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Increased sensitivity of Treg cells from patients with PBC to low dose IL-12 drives their differentiation into IFN- γ secreting cells

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Keywords

Primary biliary cholangitis, primary sclerosing cholangitis, primary Sjögren's syndrome, autoimmunity, T regulatory cells, Th1 cells

Abbreviations

ALD, alcoholic liver disease; ALP, alkaline phosphatase; EBV, Epstein bar virus induced gene 3; IFNG, interferon gamma; NASH, non-alcoholic steatohepatitis; NL, normal liver; PBC, primary biliary cholangitis; PSC, primary sclerosing cholangitis; pSS, primary Sjögren's syndrome; Res, resected liver; TLR, toll like receptor; TNF α , tumor necrosis alpha

Authors Contributions: EL and GMH contributed to study design, EL, SP, DBD, SA and MK performed experiments, EL performed data analysis and interpretation, GW, BAF, GM, DJ and FB provided patient material and contributed intellectually to the manuscript, EL drafted the manuscript. All authors critically revised the manuscript and approved the final version.

Abstract

IL-12 is a pro-inflammatory cytokine that induces the production of interferon- γ (IFN γ) and favours the differentiation of T helper 1 (Th1) cells. In the presence of IL-12 human Treg cells acquire a Th1-like phenotype with reduced suppressive activity in vitro. Primary biliary cholangitis (PBC) is an autoimmune cholestatic liver disease characterised by high Th1 and Th17 infiltrating cells, reduced frequencies of Treg cells, and a genetic association with IL-12 signalling. Herein, we sought to evaluate the IL-12 signalling pathway in PBC pathology, by studying human samples from patients with PBC, alongside those with primary Sjögren's syndrome (pSS)(autoimmune disease with IL-12 signalling gene association), primary sclerosing cholangitis (PSC) (cholestatic liver disease without IL-12 gene association) and healthy individuals. Our data revealed that TLR stimulation of PBC (n=17) and pSS monocytes (n=6) resulted in significant induction of *IL12A* mRNA (p<0.05, p<0.01, respectively) compared to PSC monocytes (n=13) and at similar levels to HC monocytes (n=8). PSC monocytes expressed significantly less IL-12p70 (108pg/ml, mean) and IL-23 (358pg/ml) compared to HC (458pg/ml and 951pg/ml, respectively) (p<0.01, p<0.05). Treg cells from patients with PBC (n=16) and pSS (n=3) but not PSC (n=10) and HC (n=8) responded to low dose (10ng/ml) IL-12 stimulation by significant upregulation of IFN γ (55 and 186pg/ml, respectively) compared to PSC Treg cells (6pg/ml)(p<0.05). This effect was mediated by the rapid and strong phosphorylation of STAT4 on Treg cells from patients with PBC and pSS (p<0.05) but not PSC and HC. In the liver of patients with PBC (n=7) a significantly higher proportion of IL-12R β 2⁺Tregs (16% on average) was detected (p<0.05) compared to other liver disease controls (5%)(n=18) which also showed ex vivo high *IFNG* and *TBET* expression. **CONCLUSION: Our data show an increased sensitivity of PBC and pSS Treg cells to low dose IL-12 stimulation, providing ongoing support for the importance of the IL12-IL-12R β 2-STAT4 pathway on Treg cells in disease pathogenesis and potentially treatment.**

1. Introduction

The interleukin-12 (IL-12) family comprises a group of heterodimeric cytokines, including IL-12, IL-23, IL-27 and IL-35, which show diverse functional effects and play key roles in immune responses [1]. In the development of a number of autoimmune diseases association with genetic variations in IL-12 family of genes [2-4] and downstream pathways has been identified. Notably, based on their respective associations with the IL-12 genes, autoimmune diseases appear to cluster in two groups that either show strong associations with the Th1/Th17 pathway or the Th1/IL-35 pathway [1].

Primary biliary cholangitis (PBC) is one such idiopathic chronic liver disease characterised by the progressive loss of intrahepatic bile ducts, leading to cholestasis and progressive fibrosis. Genetically, genome wide association studies (GWAS) identified, in addition to an HLA risk, that the IL-12 signalling pathway was a consistent gene association [2, 5].

Immunologically, a high Th1 and Th17 cell infiltration is detected at early stages of the disease with a shift towards Th17 cells being more prominent at advanced stages [6].

CD4⁺CD25⁺ T regulatory cells (Treg cells) which play a key role in self-tolerance are reduced in patients with PBC compared to controls but show no functional defect [7, 8]; although studies reporting no reduction in Treg cell numbers also exist [9, 10]. Discrepancies in the reported numbers of Treg cells in primary Sjögren's syndrome (pSS), another autoimmune disease with a genetic association implicating IL-12 signalling [11], also prevail, with the majority of studies reporting an overall reduction of peripheral blood CD25^{high} Treg cells, but with others reporting either an increase or no difference when compared with control samples [12]. In addition to the well characterised role of IL-12 in driving Th1 cell polarization, studies have shown that IL-12 can drive the differentiation of human Treg cells into Th1-like cells with reduced suppressive capacity [13, 14].

IL-12 is a heterodimer formed by IL-12p35 (encoded by *IL12A*) and IL-12p40 (encoded by *IL12B*). IL-12p40 can also bind to the IL-23p19 chain (encoded by *IL23A*) and form the pro-inflammatory cytokine IL-23, IL-12p35 can bind to EB13 (Epstein-Barr virus-induced gene 3) forming IL-35, which is believed to have a role in controlling the immune response during active inflammation [1]. IL-12 is produced by antigen presenting cells such as monocytes/macrophages, dendritic cells and B cells in response to infection [15]. In patients with PBC, in addition to Th1/Th17 cells, monocyte/macrophage infiltration is also evident, and recent studies have reported a significant increase in CD14^{low}CD16⁺ monocytes which positively correlates with Th1 cell frequency [16]. CD14^{low}CD16⁺ monocytes were also found to promote Th1 differentiation of PBC CD4⁺ T cells by IL-12 and direct cell contact [16].

In our study, we evaluated a) the ability of monocytes from patients with PBC to secrete IL-12, and b) the ability of Treg cells from patients with PBC to respond to IL-12 stimulation and differentiate into Th1-like cells. As controls, we used cells from patients with pSS (autoimmune disease in which GWAS studies have also revealed an IL-12 pathway association), primary sclerosing cholangitis (PSC) (cholestatic liver disease with no reported IL-12 gene association) and healthy samples. Our findings showed that i) there was no defect in the ability of circulating monocytes from patients with PBC and pSS to secrete IL-12, which was similar to HC, in contrary it was the PSC monocytes that were defective in their ability to produce IL-12 and IL-23 ii) the Treg cells from patients with PBC and pSS were sensitive to low dose IL-12 stimulation, with 10ng/ml being able to induce IFN γ expression, whereas PSC and HC Treg cells required a higher concentration of IL-12 to acquire a Th1-like phenotype, iii) this effect was mediated by the rapid and strong phosphorylation of STAT4 on Treg cells from patients with PBC and pSS, but not PSC and HC, iv) a

significantly higher proportion of IL-12R β 2⁺Tregs was detected in the liver of patients with PBC but not in other liver disease controls, with PBC IL-12R β 2⁺Tregs showing ex vivo Th1-like features.

2. Materials and Methods

2.1. Human Tissue and Blood

Whole blood was obtained from patients with PBC, PSC and pSS. As normal control we used blood samples from healthy volunteers (UK-PBC Cambridge) and stable hemochromatosis patients. Fresh diseased liver tissue from the Queen Elizabeth Hospital Birmingham transplant programme was available as was non-diseased liver from surgical resections. All tissue and blood samples were collected after local research ethics committee approval and patient consent (Local Research and Ethics Committee Birmingham references: 2003/242, renewed 2012; 06/Q2702/61 and Dyfed Powys Research Ethics Committee, 13/WA/0392; and NRES Research Ethics Committee North West Preston, 14/NW/1146). All PBC and PSC patients that donated their blood samples were not on any immunosuppressant treatment. Six out of eleven pSS patients were under no treatment at time of blood collection, four patients were on hydroxychloroquine (a disease-interfering drug) and only one patient was on hydroxychloroquine, methotrexate and prednisolone (the latter two considered immunosuppressants).

2.2. Cell Isolation

Peripheral blood mononuclear cells (PBMCs) and liver infiltrating mononuclear cells (LIMCs) were isolated from peripheral blood and fresh human liver tissue, respectively, as described previously [17]. Briefly, liver tissue was mechanically digested, filtered and further purified by density gradient centrifugation using Lympholyte (VH Bio).

2.3. Monocyte Enrichment and in vitro Toll-like receptor (TLR) stimulation

Using an indirect magnetic labelling system (Monocyte Isolation kit II and Pan Monocyte Isolation kit, Miltenyi) monocytes from human PBMCs and LIMCs were isolated via

negative selection (untouched) according to manufacturer's instructions. Enriched monocytes were cultured in 96-round bottom plates at 2.5×10^5 cells/0.2ml/well in RPMI 1640+10% FCS and stimulated with LPS ($1 \mu\text{g/ml}$; InvivoGen), R848 ($2.5 \mu\text{g/ml}$; Invivogen) and their combination for 24 hours prior to collection of their cell-free supernatant and cell pellets for use in ELISAs and real-time PCR analysis (described below).

2.4. CD4⁺ CD127^{low} CD25^{high} Treg Enrichment and in vitro stimulation

After PBMC and LIMC isolation CD4⁺ T cells were isolated by negative selection using the CD4⁺ T cell isolation kit (Miltenyi) following the manufacturer's instructions. After CD4⁺ T cell enrichment, CD127^{low}CD25^{hi} Treg cells were FACS-sorted into RPMI 1640+10%FCS. Treg cells were cultured at $0.5\text{--}1 \times 10^6$ cells/ml in anti-CD3 (OKT3, at $10 \mu\text{g/ml}$; eBioscience) pre-coated 96-U well plates, with recombinant human (rh)IL-2 (25IU/ml; Immunotools), anti-CD28 ($1 \mu\text{g/ml}$; BD Biosciences), with/without rhIL-12 (10ng/ml – 100ng/ml; R&D Systems) for 4 days. At day 4, cell-free supernatants and cell pellets were collected for use in real-time PCR analysis and cytokine expression analysis (described below).

2.5. CD4⁺ CD127^{low} CD25^{high} Treg co-culture with monocyte conditioned media

CD127^{low}CD25^{hi} Treg cells were FACS-sorted after CD4⁺ T cell enrichment as described above. Cells were cultured at $0.5\text{--}1 \times 10^6$ cells/ml in anti-CD3 pre-coated 96-U well plates (at $10 \mu\text{g/ml}$) with rhIL-2 (25IU/ml) and anti-CD28 ($1 \mu\text{g/ml}$). In pre-defined wells rhIL-12 (20ng/ml), TLR4 (LPS) -TLR7/8 (R848) stimulated monocyte conditioned media (at 1:1 ratio) or untreated monocyte conditioned media and the blocking antibodies anti-IL-12p70 ($20 \mu\text{g/ml}$; Antibodies Online) and anti-IL-12/IL-23 p40 ($20 \mu\text{g/ml}$; eBioscience) were added. Cells were cultured at 37°C and 5% CO₂ for 4 days. After the 4-day incubation period cell pellets were collected for mRNA expression analysis of *IFNG*, *TBET* and *IL12RB2* (see details below).

2.6. CD4⁺ Naïve T cell differentiation to iTreg and evaluation of IL-12 effects

After PBMC isolation naïve CD4⁺ T cells were isolated by negative selection using the Naïve CD4⁺ T cell isolation kit (EasySep Human T cell isolation cocktail II; StemCell Technologies), following manufacturer's instructions. Cells were cultured at 1×10^6 cells/ml in anti-CD3 (OKT3, 1 µg/ml; eBioscience) pre-coated 96-U well plates in MACs media+10%FCS, 100U/ml penicillin, 100 µg/ml streptomycin and 50 µM β-mercaptoethanol (Sigma Aldrich). All cells were treated with 100IU/ml IL-2. Separate wells were treated with 2ng/ml TGF-β with/without rhIL-12 (10 - 100ng/ml). On day 3 post plating, cells were spun down for 5min at 500 x g and without disturbing T cells on the bottom of the plate, half of the media was removed and replaced with fresh media. Thereafter, cells were incubated for another 2 days at 37°C/5% CO₂ prior to iTreg analysis based on FOXP3 and CTLA4 markers using flow cytometry (for antibody details see section below).

2.7. Flow cytometry

2.7.1 Flow cytometry analysis of in vitro differentiated iTreg cells

Cells were collected on day 5 into FACs tubes and washed in PBS. Cells were stained with live/dead marker (Zombie dye NIR, Biolegend) and incubated at room temperature for 20min protected from light. Cells were washed with PBS and further incubated with fixative solution (Foxp3 Transcription factor staining buffer set; eBioscience) at 4°C for 1 hr protected from light. Cells were washed with PBS and then with 1 x permeabilization buffer. Cells were then stained with the antibodies CD25-BB515 (clone 2A3), CTLA4-PE (CD152, clone BNI3), CD127-PECy7 (clone HIL-7R-M21), CD45RA-V421 (clone HI100) (all from BD Biosciences) and FOXP3-APC (clone PCH101; eBioscience) for 45min at 4°C. Cells were also stained with isotype matched control antibodies. Cells were washed once with permeabilization buffer followed by one wash with PBS and final resuspension in FACS buffer (PBS+1.5% FCS) and analysis in a Cyan ADP Flow Cytometer (Beckman Coulter).

2.7.2 Flow cytometry analysis of IL-12R β 2 expression on Treg cells

PBMCs and LIMCs were stained with the following antibodies to define the proportion and MFI of IL-12R β 2⁺ Treg cells: CD3-APC (clone UCHT1), CD4-PeCF594 (clone RPA-T4), CD127-PECy7 (clone HIL-7R-M21), CD25-BB515 (clone 2A3), IL-12R β 2-PE (clone 2B6/12beta 2) (all from BD Biosciences) and live/dead marker APC-Cy7 (BioLegend). Cells stained with isotype matched control antibodies and fluorescence minus one samples with cells stained with all antibodies except IL-12R β 2 were used as control.

2.7.3 Flow cytometry analysis of pSTAT4 Staining after IL-12 stimulation

Total PBMCs previously frozen in cryostor in liquid N₂ were thawed and cultured in aCD3 (10 μ g/ml) pre-coated 96-U plates at 100,000cells/100 μ l in the presence of rhIL-2 (25IU/ml), aCD28 (1 μ g/ml), with/without rhIL-12 (10ng/ml) for 15min, 45min, 2 hours and 6 hours. Cells were collected, washed in PBS and stained with live/dead APC-Cy7 marker as described above. Cells were washed in FACs buffer and stained for surface markers CD127-PECy7 and CD25-BB515 for 20min at 4°C. Cells were washed in FACs buffer and further resuspended in 100 μ l 1x BD Phosflow Fix Buffer I (stored at 4°C but pre-warmed at 37°C before use) (BD Biosciences). Cells were incubated at 37°C for 12min and washed with 1ml PBS at 2,000 rpm for 3min. Cell pellets were briefly vortexed prior to adding 500 μ l Perm Buffer III (stored at room temperature but pre-chilled at -20°C). Cells were incubated on ice for 30min and then transferred to -20°C for overnight storage. Procedure was continued the next day by washes with 2ml ice cold 2% FCS in PBS, 3 times centrifugation at 1,500rpm for 3min. After the last wash, the cells were stained with the following antibodies: CD3-PE (clone UCHT1), CD4-PECF594 (clone RPA-T4) and BD Phosflow Alexa Fluor 647 mouse anti-STAT4 (pY693) (clone 38/p-STAT4) for 60min at room temperature. FMO samples

with cells stained with all antibodies except anti-pSTAT4 were used as control. Cells were washed with 3ml 2% FCS in PBS and analysed in a Cyan Flow cytometer.

2.8. BD Cytometric Bead Array Human Th1/Th2/Th17 kit

The BD Cytometric Bead Array (CBA) Human Th1/Th2/Th17 Cytokine kit was used to measure IL-2, IL-4, IL-6, IL-10, TNF, IFN γ , and IL-17A protein levels in the supernatant of rhIL-12- treated (at 10ng/ml) Treg cells following the manufacturer's instructions. CBA samples were acquired on Cyan Flow Cytometer and data were analysed using the FCAP Array software. Concentration of cytokines was normalized to the number of cells/well.

2.9. Fluorescence associated cell sorting of intra-hepatic IL-12R β ²⁺ Treg cells

LIMCS were isolated as described above, CD4⁺ T cells were enriched by negative selection and CD127^{low} CD25^{hi} IL-12R β ²⁺ and CD127^{low} CD25^{hi} IL-12R β ²⁻ Treg cells were sorted into RPMI+10%FCS. Cells were spun down and cell pellets were used for mRNA analysis.

2.10. Quantification of mRNA expression levels by RT-PCR

Total RNA was extracted from enriched monocyte cell pellets (with/without TLR4-TLR7/8 stimulation), Treg cell pellets (with/without IL-12 stimulation) and IL-12R β ²⁺ Treg cell pellets, using Qiagen RNEasy Plus Micro kit (for <500,000cells) or Qiagen RNEasy mini kit (for >500,000 cells) according to manufacturer's instructions. RNA was transcribed into cDNA using the iScript cDNA synthesis kit (BioRad, Hercules, CA). Quantitative analysis of *IFNG* (Hs00989291_m1), *TBET* (Hs00894392_m1), *IL-12RB2* (Hs00155486_m1), *FOXP3* (Hs01085834_m1), *IL-10* (Hs00961622_m1), *IL-4* (Hs00174122_m1) and *IL-17A* (Hs00174383_m1) mRNA expression was performed using Taqman Fluorogenic 5' nuclease assays using gene-specific 5'FAM labelled probes (Life Technologies) run on ABI 7900

sequencer with *18S* (Hs03003631_g1) used as endogenous control. In case of monocytes, quantitative analysis of *IL12A* (Hs01073447_m1), *IL12B* (Hs01011518_m1), *TNFA* (Hs00174128_m1) and *EBI3* (Hs01057148_m1) mRNA expression was performed with *ACTB* (Hs01060665_g1) used as endogenous control. Values are represented as the difference in Ct values normalized to endogenous control for each sample as per the following formula: Relative RNA expression = $2^{-\Delta Ct}$ where $\Delta Ct = Ct \text{ of gene of interest} - Ct \text{ of endogenous control}$ [18].

2.11. Enzyme Linked Immunosorbent Assay (ELISA)

The expression of IL-12p70 and IL-23 cytokines was measured using the human IL-12p70 ELISA Ready-SET-Go and IL-23 Human Uncoated ELISA kit (both from eBiosciences) according to manufacturer's instructions.

2.12. Statistical Analyses

GraphPad Prism 7 software was used for statistical analysis. P values were calculated using non-parametric Wilcoxon or Mann-Whitney tests as appropriate. P values <0.05 were considered statistically significant.

3. Results

3.1. Peripheral blood monocytes from patients with PBC and pSS respond to TLR4-TLR7/8 stimulation and upregulate *IL12A* and *IL12B* genes and IL-12p70 protein

In patients with PBC elevated levels of circulating CD14^{low}CD16⁺ monocytes have been reported, and these elevations correlated with increased liver injury and Th1 polarization [16]. Therefore, we studied the ability of monocytes from patients with PBC to produce IL-12 after TLR stimulation in comparison to monocytes from patients with PSC (as a liver disease control), pSS (extrahepatic autoimmune disease with IL-12 pathway association; *IL12A*, *STAT4* [11]) and healthy controls. Stimulation of blood enriched monocytes with LPS (TLR4 agonist), R848 (TLR7/8 agonist) and their combination revealed that there is an additive effect in their ability to induce *IL12A*, *IL12B* and *TNFA* mRNA expression (**p<0.01) (Supplementary Figure 1). Therefore, the combination of LPS plus R848 was used for all downstream stimulations. PBC, PSC and pSS monocytes stimulated with LPS/R848 significantly upregulated *IL12A* and *IL12B* genes compared to untreated cells (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001). PBC and PSC monocytes stimulated with LPS/R848 significantly upregulated *TNFA* and *EBI3* (**p<0.01, ***p<0.001, ****p<0.0001) compared to untreated cells. Stimulation of healthy control blood monocytes with LPS/R848 induced a statistically significant difference in *IL12A* (*p<0.05) and *TNFA* (**p<0.01) expression but not *IL12B* and *EBI3* compared to untreated cells (Figure 1A). Comparison of gene expression levels after LPS/R848 stimulation between disease groups revealed that LPS/R848 stimulated PSC monocytes expressed significantly less *IL12A* compared to PBC and pSS samples (Figure 1A) and significantly less IL-12p70 (108pg/ml, mean value) and IL-23 (358pg/ml, mean value) protein compared to healthy control samples (419pg/ml and

908pg/ml, respectively, $**p<0.01$ and $*p<0.05$) (Figure 1B, 1C). Monocytes from patients with PBC and PSC expressed significantly lower levels of *TNFA* (both $p<0.05$) and *EBI3* ($***p<0.001$, $*p<0.05$) compared to HC monocytes. pSS monocytes also expressed significantly lower *EBI3* levels compared to HC monocytes ($*p<0.05$) (Figure 1A).

Collectively, these data suggest that PBC and pSS monocytes respond normally to TLR stimulation and express IL-12, whereas it is the PSC monocytes that are defective in their ability to respond to TLR stimulation and produce IL-12p70 and IL-23.

3.2. PBC Treg cells are sensitive to low dose IL-12 stimulation

Recent findings have revealed the role of IL-12 in polarization of Treg cells into Th1-like cells [13, 14], therefore we assessed the effect of IL-12 on Treg cells from patients with different autoimmune and immune-mediated diseases. Different concentrations of IL-12 cytokine have been used across studies, from 10ng/ml to 50ng/ml, to drive the polarization of naïve CD4⁺ T cells into Th1 cells [19-21]. In the serum of patients with PBC the median levels of IL-12p70 detected are about 10pg/ml [22] and in the inflamed liver microenvironment the mean levels of IL-12p70 detected are about 60pg/ml [10]. In this in vitro system we decided to use a range of IL-12 doses from 10ng/ml up to 100ng/ml, 1,000 – 10,000-fold higher compared to what is found in vivo and in accordance to the in vitro polarization experiments used by others. Firstly, we assessed the expression of the IL-12 receptor $\beta 2$ chain on Treg cells across the different disease and healthy control groups. Our data show that similar proportions of IL-12R $\beta 2$ ⁺ Treg cells are detected in the blood of patients with PBC (7.5% on average (mean value), PSC (5.9%), pSS (4.7%) and HC (6.0%) (Figure 2A). However, stimulation of Treg cells with different doses of IL-12 revealed that Treg cells from patients with PBC are more sensitive to low dose IL-12 stimulation; with 10ng/ml being enough to significantly upregulate *IFNG* gene expression in PBC Treg cells ($**p<0.01$). PSC Treg cells were also able to upregulate *IFNG* but this effect was significant

when cells were stimulated with $\geq 20\text{ng/ml}$ IL-12 (* $p < 0.05$, ** $p < 0.01$). HC and pSS Treg cells were unable to upregulate *IFNG* mRNA even at the highest IL-12 dose (100ng/ml) tested (Figure 2B). *TBET* expression was induced on PBC Treg cells after stimulation with IL-12 at 20ng/ml and 50ng/ml (** $p < 0.01$, * $p < 0.05$), and on PSC Treg cells when stimulated with the highest doses of IL-12 (50 and 100ng/ml) (* $p < 0.05$, ** $p < 0.01$) (Figure 2C). IL-12 stimulation had no statistically significant effect on *TBET* expression on Treg cells from pSS and HC samples (Figure 2C). IL-12 stimulation (20 and 50ng/ml) significantly induced the expression of *IL12RB2* on PBC Treg cells (* $p < 0.05$) but had no effect on PSC, pSS and HC Treg cells (Figure 2D). Overall, our data suggest that Treg cells from patients with PBC are more sensitive to low dose stimulation with IL-12.

3.3. Low dose IL-12 stimulation induces IFN γ protein expression on PBC and pSS Tregs

At low dose IL-12 stimulation (10ng/ml), Treg cells from patients with PBC expressed significantly higher levels of *IFNG* mRNA compared to PSC and HC Treg cells (* $p < 0.05$) (Figure 3A, 3B). At this low dose of IL-12 stimulation, PBC Treg cells expressed significantly higher IFN γ protein levels compared to PSC Treg cells (* $p < 0.05$), and significantly lower IL-17 (* $p < 0.05$) compared to HC Treg cells (Figure 3C). Notably, although pSS Treg cells showed no statistically significant mRNA gene expression change after IL-12 stimulation, they showed a significant induction of IFN γ protein when compared to PSC (* $p < 0.05$) and HC (* $p < 0.05$) Treg cells. pSS Treg cells also expressed significantly higher levels of IL-2 protein compared to both HC (** $p < 0.01$) and PBC Treg cells (* $p < 0.05$). No difference in IL-10, TNF α and IL-4 protein expression between disease groups was detected (Figure 3C). Collectively, these data validate further that Treg cells from patients with PBC are sensitive to low dose IL-12 stimulation, being able to produce significantly high levels of IFN γ protein. Moreover, our data show that Tregs from patients with pSS are

also able to respond to low dose IL-12 stimulation and express significantly high levels of IFN γ protein, compared to PSC and HC Treg cells.

3.4. STAT4(pY693) phosphorylation is quicker and stronger on Treg cells from patients with PBC and pSS

Following IL-12 stimulation, the Janus kinase-STAT4 signalling pathway is activated which then leads to STAT4 phosphorylation. STAT4 is considered to be one of the critical mediators of the canonical IL-12 effects, since STAT4-knockout mice have impaired Th1 differentiation and IFN γ production [23]. Therefore, we examined the phosphorylation status of STAT4 after low dose IL-12 treatment across the different disease groups and healthy controls. Treatment with 10ng/ml IL-12 increased the phosphorylation of STAT4 (pY693) in all three disease groups and healthy controls, however as early as 45 minutes after IL-12 stimulation STAT4 phosphorylation was higher on Treg cells from patients with PBC (4.6% on average, mean value) and pSS (5.0%) compared to PSC (1.7%) ($p=0.06$ and $p<0.05$, respectively) and HC Treg cells (2.6%, both $p<0.05$). After 2 hours stimulation, pSS Treg cells expressed significantly higher levels of pSTAT4 (8.0%) compared to PBC (3.4%, $p<0.05$), PSC (4.8%, $p<0.05$) and HC (1.6%, $p<0.001$) Treg cells and at 6 hours of IL-12 stimulation, both PBC (11.9%) and pSS (13.4%) Treg cells expressed significantly higher levels of pSTAT4 compared to PSC (3.6%, both $p<0.05$) Treg cells. The pSTAT4 levels in pSS Treg cells were also significantly higher compared to the levels detected on HC (4.9%) Treg cells ($p<0.05$) (Figure 4A). Notably, when total CD4⁺ T cells were studied significantly higher levels of CD4⁺ pSTAT4⁺ T cells were detected in pSS patients compared to PSC and HC cells after 45 minutes, 2 hours and 6 hours, but no difference between PBC CD4⁺ T cells and the other disease and healthy control groups was detected (Figure 4B). These data

therefore suggest that in PBC patients the increased sensitivity to low dose IL-12 is a feature of Treg cells and that IL-12 mediates its effects on Treg cells via phosphorylation of STAT4.

3.5. IL-12 interferes with the development of CD25^{hi} FOXP3⁺ CTLA4⁺ iTreg cells from naïve CD4⁺ T cells in both PBC and PSC patients

To further evaluate whether the sensitivity to respond to IL-12 is a feature of Treg cells and not of all CD4⁺ T cells, we further assessed the ability of IL-12 to interfere with the development of naïve CD4⁺ T cells to iTreg cells in vitro. Our data show that IL-12 significantly reduced the proportion of CD25^{hi} CD127⁻ FOXP3⁺ CTLA4⁺ iTreg cells from naïve CD4⁺ from both PBC and PSC patients, suggesting that IL-12 can interfere with the development of iTreg cells but this effect is the same in both cholestatic liver diseases (Figure 5A, 5B).

3.6. IL-12 secreted by monocytes drives Treg cell differentiation into Th1-like cells

In order to recapitulate the in vivo situation, we assessed the effect of monocyte conditioned media on Treg cells in terms of upregulating Th1-related markers and the effects after blocking IL-12. Culture of PBC Treg cells with PBC TLR-stimulated monocyte conditioned media significantly induced the expression of *IFNG*, *TBET* and *IL12RB2* (*p<0.05). When IL-12p70 and IL-12/IL-23 p40 were blocked this effect was significantly abolished suggesting that it is IL-12-driven (*p<0.05). Culture of Treg cells with untreated monocyte conditioned media had no effect in gene expression (Figure 4A - 4C). When PBC Treg cells were cultured with PSC TLR-stimulated monocyte conditioned media (which secrete significantly less IL-12) they also upregulated *IFNG*, *TBET* and *IL12RB2*, although this did not reach statistical significance compared to UT. There was also no statistically significant

difference between the effect of PBC TLR-stimulated monocyte conditioned media and PSC TLR-stimulated monocyte conditioned media, which agrees with our previous data showing that PBC Treg cells are able to upregulate Th1 markers even in the presence of low levels of IL-12.

Culture of PSC Treg cells with PSC TLR-stimulated monocyte conditioned media induced the expression of *IFNG*, *TBET* and *IL12B2* however, this did not reach statistically significant levels (Figure 6D - 6F). Blocking IL-12p70 significantly abolished the expression of *IFNG* and *TBET* (*p<0.05) but not *IL12RB2*, whereas blocking IL-12/IL-23 p40 was able to significantly reduce the expression of *IL12RB2* (*p<0.05) but not of *IFNG* and *TBET*.

Culture of PSC Treg cells with PBC TLR-stimulated monocyte conditioned media significantly induced the expression of *IFNG* and *IL12RB2* (*p<0.05) further agreeing with the previous results showing that PSC Treg cells can also respond to IL-12 but they need stronger dose of IL-12 to upregulate *IFNG*, *TBET* and *IL12RB2*. Collectively, these data suggest that IL-12 secreted by TLR stimulated monocytes drives the expression of Th1 markers on Treg cells, an effect that can be abolished in the presence of anti-IL-12.

3.7. In the liver of patients with PBC a higher proportion of IL-12R β 2⁺ Treg cells is detected which show Th1-like features

We next assessed the frequencies of IL-12R β 2⁺ Treg cells in the liver of patients with PBC in comparison to other liver disease and normal controls. Our data show that in the liver of patients with PBC there is a significantly higher proportion of Treg cells that express IL-12R β 2 (15.8% on average, mean value) compared to the other disease controls [4.6% in PSC, 5.6% in alcoholic liver disease (ALD) and non-alcoholic steatohepatitis (NASH) and 1.7% in normal controls (normal liver, NL and normal resected livers, Res)] (*p<0.05) (Figure 7A). IL-12R β 2⁺ Treg cells from PBC patients were further studied directly ex vivo and were found

to express higher levels of *IFNG* and *TBET* ($p=0.06$) when compared to their IL-12R β 2⁻Treg counterparts (Figure 7B). IL-12R β 2⁺ Treg cells from patients with PSC were not different compared to their IL-12R β 2⁻ counterparts (Figure 7C). IL-12R β 2⁺ Treg cells from patients with PBC expressed significantly higher levels of *IFNG* ($p=0.05$) and higher levels of *TBET* ($p=0.07$) when compared to PSC IL-12R β 2⁺ Treg cells (Figure 7D and 7E). No difference was detected between IL-12R β 2⁺ and IL-12R β 2⁻ Treg cells in gene expression of *IL-10*, *IL-17* and *FOXP3* in either PBC or PSC cases (Supplementary Figure 2). Collectively, these data suggest that a greater proportion of IL-12R β 2⁺ Treg cells is detected in the liver of patients with PBC and that these Treg cells show a Th1-like phenotype expressing higher levels of *IFNG* and *TBET*.

3.8. Intrahepatic Treg cells from patients with PBC have lost the sensitivity to low dose IL-12 treatment

We further studied the effect of different doses of IL-12 on intrahepatic Treg cells isolated from the livers of patients with PBC and PSC. Our data revealed no significant effect of IL-12 treatment on *IFNG*, *TBET*, *IL12RB2*, *IL-10*, *IL-17A* and *FOXP3* mRNA expression in both PBC and PSC intrahepatic Treg cells (compared to untreated cells) (Figure 8). PBC Treg cells stimulated with IL-12 at 20 and 50ng/ml significantly upregulated *IL12RB2* ($p=0.02$) compared to PSC Treg cells. PSC Treg cells on the other hand when stimulated with IL-12 at 20 and 50ng/ml significantly upregulated *IL10* ($p=0.05$). Overall, these data suggest that intrahepatic Treg cells from end-stage explanted liver samples respond minimally to IL-12 stimulation; at high doses of IL-12 stimulation PBC and PSC Treg cells show differential responses by upregulating *IL12RB2* and *IL10*, respectively.

3.9. Intrahepatic monocytes from different diseases respond equally to TLR stimulation and express similar IL-12p70 and IL-23 levels

We further studied the ability of intrahepatic monocytes from different liver diseases to respond to TLR stimulation and express IL-12. Our data show that intrahepatic monocytes from different liver diseases and normal controls respond equally to TLR stimulation and express similar *IL12A*, *IL12B*, *TNFA* and *EBI3* (Supplementary Figure 3A), as well as similar IL-12p70 and IL-23 protein levels (Supplementary Figure 3B, 3C).

4. Discussion

Studies have documented that IL-12 is not only able to induce the differentiation of CD4⁺ naïve T cells into Th1 cells but is also able to drive the polarization of regulatory T cells into Th1-like cells and paralyze their activity by reducing their suppressive capacity [13, 14]. In our study we evaluated the role of IL-12 in the pathophysiology of the autoimmune liver disease PBC using as comparators primary Sjögren's syndrome (pSS), another autoimmune disease with an IL-12 pathway gene association, primary sclerosing cholangitis (PSC), an immune-mediated liver disease with no reported IL-12 gene association, and healthy controls; our findings reveal an increased sensitivity of Treg cells from patients with PBC and pSS to low concentrations of IL-12, which are able to drive their differentiation into IFN γ -secreting cells.

A hallmark feature of autoimmune diseases has been the reduction in numbers and in function of Treg cells [7, 24-26], however the key mechanisms involved in driving the pathogenesis of autoimmunity, are still poorly understood. In addition, the role of IFN in autoimmunity has been controversial but is believed that a Th1 cell-mediated inflammatory response is critical for loss of tolerance. Human PBC has been recognised as reflective of Th1 mediated autoimmunity and the recent development of an animal model, based on chronic expression of IFN γ in ARE-Del^{-/-} mice [21] [27], provides evidence for a pathogenic role of IFN γ in the early stages and progression of PBC. Transfer of CD4 but not CD8 T cells from ARE-Del^{-/-} mice to B6/Rag1^{-/-} mice is able to induce moderate portal inflammation and mild parenchymal inflammation, further implying an important role of CD4 T cells in the pathological progression of the disease. In human PBC, gene expression analysis on liver biopsy tissues has also identified IFN γ signalling being significantly detectable in both early and late stages of disease [28-30] and recent GWAS studies have revealed several single

nucleotide variants enriched in CD4 T cell signalling, such as the IL12-JAK-STAT4 signalling pathway [31]. Loss of type I IFN receptor (IFN- α/β) receptor signalling using double knock out ARE-Del^{-/-}Ifnr1^{-/-} mice has been shown to reduce liver pathology, abrogate sex bias and correct germinal centre (sites implicated in loss of tolerance) abnormalities [32]. Overall, these animal and human studies provide strong evidence that IFN γ -induced Th1 responses via CD4 T cell activation plays a critical role in early stages and progression of PBC and possibly other Th1-mediated autoimmune diseases. Given that IL-12 is one of the major inducers of IFN γ production, which in turn primes additional antigen presenting cells to produce IL-12 and thus facilitate Th1 cell differentiation [15], our data confirm an important role for IL-12 in disease.

We demonstrate that although PBC and pSS are the two diseases in which, amongst others, risk variants in the *IL12A* locus have been identified, the monocytes from those patients were able to respond to TLR4 and TLR7/8 stimulation and secrete normal levels of IL-12p70 protein compared to healthy controls. In contrary, it was the monocytes from patients with PSC that were defective in their ability to express *IL12A*, *IL12B* and IL-12p70 and IL-23 proteins. PSC monocytes were able to express *TNFA* and *EBI3*, thus they were able to respond to stimulation and upregulate other inflammatory markers. The relevance of these variants in the ability of monocytes to express IL-12 cannot be answered by our current set of data and such experiments would be challenging without first identifying pathogenic variants (alone and more relevantly in association with others) should be studied. In the serum of patients with PBC significantly increased levels of IL-12p70 and IFN γ have been identified compared to healthy controls [22], and data have demonstrated that in patients with PBC circulating monocytes are increased in frequency and absolute number and are able to produce significantly increased levels of pro-inflammatory cytokines when challenged with different TLR ligands [16, 33]. Therefore, the absence of difference in IL-12 production from

PBC monocytes that was detected in our in vitro experiments could possibly be explained by the fact that equal numbers of cells were used as with the healthy control samples. In vivo the high levels of IL-12 protein in PBC patients could be attributed to the higher number of monocytes detected and not to any defect in their function. Similarly, it has been reported that in pSS patients with IgG antibodies to α -fodrin the percentage of CD14/HLA-DR⁺ monocytes is also increased compared with controls [34].

IL-12 exerts its effects through binding to its receptor, a heterodimeric type I cytokine receptor consisting of beta 1 (IL-12R β 1) and beta 2 (IL-12R β 2) subunits. IL-12R β 1 is constitutively expressed whereas IL-12R β 2 is upregulated by IFN γ in Th1 cells and plays a role in Th1 cell differentiation. Treg cells display a delayed induction of IL-12R β 2; this stringent regulation of IL-12R β 2 in Treg cells may serve as a tolerance mechanism that prevents them from undergoing IL-12-dependent functional differentiation and acquiring potentially dangerous pro-inflammatory properties such as the ability to produce IFN γ . In our study, we found no difference in the expression of IL-12R β 2 on circulating Treg cells across the different disease groups and healthy controls, however stimulation with different doses of IL-12 resulted in induction of Th1-related markers, *IFNG*, *TBET*, *IL12RB2*, with the Treg cells from patients with PBC showing a higher sensitivity and responding to low levels of IL-12 cytokine. pSS Treg cells showed no significant *IFNG*, *TBET* and *IL12RB2* mRNA expression changes after IL-12 stimulation, however, low dose IL-12 significantly upregulated IFN γ protein, similarly to PBC, and significantly higher compared to PSC and HC Treg cells. The lack of mRNA change on pSS samples could be due to quick mRNA turnover into protein in these patients [35]. These data were further verified by the increased phosphorylation of STAT4 in PBC and pSS samples after 6 hours of IL-12 stimulation, an effect not detected in PSC and healthy control samples, further suggesting that IL-12 mediates its effects via phosphorylation of STAT4.

Our findings were further supported by the co-culture experiments of patient Treg cells with the conditioned media from stimulated monocytes. PSC monocytes responded to TLR stimulation but expressed significantly low levels of IL-12 protein; however, those levels were enough to induce a Th1-differentiation on PBC Treg cells but not on PSC Treg cells. On the other hand, IL-12 levels released by PBC monocytes were also able to induce a Th1-differentiation on both PBC and PSC Treg cells, supporting our previous data that the Treg cells from patients with PBC are sensitive to low levels of IL-12 stimulation but that the same is not true for the Treg cells isolated from patients with PSC.

Although we detected no difference in the proportion of Treg cell expressing IL-12R β 2 between the blood of patients and healthy controls, in the liver of patients with PBC a significantly higher proportion of Treg cells expressed IL-12R β 2 compared to other liver diseases, cholestatic, metabolic and normal controls. Notably, when those IL-12R β 2⁺ Treg cells were studied directly ex vivo it was evident that the IL-12R β 2⁺ Treg cells from patients with PBC, but not from PSC, expressed higher *IFNG* and *TBET* levels compared to their IL-12R β 2⁻ counterparts, suggesting that in the inflamed microenvironment of patients with PBC a significant proportion of intrahepatic Tregs (~16%) shows Th1 features. In *Mdr2*^{-/-} mice, an animal model of primary sclerosing cholangitis, hepatic Treg cells that have upregulated IL-12R β 2 have also been detected [36]. Interestingly, in this model hepatic Treg cells were unable to expand in vivo and failed to improve the course of cholangitis due to the effects of hepatic IL-12 on them [36]. When we studied the ability of intrahepatic Treg cells to respond to different doses of IL-12 we detected that the sensitivity to IL-12 was lost, with intrahepatic Treg cells from patients with PBC and PSC responding equally to different concentrations of IL-12. This could be purely an effect/outcome of end-stage disease. Notably, PBC Treg cells significantly upregulated *IL12RB2* and PSC Treg cells *IL-10* when stimulated with IL-12 at

20 and 50ng/ml, suggesting that they may still have some intrinsic predisposition and sensitivity. Intrahepatic monocytes from different liver disease groups also responded equally to TLR stimulation with no significant differences in IL-12 and IL-23 protein expression detected. Again, this could be due to the fact that cells were isolated from end-stage liver samples, in which cells are more likely to be exhausted, or it could be due to the fact that intrahepatic cells express different TLRs or lastly that in the liver monocytes are not the major producers of IL-12. The observed differences between peripheral versus intrahepatic T cell responses to IL-12 stimulation could be attributed to the fact that blood samples were taken from patients at an earlier stage compared to intrahepatic cells which were retrieved from end-stage liver disease patients. Our findings on circulating cells may well reveal characteristics of cells at earlier stages of the disease.

In animal models of PBC, divergent findings regarding the role of IL-12 have been reported. In dominant negative transforming growth factor beta receptor type II (dnTGFβRII) mice deletion of IL-12p40 (strain deficient in both IL-12 and IL-23) suppressed the development of autoimmune cholangitis exhibiting dramatic reductions in histological cholangitis and significant decrease in intrahepatic proinflammatory cytokine levels [37], whereas in a more aggressive model of portal inflammation and colitis, the IL-2Rα^{-/-} mice, deletion of IL-12p40 induced a severe portal inflammation and bile duct damage, including signs of portal hypertension and liver fibrosis, but significant reduction in colitis [38]. In dnTGFβRII strain deletion of IL-12p35 (thus deficient in IL-12 and IL-35) also resulted in induction of liver fibrosis [39]. To date, ustekinumab, an anti-IL-12p40 monoclonal antibody that blocks the activity of both IL-12 and IL-23 has been tested in patients with PBC who have inadequate response to ursodeoxycholic acid. The overall study lacked significant efficacy, using a classic cholestatic based end-point (alkaline phosphatase), however, patients who reached a

>20% reduction in ALP values showed modulation of biological pathways, such as the Th17 pathway, that was related to ustekinumab response [40].

Notably, our study showed a heterogeneous response amongst patients, suggesting that within disease groups there might be individuals that behave biologically distinctly compared to the disease group as a whole. Whether this is due to the fact that those cases are carriers of the critical SNPs of the IL-12 pathway is not known but is a possibility. In our current study this was not possible to be addressed due to ethical constraints; future studies in large cohorts of patients where biological responses are correlated with their genotypes and/or in samples from healthy individuals homozygotes and/or heterozygotes for the risk variants would be interesting approaches to understand the biological implications of the risk variants.

However, even in these cases there are caveats: a) the fact that the knowledge of the true risk alleles is currently missing and b) that there are many immunomodulatory pathways associated with PBC therefore even if we studied one chosen risk allele it would not mean we are controlling for genetic variation in immunomodulatory function overall.

To conclude, our study reveals a novel feature of Treg cells in PBC and pSS, an increased sensitivity to IL-12 stimulation, with low concentrations being adequate to induce their differentiation into Th1-like cells via STAT4 phosphorylation. In the liver but not in the blood of patients with PBC we can detect a high proportion of IL-12R β 2⁺ Treg cells that already shows Th1-like features, suggesting that modulating the IL12-IL-12R β 2-STAT4 pathway on Treg cells at an early stage of disease could be a relevant therapeutic paradigm in the treatment of inflammatory diseases such as PBC.

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Figure Legends

Figure 1. Peripheral blood monocytes from patients with PBC and pSS respond normally to TLR stimulation. Circulating enriched monocytes were stimulated with LPS (1µg/ml) and R848 (2.5µg/ml) for 24hrs prior to collection of **(A)** cell pellets for *IL12A*, *IL12B*, *TNFA* and *EBI3* mRNA expression. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Statistical analysis of untreated (UT) vs LPS/R848 was performed using Wilcoxon matched-pairs signed rank test. Analysis between disease groups was performed using Mann Whitney test. Symbols indicate each individual sample and lines show median values. **(B)** Cell-free supernatant from LPS/R848 stimulated monocyte cultures was studied for the presence of IL-12p70 and **(C)** IL-23 proteins using ELISA. *p<0.05, **p<0.01 using Kruskal-Wallis test. For IL-12p70 n=19 PBC (primary biliary cholangitis), n=17 PSC (primary sclerosing cholangitis), n=6 pSS (primary Sjögren's syndrome) and n=8 HC (healthy control) samples were tested. Symbols indicate each individual sample and lines indicate median values.

Figure 2. Treg cells from patients with PBC are sensitive to low dose IL-12. **(A)** After exclusion of duplets and dead cells, CD3⁺CD4⁺ T cells were gated and further selected to define CD25^{high}CD127^{low} Treg cells as shown in the representative flow cytometry plot. IL-12Rβ2 protein expression on CD25^{high}CD127^{low} Treg cells was studied and expressed as percentage of Treg cells expressing IL-12Rβ2 (%IL-12Rβ2⁺ Treg cells). The median fluorescence intensity of IL-12Rβ2 was also evaluated. **(B-D)** Sorted CD25^{high}CD127^{low} Treg cells from patients with primary biliary cholangitis (PBC), primary sclerosing cholangitis (PSC), primary Sjögren's syndrome (pSS) and healthy controls (HC) were cultured in the presence of aCD3 (pre-coated plates, 10µg/ml), rhIL-2 (25IU/ml), anti-CD28 (1µg/ml) and different doses of recombinant human IL-12 (10-100ng/ml) for 4 days at 0.5 – 1 x10⁶ cells/ml. At the end of the stimulation period the gene expression of *IFNG*, *TBET* and

IL12RB2 was studied. * $p < 0.05$, ** $p < 0.01$ using Wilcoxon matched-pairs signed rank test. Symbols represent the median value and lines indicate the 95% CI (for some points the error bars are shorter than the height of symbol, thus not shown). For PBC, $n=18$ for UT, 10 and 20ng/ml and $n=5$ for 50 and 100ng/ml IL-12 stimulations were studied. For PSC, $n=12$ for UT, 10 and 20ng/ml and $n=5$ for 50 and 100ng/ml IL-12 stimulations were studied. For pSS, $n=3$ for all doses and for HC $n=8$ for all IL-12 dose stimulations were studied.

Figure 3. At low dose IL-12 stimulation Treg cells from patients with PBC and pSS express significantly higher levels of IFN γ compared to PSC Treg cells. (A, B) Sorted Treg cells were stimulated with 10ng/ml IL-12 in the presence of IL-2 (25IU/ml), anti-CD28 (1 μ g/ml) and aCD3 (pre-coated plates, 10 μ g/ml) for 4 days at $0.5 - 1 \times 10^6$ cells/ml. *IFNG* mRNA expression analysis was studied in $n=18$ PBC (primary biliary cholangitis), $n=12$ PSC (primary sclerosing cholangitis), $n=3$ pSS (primary sclerosing cholangitis) and $n=8$ HC (healthy control). * $p < 0.05$ using Mann Whitney test. Symbols represent each individual sample and lines indicate median values. **(B-C)** Cell free supernatant from Treg cell cultures was tested for the presence of IFN γ , IL-10, IL-2, IL-17, TNF α and IL-4 cytokines using the BD Cytokine Bead Array. **(B-C)** Representative flow cytometry plots across the different diseases and healthy controls. * $p < 0.05$, ** $p < 0.01$ using Mann Whitney test. Symbols represent each individual sample and lines indicate median values from $n=16$ PBC (primary biliary cholangitis), $n=11$ PSC (primary sclerosing cholangitis), $n=3$ pSS (primary Sjögren's syndrome) and $n=4$ HC (healthy controls).

Figure 4. STAT4 phosphorylation is quicker and stronger on Treg cells from patients with PBC and pSS. Peripheral blood mononuclear cells (PBMCs) at 100,000cells/100 μ l were stimulated with aCD3 (10 μ g/ml; pre-coated 96-U plates) in the presence of rhIL-2 (25IU/ml), aCD28 (1 μ g/ml), with/without rhIL-12 (10ng/ml) for 15min, 45min, 2 hours and 6 hours. Cells were collected and stained for Treg cell markers (high CD25 and low CD127 expression) and STAT4 phosphorylation (pY693). **(A)** Representative flow cytometry plots showing the % of pSTAT4 on alive CD3⁺CD4⁺ CD25^{high}CD127^{low} Treg cells on n=7 PBC (primary biliary cholangitis), n=7 PSC (primary sclerosing cholangitis), n=6 pSS (primary Sjögren's syndrome) and n=7 HC (healthy control) samples. Statistical analysis using Mann Whitney test. **(B)** Data show the % of pSTAT4 on alive CD3⁺CD4⁺ T cells. **Statistical analysis using Mann Whitney test.** Symbols indicate values from each patient and lines represent median values.

Figure 5. IL-12 interferes with the development of iTreg cells from naïve CD4⁺ T cells in both PBC and PSC patients. Naïve CD4⁺ T cells were differentiated into iTreg cells in the presence of low aCD3 stimulation (1 μ g/ml, pre-coated), 100IU/ml IL-2, 2ng/ml TGF- β with/without rhIL-12 (10ng/ml- 100ng/ml). After 5 days in culture the percentage of alive CD25^{high} FOXP3⁺ CTLA4⁺ cells was calculated. **(A)** Representative flow cytometry contour plots from one PBC sample showing the gating strategy followed. **(B)** Data show the fold change of % of FOXP3⁺ CTLA4⁺ cells in CD25^{hi} T cells after IL-12 stimulation compared to non-IL-12 stimulated cells. Data show mean $\pm$ SEM from n=4 PBC (primary biliary cholangitis) and n=4 PSC (primary sclerosing cholangitis) samples.

Figure 6. IL-12 secreted by TLR stimulated monocytes drives the induction of Th1 markers on Treg cells. Circulating enriched monocytes from patients with PBC (primary biliary cholangitis) and PSC (primary sclerosing cholangitis) were cultured with LPS (1µg/ml) and R848 (2.5µg/ml) (PBC MoCM T and PSC MoCM T, respectively) for 24hrs or left untreated (PBC MoCM UT and PSC MoCM UT). At the end of the stimulation period the cell-free supernatant was collected and used to culture sorted Treg cells from patients with (A-C) PBC and (D-F) PSC, in the presence/absence of anti-IL-12p70 (20µg/ml) and anti-IL12/anti-IL23 (20µg/ml). *IFNG*, *TBET* and *IL12RB2* mRNA expression analysis was studied. *p<0.05 using Wilcoxon matched-pairs signed rank test.

Figure 7. Intrahepatic IL-12Rβ2⁺ Treg cells from patients with PBC show a Th1-like phenotype. (A) Liver-infiltrating mononuclear cells were isolated and the presence of IL-12Rβ2⁺ Treg cells was studied by flow cytometry. Cells were gated on singlets and alive cells, then on CD3⁺CD4⁺ and further on CD25^{high}CD127^{low} Treg cells. IL-12Rβ2 protein expression on CD25^{high}CD127^{low} Treg cells was studied and expressed as percentage of Treg cells expressing IL-12Rβ2 (%IL-12Rβ2⁺ Treg cells). Representative flow cytometry dot plots showing the expression of IL-12Rβ2 in one PBC and one PSC liver. In grey it is shown the IL-12Rβ2 staining and in black the isotype matched controls (IMC). *p<0.05 using Mann Whitney test. N=7 PBC (primary biliary cholangitis), n=9 PSC (primary sclerosing cholangitis), n=2 NASH (non-alcoholic steatohepatitis), n=7 alcoholic liver disease, 2 ND (normal liver donor) and 2 Res (resected liver) samples were studied. (B) The expression of *IFNG* and *TBET* was studied on sorted IL-12Rβ2⁺ and IL-12Rβ2⁻ Treg cells from patients with PBC (n=5) and (C) from patients with PSC (n=5). Statistical analysis using Wilcoxon matched-pairs signed rank test. (D, E) The expression of *IFNG* and *TBET* on sorted IL-12Rβ2⁺ Treg cells was compared between PBC and PSC liver samples. Statistical analysis using Mann Whitney test.

Figure 8. Intrahepatic Treg cells from patients with PBC have lost the sensitivity to low dose IL-12 treatment. Intrahepatic CD25^{high}CD127^{low} Treg cells from patients with primary biliary cholangitis (PBC) and primary sclerosing cholangitis (PSC) were sorted and cultured in the presence of aCD3 (pre-coated plates, 10µg/ml), rhIL-2 (25IU/ml), anti-CD28 (1µg/ml) and different doses of recombinant human IL-12 (10-100ng/ml) for 4 days at 0.5 – 1 x10⁶ cells/ml. At the end of stimulation period the gene expression of *IFNG*, *TBET*, *FOXP3*, *IL12RB2*, *IL-10* and *IL-17A* was studied. *p<0.05 using Mann Whitney test. Symbols represent each individual sample and lines indicate the median values from n=PBC and n=4 PSC liver samples.

Figure 1

Figure 2

A

stimulation with
rhIL-12 at 10ng/ml

B

C

Figure 3

A **B** **Figure 4**

Figure 5

A

PBC Treags

B

C

Figure 6

D

PSC Treags

E

F

Figure 7

Figure 8

Supplementary Figure Legends

Supplementary Figure 1. Stimulation of peripheral blood monocytes from patients with PBC with LPS and R848 has an additive effect in their ability to induce *IL12A*, *IL12B* and *TNFA* mRNA expression. Circulating enriched monocytes were stimulated with LPS (1µg/ml), R848 (2.5µg/ml) and their combination for 24hrs prior to collection of their cell pellets for *IL12A*, *IL12B* and *TNFA* mRNA expression. **p<0.01. Statistical analysis of untreated (UT) vs LPS/R848 was performed using Wilcoxon matched-pairs signed rank test. Symbols represent each individual sample and lines indicate mean ± SEM.

Supplementary Figure 2. Ex vivo analysis of intrahepatic IL-12Rβ2⁺ and IL-12Rβ2⁻ Treg cells. The expression of *IL-10*, *IL-17A* and *FOXP3* was studied on sorted IL-12Rβ2⁺ and IL-12Rβ2⁻ Treg cells from patients with (A) PBC (n=5) and (B) from patients with PSC (n=5). Symbols represent each individual sample and lines indicate median values.

Supplementary Figure 3. Intrahepatic monocytes from patients with different liver diseases respond equally to LPS/R848 stimulation. Intrahepatic enriched monocytes were stimulated with LPS (1µg/ml) and R848 (2.5µg/ml) for 24hrs prior to collection of (A) cell pellets for *IL12A*, *IL12B*, *TNFA* and *EBI3* mRNA expression. Symbols indicate each individual sample and lines show median values. (B) Cell-free supernatant from LPS/R848 stimulated monocyte cultures was studied for the presence of IL-12p70 and (C) IL-23 proteins using ELISA. For IL-12p70 n=12 PBC (primary biliary cholangitis), n=13 PSC (primary sclerosing cholangitis), n=6 ALD/NASH (alcoholic liver disease and non-alcoholic steatohepatitis) and n=6 ND/Res (normal donor and resected liver) samples were tested.

Symbols indicate each individual sample and lines indicate median values. For IL-23, n=8 PBC, n=9 PSC, n=8 ALD/NASH and n=6 ND/Res liver samples were used.

Supplementary Figure 1

A

B

Supplementary Figure 2

Supplementary Figure 3