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Original Article

STING-mediated IL-6 Inhibits OATP1B1 Expression via the TCF4 Signaling Pathway in Cholestasis

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Abstract

Background and Aims: Organic anion-transporting polypeptides (OATPs) play a crucial role in the transport of bile acids and bilirubin. In our previous study, interleukin 6 (IL-6) reduced OATP1B3 levels in cholestatic disease. However, it remains unclear whether IL-6 inhibits OATP1B1 expression in cholestatic diseases. This study aimed to investigate whether IL-6 can inhibit OATP1B1 expression and explore the underlying mechanisms. ***Methods:*** The effect of stimulator of interferon genes (STING) signaling on inflammatory factors was investigated in a cholestatic mouse model using RT-qPCR and enzyme-linked immunosorbent assay. To assess the impact of inflammatory factors on OATP1B1 expression in hepatocellular carcinoma, we analyzed OATP1B1 expression by RT-qPCR and Western Blot after treating PLC/PRF/5 cells with TNF-α, IL-1β, and IL-6. To elucidate the mechanism by which IL-6 inhibits OATP1B1 expression, we examined the expression of the OATP1B1 regulator TCF4 in PLC/PRF/5 and HepG2 cells using RT-qPCR and Western Blot. The interaction mechanism between β-catenin/TCF4 and OATP1B1 was investigated by knocking down β-catenin/TCF4 through siRNA transfection. ***Results:*** The STING inhibitor decreased inflammatory factor levels in the cholestatic mouse model, with IL-6 exhibiting the most potent inhibitory effect on OATP1B1. IL-6 downregulated β-catenin/TCF4, leading to decreased OATP1B1 expression. Knocking-down β-catenin/TCF4 counteracted the β-catenin/TCF4-mediated repression of OATP1B1. ***Conclusions:*** STING-mediated IL-6 up-regulation may inhibit OATP1B1, leading to reduced transport of bile acids and bilirubin by OATP1B1. This may contribute to altered pharmacokinetics in patients with diseases associated with increased IL-6 production.

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Introduction

The cyclic GMP–AMP synthase (cGAS)–stimulator of interferon genes (STING) signaling plays an essential regulatory role in antibacterial, antiviral, and anti-tumor immunity via the induction of cytokines, especially type I interferons (IFNs).[1,2] Several recent studies have demonstrated a close association between inflammatory diseases (such as heart failure, myocardial infarction, cardiac hypertrophy, non-alcoholic fatty liver disease, aortic aneurysm, and obesity) and aberrant, dysregulated cGAS-STING axis signaling.[3,4] Engulfing large amounts of DNA by macrophages, including mitochondrial DNA, extracellular vesicular DNA, and apoptotic hepatocyte DNA, has been shown to induce STING activation in macrophages.[5,6] As early as 2016, ER stress was shown to mediate liver fibrosis through the induction of liver cell death and the activation of STING.[7] In 2018, fibrosis and inflammation in non-alcoholic fatty liver disease patients were shown to be associated with STING, and this phenomenon was confirmed to be mediated by macrophages.[3,8] STING activation has been shown to enhance fibrogenesis by inducing the release of IL-1β from macrophages to activate hepatic stellate cells.[9] Additionally, various inflammatory factors caused by activation of the STING pathway, particularly IL-6 and IFN-β, can directly target cells and cause damage.[5,10] Although hepatic fibrosis and cirrhosis are the endpoints of cholestatic diseases, the activation of STING and inflammatory factors in cholestatic diseases has not been well described.

Cholestasis is a pathophysiological process caused by bile secretion and excretion disorders. It manifests as the ex-

Keywords: Inflammation; Bile acid; Bilirubin; Cholestasis; STING; IL-6; β-catenin; TCF4.

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cessive accumulation of bile components such as bile acids, cholesterol, and bilirubin in the liver and circulation, resulting in damage to liver cells and organisms.[11,12] The transport of compounds in the blood (bile acids, bilirubin) and drugs (statins) to hepatocytes occurs via members of the organic anion transporting polypeptide (OATP) family.[12,13] Currently, the most prominent functional members are OATP1B1 and OATP1B3 in hepatocytes, and their deficiency can cause Rotor syndrome.[14,15] There is limited information regarding the regulation of the function and expression of OATP1B1 and OATP1B3 under cholestatic conditions in humans. Our previous study demonstrated the important role of OATP1B3 under cholestasis and the role of IL-6 in the regulation of OATP1B3.[16] However, the molecular mechanism by which IL-6 regulates OATP1B1 remains unclear.

To investigate the regulatory mechanism of OATP1B1 under cholestasis, we treated mice with cholestasis with C170 (a STING inhibitor) and found that it inhibited inflammatory factors (such as TNF-α and IL-6) and reduced the accumulation of bile acids and bilirubin in the serum. The mechanism of IL-6 regulation of OATP1B1 via β-catenin was further confirmed in HepG2 and PLC5/PRF/5 cells.

Methods

Animals and treatment

All animal experiments were approved by the Animal Care and Use Committee at the Medical Research Center (Southwest Hospital, Chongqing, China; approval number: AMUWEC20223955). Seven-week-old male C57BL/6J mice were purchased from the Center of Laboratory Animals of the Southwest Hospital. These mice were maintained under SPF conditions in the hospital. After a one-week acclimatization period, the mice were randomly assigned to the following four groups: (1) the chow diet and corn oil intraperitoneal injection group; (2) the chow diet and 10M C170 intraperitoneal injection group; (3) the 0.1% 3,5-diethoxycarbonyl-1,4-dihydroxychollidine (DDC) diet and corn oil intraperitoneal injection group; and (4) the 0.1% DDC diet and 10M C170 intraperitoneal injection group. The 0.1% DDC diet is a solid feed prepared by adding DDC powder to pulverized, filtered, and evenly mixed feed powder. C170 is a potent covalent inhibitor of STING that inhibits mouse STING (mmSTING) and human STING (hsSTING).[17] C170 (MedChemExpress, Princeton, NJ, USA) was dissolved in corn oil to prepare a working solution at a concentration of 10M. Mice in the normal administration groups (Oil groups) were administered an intraperitoneal injection of 200 μL/day of corn oil, and mice in the C170 groups were administered an intraperitoneal injection of 200 μL/day of the working solution. The drug was administered continuously for 14 days. After two weeks, all mice were fasted overnight before being euthanized.

Human liver samples

This study was conducted in accordance with the principles enshrined in the World Medical Association's Declaration of Helsinki (2013). The study protocol was approved by the Ethics Committee of the First Affiliated Hospital of Third Military Medical University, Chongqing, China (approval number: (B) KY2021157). Written informed consent for participation in the study and the publication of images was obtained from all patients. Fourteen liver samples (n = 14) were obtained from patients with cholestasis due to suspected pancreatic or periampullary cancer. These patients were diagnosed based on the criteria of an alkaline phosphatase level >1.5× upper limit of normal and a gamma-glutamyl transferase (GGT) level > 3× upper limit of normal before treatment. Additionally, liver tissues from fourteen patients (n = 14) without cholestasis who underwent resection of liver metastases were used as the control group. Supplementary Table 1 lists the biochemical characteristics of the patients. The liver samples from all patients were immediately sectioned and immersed in liquid nitrogen.[18]

Cell lines and reagents

The human hepatoma PLC/PRF/5 cell line (ATCC) and PLC/PRF/5-ASBT cells were used as described elsewhere.[19] The human hepatoma HepG2 cell line[20] and THP1 cell line[21] were also used as described elsewhere. The PLC/PRF/5 and THP1 cell lines were cultured in RPMI 1640 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum. The HepG2 hepatocellular carcinoma cell lines were maintained in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum in a 5% CO_2 environment. The cells were starved in Dulbecco's Modified Eagle's Medium with 2% fetal bovine serum for 12 h before treatment with gradient concentrations of IL-6 (0, 5, 10, and 25 ng/mL). LiCl, an agonist of β-catenin, was added to IL-6 to reverse the effect of IL-6.

Transfection of PLC/PRF/5 cells

The plasmids of shTCF7L2 were generated by Shanghai Jikai Company.

The siRNAs targeting β-catenin were: siRNA#1 (GATGGTGTCTGCTATTGTA), siRNA#2 (GACTACCAGTTGTGGTTAA), and siRNA#3 (GGACAAGGAAGCTGCAGAA). Lipofectamine™ 2000 was obtained from Invitrogen (Carlsbad, CA). PLC/PRF/5 cells were transfected with these constructs using the transfection reagents according to the manufacturer's protocol.[20]

Cell co-culture and enzyme-linked immunosorbent assay (ELISA)

The co-culture transwell setup was utilized to recreate the inflammatory microenvironment by segregating THP1 cells and PLC/PRF/5 cells using a polycarbonate membrane to evaluate cell-cell interactions. The co-culture model was established as follows: PLC/PRF/5 cells (1×10^5 cells) were plated on the bottom of 24 plates. THP1 cells were seeded at a density of 5×10^4 cells on polycarbonate membranes with a pore size of 0.4 μm. PMA at a concentration of 100 ng/mL was added to THP1 cells for the experiment.[22] Transwells were placed into the same 24 multiple plates in the presence or absence of the STING agonist, SR-717. SR-717 (MedChemExpress, Princeton, NJ, USA) was added at a concentration of 10 μM. The top and bottom cells, along with the culture medium, were collected.

ELISA for IL-6 and IFN-β was performed following the manufacturer's protocol (MULTI SCIENCES, Zhejiang, China).[23] Culture supernatants were diluted 5-fold with medium, and then mixed with recombinant standards and added to the enzyme immunoassay plate with a 1:100 antibody dilution, respectively. The plate was subsequently incubated for 2 h at room temperature and washed six times, followed by the addition of streptavidin-horseradish peroxidase at a 1:100 dilution for 45 m at room temperature. The plate was then washed six times, and 0.1 mL of TMB substrate was added to each well. The experiment was terminated by adding 0.1 mL of termination substrate, and the absorbance was read at 450 nm.

RNA extraction and RT–qPCR

Total RNA was extracted using Trizol reagent (Takara Bio Inc.,

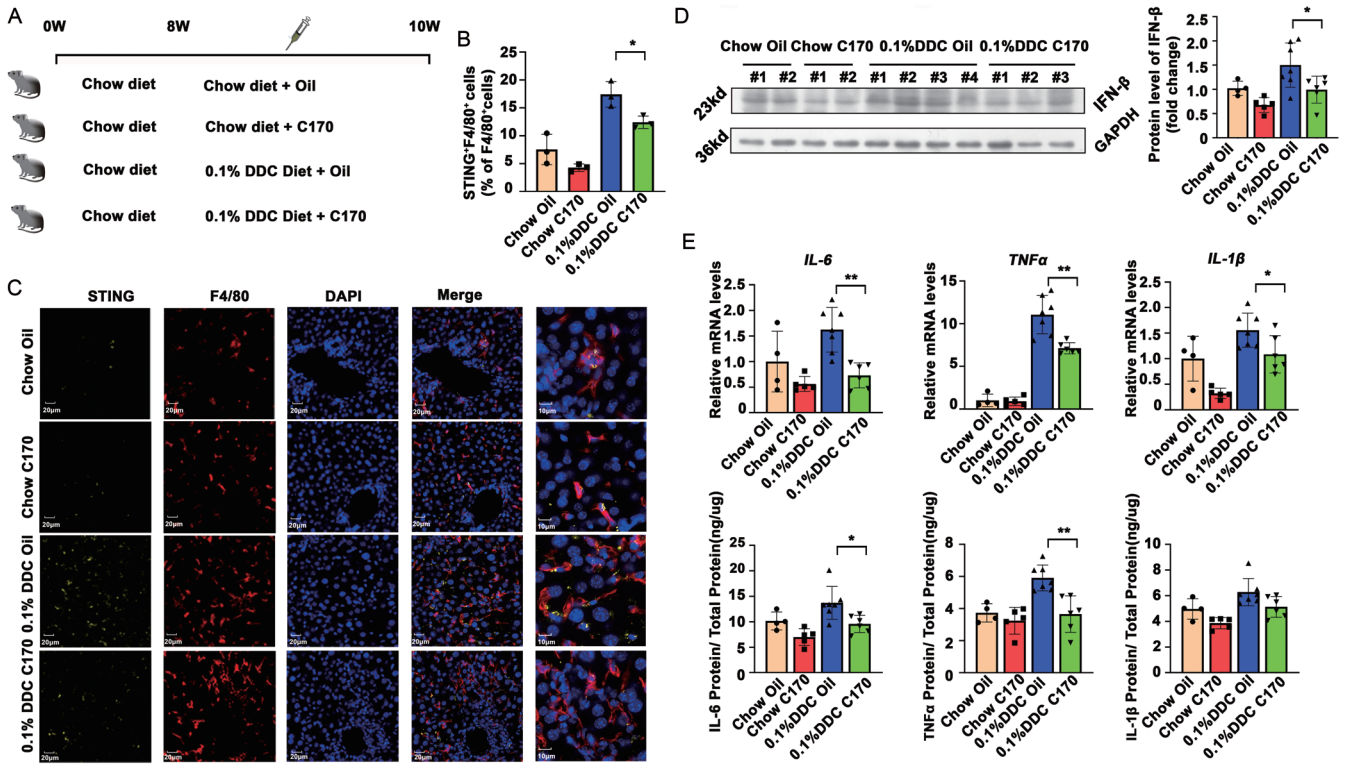

Fig. 1. STING signaling pathway is activated in 0.1% DDC cholestasis mice. (A) Schematic illustration of the generation of C57/6J mice model of cholestasis. The experimental procedure for investigating the protective role of C170 in mice administered a 0.1% DDC diet at week 8 for 14 days. Intraperitoneal injections of corn oil or 10M C170 were administered daily to the treatment group mice (Chow Oil n = 4; Chow C170 n = 5; DDC Oil n = 7; DDC C170 n = 6). (B, C) Representative images (original magnification: 20×) and quantitative analysis of immunofluorescence staining for STING (yellow) and F4/80 (red). (scale bar: 50 μm). (D) Western blot of IFN-β in mouse livers. Expression levels were normalized to those of GAPDH. (E) Quantitative analysis of qPCR (top panel) and ELISA (lower panel) data for TNF-α, IL-6, and IL-1β. Data presented as mean ± standard deviation (SD). *$p < 0.05$, **$p < 0.01$. STING, stimulator of interferon genes; DAPI, 4′,6-Diamidino-2′-phenylindole; IFN-β, interferon-beta; GAPDH, glyceraldehyde -3-phosphate dehydrogenase; DDC, 3,5-diethoxycarbonyl-1,4-dihydroxychollidine; C170, a STING inhibitor; IFN-β, interferon-beta; IL-6, interleukin-6; TNF-α, tumor necrosis factor-alpha; IL-1β, interleukin-1 beta.

Shiga, Japan) and reverse transcribed into cDNA for RT-qPCR analysis, using TaqMan probes (Life Technologies, Carlsbad, CA, USA).[24] The primers used are listed in Supplementary Table 2.

Western blot analysis

Total protein was extracted using radioimmunoprecipitation assay buffer (Sigma Aldrich, MO, United States) containing phosphatase and protease inhibitors (Roche, Basel, Switzerland), and cytoplasmic extracts were isolated using NE-PER nuclear and cytoplasmic extraction reagents (ThermoFisher, Waltham, MA, USA). Protein concentrations were measured with a BCA protein assay kit (ThermoFisher, Waltham, MA, USA). Cell lysates or protein solutions were mixed with an equal volume of 2× loading buffer and boiled at 100°C for 5m. The proteins were separated using SDS-PAGE and subsequently transferred to a PVDF membrane. The PVDF membrane was blocked in 5% (w/v) semi-skimmed milk in TBST. The primary antibodies and their respective dilutions are listed in Supplementary Table 3. After washing three times, protein bands on the membrane were detected with the ECL Western blotting substrate (Affinity Bioscience, Jiangsu, China).[25]

Immunofluorescence staining cells and tissues

Human cell lines: HepG2 and PLC/PRF/5 cells were plated in 6-well tissue culture plates (CORNING, NY, USA) coated with collagen. After treatment with IL-6, cells were fixed at room temperature in 4% paraformaldehyde for 30 m.

Human and mice tissues: Immunofluorescence was performed as described elsewhere.[26] The specimens were fixed in 4% paraformaldehyde, paraffin-embedded, and sectioned.

Statistical analysis

Data analyses were performed using GraphPad Prism (version 6.01; GraphPad Prism Software, CA, USA). All variables were expressed as mean ± standard deviation (SD) and analyzed using one-way ANOVA and independent-samples Student's *t*-test. Two-tailed *p*-values < 0.05 were considered indicative of statistical significance.

Results

The STING signaling pathway is activated in 0.1% DDC cholestasis mice

We used a 0.1% DDC cholestasis mouse model to demonstrate activation of the STING signaling pathway under cholestasis *in vivo*. C170 (10M/kg/day), an inhibitor of STING, was injected intraperitoneally to suppress the activation (Fig. 1A). Mice in the STING inhibitor group exhibited less hepatic inflammation, fibrosis, and ductal responses than those in the normal administration group (Supplementary Fig. 1). Immunofluorescence staining demonstrated higher expres-

sion of STING in the liver tissue of cholestatic mice compared to controls, which was inhibited by C170 (Fig. 1B, C). We also analyzed downstream I-IFNs and pro-inflammatory cytokines of the STING pathway, including IFN-β, IL-6, TNF-α, and IL-1β, using Western blot, RT-qPCR, and ELISA assays. The expression of IFN-β in the 0.1% DDC diet group was higher than that in the control group, while C170 injection led to the downregulation of IFN-β (Fig. 1D). Similar findings were observed regarding IL-6, TNF-α, and IL-1β levels (Fig. 1E and Supplementary Fig. 2). These data indicate activation of the STING signaling pathway after the 0.1% DDC diet, with higher expression levels of STING downstream I-IFNs and pro-inflammatory cytokines compared to the control group.

Patients with cholestasis had increased expression of STING

Next, we evaluated the expression level of STING in liver tissues of cholestatic patients (n = 14) and control subjects without cholestasis (n = 14). Immunohistochemistry staining showed higher STING expression levels in patients with cholestasis compared to controls. Specifically, STING expression levels were 3.153-fold higher ($p < 0.001$) in the cholestatic group (n = 5) than in the control group (n = 6) (Fig. 2A). A high level of STING expression was observed in the portal region, where immunological cells such as macrophages cluster. To further elucidate the activation of STING in liver macrophages, we assessed the co-localization of STING expression and CD68 (macrophage-specific surface antigen) using immunofluorescence assays (Fig. 2B, C). Consistent with the immunohistochemical results, STING expression aggregated in the hepatic portal area and co-localized with CD68 in cholestatic patients. Additionally, we performed RT-qPCR analysis to examine the expression of STING-induced pro-inflammatory cytokines. The mRNA levels of *TNF-α*, *IL-6,* and *IL-1β* were significantly higher in liver tissues of cholestatic patients (n = 14) than in control patients (n = 14) (3.0-fold, $p < 0.01$; 4.6-fold, $p < 0.05$; and 3.0-fold, $p < 0.05$, respectively) (Fig. 2D). These data indicated that mRNA expressions of STING-induced downstream pro-inflammatory cytokines were significantly elevated in cholestatic patients compared to controls. This explained the increased expression of STING and STING-induced cytokines.

Patients with cholestasis had decreased hepatic OATP1B1 expression

We have previously shown increased levels of serum TBA, serum TBIL, and serum DBIL in 0.1% DDC mice (Supplementary Fig. 3) and in cholestatic patients (Supplementary Table 1) compared with chow oil mice and control patients. OATP1B1 is a specific bilirubin transporter that can also serve as a secondary bile acid transporter.[13] Therefore, OATP1B1 may regulate bilirubin and bile acid levels. We hypothesized whether OATP1B1 expression is diminished in patients with cholestasis, leading to the accumulation of bilirubin and bile acids. To address this, we performed RT-qPCR and Western blot analyses to detect OATP1B1 expression levels in liver tissue samples from obstructive cholestatic patients and controls. The results showed significantly decreased mRNA and protein expressions of OATP1B1 in hepatic tissue of obstructive cholestatic patients relative to controls. Specifically, compared to control patients, the hepatic tissues of cholestatic patients showed significantly lower mRNA levels (0.46-fold, $p < 0.01$) of *OATP1B1* (Fig. 3A) and OATP1B1 protein levels (0.41-fold, $p < 0.001$) (Fig. 3B, C). Furthermore, we performed immunofluorescence to detect the localization of OATP1B1 expression (Fig. 3D, E) and found that OATP1B1 was mainly localized to hepatocyte basolateral membranes and was down-regulated, consistent with the results of RT-qPCR and Western blot assay.

IL-6 induced the down-regulation of OATP1B1

Numerous studies have reported on the regulation of liver transporters by a wide spectrum of I-IFNs and pro-inflammatory cytokines in liver disease models. However, the underlying regulatory mechanisms are not well-characterized.[27–30] Our previous experiments demonstrated the activation of STING and STING-induced I-IFN (IFN-β) and cytokines (TNF-α, IL-1β, and IL-6) in patients and mice with cholestasis. To further elucidate the degradation mechanism of OATP1B1, we applied TNF-α, IL-1β, and IL-6 treatments to measure the mRNA expression of OATP1B1 in PLC/PRF/5 hepatoma cells. The results showed that TNF-α, IL-1β, and IL-6 down-regulated OATP1B1 (Fig. 4A–C). Furthermore, to screen the strongest regulators of OATP1B1, we compared the inhibitory effects of the aforementioned I-IFNs and inflammatory factors on OATP1B1 by calculating the suppression ratio of these factors on OATP1B1. The results demonstrated that IL-6 had the greatest inhibitory effect on OATP1B1. As shown in Figure 4, in PLC/PRF/5 hepatoma cells treated with IL-6 (25 ng/mL), the mRNA level of OATP1B1 was 0.4-fold compared to that in the control group (Fig. 4C). This indicates that IL-6 has a greater inhibitory effect compared to TNF-α and IL-1β. To further validate this conclusion, we performed Western blot analysis under the same conditions of IL-6 treatment on PLC/PRF/5 cells (Fig. 4D). To exclude the influence of IFN-β, a major secretory factor produced by STING stimulation, PLC/PRF/5 cells were treated with IFN-β at the same gradient concentration. Western blot analysis showed that compared to IL-6, IFN-β had almost no effect on OATP1B1 expression (Fig. 4E, F). These data indicate that IL-6 is the most significant factor regulating OATP1B1 in PLC/PRF/5 cell lines. These findings suggest that IL-6 plays a crucial role in down-regulating OATP1B1 in patients with cholestatic disease in the context of inflammation.

Over-expressed STING enhanced IL-6 induced inhibition of OATP1B1

Previously, we demonstrated the localization of STING expression in hepatic macrophages and the upregulation of STING-induced pro-inflammatory cytokine expression in 0.1% DDC mice and patients with cholestasis. Additionally, IL-6 was found to induce down-regulation of the transporter OATP1B1 expression. To further validate that STING-stimulated IL-6 mediates OATP1B1 inhibition, we performed co-culture of THP1 macrophages with PLC/PRF/5 hepatoma cells with STING agonist treatment (Fig. 5A). Western blot analysis showed a significant increase in protein levels of P-STING in THP1 cells and, conversely, a significant decrease in OATP1B1 levels in PLC/PRF/5 cells after treatment with the STING agonist SR-717 (Fig. 5B, E–G). ELISA assay demonstrated significantly increased expression levels of IL-6 and IFN-β after SR-717 treatment (Fig. 5C, D). Moreover, co-culture of STING-overexpressed THP1 cells with HepRG cells showed increased protein levels of IL-6, and the levels of OATP1B1 expression were significantly restored after IL-6 inhibition treatment (Supplementary Fig. 4A–E). Knockdown of NF-κB alleviated the regulation of IL-6 (Supplementary Fig. 4F, G). These data suggest that STING overexpression enhances IL-6 expression, leading to the inhibition of OATP1B1.

IL-6 inhibited OATP1B1 expression by β-catenin/TCF4

Our previous experiments showed that STING-mediated IL-6

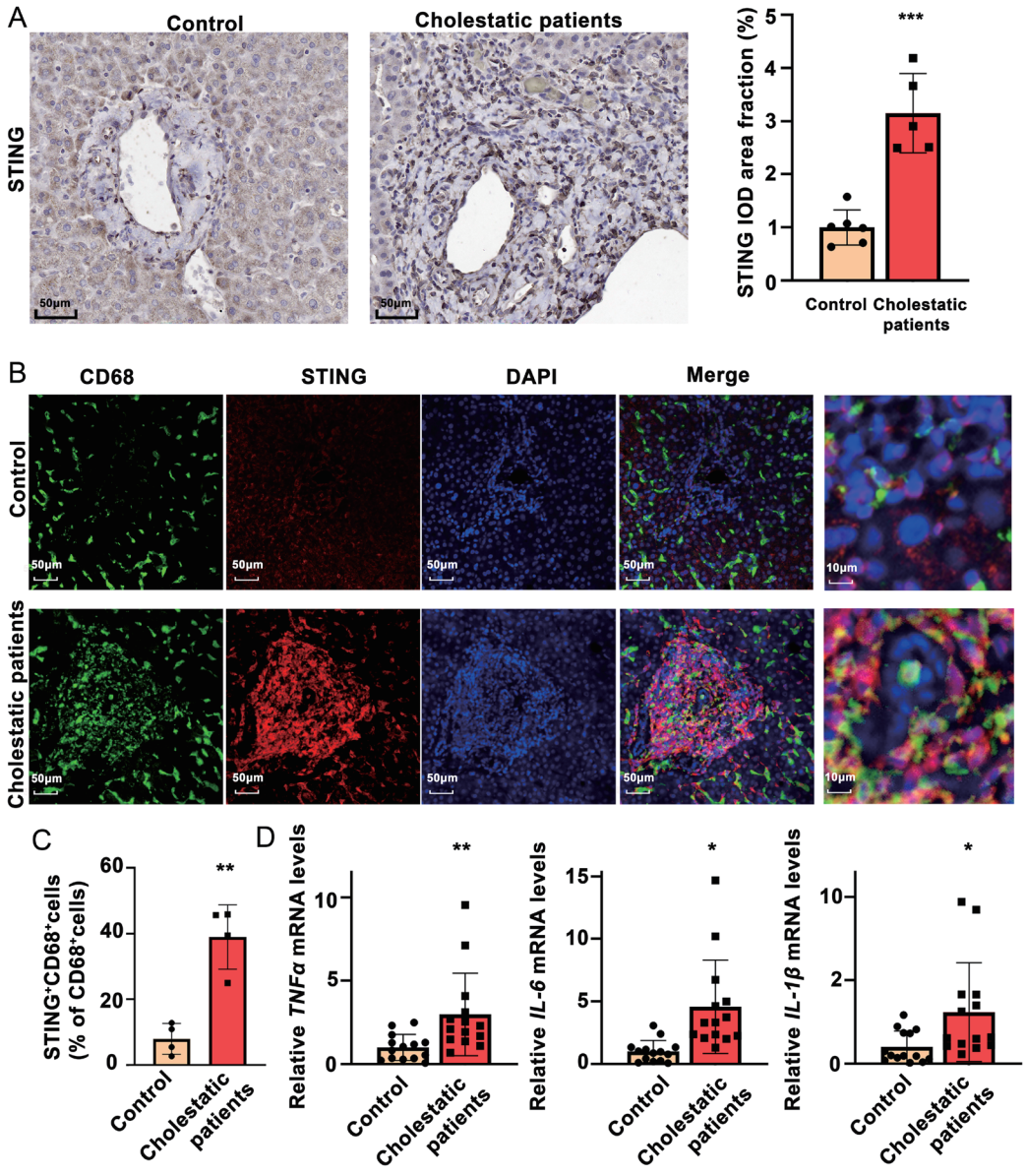

Fig. 2. Patients with cholestasis had increased expression of STING. (A) Representative images (original magnification: 40×) and quantitative analysis using Image J (cholestatic patients, n = 5; control group, n = 6) of immunohistochemical staining for STING (scale bar: 50 μm). (B, C) Representative images (original magnification: 20×) and quantitative analysis of multiplex immunofluorescence (IF) with antibodies against CD68 (green), STING (red), and DAPI (blue) in cholestatic patients and the control group (n = 4 per group) (scale bar: 50 μm). (D) qPCR evaluation of TNF-α, IL-6, and IL-1β mRNA levels in cholestatic patients (n = 14) and the control group (n = 14). The target mRNA levels were normalized to that of 18S in each group. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$. STING, stimulator of interferon genes; DAPI, 4′,6-Diamidino-2′-phenylindole; IL-6, interleukin-6; TNF-α, tumor necrosis factor-alpha; IL-1β, interleukin-1 beta.

increased OATP1B1 mRNA and protein levels in hepatoma PLC/PRF/5 cell lines. However, the mechanism underlying IL-6-stimulated OATP1B1 expression in cholestasis is not clear. To address this, potential regulatory molecules for OATP1B1 were explored by querying transcription factor databases (*JASPAR*, PROMO, Cistrome DB). TCF4/*TCF7L2* was identified as the transcription factor of OATP1B1 in these databases (Fig. 6A). To further investigate the potential interactions of IL-6 and TCF4/*TCF7L2*, we visualized the IL-6 and TCF4/*TCF7L2* interaction network using the *STRING* database. The interaction diagram revealed a strong association of IL-6 with β-catenin, suggesting that IL-6 may regulate TCF4 through β-catenin (Fig. 6B). To validate the expression of β-catenin and TCF4 in patients with cholestasis, we performed Western blot analysis on liver tissue samples from control and obstructive cholestasis patients. The protein levels of TCF4 and β-catenin significantly decreased in obstructive cholestatic patients compared to control patients (Fig. 6C, D). Additionally, we treated PLC/PRF/5 and HepG2 cells with different concentrations of IL-6 (0 ng/mL, 5 ng/mL, 10 ng/mL, and 25 ng/mL) and performed Western blot assays to detect the protein expression of β-catenin/TCF4. The results revealed that recombinant IL-6 caused a dose-dependent inhibition of β-catenin expression in PLC/PRF/5 and HepG2 cells (Fig. 6E, F). Based on these findings, we hypothesized that reduced expression of β-catenin and TCF4

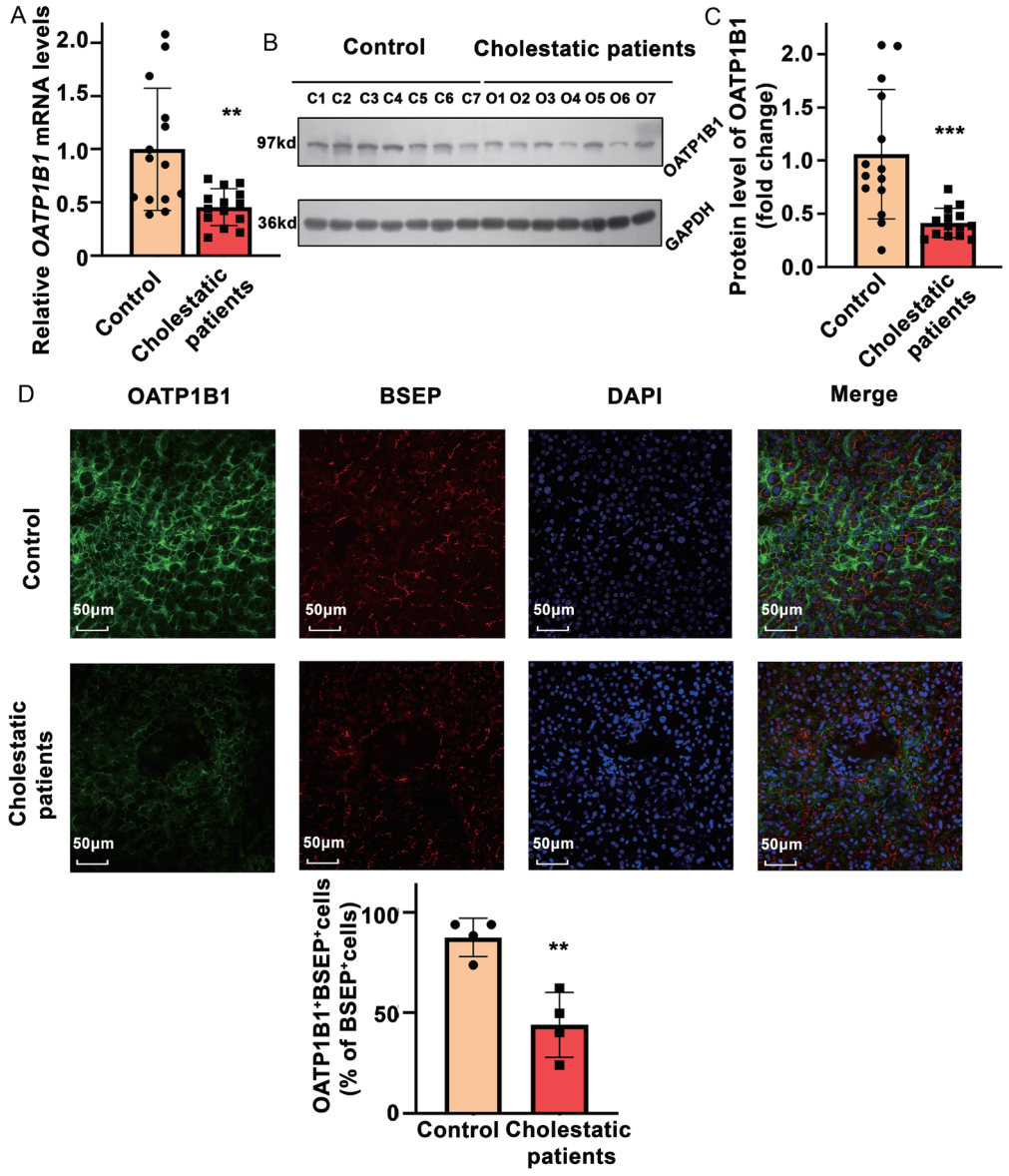

Fig. 3. Patients with cholestasis had diminished hepatic expression of OATP1B1. (A) Quantitative analysis of qPCR data for *OATP1B1* expression in cholestatic patients (n = 14) and control group (n = 14). The target mRNA level was normalized to that of *18S* in each group. (B) Western blot analysis of OATP1B1 levels in cholestatic patients (n = 14) and the control group (n = 14). (C) Band intensity in Western blotting was quantitatively measured using ImageJ software and normalized to that of the control group. (D) Representative images (original magnification: 20×) and quantitative analysis of immunofluorescence staining for OATP1B1 (green) and BSEP (red) (scale bar: 50 μm). Data presented as mean ± standard deviation (SD). $^{**}p < 0.01$, and $^{***}p < 0.001$. OATP1B1, organic anion transporting polypeptide 1B1; BSEP, bile salt export pump; DAPI, 4′,6-Diamidino-2′-phenylindole.

would decrease their nuclear translocation, ultimately leading to downregulation of OATP1B1 expression, which in turn induces cholestatic disease.

IL-6 attenuated OATP1B1 expression in PLC/PRF/5 with nuclear deletion of β-catenin

In previous studies, β-catenin and TCF4 protein levels were decreased after recombinant IL-6 treatment. To explore the regulatory mechanism of β-catenin on the transcription factor TCF4, we performed an immunofluorescence assay to detect the location of β-catenin expression that regulates TCF4. Compared to the control, IL-6 decreased the fluorescence intensity of β-catenin, which was localized in the nuclei of HepG2 and PLC/PRF/5 cells (Fig. 7A–C). Next, we investigated nuclear proteins extracted using nuclear and cytoplasmic extraction reagents. Western blot assays were performed to verify the potential effect of IL-6. Compared with the control, IL-6 reduced the protein levels of β-catenin and TCF4 in the nucleus in both HepG2 (Fig. 7D) and PLC/PRF/5 (Fig. 7E) cells. However, the use of the β-catenin agonist LiCl reversed the IL-6-induced down-regulation of TCF4 and OATP1B1 in the nuclei of HepG2 and PLC/PRF/5 cells. This provided further evidence that IL-6 inhibited OATP1B1 by suppressing nuclear β-catenin expression (Fig. 7F). To further validate the role of β-catenin and TCF4 in IL-6-induced OATP1B1 down-regulation, we performed β-catenin-siRNA and TCF4-

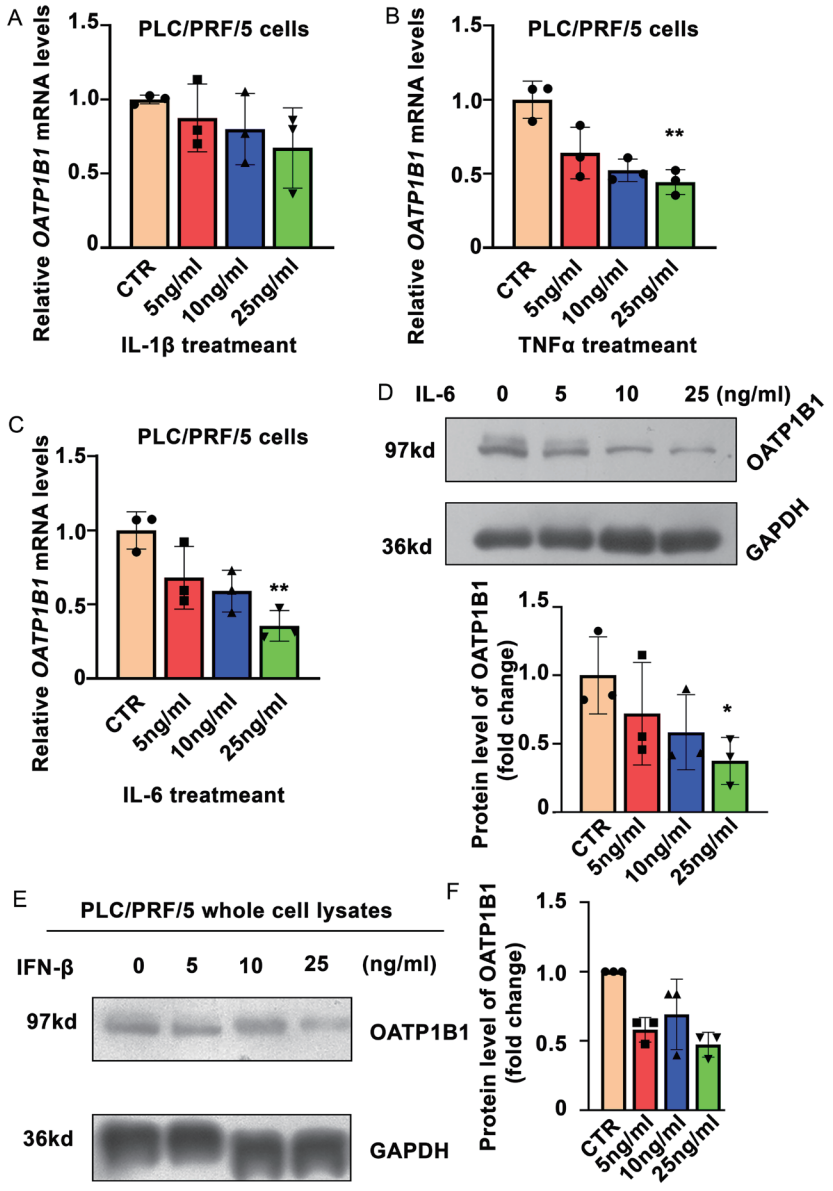

Fig. 4. IL-6 induced the down-regulation of OATP1B1. (A) RT–qPCR analysis showing increased mRNA expression of OATP1B1 in PLC/PRF/5 cells with IL-1β treatment (25 ng/mL). (B) RT–qPCR analysis shows a similar facilitating effect of TNF-α and (C) IL-6. (D) Western blot analysis of the relative levels of OATP1B1 protein in IL-6-treated PLC/PRF/5 cells. Expression levels were normalized to those of GAPDH. (E) Western blot analysis of the relative levels of OATP1B1 protein expression in IFN-β-treated PLC/PRF/5 cells. (F) Band intensity in Western blotting was quantitatively measured using ImageJ software, and the intensity was normalized to that of the control group. Data presented as mean ± standard deviation (SD). *$p < 0.05$, **$p < 0.01$. OATP1B1, organic anion transporting polypeptide 1B1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CTR, control; IL-6, interleukin-6; TNF-α, tumor necrosis factor-alpha; IL-1β, interleukin-1 beta; IFN-β, interferon-beta.

shRNA transfections to silence the expression of β-catenin and TCF4. After knocking down β-catenin and TCF4, the expression of OATP1B1 was attenuated compared to the control (Fig. 7F, G). These findings suggest that IL-6 decreases the expression of OATP1B1 by inhibiting the nuclear β-catenin/TCF4 signaling pathway.

Discussion

Patients with cholestasis typically have elevated bile acids and bilirubin.[31,32] The sodium taurocholate co-transporting polypeptide (NTCP), as well as OATPs, work in concert to remove bile acids from plasma.[33,34] OATPs, which are present only in the human liver, are specific transporters of direct bilirubin.[35] OATPs and NTCP are the major uptake transporters for bile salts into hepatocytes. Many early trials have shown that pro-inflammatory cytokines can inhibit the expression of transporters OATPs and NTCP.[27] For instance, Le Vee *et al*. reported that TNF-α and IL-6 altered the expression of OATP1B1.[36] Such pronounced changes in hepatic transporter expression were likely attributable to inflammation in cholestasis. Inflammatory factors can regulate NTCP expression by affecting the protein level of RXR:RAR.[37] However, the mechanism by which inflammatory cytokines diminish the

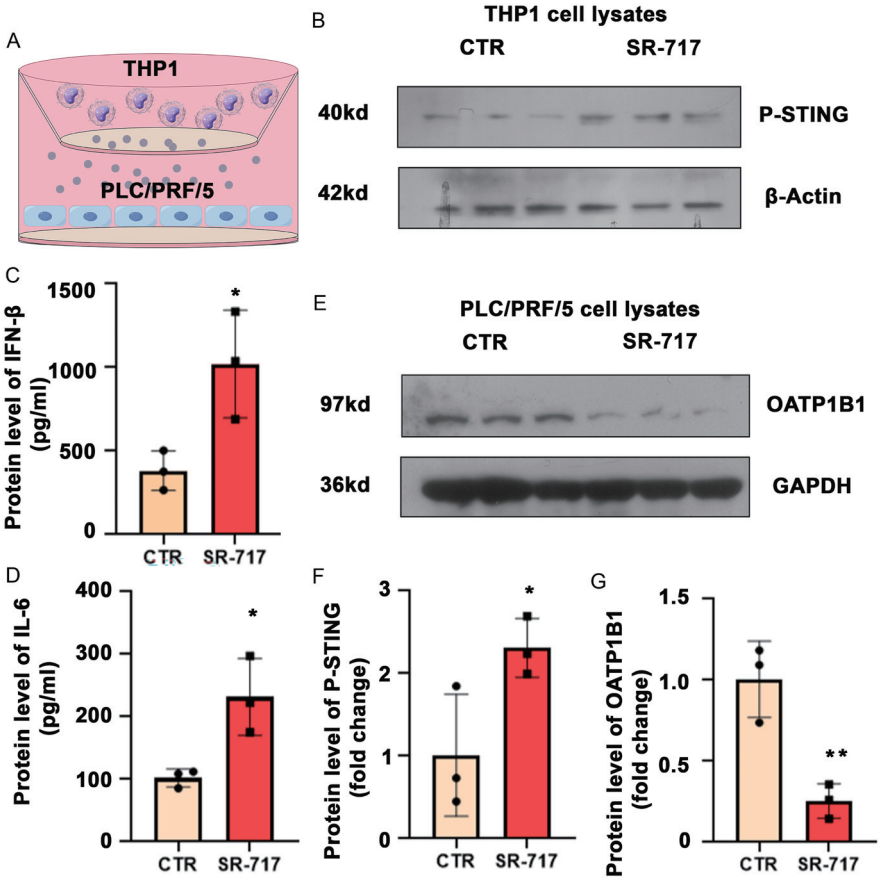

Fig. 5. Over-expressed STING enhanced IL-6 induced inhibition of OATP1B1. (A) THP1 and PLC/PRF/5 cells were co-cultured with and without STING agonist treatment. (B) Western blotting analysis of P-STING levels in THP1 cells. Expression levels are normalized to those of β-Actin. (C) IFN-β levels and (D) IL-6 levels measured by ELISA after five-fold dilution of the lower cell supernatants; (E) Western blotting analysis of OATP1B1 levels in PLC/PRF/5 cells. Expression levels are normalized to those of GAPDH. (F) Gray scale values of western blotting bands of THP1 cells of both groups were calculated and counted. (G) Gray scale values of Western blotting bands of PLC/PRF/5 cells in both groups. *$p < 0.05$, **$p < 0.01$. SR-717, a STING agonist; STING, stimulator of interferon genes; P-STING, phospho-STING; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CTR, control; IFN-β, interferon-beta; IL-6, interleukin-6.

expression of OATP1B1 is unclear. In our previous study, we demonstrated the regulation of OATP1B3 by IL-6.[16] IL-6 also has a regulatory effect on OATP1B1, but the exact mechanism is not yet clear. In this study, we performed a range of *in vivo*, *in vitro*, and clinical assays to further investigate the source of IL-6 in cholestasis and its mechanism of action in regulating OATP1B1. Our findings suggest that the activation of the cGAS/STING signaling pathway induces the production of IL-6 by macrophages, which subsequently triggers the downregulation of OATP1B1 by the β-catenin/TCF4 signaling pathway in hepatocellular carcinoma cells and patients with cholestasis. Therefore, inhibition of the STING pathway and regimens based on IL-6 therapy may be effective in improving clinical symptoms of cholestasis.

The role of pro-inflammatory cytokines in liver disease-related inflammatory responses is well documented.[38,39] In studies by Wang and Liu *et al*., STING signaling pathway activation in macrophages was shown to induce liver fibrosis by increasing IL-1β in mice with liver injury.[9,40] In addition, Le Vee *et al*. found that the cytokine oncostatin M (OSM), a member of the interleukin-6 family, concomitantly reduced OATP1B1 protein expression and OATP transport activities. Furthermore, OSM-mediated repression of OATP1B1 was counteracted by knocking down the expression of OSM receptors through siRNA transfection.[41] This study is the first to demonstrate the activation of STING signaling in 0.1% DCC mice and patients with cholestasis. Additionally, we examined the effects of several STING-stimulated pro-inflammatory cytokines in cholestasis on hepatocyte expression and found that most pro-inflammatory factors inhibited the expression of OATPs. *In vivo* experiments are deficient because OATP1B1 can only be expressed in human hepatocytes. To compensate for this deficiency, we conducted numerous cellular experiments and screened all inflammatory factors that have the potential to affect OATP1B1. We found a more significant inhibitory effect of IL-6 on OATP1B1 compared to IL-1β and TNF-α. However, to date, the mechanism by which pro-inflammatory cytokines, particularly IL-6, regulate OATP1B1 remains unclear.

To clarify the underlying mechanism, we assayed the expression of OATP1B1 in HepG2 and PLC/PRF/5 human hepatoma carcinoma cells after treatment with inflammatory factors. The results showed that IL-6, TNF-α, and IL-1β significantly inhibited hepatic OATP1B1 mRNA levels, with IL-6 exhibiting the strongest inhibitory effect. These results are consistent with those reported by Le Vee M and Hiroyuki Ono *et al*.[26,36] Akihisa Ueno *et al*. found a strong association between the expression of OATP1B1 and β-catenin target genes in hepatocellular carcinoma.[42] Our findings provide additional insights into the molecular mechanisms by which IL-6 down-

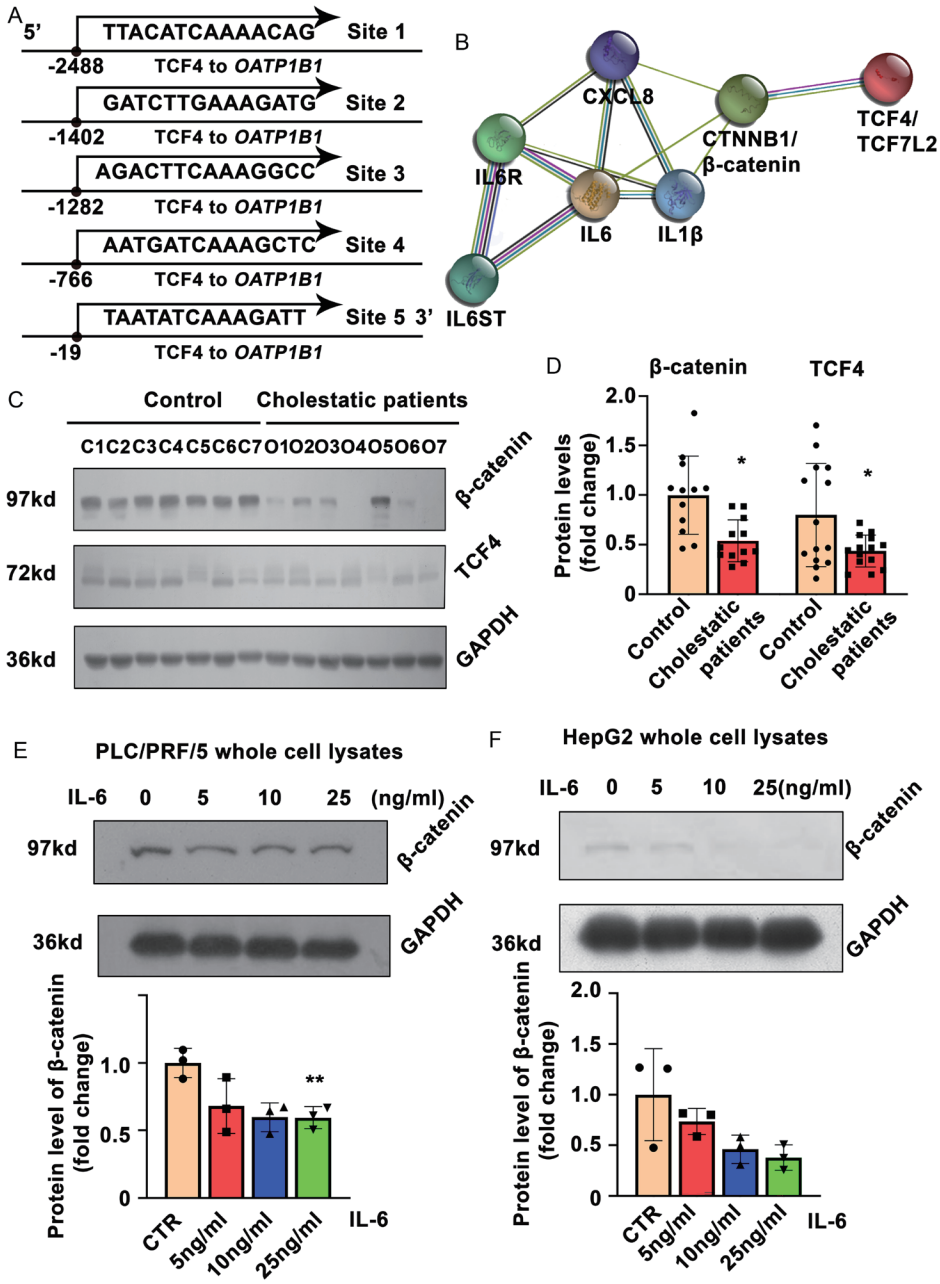

Fig. 6. IL-6 inhibited OATP1B1 expression via β-catenin/TCF4. (A) The OATP1B1 promoter site that can bind to TCF4 was predicted using the *JASPAR* database. (B) Prediction of the interaction of IL-6 affecting β-catenin and TCF4 in the *STRING* database; (C, D) Western blot images for β-catenin and TCF4 and their densitometric values normalized to the control group; (E) Western blot analysis of the relative levels of β-catenin and TCF4 protein expression in PLC/PRF/5 whole cell lysates. Expression levels are normalized to those of GAPDH. (F) Western blot analysis of the relative protein levels of β-catenin and TCF4 in HepG2 whole cell lysates. Data presented as mean ± standard deviation (SD). *$p < 0.05$, **$p < 0.01$. IL6ST, interleukin 6 signal transducer; CXCL8, Interleukin-8; IL-1β: interleukin-1 beta; IL-6R: Interleukin-6 receptor; TCF4, T-cell factor 4; GAPDH: glyceraldehyde -3-phosphate dehydrogenase; CTR, control.

regulates the expression of OATP1B1. Specifically, IL-6 inhibited β-catenin/TCF4 expression in PLC/PRF/5 and HepG2 cells in a dose-dependent manner. Moreover, the downregulation of β-catenin may be influenced by ubiquitination. We further found that LiCl, an agonist of β-catenin, reverses the IL-6-induced down-regulation of OATP1B1. Therefore, we speculate that IL-6 inhibits hepatic OATP1B1 expression by depressing the β-catenin/TCF4 signaling pathway. Such pronounced changes in hepatic transporter expression are likely to contribute to both cholestasis and altered pharmacokinetics caused by inflammation in humans. Currently, the mechanism of TCF4 involvement in the liver is unclear. Our results show that IL-6 increases the degradation of TCF4 by suppressing β-catenin expression. This reduces the nuclear localization of β-catenin/TCF4, thereby inhibiting the transport function of OATP1B1 for bile acids and bilirubin. This is a novel explanation of the regulatory mechanism of β-catenin/TCF4 under cholestasis.

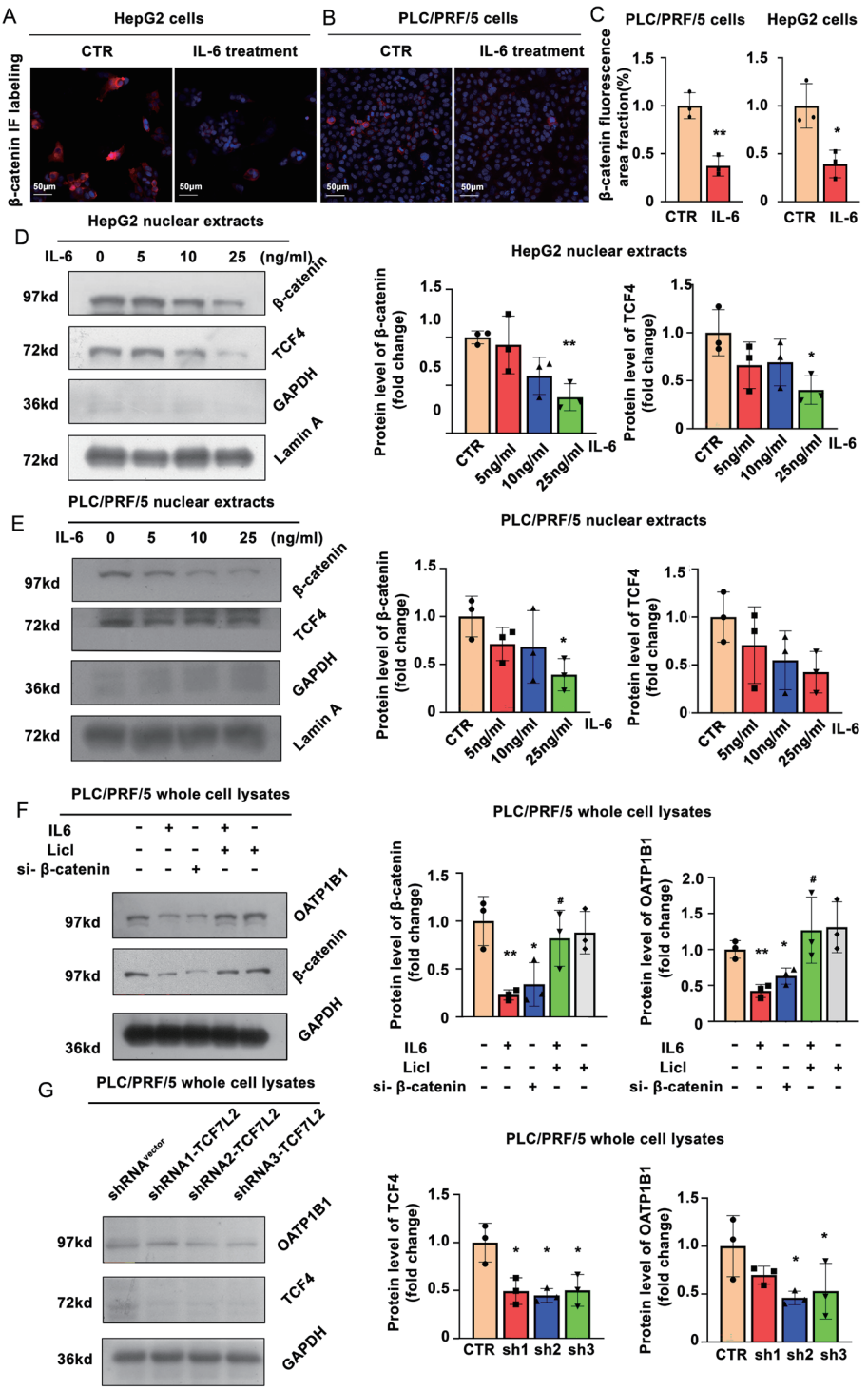

Fig. 7. IL-6 attenuated OATP1B1 expression in PLC/PRF/5 and HepG2 with nuclear deletion of β-catenin. (A-C) Representative images of immunofluorescence staining of β-catenin in HepG2 cells (A) and PLC/PRF/5 cells (B). After treatment with IL-6 (25ng/mL), the β-catenin level is reduced in the nucleus (scale bar: 50 μm). (D) Western blot images for β-catenin and TCF4 nuclear protein expression in HepG2. Expression levels are normalized to those of Lamin A. (E) Western blot images for β-catenin and TCF4 nuclear protein expression in PLC/PRF/5. Expression levels are normalized to those of Lamin A. (F) OATP1B1 expression is down-regulated after treatment with IL-6 (25ng/mL) and si-β-catenin transfection. LiCl, an agonist of β-catenin, reversed the inhibitory effect of IL-6 on OATP1B1. Western blot analysis of the relative protein levels of β-catenin and OATP1B1 in PLC/PRF/5 whole cell lysates. (G) Western blot data for TCF4 and OATP1B1 levels in PLC/PRF/5 cells transduced with three shTCF7L2 (protein also known as TCF4) shRNAs. Western blot analysis of the relative levels of TCF4 and OATP1B1 protein in PLC/PRF/5 whole cell lysates. *$p < 0.05$, **$p < 0.01$. * represents $p < 0.05$ as compared to control group, and # represents $p < 0.05$ as compared to IL-6 group. + means that the substance is added, - means that the substance is not added. IL-6, interleukin-6; OATP1B1, organic anion transporting polypeptide 1B1; TCF4, T-cell factor 4; GAPDH, glyceraldehyde -3-phosphate dehydrogenase; CTR, control.

Conclusions

This study provides a new understanding of the mechanism by which STING-mediated IL-6 inhibits OATP1B1 expression to alter bile acid transporters. IL-6 was shown to reduce OATP1B1 expression by down-regulating β-catenin/TCF4 signaling. Furthermore, this is the first study to demonstrate the activation of STING in patients with cholestasis.

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Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Experiment design (JC, CET, XZ), experiment performance and data analysis (YG), contributing critical reagents, materials, and analysis tools (NZ, DS, HZ, YP), and writing and revising the manuscript (JC, CET, XZ, YC, YG). All authors reviewed and approved the manuscript for publication.

Ethical statement

This study was conducted in accordance with the principles enshrined in the World Medical Association's Declaration of Helsinki (2013). The study protocol was approved by the Ethics Committee of the First Affiliated Hospital of Third Military Medical University, Chongqing, China (approval number: (B) KY2021157). Written informed consent for participation in the study and the publication of images was obtained from all patients. All animal experiments were approved by the Animal Care and Use Committee at the Medical Research Center (Southwest Hospital, Chongqing, China; approval number: AMUWEC20223955). All animals received human care.

Data sharing statement

Data supporting the findings of this study are available from the corresponding authors (JC and CET) upon reasonable request.

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