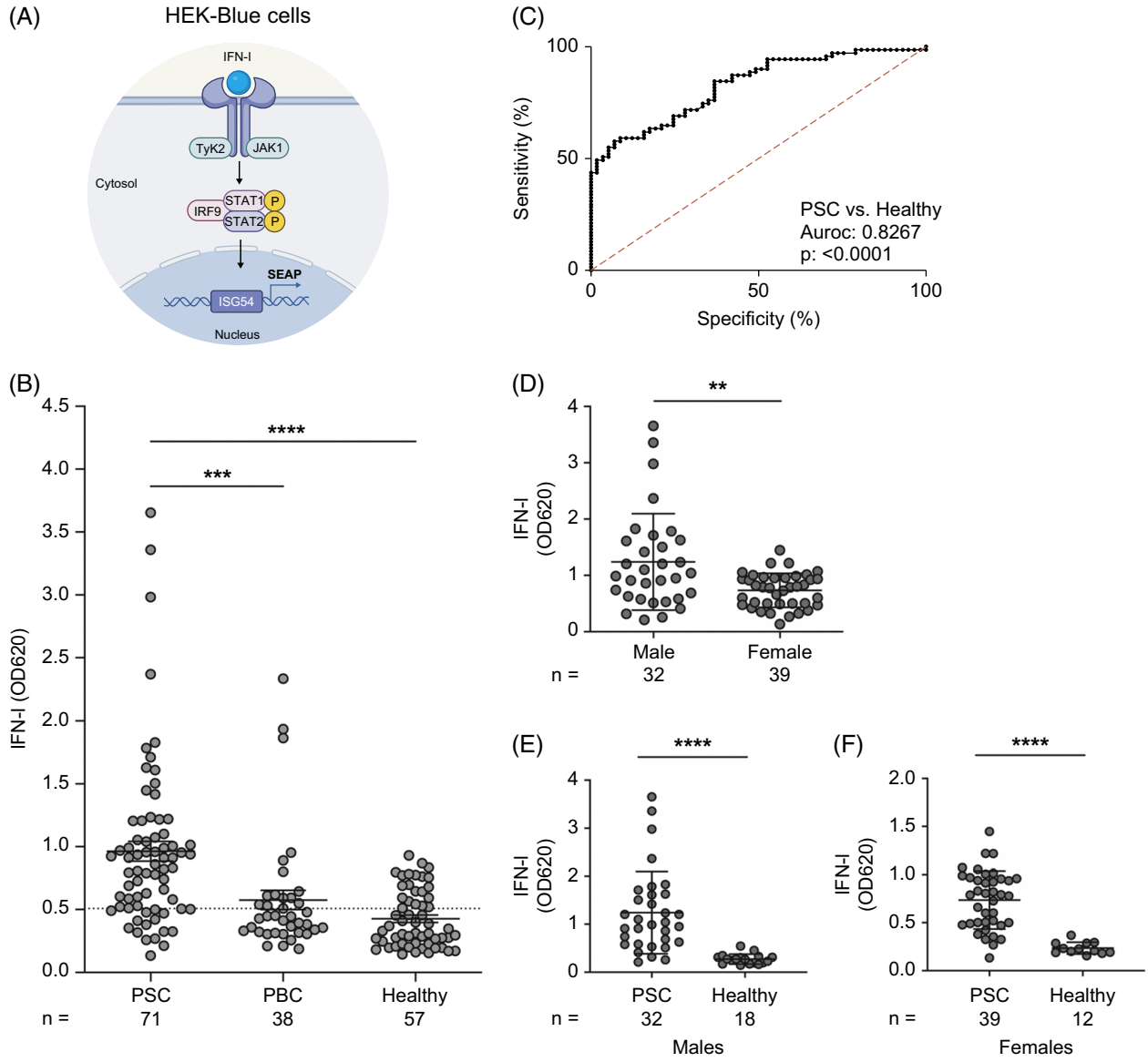

FIGURE 2 Bioactive type-I IFNs are elevated in human PSC sera. (A) HEK-Blue reporter cell line capable of detecting bioactive type-I IFNs through IFNAR. (B) Bioactive type-I IFN levels as OD (optical density) values measured at 620 nm are depicted in the serum of patients with PSC and PBC and healthy controls. The line represents the cutoff value. Posthoc calculated power (1−β err prob) is 0.9891 with a calculated overall effect size (f) of 0.3597. (C) Performance of type-I IFN assay in distinguishing PSC from healthy controls (ROC curve and AUROC value). (D) The type-I IFN distribution in the different genders in patients with PSC. (E) Bioactive type-I IFN levels as OD (optical density) values measured at 620 nm are depicted in the serum of male patients (E) and female patents (F) with PSC and in healthy controls. Mann-Whitney test mean ± SEM, ** p < 0.001, ***$p < 0.0001$, ****$p \leq 0.0001$. Abbreviations: IFN, interferons; IFNAR, type-I interferon receptor; PBC, primary biliary cholangitis; PSC, primary sclerosing cholangitis; ROC, receiver operating characteristic curve.

aminotransferase ($r = 0.0418$, $p = 0.0005$), and gamma-glutamyl transpeptidase ($r = 0.6629$, $p = 0.0001$) showed also moderate correlations with serum ODs (Figures 3C–E). Notably, there was no correlation between serum bilirubin and serum bioactive type-I IFN levels (Figure 3F).

Since ALP levels naturally fluctuate in PSC and could become normal during the course of the disease,[1] we have looked at the fraction of the PSC cohort that was recently diagnosed (within 2 y of diagnosis) with PSC and therefore exhibited more elevated ALP (Figure 3B). In this case, type-I IFNs depicted a moderate but significant correlation with these patients ($r = 0.5065$, $p = 0.02$, n = 19; Figure 3B).

Bioactive type-I IFNs show concurrence with liver fibrosis but not with IBD

To determine whether type-I IFNs would relate to disease severity and progression, we looked first at whether the duration of the disease could relate to

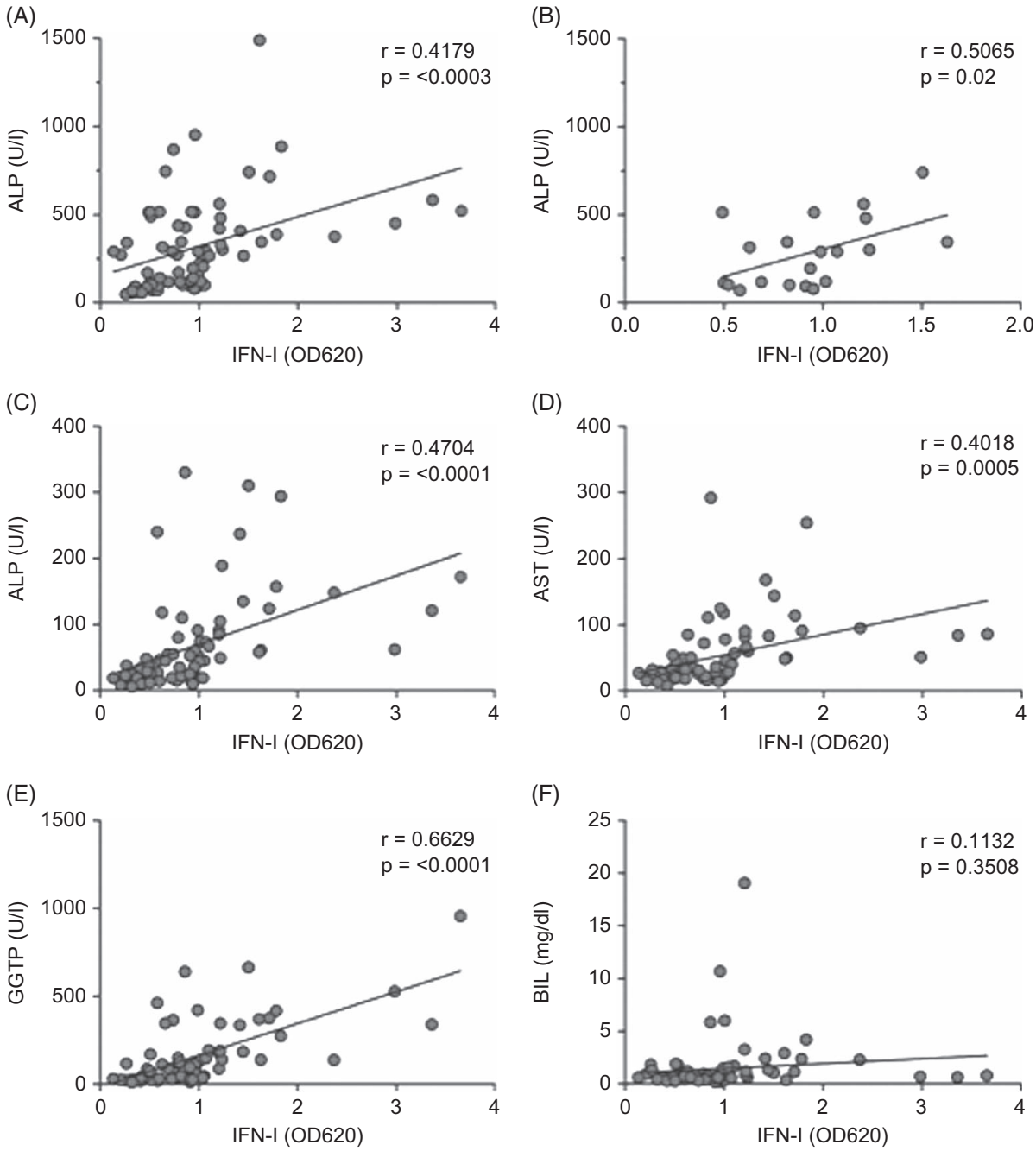

FIGURE 3 Correlations of bioactive type-I IFNs with laboratory parameters. (A, C-E) Liver enzymes ALP, AST, ALT and GGTP, as well as serum bilirubin values (F) of all PSC patients (n = 71) were correlated with type I IFN levels using Pearson correlation. The two-tailed Pearson correlation coefficient (*r*) and *p* value are given. (B) Correlation of ALP levels of recently (≤ 2 y) diagnosed patients with PSC with type-I IFN levels. These patients are highlighted with color in (A). Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BIL, bilirubin; GGTP, gamma-glutamyl transpeptidase; IFN, interferon; OD, optical density; PSC, primary sclerosing cholangitis.

serum bioactive IFN levels. The duration of PSC and type-I IFNs did not show a significant correlation ($r = 0.1758$, $p = 1475$, $n = 71$). This could indicate that patients under various therapies represent rather stable or slow disease progression but could also indicate that serum IFNs might not necessarily reflect the worsening inflammation in the liver microenvironment (Figure 4A).

Chronic liver inflammation in PSC triggers the destruction of bile ducts which ultimately activate wound healing pathways and lead to tissue remodeling and fibrosis. The latter can result in permanent scarring of the liver and liver cirrhosis.[1,3] We have divided the PSC patient cohort based on the presence of cirrhosis and looked for correlation with serum OD values (Table 1, Figure 4B). Accordingly, serum type-I IFNs were slightly

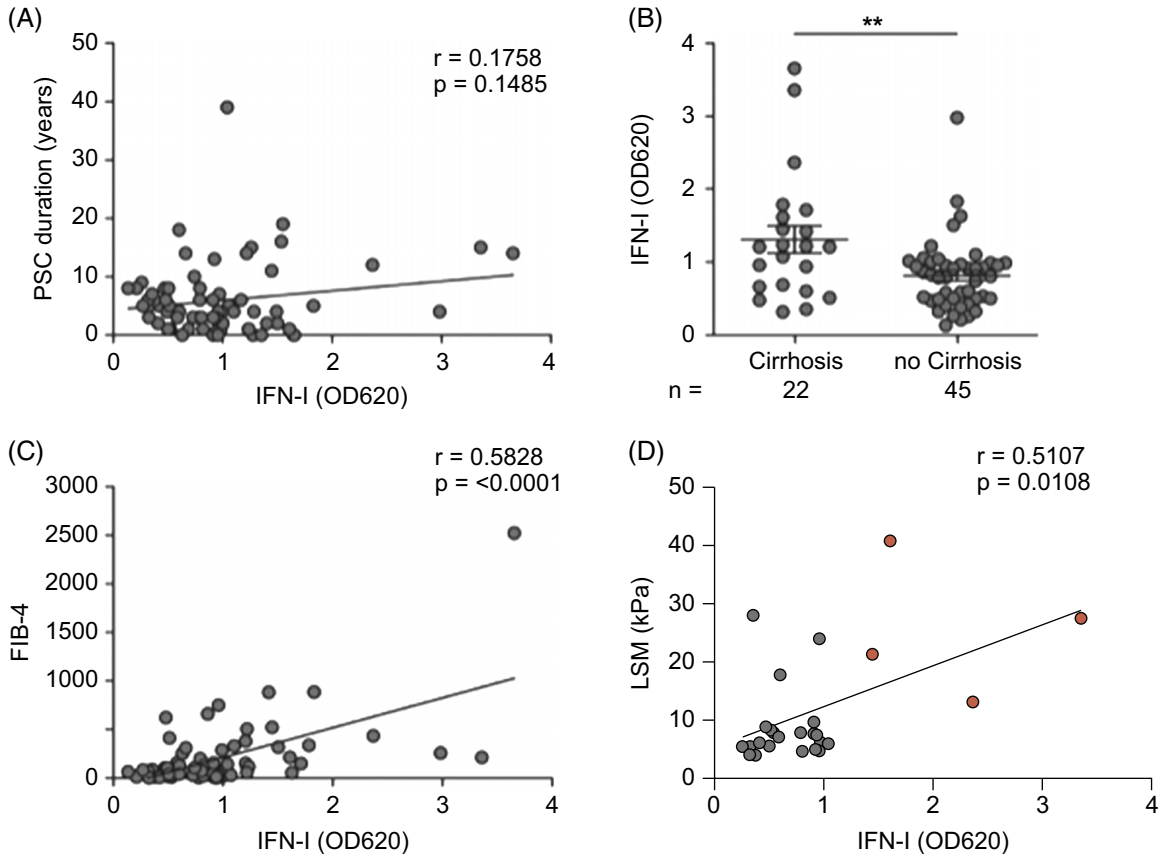

FIGURE 4 The link between bioactive type-I IFNs and fibrosis. (A) The time from PSC diagnosis (years) is correlated with type-I IFNs. (B) Patients with cirrhosis and without cirrhosis and their type-I IFN levels are depicted. (C) FIB-4 parameter score was calculated and subsequently correlated with type-I IFNs (Pearson correlation). (D) LSM available for the part of the PSC cohort (n = 24) correlated with type-I IFNs (Pearson correlation). Patients with above 7.4 kPa threshold and high OD values are highlighted in color. Mann-Whitney test, $*p < 0.05$, $**p < 0.001$, $***p < 0.0001$, $****p \leq 0.0001$. Abbreviations: FIB-4, fibrosis 4 score; IFN, interferon; LSM, liver stiffness measurements; OD, optical density; PSC, primary sclerosing cholangitis.

elevated in patients with cirrhosis and PSC (Figure 4B). The fibrosis 4 (FIB-4) scoring system uses a combination of patient age, platelet count, aspartate aminotransferase, and ALT levels to create a score.[30] This score has a negative predictive value of over 90% for advanced liver fibrosis in various liver diseases with different etiologies.[31] The FIB-4 score could differentiate among PSC samples (Figure 4C) and showed correlations with serum OD values (Figure 4C). For a fraction of the PSC patient cohort, liver stiffness measurements (LSMs) based on vibration-controlled transient elastography were undertaken. LSM has been shown to correlate with fibrosis stage and severity of portal hypertension in various liver diseases.[32,33] Indeed, LSM data representing the higher fibrosis stage were only associated with higher OD values for a fraction of the patients (Figure 4D, colored dots).

Among clinical features, one of the most intriguing is the association of the large proportion of patients with PSC with IBD.[1,2] Interestingly, the presence of IBD did not correlate with differences in bioactive type-I IFN levels compared to patients with PSC without IBD (Figure 5A). Also, the serum levels of IL-22, indicative of active IBD,[34] did not demonstrate a positive correlation with serum OD values of type-I IFNs (Figure 5B).

Dissecting the abundance of serum type-I IFN subclasses in PSC

To evaluate the subtype of IFNs detected by the reporter cell line, we have determined serum levels of IFNα, IFNβ, and IFNω using the ELISA technique (Figure 6A). All 3 types of IFNs bind to the same IFNAR and are therefore indistinguishable by the reporter cell line.[24] In the PSC cohort, IFNα was detected at a very low concentration (close to the lowest detection limit of the assay; all positive samples were lower than 11 pg/mL) and only was present in 16% of patients. IFNβ was more often detectable than IFNα and represented 24% of the samples (Figures 6A, B). On the other hand, IFNω was highly abundant in PSC serum that showed elevated type-I IFNs according to their elevated OD values (Figures 6A, B). IFNω could be measured in 60% of PSC samples (Figure 6B). Interestingly, only 4 patients

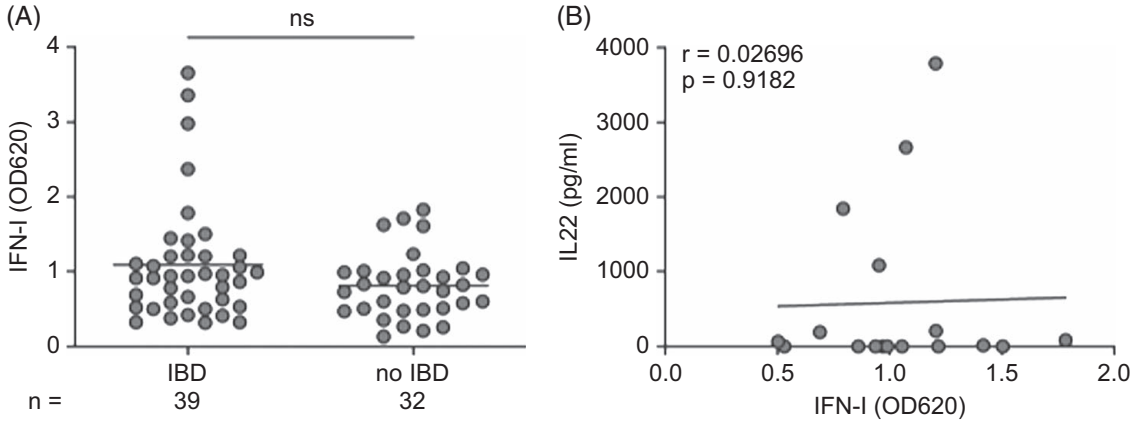

FIGURE 5 The link between bioactive type-I IFNs and IBD. (A) The connections among the presence of IBD and (B) the correlation of IL-22 and type-I IFN levels are depicted. The two-tailed Pearson correlation coefficient (r) and p value are given. Mann-Whitney test, mean ± SEM. *$p < 0.05$, **$p < 0.001$, ***$p < 0.0001$. ****$p \leq 0.0001$. Abbreviations: IBD, inflammatory bowel disease; IFN, interferon; ns, not significant, OD, optical density.

had more than 1 subtype of type-I IFNs (either IFNα and IFNω or IFNβ and IFNω) present in their serum (data not shown). In our cohort, 33 patients did not have any IFN alpha, beta, or omega in their serum. However, based on the OD values measured in the cell assay, only 11 patients were under the cutoff value of type-I IFN detection in comparison with healthy samples (Figure 2B). Since the cell assay measures all subtypes of type-I IFNs, this suggests that other type-I IFN subtypes might be present in PSC serum samples that are not measured by the subtype-specific ELISAs.

DISCUSSION

Emerging evidence indicates the role of type-I IFNs in various autoimmune conditions such as lupus, psoriasis, and systemic sclerosis.[35] Moreover, recent studies discovered a crucial role of these molecules also in metabolic disorders, where type-I IFNs (specifically IFNα) represent key initial factors in promoting inflammation in the adipose tissue (obesity) or pancreas (type I diabetes).[35] Within the liver environment, type-I IFNs exert divergent functions: promote inflammation in metabolic dysfunction–associated steatotic liver disease or protect during alcoholic liver damage or viral hepatitis.[10,12,36] PSC, a chronic inflammatory disorder, has not been previously associated with type-I IFN signature. This is the first study where a positive association between type-I IFN levels and liver damage is demonstrated through the identification of IFN in the liver and serum of a murine model of PSC. Accordingly, bioactive type-I IFNs are elevated in patients with PSC and showed moderate correlations with tissue damage

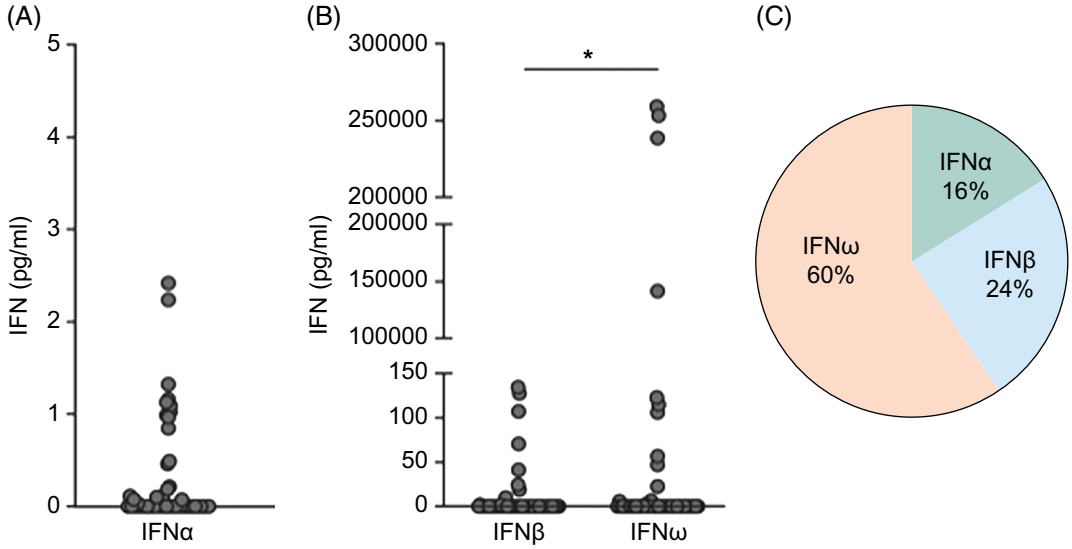

FIGURE 6 The type-I IFN subtype distribution in PSC sera. (A) IFNα, (B) IFNβ, and IFNω was measured in PSC sera using ELISA. (C) The distribution of IFN subtypes among patients is depicted. Mann-Whitney test, mean ± SEM. *$p < 0.05$, **$p < 0.001$, ***$p < 0.0001$, ****$p \leq 0.0001$. Abbreviations: IFN, interferon; ns, not significant; PSC, primary sclerosing cholangitis.

and fibrosis. Patients with cirrhosis exhibited higher OD values which was consequent with the correlation found with FIB-4 score. LSM measurement is a powerful tool for identifying liver fibrosis and can distinguish severe from nonsevere fibrosis with high accuracy.[32] Unfortunately, only a part of our cohort had LSM measurements available which represented a small group of patients ($n = 24$). Although several patients with advanced fibrosis (LSM > 10 kPa) had higher bioactive IFN levels, additional data are necessary for a more accurate conclusion on how LSM and type-I IFNs would relate to each other. This could especially be interesting to follow as the progression rate of LSM increases exponentially with higher fibrosis stage and is ultimately associated with clinical outcomes.[32]

We observed a higher degree of fibrosis based on histology (cirrhosis) or FIB-4 score or LSM measurements in males with higher OD values than females. The slight male tendency could indicate that females seem to be more protected from liver fibrosis than males, at least before menopause.[37,38] A larger cohort size and longitudinal studies are necessary in the future to evaluate clear gender differences during the progression of PSC.

A recent study found increased type-II IFN (IFNγ) levels and elevated frequencies of hepatic $CD56^{bright}$ natural killer cells in human patients with PSC. Using an animal model of PSC, authors demonstrated that genetic deletion of IFNγ in Mdr2 KO mice attenuated liver fibrosis[6] suggesting a pathogenic disease–promoting role of IFNγ in PSC. Notably, some of the patients with high LSM values (above 7.4 kPa cutoff) in our PSC cohort showed indeed elevated IFNγ in their serum (data not shown). Regarding type-I IFNs, studies suggest a protective role in fibrosis development for IFNα in HCV-associated fibrosis and IFNβ in mouse models of fibrosis.[39–41] IFNω has not been associated with liver fibrosis but demonstrates an antiproliferative capacity on multiple different cell lines.[9,42] Thus, type-I IFNs could represent additional molecular pathways accompanying the developing fibrosis and could signify mechanisms to counteract tissue remodeling and fibrosis during PSC progression. It remains to be elucidated what their exact role is during PSC development.

Type-I IFNs are comprised of multiple species, which signal through the same heterodimeric receptor (IFNAR).[8] In humans there are 12 subtypes of IFNα and 1 from each IFNβ, IFNω, and IFNκ. Here, most patients with PSC demonstrated IFNβ or IFNω in their serum. Further experiments are needed to understand what effects IFNω have within the liver microenvironment and how their presence impacts liver pathophysiology. While IFNω was the dominating subtype, it is entirely possible that other type-I IFN subtypes that our ELISA assay has not addressed but could be detected using the reporter cell line are present in patients with PSC.

We have observed higher bioactive type-I IFN values for male patients with PSC than female ones. Interestingly, this bias we could also identify between healthy male and female individuals. Gender bias regarding IFNα production is well documented in viral infection or upon TLR7 stimuli.[43] It is less known about other IFN subtypes. Since the cell model measures multiple IFN subtypes, additional analysis is needed to evaluate what contributes to this difference in healthy individuals and patients with PSC. Notably, age based on our cohort composition was not found as confounder for type-I IFN levels.

It is an intriguing question as to what is the cellular source of type-I IFNs detected in the serum. Type-I IFNs could be induced by innate immune activation through TLRs recognizing PAMPs or damage-associated molecular patterns.[15] The cellular source could be broad: for example, plasmacytoid dendritic cells are able to produce 1000-fold more type-I IFNs per cell than any other cell type.[44] We have identified an increased frequency of plasmacytoid dendritic cells in Mdr2 KO animals (data not shown) but other immune cells such as myeloid plasmacytoid dendritic cells, fibroblasts, and epithelial cells could also produce type-I IFNs and thus could not be ruled out as significant sources for these molecules detected in the serum samples. Reactive cholangiocytes secrete a wide variety of bioactive molecules involving IFNγ; however, their capacity for type-I IFN production is not yet entirely uncovered.[45]

Patients with PBC mostly did not show elevated levels of type-I IFNs in serum despite the fact that early liver biopsies from patients with PBC depicted some specific stainings for type-I IFNs demonstrating that not all local inflammatory changes could be detected systemically in serum.[13,14,28] In early human PBC, inflammatory macrophages and hepatocytes demonstrated type-I IFN-producing capacity[13,28]; nevertheless, it remains to be evaluated how IFN levels alter during the course of the disease. Interestingly, Bae et al[13] implicated type-I IFNs as a required factor for gender bias in a murine model of autoimmune cholangitis. They postulate that by hindering IFNAR receptor signaling, there is a reduction in the overexpression of IFNγ, suggesting a crosstalk between type-I and type-II IFNs.[13] Taking into consideration the study conducted by Ravichandran et al[6] that implicates IFNγ in the progression of PSC, the interplay with type-I IFNs and its role in PSC development could be an interesting theme for future studies.

Many previous studies investigated serum alterations in PSC. Recent studies identified inflammatory biomarkers such as calprotectin (bile) and IL-8 (bile and serum) as important indicators of disease severity and transplant-free survival in PSC.[46]) In the current study, serum type-I IFNs were moderately correlated with ALP, ALT, and gamma-glutamyl transpeptidase serum activity but we did not observe a correlation with serum bilirubin or with disease duration. This could indicate that type-I IFNs are accompanying the inflammatory process and yet have limited power for prognosis prediction.

The prevalence of IBD in patients with PSC is ~80%[47] with some studies suggesting that the correlation is

indicative of a clinically distinct milder form of the disease.[48] Our study depicted a positive correlation between type-I IFN levels and liver fibrosis, but no differences were found when comparing patients with PSC-IBD and patients without IBD. The role of type-I IFN in IBD is unclear: a study of the T-cell transfer model of colitis showed that induced type-I IFN signaling can enhance gut inflammation[49] while other studies indicate dysregulation of IFNAR signaling in IBD.[50,51] For the combination of PSC and IBD, a larger patient cohort is needed to investigate the exact function of type-I IFNs in this particular category of patients.

Taken together, bioactive type-I IFNs could represent a novel previously unrecognized pathway that could operate during disease development and could reflect the unique immune and micromilieu changes. Elucidating the consequences of type-I IFNs could help improve the pharmacological targeting of PSC in the future.

AUTHOR CONTRIBUTIONS

Christina Krötz, Rebekka J.S. Salzmann, Cindy M. Keller, Sophia Rottmann, and Ramona Reichelt performed experiments; Christina Krötz and Rebekka J.S. Salzmann analyzed and interpreted the results and prepared figures. Tudor Mocan, Lavinia P. Mocan, Bettina Langhans, Frederik Schünemann, Alexander Pohl, Thomas Böhler, Käthe Bersiner, Marcin Krawczyk, Piotr Milkiewicz, Zeno Sparchez, Sebastian Gehlert, Maria A. Gonzalez-Carmona, Arnulf Willms, Christian P. Strassburg, and Leona Dold collected samples and managed patient data. Bettina Langhans, Marcin Krawczyk, Tudor Mocan, Piotr Milkiewicz, Sebastian Gehlert, Frank Lammert, Arnulf Willms, Christian P. Strassburg, Miroslaw T. Kornek, and Leona Dold critically read the manuscript. Miroslaw T. Kornek cosupervised the students and Veronika Lukacs-Kornek designed and directed the study, interpreted the results, and wrote the manuscript.

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CONFLICTS OF INTEREST

Zeno Sparchez is on the speakers' bureau and received grants from Gilead. He is on the speakers' bureau for AbbVie and Genesis. The remaining authors have no conflicts to report.

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