

Spatial proteomics and cell-cell crosstalk analysis reveal PD-L1 and IL-6 interaction in human primary sclerosing cholangitis

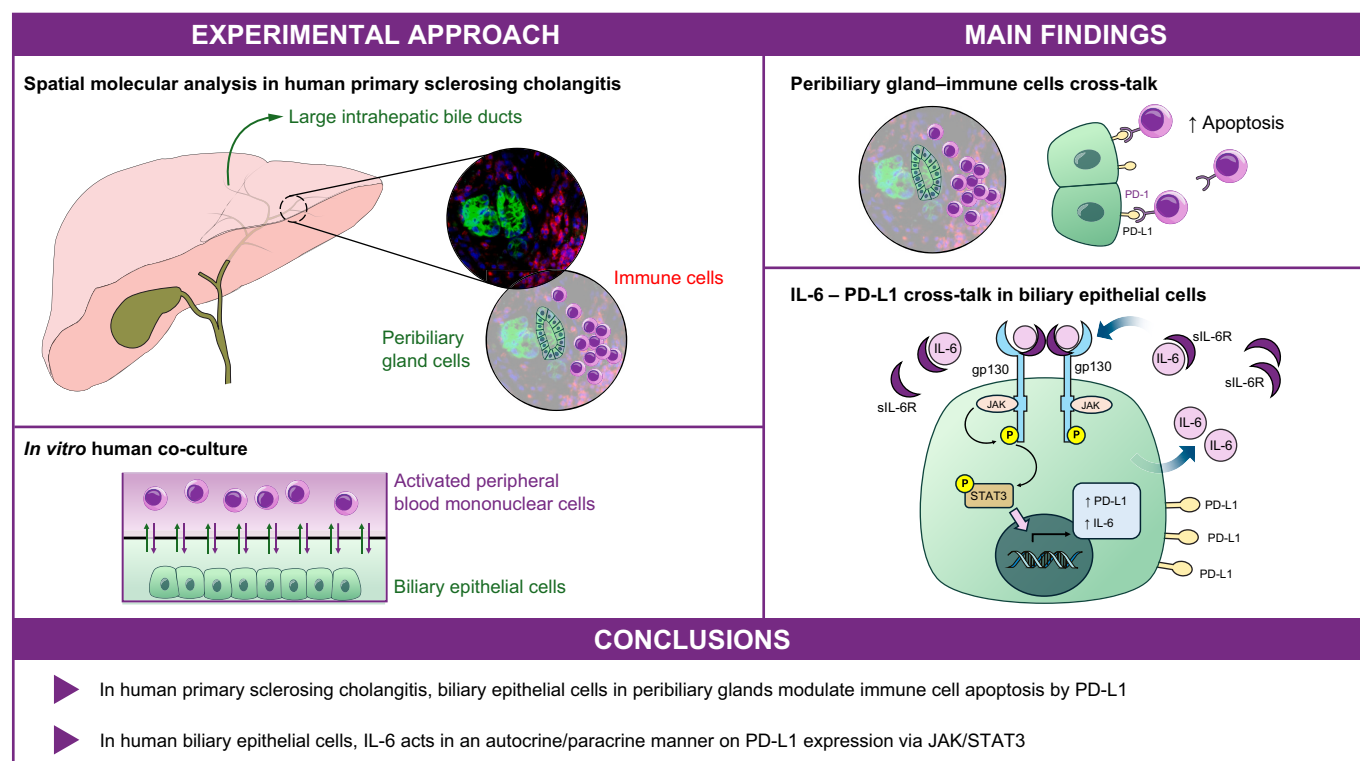
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Graphical abstract



Highlights:

- Biliary epithelial cells influence immune response in primary sclerosing cholangitis.
- Biliary epithelial cells promote apoptosis in PD-1⁺ immune cells via PD-L1.
- IL-6 induces PD-L1 expression by trans-signalling in biliary epithelial cells.
- JAK/STAT3 and NF-κB pathways mediate the effects of IL-6 on biliary epithelial cells.

Impact and implications:

The pathogenic basis of primary sclerosing cholangitis (PSC) is poorly understood, hampering the development of specific therapeutic approaches. We identified IL-6 as a key upstream regulator of PD-L1 via the JAK/STAT3 and NF-κB pathways in biliary epithelial cells, highlighting its central role in regulating epithelial-immune crosstalk during biliary injury. The identification of the PD-L1 pathway in epithelial-immune cell interactions in PSC may improve understanding of the disease, as well as inform the development of therapeutic strategies.

Spatial proteomics and cell-cell crosstalk analysis reveal PD-L1 and IL-6 interaction in human primary sclerosing cholangitis

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Background & Aims: Primary sclerosing cholangitis (PSC) is a chronic, immune-mediated cholangiopathy characterized by persistent inflammation and fibrotic remodelling of the biliary tree. We combined spatial proteomics of PSC-affected large bile ducts with a co-culture system of biliary epithelial cells (BECs) and activated peripheral blood mononuclear cells (aPBMCs) to dissect the molecular crosstalk at the epithelial-immune interface.

Methods: Spatial proteomic analysis was performed on six PSC-affected bile ducts and five healthy controls by GeoMx[®] DSP. Single-cell RNA-sequencing analysis from publicly available datasets of human control (n = 22) and PSC-affected (n = 8) livers was performed. We developed an *in vitro* inflammatory model of PSC by transwell co-culture of BECs (*i.e.* H69 and primary human biliary tree stem cells) and primary human PBMCs (n = 6). Co-cultures were analysed by transcriptomics on nCounter[®], by immunoassays and by culture with/without blocking agents.

Results: Peribiliary glands showed higher PD-L1 expression in PSC (score = 1.2 ± 0.4) compared to controls (0.3 ± 0.5 ; $p < 0.05$), which was associated with apoptosis in neighbouring immune cells on spatial proteomics. *In vitro*, aPBMCs induced the upregulation of 68 immunoregulatory genes in BECs; PD-L1 and IL-6 upregulation was confirmed at the protein level. BECs induced increased apoptosis in T (CD4⁺/CD8⁺) and NK cells. In co-culture, the exposure of BECs to the IL-6/sIL-6R complex induced significant PD-L1 upregulation (12.7 ± 1.3 ; $p < 0.001$), which was inhibited by IL-6 receptor blockade (2.6 ± 1.2 ; $p < 0.001$) or JAK1/2 inhibition.

Conclusions: These findings define a critical immunosuppressive axis in PSC driven by BEC-derived IL-6 and PD-L1. Mechanistically, IL-6 acts as an upstream regulator of PD-L1 via JAK/STAT3 and NF- κ B signalling, predominantly through IL-6 trans-signalling. This study provides a rationale for targeting epithelial-immune cell interactions as a therapeutic strategy in PSC.

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Introduction

Primary sclerosing cholangitis (PSC) is a chronic, immune-mediated cholangiopathy characterized by progressive inflammation and fibrosis of the intra- and extrahepatic bile ducts.¹ The primary site of disease is the large intrahepatic bile ducts and the extrahepatic biliary tree, where chronic inflammation triggers complex cellular crosstalk within the duct wall involving (myo)fibroblasts and epithelial cells, ultimately driving progressive fibrosis and biliary stricture formation.^{2–4} The resulting ascending cholestasis causes chronic injury to the hepatic parenchyma and progressive liver fibrosis, eventually leading to cirrhosis.

In the pathogenesis of the disease, major modifications occur in the bile duct wall where peribiliary glands (PBGs) have

been shown to drive fibroblast activation and can undergo metaplastic and dysplastic changes towards malignant traits.^{3,5–7} The presence of a chronic inflammatory microenvironment determines the injury of biliary epithelial cells (BECs) within PBGs but also creates a pro-tumorigenic niche that predisposes patients with PSC to cholangiocarcinoma.^{2,8} In turn, BECs release a variety of chemokines, cytokines and growth factors, responsible for shaping the inflammatory milieu.^{9,10} Factors secreted by BECs can either enhance local inflammation or have immunomodulatory effects.¹¹ Interestingly, BECs within PBGs can influence inflammation in PSC via the release of Fas Ligand (FasL), which induces apoptosis in T cells by modulating the Fas/FasL pathway.¹² Also, BECs are a source of IL-6 in human PSC.² Interestingly, IL-6 together with tumour necrosis factor- α (TNF- α) and IL-8 are major players in

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regulating hepatobiliary immunity in a paracrine and/or auto-crine manner.^{13,14}

The study of the interplay between BEC activation and inflammatory cells in PSC is of considerable interest,¹¹ as its dysregulation may contribute to the establishment of chronic inflammation and disease progression.¹⁴ An important axis in the epithelial-inflammatory crosstalk is represented by the programmed cell death protein 1 (PD-1) and programmed cell death 1 ligand 1 (PD-L1) system. PD-L1 is known to restrain T-cell hyperactivity by acting as a negative regulator of inflammation. Recent studies have highlighted a mechanistic link between chronic inflammatory signalling and PD-L1 expression in liver cancers.¹⁵ However, this axis remains poorly explored in the context of chronic biliary inflammatory conditions, including PSC.¹⁵

Therefore, the aims of this study were: i) to investigate the expression of PD-L1 by BECs in the course of PSC; ii) to decipher the role of the PD-1/PD-L1 system in the molecular interplay between inflammatory cells and BECs within PBGs via a spatial molecular analysis approach; iii) to define the mechanisms of PD-1/PD-L1 activation and their molecular crosstalk with the IL-6/JAK/STAT3 and NF- κ B pathways using a co-culture system mimicking the PSC microenvironment.

Materials and methods

Full methods are reported in the supplementary materials and methods.

Patient samples

Specimens containing large intrahepatic bile ducts were collected from patients with PSC undergoing liver transplantation for advanced liver disease ($n = 6$) at the Policlinico Umberto I Hospital (Sapienza University of Rome, Rome, Italy). As controls, liver and biliary tree with normal histology were obtained from organ donors discarded from liver transplantation procedures ($n = 6$). Demographics did not differ between controls and patients with PSC. Median age was 57 (IQR: 16) for controls and 57 (IQR: 4) for people with PSC. The control group included two females (2/5), and the PSC group included two females (2/6).

Written informed consent was obtained from each patient for research purposes. The research protocol was reviewed and approved by the Ethic Committees of Policlinico Umberto I. All research was conducted in accordance with both the Declarations of Helsinki and Istanbul as reflected in a prior approval by the institution's human research committee.

Spatial molecular profiling

FFPE PSC samples ($n = 6$) were selected for analysis on the GeoMx[®] Digital Spatial Profiler (DSP; NanoString, Bruker Spatial Biology, Bothell, WA, USA). Samples ($n = 5$) obtained from livers with normal histology were included as controls. Samples were processed according to manufacturer's protocols, as previously described.¹⁶ Whole slides were imaged using the GeoMx[®] DSP and the integrated software suite used to select regions of interest (ROIs) within the large intrahepatic biliary tree to sample PBGs and surrounding inflammatory cells based on their anatomical location. Homogeneous ROIs (similar size and histological disease activity) were selected

within the bile duct wall; one-to-three representative areas (total $n = 12$ for PSC and $n = 5$ for controls) were selected from each case. ROI size was based on the maximum size that could be collected by the instrument (*i.e.* approximately 650x750 μ m), and it was adapted in order not to include undesired areas (*e.g.* large blood vessels within the bile duct wall). The readout was performed using the nCounter[®] Sprint profiler. Data were processed by GeoMx[®] DSP Analysis Suite (v3.0.0113). Based on data quality control, housekeeper normalization was performed before data analysis.

scRNA-seq analysis from publicly available datasets

Human single-cell RNA sequencing (scRNA-seq) datasets generated by Tallulah *et al.* from control ($n = 22$; GSE243977) and PSC-affected ($n = 8$; GSE247128) livers were downloaded from the GEO database. The scRNA-seq dataset was processed, explored and visualized using Trailmaker[™] software (<https://app.trailmaker.parsebiosciences.com/>; Parse Biosciences, Seattle, WA, USA).

Co-culture of human BECs and human PBMCs

For co-culture experiments, the H69 human biliary epithelial cell line, primary human biliary tree stem cells (hBTSCs) isolated from extrahepatic biliary tree of healthy organ donors ($n = 3$), and human peripheral blood mononuclear cells (PBMCs) isolated from peripheral blood of $n = 6$ healthy donors¹⁷ were used. Biliary epithelial cells (BECs) or BTSCs were seeded at a density of 6,000 cells/cm² in growth medium. After cell adhesion, the medium was replaced with activated PBMCs (aPBMCs) standard culture medium, and aPBMCs were added to the BEC culture using a transwell culture system (0.4 μ m transwell inserts; Corning) at a 1:10 BECs:aPBMCs ratio, in the presence or absence of the IL-6R inhibitor (100 μ g/ml; tocilizumab, Hoffmann-La Roche, Basel, Switzerland), JAK1/2 inhibitor (10 μ M; baricitinib, APEXBio Technology LLC, Houston, TX, USA), p300 inhibitor (3 μ M; A485, MedChemExpress, NJ, USA), or PD-1 antibody (10 μ g/ml; pembrolizumab, Bio X Cell InVivoSIM, Lebanon, NH, USA) or the recombinant IL-6/sIL-6R complex (RnD Systems, Minneapolis, MN, USA, 120 ng/ml). After up to 48 h, cells and supernatants were harvested and collected separately for subsequent analyses. For the co-culture studies, experiments were performed in triplicate using PBMCs isolated from three independent donors ($n = 3$).

Transcriptomic analysis of cell cultures

One-hundred nanograms of RNA per sample was processed with the nCounter[®] using the Tumor Signaling 360[™] Panel. Data were analysed by ROSALIND[®] (<https://rosalind.bio/>) and nSolver[™] Analysis Software (NanoString, v4.0).

Quantitative real-time PCR and western blotting

Quantitative PCR was carried out in triplicate with QuantStudio[™] 3 Real-Time PCR System using PowerTrack[™] SYBR Green Master Mix (Thermo Fisher Scientific). Primer sequences are reported in Table S1. The fold changes in gene expression were normalized to the internal control gene RPLP0. Western blot analyses were performed by the BCA Protein Assay (Thermo Fisher Scientific). Membranes were incubated with the primary antibodies (Table S2) and then with

HRP-conjugated anti-mouse and anti-rabbit secondary antibodies. Anti-GAPDH antibody (Thermo Fisher Scientific) was used as control of protein loading. Fiji ImageJ software was used to perform densitometry analysis as previously described.¹⁷ Uncropped blots are shown in [Figs. S1 and S2](#).

Confocal microscopy

Immunofluorescence was performed as specified in the supplementary materials and methods. Image acquisition was performed using a Nikon A1 confocal laser scanning microscope (Nikon, Minato, Tokyo, Japan), as previously described.¹⁸ The confocal serial sections were processed with ImageJ software to acquire three-dimensional projections, and image rendering was performed using Adobe Photoshop Software.

Caspase-3/7 activity assay by flow cytometry

Apoptotic cell populations were evaluated by detecting caspase-3/7 activity using the CellEvent™ Caspase-3/7 Green Flow Cytometry Assay Kit (Thermo Fisher Scientific, Cat. No. C10740). SYTOX™ AADvanced Dead Cell Stain was used. Cells were analysed on a Xenith spectral acoustic flow cytometer (ThermoFisher). Caspase-3/7⁺/SYTOX⁻ cells were classified as early apoptotic, caspase-3/7⁺/SYTOX⁺ cells as late apoptotic, while caspase-3/7⁻/SYTOX⁺ cells were considered as very late apoptotic or necrotic cells.

Quantification of cytokines in cell culture media

Cytokines in supernatants were quantified using an automated immunoassay workflow. Specifically, IL-6, TNF- α , IL-1 β , and IL-8 were measured using the Simple Plex ELLA automated ELISA platform (ProteinSimple, Bio-Techne, Minneapolis, MN, USA), a microfluidics-based system that performs fully automated sandwich immunoassays in nanoliter-scale cartridges, enabling high sensitivity, reproducibility, and minimal hands-on time. Instrument calibration was conducted using the in-cartridge factory standard curve.

Statistical analysis

Statistical analysis was performed using GraphPad Prism Software (v8.0, San Diego, CA, USA). Pairwise comparisons between two experimental groups were conducted using an unpaired two-tailed Student's *t* test or Mann-Whitney *U* test. For comparisons involving three or more groups, one-way ANOVA was employed, followed by the Tukey or Dunnett multiple comparisons *post hoc* test to identify statistically significant differences. A significance threshold was set at *p* < 0.05 for all statistical analyses. The Pearson's test was used for correlation analysis. Each experiment was performed in triplicate.

Results

In human PSC, BECs within PBGs express PD-L1 and escape immune attack

PD-L1 positivity in BECs within PBGs was evaluated by immunohistochemistry both in controls (*n* = 6) and in PSC (*n* = 6) ([Fig. 1A](#)). PBGs can be identified based on their morphology

and location within large bile duct walls. In controls, PBG cells were negative or only occasionally positive for PD-L1. In PSC, PD-L1 expression significantly increased in PBGs compared to control livers. In large intrahepatic bile ducts in PSC, PD-L1 positivity was also observed in some neighbouring inflammatory cells.

The putative role and effects of PD-L1 expressed by BECs on biliary inflammation in PSC were explored by spatial proteomic analysis by GeoMx® DSP platform ([Figs 1B-D](#) and [S3](#); [Table S3](#)). In large bile ducts, BEC and inflammatory cell fractions within PBGs were segmented by immunofluorescence markers (*i.e.* pan-cytokeratin and CD45, respectively) and separately analysed. In the BEC fraction ([Fig. 1C](#), [Table S3](#)), the principal component analysis revealed a separate clustering of PSC samples compared to control ones, which was also confirmed by hierarchic clustering analysis. Interestingly, protein analysis indicated the increased expression of immune checkpoints including PD-L1, PD-L2 and CTLA-4. BECs also showed increased expression of both the proliferation marker Ki-67 and the apoptosis marker cleaved caspase 9; interestingly, Ki-67 clustered together with PD-L1 and PD-L2 expression in the hierarchic cluster analysis.

Then, we analysed the CD45⁺ cell fraction ([Fig. 1D](#), [Table S3](#)). As seen for BECs, PSC samples showed a distinct clustering pattern compared with control samples. Remarkably, inflammatory cells expressed higher levels of PD-1 and several apoptosis markers, but not of the proliferation marker Ki-67.

To further validate the findings from spatial molecular analysis, we explored published human scRNA-seq datasets from control and PSC-affected livers. The integrated cell map clusters were classified into eight general classes (#1 to #8; [Fig. S4](#)). Cells from PSC livers showed upregulation of *CD274* (*i.e.* PD-L1) and *IL6* genes as well as immune checkpoints, compared to those isolated from control samples ([Fig. 2A](#)). The cell annotation based on ScType¹⁹ resulted in the identification of liver cell types within the general classes, with “cholangiocytes” mostly corresponding to cluster #7 ([Fig. S4](#)). Based on custom annotation, we next identified putative PBG-derived cells (*n* = 1,624) defined by expression of *MUC5AC*, *MUC5B*, *MUC6*, and *MUC13*. These cells were located in cluster #7 alongside other BEC subpopulations, including cholangiocytes (*n* = 6,647).

In PSC samples, we examined the expression of genes of interest in the established cell subpopulations. PBG cells showed the highest expression of *CD274* compared to all other cell types ([Fig. 2C](#)); they also presented increased *IL6* gene expression compared to all other cell types except B cells ([Fig. 2C](#)). Interestingly, T cells showed the highest expression of immune checkpoint and immune exhaustion genes (*PDCD1*, *CTLA4*, *TIGIT*, *LAG3*) compared to other cell sets ([Fig. 2D](#)). Thus, we performed a sub clustering of T cells based on the co-expression of *ITGA4/ITGB7* (gut-homing receptor) and *CXCR6* (liver-homing receptor) to identify putative large intrahepatic bile duct-homed T cells (1,213 cells)¹⁰ from liver T cells (18,432 cells). Interestingly, immune checkpoint, immune exhaustion, and pro-apoptotic genes were significantly upregulated in T cells putatively homed to large intrahepatic bile ducts compared with liver-resident T cells in PSC samples ([Fig. 2E](#)).

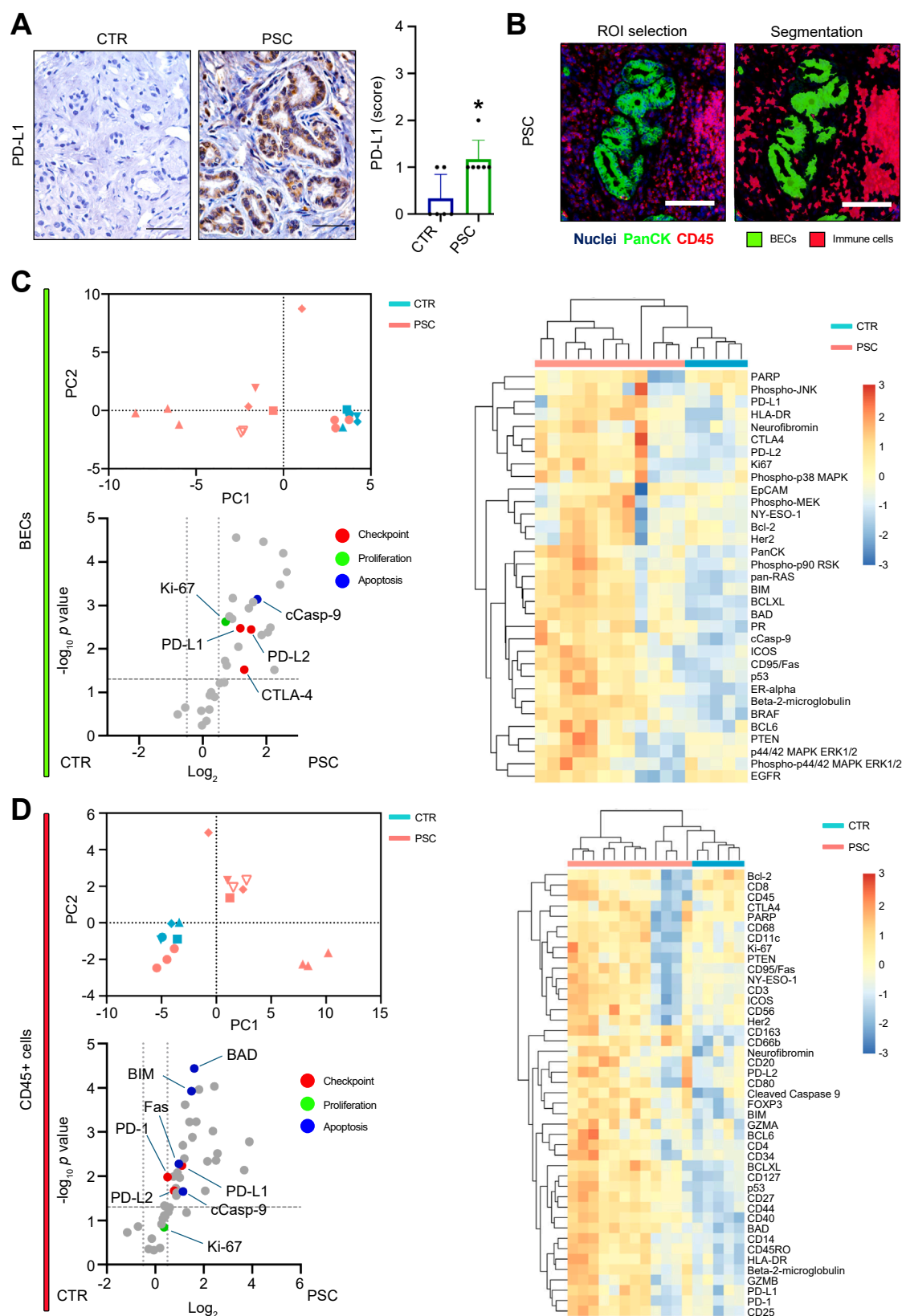


Fig. 1. Immunohistochemistry and spatial molecular analysis on human PSC and controls. (A) PD-L1 immunohistochemistry. Scale: 50 μ m. Histograms show means and SD for PD-L1 positivity score. Mann-Whitney *U* test. (B) On the left, immunofluorescence for pan-cytokeratin (PanCK) and CD45 (nuclei in blue); on the right, segmentation mask for collection on GeoMx[®] DSP. Scale: 100 μ m. PanCK⁺ BECs (green) and CD45⁺ immune cells (red) were segmented. (C-D) PC analysis, volcano plot and hierarchical clustering heat map for spatial proteomic analysis. Horizontal dotted line in volcano plots indicates statistical significance ($p < 0.05$). In PC analysis, different patients are indicated by different symbols. BECs, biliary epithelial cells; CTR, controls; PC, principal component; PSC, primary sclerosing cholangitis.

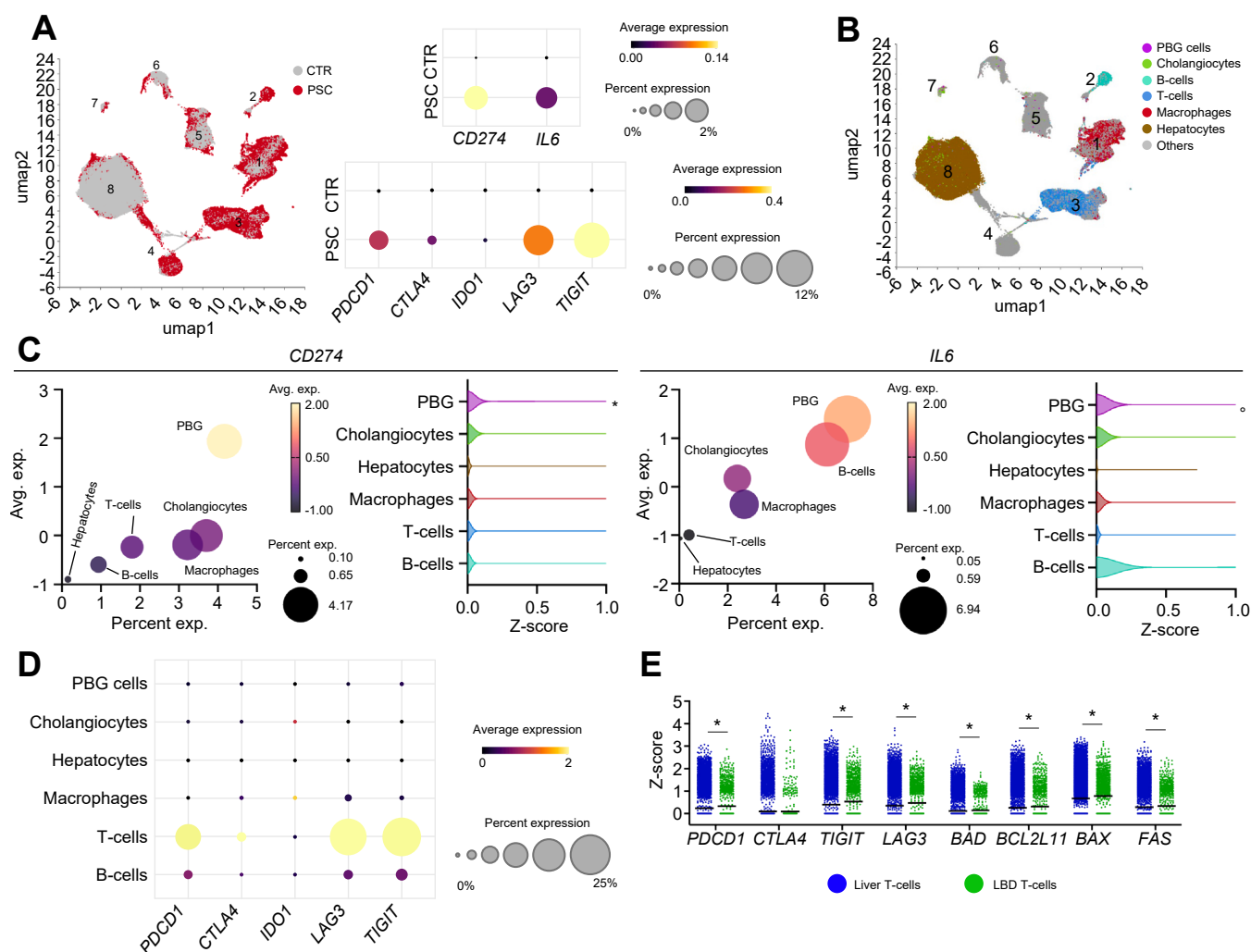


Fig. 2. scRNA-seq analysis of human PSC and control livers. (A) Integrated UMAP and matrix bubble plot of selected genes from scRNA-seq analysis. UMAP shows eight Leiden clusters annotated as CTR (grey) or PSC (red). (B) Integrated UMAP showing numbered Leiden clusters annotated to seven major custom cell types. (C) *CD274* and *IL6* expression at scRNA-seq analysis of PSC samples. * $p < 0.05$ vs. all other cell types; ° $p < 0.05$ vs. other cell types except for B cells. (D) Matrix bubble plot of selected genes from scRNA-seq analysis of PSC samples. (E) Dot plots of selected genes from scRNA-seq analysis of PSC samples comparing liver T cells and large bile duct T cells. * $p < 0.05$. Bubble plots in (A/C/D) report average expression (colour) and percentage of positive cells (size). CTR, controls; PSC, primary sclerosing cholangitis; scRNA-seq, single-cell RNA sequencing; UMAP, uniform manifold approximation and projection.

***In vitro* inflammatory microenvironment induces immunomodulatory capabilities in human biliary epithelial cells**

The human BEC cell line H69 was indirectly co-cultured with CD3/CD28 activated PBMCs (aPBMCs) using a transwell culture system. The use of aPBMCs was supported by their similar activation profile compared to inflammatory cells in PSC, as investigated by scRNA-seq (Fig. S5). The influence of aPBMCs on BEC behaviour was studied by comparing gene expression analysis in BECs alone and after co-culturing. Using a 780-gene nCounter® panel, we identified 70 differentially expressed genes (DEGs), 68 of which were upregulated in BECs after co-culture (Fig. 3A; Table S4). The Gene Ontology and KEGG analyses (Fig. 3B) revealed DEG enrichment in pathways related to cytokine production, immune system regulation and cell activation, including Toll-like receptor, TNF,

NF- κ B and JAK-STAT signalling pathways, as well as the PD-1/PD-L1 immune checkpoint. Of interest, genes associated with T-cell co-stimulation, IL-6 signalling, and NF- κ B signalling were upregulated, as shown in Fig. 3C. Accordingly, pathway scores of T cell co-stimulation, IL-6/JAK/STAT and NF- κ B signalling were increased after co-culturing with aPBMCs (Fig. 3D). Expression of PD-L1, IL-6, and NF- κ B p65 in BECs after exposure to aPBMCs was confirmed by confocal microscopy (Fig. 3E) and western blot analysis (Fig. 3F).

Effects of human BECs on aPBMCs

Among DEGs identified by transcriptomics, *CXCL11*, *CXCL10*, *CXCL2* genes were upregulated in BECs after aPBMC culturing (Fig. 4A); these chemokines are known to specifically recruit CD8⁺ T cells and natural killer (NK) cells to the site of inflammation.²⁰ Next, we performed an automated

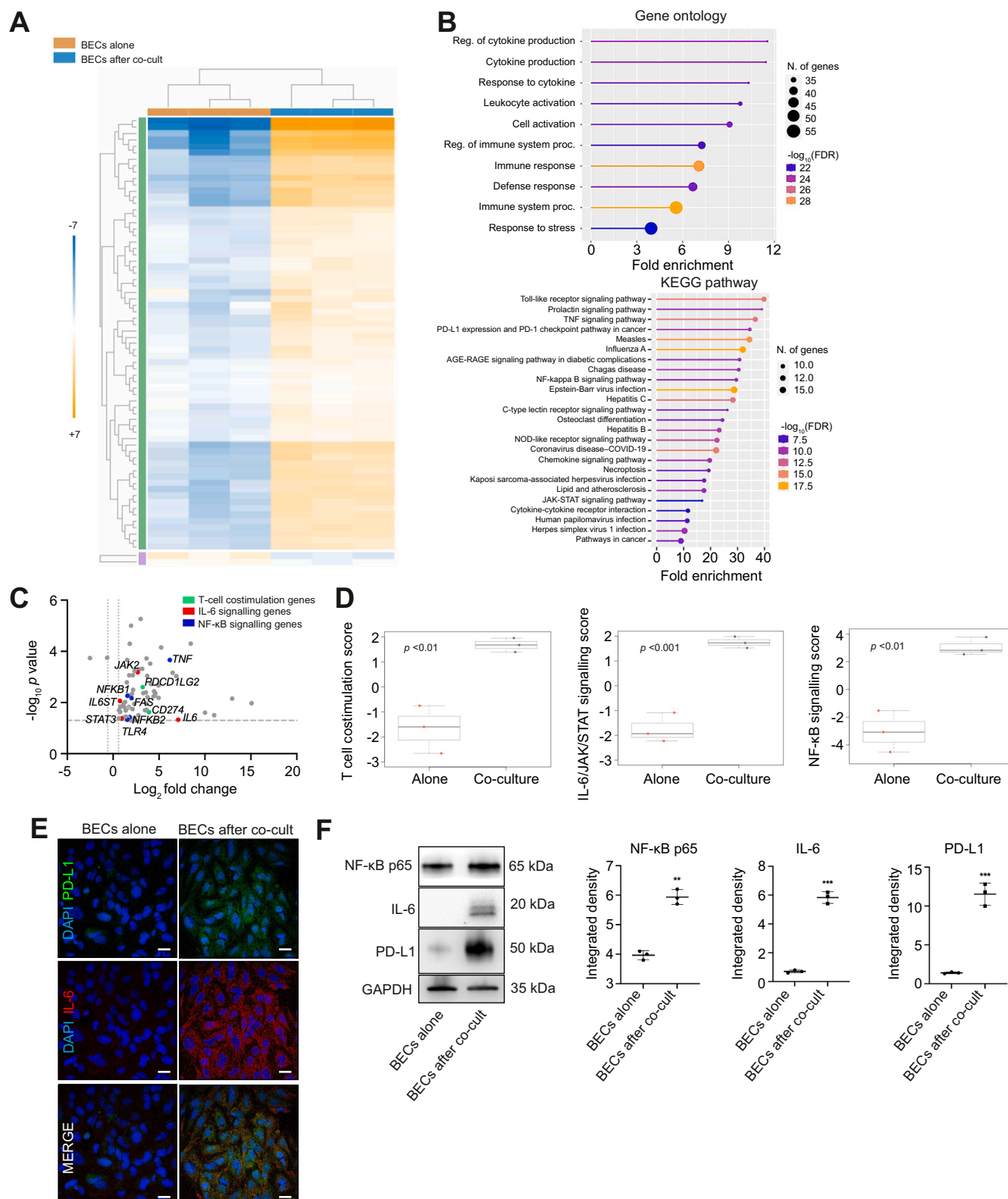


Fig. 3. Transcriptomic analysis and protein expression of BECs alone and after co-culture with aPBMCs. (A) Heatmap of 70 DEGs (≥ 1.5 fold-change, $p < 0.05$) between BECs alone and after co-culture with aPBMCs. (B) Top GO biological processes and KEGG pathway enriched in the 70 DEGs. (C) Volcano plot showing DEGs in BECs alone and after co-culture. Unpaired t test ($n = 3$). (D) Pathway scoring in BECs alone and following co-culture. Unpaired t test ($n = 3$). (E) Confocal immunofluorescence in BECs alone and after co-culture. Nuclei counterstained with DAPI. Scale: 10 μ m (40x). (F) Western blots and quantifications in BECs alone and after co-culture. Unpaired t test, $n = 3$; ** $p < 0.01$, *** $p < 0.001$. aPBMCs, activated peripheral blood mononuclear cells; BECs, biliary epithelial cells; DEGs, differentially expressed genes; GO, gene ontology.

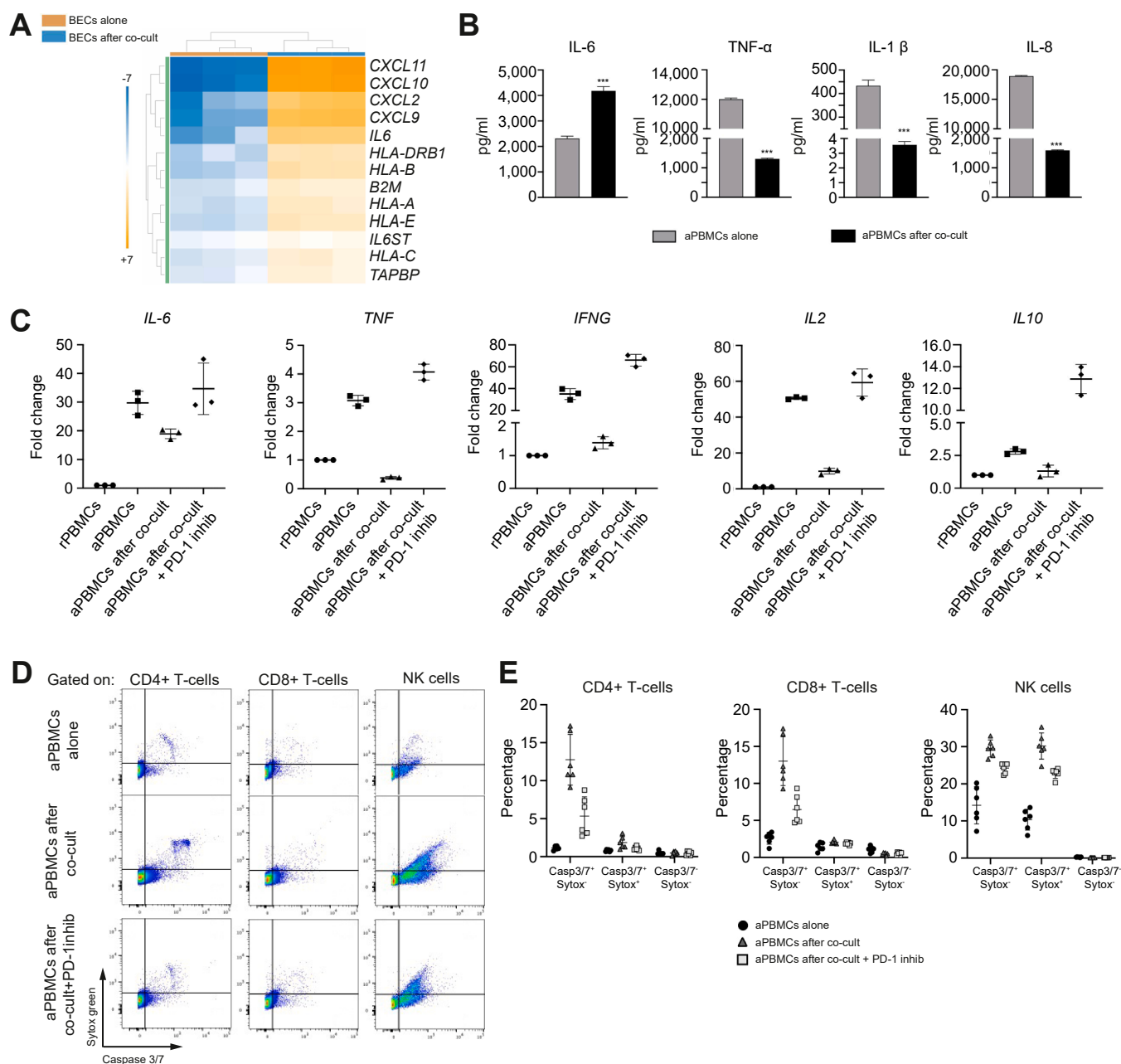


Fig. 4. Immunomodulatory effects of BECs on co-cultured aPBMCs. (A) Heatmap shows selected DEGs in BECs alone and after aPBMC co-culture. (B) Means/SD of chemokine concentration in media of aPBMCs alone and after co-culture with BECs. Unpaired *t* test (***p* < 0.001). (C) Means/SD of proinflammatory cytokines in aPBMCs alone and following BEC co-culture, with/without anti-PD-1 (pembrolizumab). Resting (r)PBMCs used as controls. **p* < 0.05/***p* < 0.001 vs. aPBMCs. °*p* < 0.05/°°*p* < 0.001 vs. aPBMCs after co-culture. (D) Representative FACS analysis of caspase-3/7/SYTOX on CD4⁺, CD8⁺ T cells and NK cells from leukocytes cultured alone and after BEC co-culture, with/without anti-PD-1. (E) Percentages of cell apoptosis detected by FACS experiments described in D. ***p* < 0.01/**°*p* < 0.001 vs. aPBMCs alone; °°*p* < 0.01/°°°*p* < 0.001 vs. aPBMCs after co-culture. In (C)-(D), one-way ANOVA and Tukey *post hoc* test. aPBMCs, activated peripheral blood mononuclear cells; BECs, biliary epithelial cells; DEGs, differentially expressed genes; NK, natural killer.

immunoassay (ELLA system) on culture supernatants to investigate pro-inflammatory cytokines secreted by aPBMCs. After co-culturing with BECs, aPBMC supernatant showed significantly lower levels of TNF- α , IL-1 β , and IL-8 but increased IL-6 levels compared to aPBMCs alone (Fig. 4B). On reverse-transcription quantitative PCR, a reduction of all studied cytokine genes was observed in aPBMCs co-cultured with BECs (Fig. 4C). Notably, cytokine expression was restored

when PD-1 antibody (pembrolizumab) was added to the co-culture system (Fig. 4C). Taken together, these results suggest that increased IL-6 levels in the supernatant are mainly derived from BECs, and that cytokine modulation in aPBMCs may be attributed to PD-1/PD-L1 activation.

FACS analysis was performed to quantify apoptosis occurring in CD4⁺, CD8⁺ T cells and NK cells after BECs co-culture with or without PD-1 antibody (Figs 4D,E and S6).

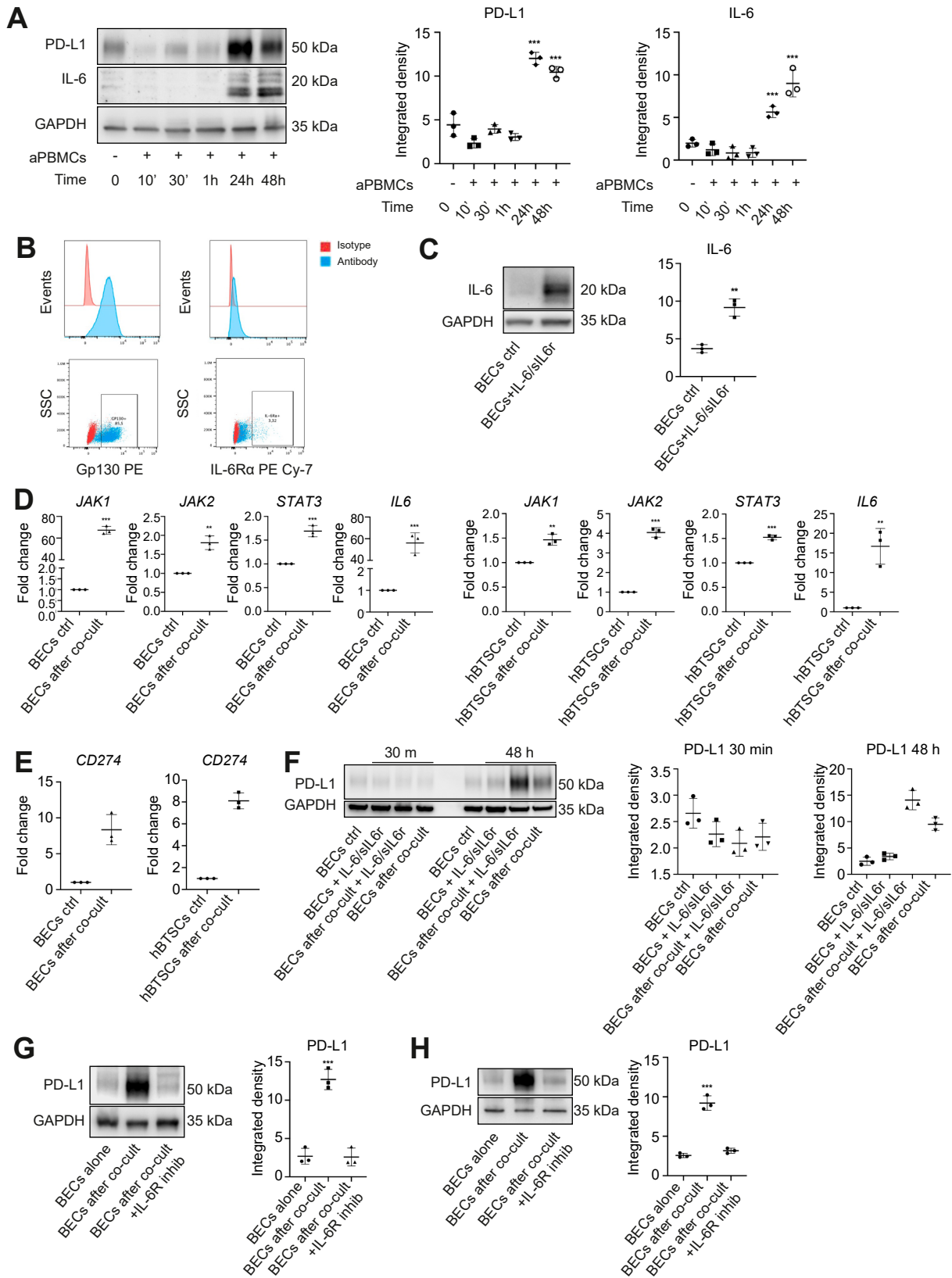


Fig. 5. IL-6/JAK/STAT3 pathway supports PD-L1 expression in BECs. (A) WB in BECs after aPBMC co-culture at different time points (one-way ANOVA, Dunnett *post hoc*; ****p* < 0.001 vs. BECs alone). (B) BECs flow cytometric analysis. (C) WB in BECs alone (ctrl) and after IL-6/sIL-6r stimulation (*t* test; ***p* < 0.01). (D/E) RT-qPCR in BECs/hBTSCs alone and after aPBMC co-culture (*t* test; ***p* < 0.01, ****p* < 0.001). (F) WB in BECs alone and after co-culture, with/without IL-6/sIL-6r (one-way ANOVA, Tukey *post hoc*; ***p* < 0.01/****p* < 0.001 vs. BECs; °°°*p* < 0.001 vs. BECs after co-culture). (G) WB in BECs alone and after co-culture, with/without IL-6R

Co-culture of leukocytes with BECs significantly increased the percentage of early apoptotic cells (Casp3/7⁺/SYTOX⁻ cells) in all cell subtypes compared to leukocytes cultured alone; a significant increase in late apoptosis (Casp3/7⁺/SYTOX⁺ cells) was observed only in NK cells. Apoptotic events were reduced in the presence of PD-1 antibody when compared to leukocytes co-cultured with BECs. No significant changes were observed in necrotic cells (Casp3/7⁻/SYTOX⁺) under any condition.

These data confirmed that BECs can modulate the immune response through PD-L1-mediated mechanisms targeting cytotoxic immune cell subsets.

Inflammatory microenvironment induces the activation of IL-6 pathway in BECs

The mechanistic role of the IL-6 pathway in BECs was characterized by a series of *in vitro* experiments. At different time points, co-culture with aPBMCs induced a statistically significant upregulation of IL-6 and PD-L1 in BECs after 24 h and 48 h (Fig. 5A). We then investigated through flow cytometry whether the IL-6 classical or trans-signalling pathway is involved in BECs.²¹ Interestingly, almost all BECs expressed Gp130, while only a small fraction expressed the membrane bound IL-6R α (Fig. 5B). To investigate the activation of IL-6 trans-signalling, BECs were treated with the IL-6/sIL-6R complex. Western blot analyses revealed a significant upregulation of IL-6 in treated BECs (Fig. 5C). Moreover, increased mRNA levels of the tyrosine Janus kinases (*JAK1*, *JAK2*) and of the signal transducer and activator of transcription 3 (*STAT3*) and *IL6* were observed in BECs after aPBMC co-culturing (Fig. 5D). These data were also confirmed in human primary cells isolated from PBGs (*i.e.* hBTSCs; Fig. 5D). Taken together, these findings suggest that BECs can activate the IL-6 trans-signalling pathway upon exposure to an inflammatory microenvironment, likely acting in an autocrine/paracrine manner.

Additionally, a significant increase in PD-L1 gene expression (*i.e.* *CD274*) was observed in both H69 BECs and hBTSCs following co-culture with aPBMCs (Fig. 5E). Based on these observations, we investigated the correlation between IL-6 and PD-L1 by exposing BECs to the IL-6/sIL-6R complex, with or without aPBMC co-culture, at different time points (Fig. 5F). After 30 min, no differences were detected in PD-L1 expression among conditions. After 48 h, BECs displayed a significant upregulation of PD-L1, which was further enhanced when the IL-6/sIL-6R complex was added to co-cultures. Conversely, treatment of BECs with the IL-6/sIL-6R complex alone had no effect on PD-L1 expression compared with controls.

Then, the effect of IL-6 on PD-L1 expression was confirmed by inhibiting IL-6 pathways in BECs co-cultured with aPBMCs. IL-6R inhibitor (tocilizumab) and JAK1/2 inhibitor (baricitinib) were added to the co-culture system. Both inhibitors blocked the increase in PD-L1 expression induced by aPBMC co-culture (Fig. 5G,H). Taken together, these data indicate a

significant role of the trans IL-6 signalling pathway in PD-L1 regulation in BECs.

Crosstalk between NF- κ B, STAT3 and IL-6 in BECs

Since NF- κ B and STAT3 act as “rapid-acting” primary transcription factors, regulating many cellular responses, including inflammation, we explored the interaction between IL-6/JAK/STAT3 and NF- κ B pathways in our *in vitro* system. Pathway score analysis demonstrated a positive correlation between IL-6/JAK/STAT3 and NF- κ B signalling in BECs alone and after co-culture with aPBMCs (Fig. 6A). Western blot analysis of BECs co-cultured with aPBMCs revealed that both NF- κ B p65 expression and the pSTAT3/STAT3 ratio were significantly increased at 30 min and 24 h compared with BECs cultured alone (Fig. 6B). Next, we evaluated the expression of NF- κ B p65 and IL-6 in BECs following co-culture with/without IL-6R and JAK1/2 inhibitors. The NF- κ B p65 and IL-6 upregulation in BECs after aPBMC co-culturing was reverted by IL-6R or JAK1/2 inhibitor administration (Fig. 6C,D). Immunofluorescence analysis confirmed the nuclear localization of pSTAT3 and NF- κ B p65 in BECs co-cultured with aPBMCs compared to BECs alone. Interestingly, the addition of an IL-6R inhibitor to the co-culture system induced the nuclear-to-cytoplasm transition of NF- κ B p65 and the absence of pSTAT3 staining compared to BECs after co-culture (Fig. 6E).

Remarkably, JAK1/2 inhibition reduced pSTAT3 expression induced in BECs following aPBMC co-culture (Fig. 6F). Given the role of histone acetyltransferase p300 and histone deacetylase (HDAC)2 in promoting the interaction between activated STAT3 and NF- κ B p65,^{22,23} we assessed their expression in BECs after aPBMC co-culture with or without IL-6R inhibition. Immunofluorescence demonstrated that BECs exhibited increased nuclear p300 localization after co-culturing with aPBMCs compared to BECs alone, which was reverted after IL-6 pathway inhibition (Fig. 6G). In parallel, co-culture conditions led to a downregulation of HDAC2 expression in BECs, which was reverted upon IL-6R inhibition (Fig. 6H). Finally, addition of the p300 inhibitor A485 significantly reduced the upregulation of NF- κ B p65, PD-L1, and IL-6 in BECs induced by exposure to aPBMCs (Fig. 6I). Overall, our findings suggest that IL-6-mediated activation of STAT3 may account for the activation and nuclear retention of NF- κ B p65, which in turn promotes the transcription of various genes involved in inflammation.

IL-6 induces EMT in BECs

We then explored the potential acquisition of epithelial-mesenchymal transition (EMT) traits in BECs under inflammatory conditions. Co-culture with aPBMCs induced the upregulation of several EMT-related genes in BECs, with an overall increase in EMT score compared with BECs alone, as demonstrated by gene set enrichment analysis (Fig. 7A). Then, western blot analysis (Fig. 7C) showed that BECs co-cultured with aPBMCs displayed decreased expression of EpCAM and increased expression of the mesenchymal marker SNAIL,

inhibitor (tocilizumab). (H) WB in BECs alone and after co-culture, with/without JAK1/2 inhibitor (baricitinib). In (F–H), graphs show means/SD; one-way ANOVA, Tukey *post hoc*; ***p* < 0.01/***p* < 0.001 vs. others. aPBMCs, activated peripheral blood mononuclear cells; BECs, biliary epithelial cells; WB, western blot.

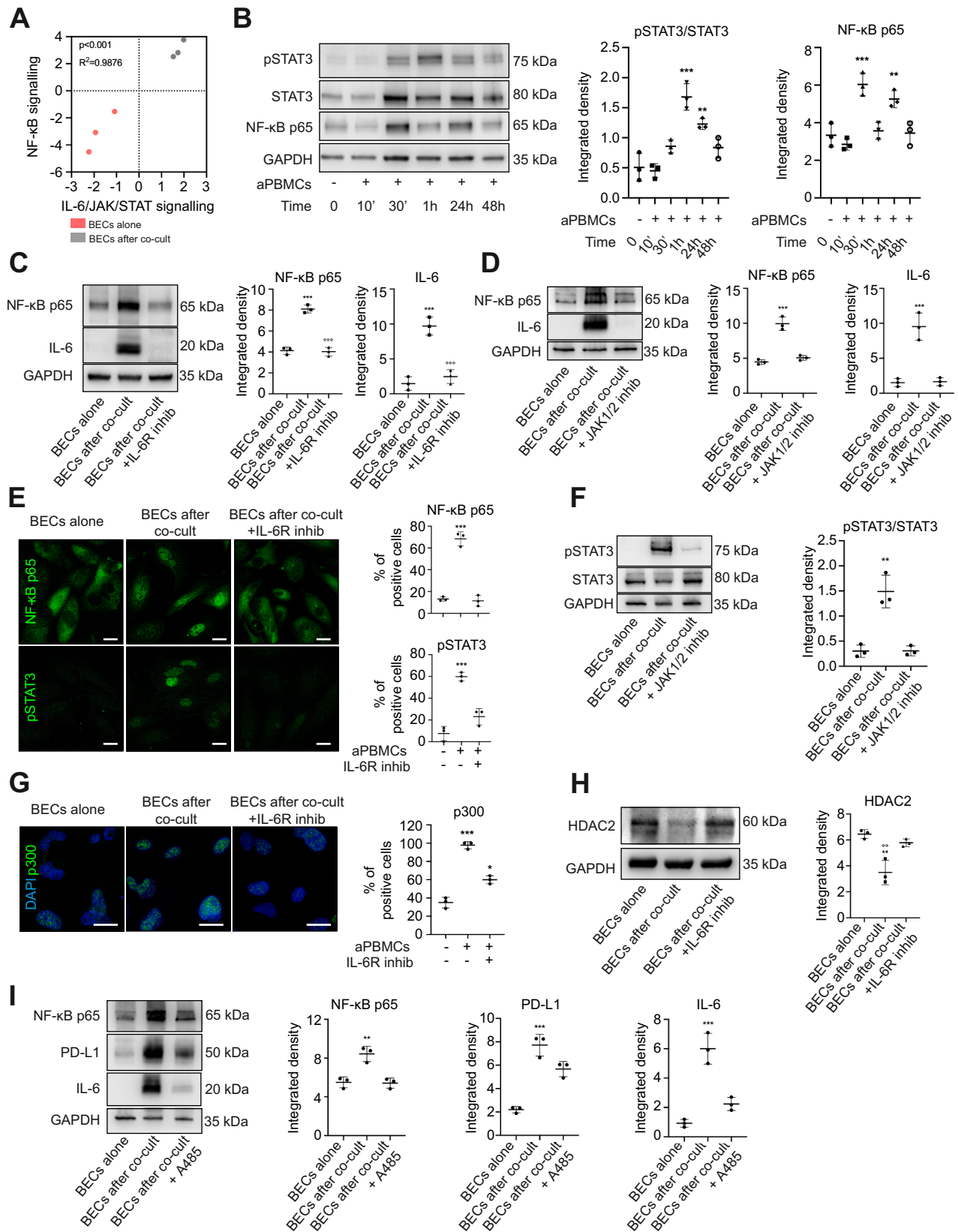


Fig. 6. Association between IL-6/JAK/STAT3 and NF-κB signalling. (A) Correlation between pathway scores in BECs alone and after co-culture with aPBMCs (Pearson's test). (B) WB in BECs after co-culture with aPBMCs (one-way ANOVA, Dunnett *post hoc*; ** $p < 0.01$ /** $p < 0.001$ vs. BECs alone). (C-D) WB in BECs alone and after co-culture, with/without IL-6R inhibitor (tocilizumab) or JAK1/2 inhibitor (baricitinib). *** $p < 0.001$ vs. BECs alone; °°° $p < 0.001$ vs. BECs after co-culture. (E) Confocal immunofluorescence in BECs alone and after co-culture, with/without IL-6R inhibitor. *** $p < 0.001$ vs. others. (F) WB in BECs alone and after co-culture, with/

compared to BECs alone; this was reverted by IL-6R inhibition. These data were confirmed by immunofluorescence staining of EpCAM and lamin A/C (Fig. 7D).

Discussion

The main findings of the present study, which focused on epithelial and inflammatory cell interactions in PSC, led to the identification of: (i) the role of PD-L1 expression in the crosstalk between PBGs and inflammatory cells, as revealed by spatial molecular analysis; (ii) the role of the PD-1/PD-L1 axis in regulating immune cell activation by BECs in an *in vitro* model that recapitulates epithelial–inflammatory interactions in PSC; (iii) a molecular interaction between the PD-1/PD-L1 axis and the JAK/STAT3 and NF- κ B pathways; and (iv) the specific role of IL-6 in the molecular cascade driven by BECs in an inflammatory milieu.

PSC is a chronic, immune-mediated cholangiopathy characterized by progressive inflammation and fibrosis of the intra- and extrahepatic bile ducts.¹ PSC pathogenesis is still poorly defined, and the lack of *in vivo* experimental models that recapitulate immune injury hampers the understanding of the cellular and molecular mechanisms underlying disease progression.²⁴ To overcome these issues, the present study applied two innovative methodological approaches. First, the spatial molecular analysis specifically performed at the primary site of biliary inflammation (*i.e.* large intrahepatic bile ducts) revealed the PD-1/PD-L1 axis as relevant in the crosstalk between BECs within PBGs and immune cells; then, a BEC-immune cell co-culture system was developed to further elucidate the actual molecular mechanisms of this interaction.

Through spatial molecular analysis, we explored molecular traits in the epithelial cell fraction and neighbouring inflammatory cells, precisely at the anatomical site of damage (*i.e.* PSC-affected bile duct). Our analysis highlights the increased expression of immune checkpoints (*e.g.* PD-L1, PD-L2) by PBG cells together with increased apoptosis in neighbouring CD45⁺ cells. In previous studies, the immunological landscape of the PSC liver was characterized using spatial molecular approaches, indicating exhaustion of myeloid cell lineages and downregulation of the inflammatory response in macrophage subpopulations.^{25,26} The novelty of the present study resides in focusing on the morpho-molecular changes in the large bile duct wall instead of liver tissue. Since large intrahepatic and extrahepatic bile ducts represent the main site of PSC damage, our study highlights pathogenic processes leading to biliary strictures. Of note, the analysis of scRNA-seq repositories obtained from PSC livers confirmed the scarcity of PBG cells sampled from intrahepatic large bile ducts and supports the importance of the results obtained from our spatial approach. Interestingly, Zimmer *et al.*¹⁰ obtained an immune landscape map of the extrahepatic biliary tree in patients with PSC by combining endoscopy-guided sampling with a high dimensional analysis approach. Remarkably, our findings further provide spatial and cellular insight into

immune–epithelial cell interactions by identifying PD-L1 expression in PBG cells and apoptotic effects on neighbouring PD-1⁺ immune cells. Methodologically, the spatial proteomic platform also allowed us to select the anatomical regions and cells to be sampled, describing cellular interactions within an anatomical niche. Results obtained from this approach indicated that inflammatory cell exhaustion could be ascribed to PD-1/PD-L1 axis modulation by BECs.¹⁰

To functionally validate the *in situ* observations, we recapitulated the PSC inflammatory microenvironment by co-culturing BECs with aPBMcs. Our transcriptomic analysis delineated the activation program undertaken by BECs in response to inflammatory stimuli. Immune cells are regulated by a variety of cytokines, including chemokines, which govern their differentiation and their infiltration into specific tissues in response to chemotactic gradients.²⁷ Our results indicated that BECs, under inflammatory conditions, upregulated the expression of several chemokines, and modulated cytokine secretion by immune cells, supporting their role in shaping immune infiltration and activation. In a previous study, BECs were shown to activate immune-escaping mechanisms through the Fas/FasL pathway in PSC.¹² In the present study, upregulation of Fas expression, together with apoptotic markers, was observed in the CD45⁺ cell fraction within PSC-affected bile ducts in spatial molecular analysis; *in vitro*, we also observed increased caspase-3/7 activity in CD8⁺ T cells and NK cells. Taken together, our data support the concept that BECs can shape immune response by modulating cytokine release and suppressing specific inflammatory cell subpopulations.^{10,11}

Remarkably, our results further revealed the involvement of immune checkpoints in the inflammatory milieu in PSC, where increased expression of PD-L1, PD-L2, and CTLA-4 by BECs was observed and associated with increased apoptosis in neighbouring PD-1⁺ inflammatory cells. PD-1/PD-L1 signalling represents an important effector in the suppression of immune system activation.^{28–30} It is well established that PD-1 is expressed on activated T cells,²⁷ and anti-PD-1 and/or anti-PD-L1 therapy can inhibit “immune escape” and strengthen immune activation.²⁷ Interestingly, in our *in vitro* system, treatment with a PD-1 inhibitor partially restored pro-inflammatory cytokine expression and reduced the proportion of apoptotic immune cells, suggesting a potential mechanism underlying immune exhaustion in biliary inflammatory diseases.³¹

The expression of PD-L1 is primarily regulated by interferon signalling, which includes the kinases JAK1/2, as well as the transcription factors STAT1/2/3.^{32,33} In chronic inflammation, JAK/STAT pathway activation is promoted by IL-6, which can be locally produced by infiltrating immune cells and/or resident cells, thus generating a positive feedback loop; moreover, the IL-6/JAK/STAT3 pathway can affect the tumour microenvironment by suppressing the immune response.^{32,33} In patients with PSC, systemic and local activation of IL-6/STAT3 signalling was detected;^{34,35} in our *in vitro* model, the activation/

without JAK1/2 inhibitor. (G) Immunofluorescence in BECs alone and after co-culture, with/without IL-6R inhibitor. **p* < 0.05/***p* < 0.001 vs. BECs alone; °*p* < 0.01 vs. BECs after co-culture + IL-6R inhibitor. (H) WB in BECs alone and following co-culture, with/without IL-6R inhibitor. ***p* < 0.01 vs. others. (I) WB in BECs alone and after co-culture, with/without A485, (p300 inhibitor). ***p* < 0.01/***p* < 0.001 vs. others. Graphs report means/SD. In (B)–(I), one-way ANOVA and Tukey *post hoc*. In (E)–(G), scale: 10 μ m (40x). aPBMcs, activated peripheral blood mononuclear cells; BECs, biliary epithelial cells; WB, western blot.

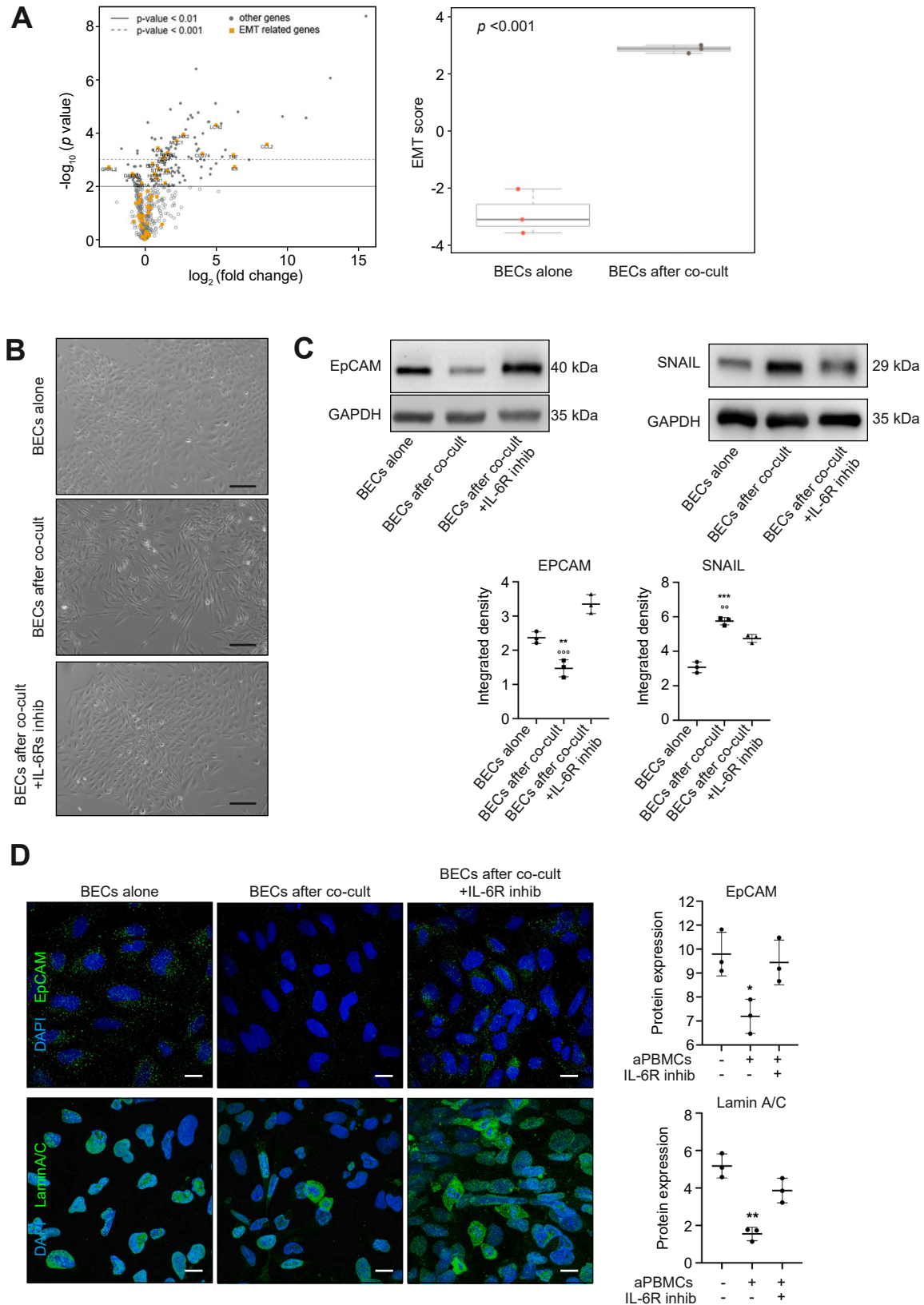


Fig. 7. Role of IL-6 in sustaining EMT transition of BECs. (A) GSEA volcano plot of EMT-related genes (yellow). Names are shown for differentially expressed genes ($p < 0.01$). Plot reports EMT scores in BECs alone and after aPBMC co-culture ($p < 0.001$). (B) Phase contrast of BECs alone or after co-culture, with/without IL-6R inhibitor (tocilizumab). Scale: 50 μm (20x). (C) WB in BECs alone and after co-culture, with/without IL-6R inhibitor (one-way ANOVA, Tukey *post hoc*; $**p < 0.01$, $***p < 0.001$ vs. BECs alone; $^{\circ}p < 0.01$, $^{\circ\circ}p < 0.001$ vs. BECs after co-culture + IL-6R inhibitor). (D) Immunofluorescence in BECs alone and after co-culture, with/without IL-6R inhibitor. Scale: 10 μm (40x) (one-way ANOVA, Tukey *post hoc*; $*p < 0.05$, $**p < 0.01$ vs. others). Graphs report means/SD. aPBMCs, activated peripheral blood mononuclear cells; BECs, biliary epithelial cells; EMT, epithelial-mesenchymal transition; GSEA, gene set enrichment analysis; WB, western blot.

inhibition of IL-6/JAK/STAT3 signalling in BECs was functionally associated with PD-L1 expression. This is in accordance with the role of IL-6 in driving PD-L1 expression by increasing its stability at the post-transcriptional level.^{36,37}

We also characterized IL-6 signalling in BECs through both classic and trans-signalling pathways.²¹ Although the IL-6 signalling pathway has already been implicated in cholangiopathies, our results demonstrate a predominant role for IL-6 trans-signalling mediated by the soluble form of IL-6R (sIL-6R), which is typically released by activated T lymphocytes. These data were further supported by the response of BECs to IL-6/sIL-6R complex administration.

From a molecular perspective, we further explored the association between IL-6/JAK/STAT3 and NF- κ B pathways.^{38,39} Our data revealed that NF- κ B p65 in BECs follows the expression of pSTAT3, which was characterized by a cytoplasmic-to-nucleus translocation. pSTAT3 might prolong the presence of active NF- κ B p65 in the nucleus by modulating its acetylation through the acetyltransferase p300,⁴⁰ which could be reverted by HDAC2,⁴¹ as observed in our model after administration of IL-6R and p300 inhibitors. Therefore, our data support the interplay between pSTAT3 and NF- κ B by acetylation status modulation in BECs under inflammatory conditions.

Finally, we investigated the potential role of IL-6 in inducing EMT in BECs. PSC pathogenesis is characterized by a fibrogenic process within the bile ducts, which determines the formation of biliary strictures;¹ this process is also influenced by cells within PBGs and is associated with the acquisition of EMT traits in BECs.³ Interestingly, our data showed upregulation of EMT-related genes in BECs under an inflammatory milieu and the acquisition of specific morpho-molecular changes, which were reverted by IL-6R inhibition.

The sustained inflammatory injury of the bile ducts in PSC is also a recognized risk factor for fibrogenic progression and the development of cholangiocarcinoma. Prolonged inflammatory injury, together with the immunomodulatory responses of BECs and PBGs, may contribute to neoplastic transformation and immune escape and exhaustion mechanisms.² The combination of a standard chemotherapy regimen and immune checkpoint inhibitors is approved as a first-line treatment for advanced and locally advanced CCA.⁴² Notably, in a large retrospective cohort, immunotherapy in PSC-associated cholangiocarcinoma was shown to be a well-tolerated and potentially effective treatment.⁴³ Future studies should address how the IL-6/PD-L1 axis might also be involved in cholangiocarcinogenesis in patients with PSC, with

implications both for the definition of disease pathogenesis and the refinement of therapeutic strategies.

Our experimental approaches have some limitations. Regarding *in situ* analyses, our aim of characterizing phenotype and interactions at the primary site of injury, *i.e.* the large bile ducts, requires the use of surgical specimens which were explants from liver transplantation for advanced liver disease. Despite this, large intrahepatic bile ducts in PSC display highly variable degrees of damage, ranging from ducts with minimal inflammation (early damage) to ducts with fibrotic strictures within the same samples;³ accordingly, our *in situ* findings, combined with *in vitro* models, are consistent with those reported by others in earlier disease stages, supporting the pathogenetic relevance of our results. Also, in this phase, we did not include other biliary diseases for comparison with PSC, as surgical specimens of large bile ducts from benign, non-terminal conditions are scarcely available. Regarding *in vitro* studies, further insights could be provided by performing co-culture experiments using primary BECs and PBMCs obtained from the same patient. Nevertheless, activated PBMCs have a phenotype⁴⁴ which mirrors PSC-derived immune cells, as observed by scRNA-seq, supporting their validity for our aims.

In conclusion, the present study identifies the involvement of the PD-1/PD-L1 pathway in the interaction between PBGs and inflammatory cells in PSC. In BECs, under an inflammatory milieu, IL-6 acts in an autocrine/paracrine manner, sustaining PD-L1 expression through the functional interaction between NF- κ B p65 and STAT3, thus promoting an immunosuppressive phenotype. The understanding of molecular crosstalk between inflammatory and immunoregulatory pathways may offer novel insights into PSC pathogenesis and identify potential therapeutic targets for intervention. The consistency of our *in vitro* findings with observations obtained from human PSC validates our co-culture system as a valid approach to study PSC pathogenesis, enabling the identification of mechanistic links underlying immune modulation. From a clinical perspective, our results may represent a potential therapeutic avenue. Recent findings on norUDCA,⁴⁵ which has been shown to reduce inflammation in multiple mouse models,⁴⁶ highlight the importance of elucidating PSC immune pathogenesis in order to identify new therapeutic targets for investigational PSC therapies. Moreover, the present study highlights the need for early intervention in PSC to prevent profound and potentially progressive immune dysregulation, which may drive progression from chronic inflammation to cholangiocarcinoma.

Affiliations

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Abbreviations

aPBMCs, activated peripheral blood mononuclear cells; BECs, biliary epithelial cells; BTSCs, biliary tree stem cells; DEGs, differentially expressed genes; DSP, digital spatial profiler; EMT, epithelial-mesenchymal transition; EpCAM, epithelial cell adhesion molecule; FasL, Fas Ligand; FFPE, formalin-fixed, paraffin-embedded; GEP, gene expression profiling; GSEA, Gene set enrichment analyses; hBTSCs,

human BTSCs; HDAC2, histone deacetylase 2; IL-6R, IL-6 receptor; JAK, Janus kinase; NK, natural killer; PBGs, peribiliary glands; PBMCs, peripheral blood mononuclear cells; PD-1, programmed cell death protein 1; PD-L1, programmed cell death 1 ligand 1; PSC, primary sclerosing cholangitis; ROI, region of interest; scRNA-seq, single-cell RNA sequencing; sIL-6R, soluble IL-6R; STAT, signal transducer and activator of transcription; TNF, tumour necrosis factor; UDCA, ursodeoxycholic acid.

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Conflict of interest

The authors declare no conflicts of interest pertaining to this manuscript.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

Concept and design: RDT, AP, GC, GuC; Case/data acquisition: GO, GB, SDB, AP, AF, PO, RDT, SM, SL, MF, TDL, LC, SP; Data analysis and interpretation: RDT, AP, GC, DO, LC, GuC, EG, DA, VC; Writing of the manuscript: RDT, AP, GC, GO, DO, GuC, VC; Final approval: all authors; Financial Support: GC, EG, VC, DA, DO, SM, AF; Project Supervisors: GC, GuC.

Data availability

The data that support the findings of this study are available from the authors upon reasonable request.

Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jhepr.2026.101903>.

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Author names in bold designate shared co-first authorship

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