

RESEARCH ARTICLE

Interleukin-33 drives hepatic fibrosis through activation of hepatic stellate cells

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Liver fibrosis is a consequence of chronic liver disease, causing morbidity and mortality. Interleukin-33 (IL-33) is a critical mediator of inflammation, which may be involved in the development of liver fibrosis. Here, we investigated the role of IL-33 in human patients and experimental bile-duct ligation (BDL)-induced fibrosis in mice. We report increased hepatic IL-33 expression in the murine BDL model of fibrosis and in surgical samples obtained from patients with liver fibrosis. Liver injury, inflammatory cell infiltration and fibrosis were reduced in the absence of the IL-33/ST2 receptor, and the activation of hepatic stellate cells (HSCs) was decreased in ST2-deficient mice. Recombinant IL-33 activated HSCs isolated from C57BL/6 mice, leading to the expression of IL-6, TGF- β , α -SMA and collagen, which was abrogated in the absence of ST2 or by pharmacological inhibition of MAPK signaling. Finally, administration of recombinant IL-33 significantly increased hepatic inflammation in sham-operated BL6 mice but did not enhance BDL-induced hepatic inflammation and fibrosis. In conclusion, BDL-induced liver inflammation and fibrosis are dependent on ST2 signaling in HSCs, and therefore, the IL-33/ST2 pathway may be a potential therapeutic target in human patients with chronic hepatitis and liver fibrosis.

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INTRODUCTION

Hepatic fibrosis results from chronic hepatocyte damage, leading to remodeling and formation of pseudoacinar-structured repair with proliferation and deposition of fibers and extracellular matrix (ECM), known as cirrhosis.¹ Cirrhosis is a common consequence of chronic liver diseases associated with hepatocellular dysfunction and portal vein hypertension, leading to liver failure.² Hepatocellular injury leads to inflammation, with the recruitment and activation of neutrophils, innate lymphocytes, T lymphocytes and hepatic stellate cells (HSCs).³ Activated HSCs produce inflammatory chemokines, express cell adhesion molecules, activate lymphocytes and secrete ECM, contributing to liver fibrosis.^{4–6} Importantly,

activation of a Th2 response is known to causes fibrosis of the liver and other organs.^{7–9} Activation of innate lymphoid cells (ILCs), a subset of lymphoid cells lacking T- and B-antigen receptors, is critical for an immediate innate response and, in the context of helminthic infection and liver injury, produced type 2 cytokines and IL-13, which are crucial for the fibrotic response.^{10,11}

Interleukin-33 (IL-33), an IL-1-related cytokine, has emerged as an important cytokine for inducing Th2 cytokine production. IL-33 is released on cell death, and increased IL-33 can be detected in severe inflammation *in vivo*.^{3,9} IL-33 binding to the IL-33/ST2 receptor produces pro-inflammatory cytokines and Th2 cytokines.¹² McHedlidze *et al.*¹¹ demonstrated

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increased IL-33 production on liver injury, activating ILC2s with IL-13 production signaling via the IL-4 receptor and transcription factor STAT6 activation, as a critical downstream pathway of IL-33-dependent pathologic tissue remodeling and fibrosis.

Here, we investigated the role of IL-33/ST2 signaling in an experimental model of hepatic inflammation and fibrosis using the bile-duct ligation (BDL) model and report that IL-33/ST2 signaling via HSCs causes hepatic fibrosis. Therefore, targeting IL-33/ST2 might be considered therapeutically to attenuate ongoing hepatic inflammation and fibrosis in human patients.^{13,14}

MATERIALS AND METHODS

Patients

A total of 24 patients who underwent partial hepatectomy due to early-stage hepatocellular carcinoma with liver cirrhosis at The First Affiliated Hospital of Nanjing Medical University from January 2012 to December 2013 were involved in the study. Normal liver tissues were obtained as controls from 20 Chinese patients with benign disease, such as hemangioma. The clinical characteristics are summarized in Table 1. Informed consent for sample analysis was obtained from all patients before surgery, and the study was approved by the institutional ethics committee of The First Affiliated Hospital of Nanjing Medical University and performed according to the Declaration of Helsinki.

Animals

ST2-deficient mice backcrossed to C57BL/6 J for 10 generations¹⁵ were bred in the Transgenose Institute animal facility (UMR 7355 CNRS, Orleans, France). Wild-type (WT) C57BL/6 J mice were used as controls, bred in Orleans or Nanjing Medical University. Six- to ten-week-old male mice were maintained in sterile, isolated, ventilated cages with controlled temperature (21–23 °C) and light (12-h light/12-h dark cycle) conditions and access to food and water *ad libitum*. All experimental procedures were performed according to the local Animal Care Committee following guidelines that comply with those of the French and Chinese government, and the experimental protocol was approved by the local ethics committee.

Table 1 Clinical characteristics

Characteristics	Fibrotic liver	Control liver
Number	24	20
Sex (F/M)	17-Jul	11-Sep
Age (year)	55.2 (38–61)	46.6 (36–51)
HBV Infected	19/24	20-Apr
ALT level (IU/l)	142.4 (41–336)	31.1 (23–68)
AST level (IU/l)	112.7 (54–411)	28.6 (11–54)
Total bilirubin level	18.5 (11–45)	11.7 (8–21)
Serum albumin (g/l)	30 (22–36)	34 (31–39)
Primary mass size (cm)	5.1 (3.5–10.5) (tumor)	7.9 (5–11) (hemangioma)

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; HBV, hepatitis B virus. Data are presented as mean (minimum–maximum).

Animal model of BDL

Mice were anesthetized with pentobarbital sodium (1 vol %). BDL was performed after midline laparotomy. The common bile duct was ligated with 5–0 silk and transected between two ligations. Sham operation was performed similarly, except for ligation and transection of the bile duct. All surgical procedures were performed under aseptic conditions. Animals were allowed to recover from anesthesia and surgery under a warming lamp and were held in single cages until subsequent experiments, performed at postoperative days 1, 3, 10 and 21 ($n = 8$ animals per time point). Sham-operated animals without BDL served as controls ($n = 5$). In preliminary trials, mice were given an intraperitoneal injection of recombinant mouse IL-33 (1, 5 or 10 µg per day, R&D Systems, Abingdon, UK) 3 days before BDL, and 5 µg was finally used in the experiments.

Cytokine analysis

The homogenized tissue was centrifuged at 10,000 r.p.m. for 15 min, and the supernatant was recovered and stored at –80 °C until further analyses. The levels of cytokines were measured by ELISA using commercial kits (R&D Systems), according to the manufacturer's instructions.

Assay for serum transaminase activity

Serum samples from mice were obtained at different times. Serum alanine aminotransferase (ALT) activity was determined using a serum transaminase test kit (Rong Sheng, Shanghai, China), based on methods recommended by the International Federation of Clinical Chemistry.

Liver homogenization and myeloperoxidase activity

A myeloperoxidase (MPO) activity assay was used as an indirect index of the neutrophil presence in lung and intestinal tissues.¹⁶ Briefly, 200 mg of tissue in 1 ml of phosphate-buffered solution (PBS) was homogenized and resuspended in PBS (1 ml) containing 0.5% hexadecyltrimethyl ammonium bromide and 5 mM ethylenediaminetetraacetic acid. Following centrifugation, aliquots of 50 µl of supernatants were placed in test tubes containing 2 ml of Hanks' balanced salt solution, 100 µl of *o*-dianisidine dihydrochloride (1.25 mg/ml), and 100 µl of H₂O₂ 0.05%. After 15 min of incubation at 37 °C with shaking, the reaction was stopped by adding 100 µl of NaN₃ 1%. Absorbance was determined at 460 nm using a microplate reader.

Microscopic investigation

Liver tissue was fixed in 4% buffered formaldehyde, embedded in paraffin, cut at 3 µm and stained with hematoxylin and eosin (H&E). Liver H&E sections were graded for BDL-induced liver injury, inflammation and fibrosis under a Leica DM4000 B Upright Research Microscope (×200, ×400; Stuttgart, Germany) using the scoring system proposed by Suzuki *et al.*¹⁶ In this classification, three liver injury indices—sinusoidal congestion (score: 0–4), hepatocyte necrosis (score: 0–4) and ballooning degeneration (score: 0–4)—were graded for a total score of 0–12.

Table 2 Primers used

Gene	Forward	Reverse
<i>Il-1β</i>	GCAACTGTTCCTGAACTCAACT	ATCTTTTGGGGTCCGTCAACT
<i>Il-1α</i>	TCTATGATGCAAGCTATGGCTCA	CGGCTCTCCTTGAAGGTGA
<i>Lcn2</i>	TGGCCCTGAGTGCATGTG	CTCTTGTAGCTCATAGATGGTGC
<i>Gm-csf</i>	TGCACCCCTGACTGGAGTTAC	TGAAATCTCGATGTGTCCACAG
<i>Tslp</i>	ACGGATGGGGCTAACTTACAA	AGTCCTCGATTGCTCGAACT
<i>Il-33</i>	ATTTCCCGGCAAAGTTCAG	AACGGAGTCTCATGCAGTAGA
<i>Collagen 1a1</i>	GCTCCTCTTAGGGGCCACT	ATTGGGGACCCCTTAGGCCAT
<i>Il-6</i>	TAGTCCTTCCTACCCCAATTTCC	TTGGTCCTTAGCCACTCCTTC
<i>α-sma</i>	GTCCCAGACATCAGGGAGTAA	TCGGAATCTCAGCGTCAGGA
<i>Tgf-β</i>	CTCCCGTGGCTTCTAGTGC	GCCTTAGTTGGACAGGATCTG
<i>Gapdh</i>	AGGTCGGGTGAACGGATTG	TGTAGACCATGTAGTTGAGGTCA

HSC isolation and culture

Primary mouse HSCs were isolated from livers of C57BL/6 mice according to a modified method previously described.¹⁷ The isolated cells were plated on uncoated plastic at a density of 5×10^6 per 10-cm-diameter plate. After the first 24 h, non-adherent cells and debris were removed by washing. Cell viability was greater than 90%, as assessed by trypan blue exclusion. Purity was 90–95%, as assessed by a typical light microscopic. Then, cells were trypsinized and plated on a 6-well plate at 2×10^5 per well. After 24 h, cells were considered to be quiescent by the light microscopic appearance of the lipid droplets. Recombinant murine IL-33 in DMEM containing penicillin, streptomycin and 10% FBS, at 0, 1, 10, 50 or 100 ng/ml concentrations, was added. (Preprotech #210-33, Rocky Hill, NJ, USA). The restimulation was performed on the first passage of HSCs to ensure the highest viability and plating efficiency. In some experiments, the ERK/MEK1 inhibitor PD98059 (20 μ M, Cell Signaling, Beverly, MA, USA, #9900), p38 inhibitor SB203580 (10 μ M, Cell Signaling, #5633) and JNK inhibitor SP600125 (25 μ M, Cell Signaling, #8177) were prepared in DMSO as the mother liquor, and then, 2 μ l of the mother liquor was added to 2 ml of DMEM containing penicillin, streptomycin and 10% FBS, and this was used for cell culture for 1 h. Cells were then restimulated with 10 or 100 ng/ml rmIL-33 for another 24 h.

Collagen detection assay

Supernatants of liver homogenate samples were collected from control and BDL groups 21 days after surgery on the mice or 1 day after rmIL-33 injection; supernatants were obtained from cells restimulated with 0, 1, 10, 50 or 100 ng/ml rmIL-33 for 24 h (1×10^5 cells/ml in a 6-well plate). The collagen concentration was measured using a Sircol assay, according to the manufacturer's recommended procedure (Biocolor S1000, Carrickfergus, UK).

Real-time polymerase chain reaction

After rIL-33 stimulation, total RNA was extracted from isolated HSCs. Reverse transcription reactions were performed using a SuperScript First-Strand Synthesis System (Invitrogen, Foster City, CA, USA). RNA templates were treated with

DNase to avoid genomic DNA contamination. To quantify cDNA in the reverse transcribed samples, real-time polymerase chain reaction (PCR) analysis was performed using a 7300 Detection System (Applied Biosystems, Foster City, CA, USA). Real-time PCR was performed according to the manufacturer's instructions with a SYBR Premix Ex Taq kit (Takara, Japan). The primers to detect various genes are described in Table 2. The data were normalized with glyceraldehyde 3-phosphate dehydrogenase, levels in the samples.

Western blot analysis

Proteins were extracted from mouse tissues and cells and quantitated using a protein assay (Bio-Rad Laboratories, Berkeley, CA, USA). Protein samples (30 μ g) were fractionated by SDS-PAGE and transferred to a nitrocellulose membrane. Immunoblotting was conducted using antibodies against murine IL-33 (ab54385, Abcam, Cambridge, UK), anti α -smooth muscle actin (ab5694, Abcam, HK), anti ERK1 (sc-93), anti p-ERK1/2 (sc-16982R, Thr-202/Tyr-204), anti p38 (sc-728), anti p-p38 (sc-7975R, Tyr-182), JNK (sc-571) and anti p-JNK1 (sc-6254) (Santa Cruz Biotechnology, Dallas, TX, USA). The results were visualized using a chemiluminescent detection system (Pierce ECL Substrate Western blot detection system, Thermo Scientific, Chicago, IL, USA) and exposure to autoradiography film (Kodak XAR film, Xiamen, China). Levels of JNK, ERK or p38 phosphorylation were quantified with ImageJ software (version 1.44p).

Isolation of liver-infiltrating mononuclear cells and flow cytometry analysis

Liver-infiltrating mononuclear cells were isolated from mice 24 h after the BDL challenge. Liver-infiltrating mononuclear cells were isolated and plated at 1×10^5 /ml, followed by restimulation for 6 h *in vitro* with phorbol 12-myristate 13-acetate (PMA) (50 ng/ml) and ionomycin (750 ng/ml) in complete medium (IMDM supplemented with 5% (vol/vol) FCS, L-glutamine (2 μ M), penicillin (100 U/ml), streptomycin (100 μ g/ml) and β -mercaptoethanol (50 nM); all from Invitrogen). The antibodies used for fluorescence activated cell sorting analysis were Pacific blue-anti-CD4, PE-anti-IL-4,

APC-anti-IFN- γ , PE-Cy7-anti-Gr-1, FITC-anti-CD11b, APC-cy7-anti-F4/80, APC- Mouse Lineage Antibody cocktail, FITC-anti-Sca-1 (Ly-6A/E), PE-anti-anti-c-kit (CD117) and isotype-matched controls were purchased from BD Pharmingen (San Diego, CA, USA). The cells were counted and stained for extracellular and intracellular markers and analyzed by FlowJo 7.6.4 software. (Tree Star, Ashland, OR, USA).

Statistical analysis

The results are expressed as the mean \pm standard deviation (s.d.). Comparisons between the two groups were performed using unpaired Student's *t*-test. All statistical analyses were

performed using GraphPad Prism 6.01 (San Diego, CA, USA), and two-tailed tests were applied to all data unless otherwise specified; a *P*-value of <0.05 (95% CI) was considered indicative of a statistically significant result. In some experiments, the data are expressed as the median and range (0, 25, 50, 75 and 100%).

RESULTS

Increased IL-33 expression in experimental BDL-induced fibrosis and human cirrhotic tissues

First, we asked whether IL-33 is expressed in BDL-induced liver fibrosis compared with sham-operated controls. We

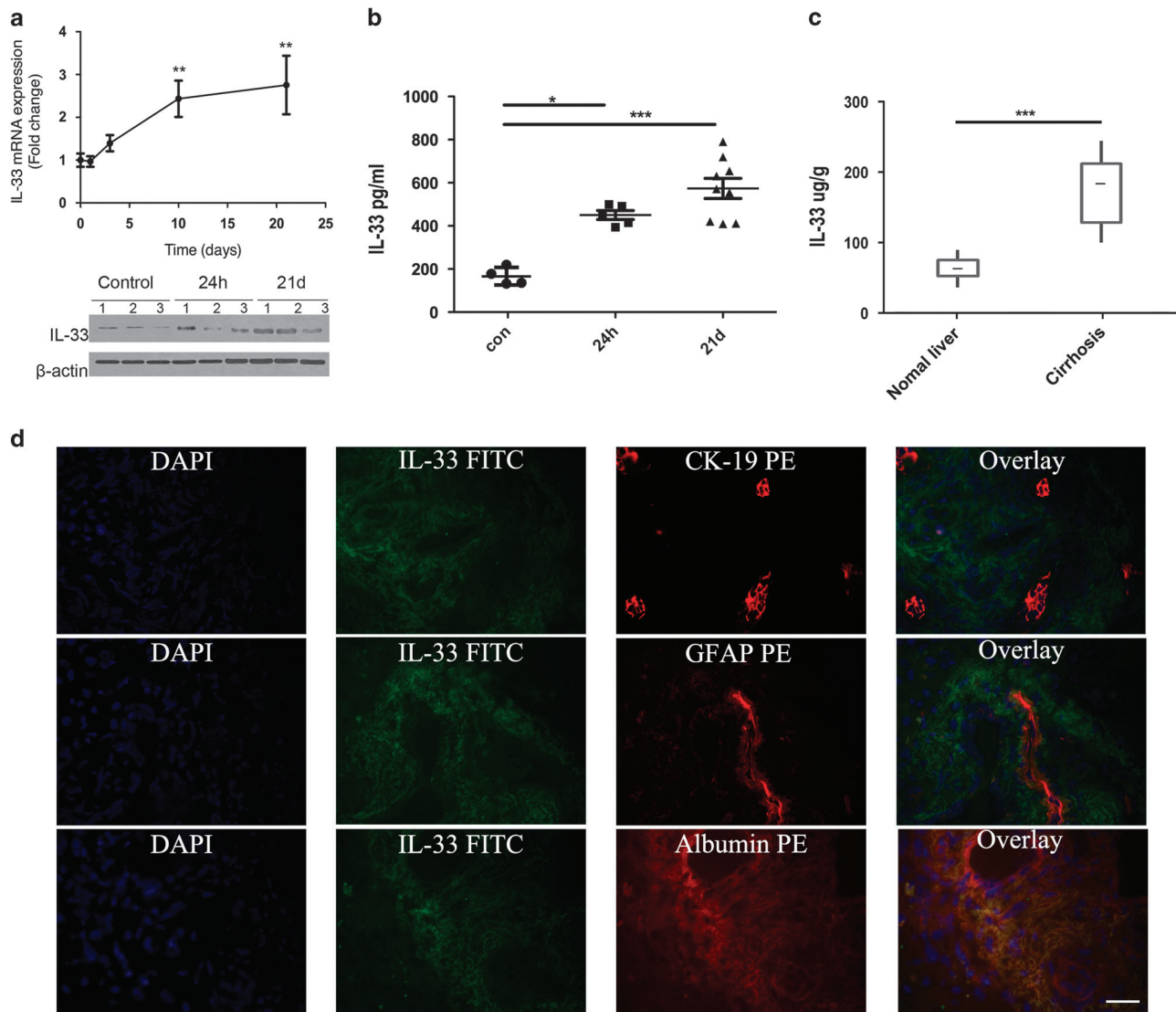


Figure 1 IL-33 expression in acute experimental and clinical liver fibrosis. (a) The hepatic IL-33 expression following bile-duct ligation (BDL) was assessed in C57BL/6 mice at several time points by real-time PCR and western blot, and (b) IL-33 serum levels were determined by ELISA. Mean values \pm s.e.m. are given of two independent studies with groups of eight mice ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$). (c) IL-33 in human liver tissue homogenates (ELISA) after partial hepatectomy of early-stage hepatocellular carcinoma with fibrosis or controls with hepatic hemangioma. The data are expressed as the median and range (0, 25, 50, 75 and 100%) from 24 fibrotic and 20 normal liver tissues ($***P < 0.001$). (d) Cellular expression of IL-33 in fibrotic human livers was identified by immunofluorescence (IF) using FITC-labeled anti huIL-33 antibody, PE-labeled CK-19 (cholangiocytes), PE-labeled GFAP (HSC) or PE-labeled albumin (hepatocytes). Nuclei were counterstained with DAPI (magnification $\times 400$, scale bar, 50 μ m).

detected IL-33 mRNA in the livers, which was significantly increased at day 10 and persisted till day 21 after BDL (Figure 1a). Hepatic IL-33 protein (31 kDa)¹⁸ was determined by western blot at 24 h and 21 days after BDL, representing the acute phase (24 h) and chronic phase (21 days) of BDL-induced liver pathology (Figure 1a). Furthermore, an increase in IL-33 was also detected in serum by ELISA at 24 h and was significantly higher 21 days after BDL (Figure 1b).

IL-33 levels in the liver of 24 patients with cirrhotic liver and 20 patients with normal liver function were significantly increased in cirrhotic livers compared with the normal controls, suggesting a potential role of IL-33 in hepatic fibrosis (Figure 1c).

To track the cellular sources of IL-33, immunofluorescence (IF) was used on frozen human fibrotic liver specimens. We found that PE-labeled, albumin⁺ hepatocytes were the primary cellular sources of IL-33, whereas neither CK-19-positive cholangiocytes nor GFAP-positive HSCs expressed IL-33 (Figure 1d).

BDL-induced liver injury and fibrosis are diminished in the absence of ST2

As IL-33 levels were significantly elevated in BDL-induced liver inflammation and fibrosis, we asked whether IL-33/ST2 signaling has a role in the development of BDL-induced hepatic pathology using ST2-deficient mice. Representative macro- and microscopic examinations of liver injury from day 3 to day 21 after BDL are shown in Figure 2a. Liver damage and hemorrhage were significantly attenuated in ST2-KO mice. Microscopically, hepatic inflammation, inflammatory cell infiltration, hepatocyte degeneration and hepatocellular necrosis are more pronounced in WT mice compared with ST2-deficient mice. At 21 days after BDL, liver fibrosis was detectable by macroscopic examination and Masson-stained slides. The representative results showed that ECM deposition and collagen staining in periportal areas were less prominent in the absence of ST2 compared with the WT controls.

Serum ALT and aspartate aminotransferase (AST) levels were significantly decreased in ST2-deficient mice compared with WT littermate-control mice after BDL (Figure 2b). We also found reduced IL-1 β , CXCL1 (C-X-C Motif Chemokine Ligand 1)/KC and thymic stromal lymphopoietin in livers at the indicated times (Figure 2c). Moreover, the total collagen was significantly lower in ST2-deficient livers, as assessed by Sircol assay. Similarly, collagen type I and α 1 (COL1A1) transcripts were drastically upregulated in WT mice after BDL compared with that in ST2-KO mice (Figure 2d).

Hepatic inflammation is reduced in the absence of ST2 following BDL

To study whether diminished inflammation in ST2-deficient mice correlated with reduced inflammatory cell migration, we isolated infiltrating liver mononuclear cells at 1 day following BDL for analysis by flow cytometry.

First, among CD4-positive cells, we found an increase in IL-4⁺ Th1 and IFN γ ⁺ Th2 cells at 1 day after BDL. However, ST2 deficiency downregulated Th1 and Th2 cell infiltration compared

with WT mice, suggesting an important role of IL-33 in immune regulation. Furthermore, BDL-induced neutrophil and macrophage infiltration was increased after BDL, but significantly abrogated in ST2-deficient mice (Figure 3a). Because ILC2s are able to produce type 2 cytokines and to regulate type 2 immune responses, we next investigated the proportion of ILC2s in BDL-challenged WT mice. We identified ILC2s as the Sca-1⁺ and c-kit⁺ group from the lineage-negative population. We demonstrated that liver-infiltrating ILC2s were significantly increased on BDL, especially at 21 days (1.42, 1.48 and 3.01%) (Figure 3b), indicating an important role of IL-33 related to ILC2s in liver fibrogenesis.

Intraperitoneal injection of IL-33 causes inflammation but does not enhance BDL-induced inflammation in WT mice

BDL-induced hepatic inflammation was diminished in the absence of ST2 signaling, suggesting that endogenous IL-33 has a role in hepatic injury and fibrosis. We then asked whether exogenous IL-33 could augment BDL-induced liver pathology. Therefore, BL6 mice were injected with murine IL-33 intraperitoneally 3 days before BDL surgery (1, 5 and 10 μ g per day), and we found increased liver enzyme release after injection. However, no significant differences were observed between the 5 and 10 μ g group (Figure 4a). Then, we chose 5 μ g as the final irritant concentration. We found upregulated liver inflammation in both naive and operated BDL BL6 mice 3 days after sham or BDL surgery. Thus, preoperative IL-33 alone could induce hepatitis in sham-operated BL6 mice, as expected by the pro-inflammatory effect of IL-33. However, IL-33 injection did not enhance liver pathology in BDL-operated BL6 mice (Figure 4b). Suzuki score and MPO levels, reflecting infiltrating of neutrophils, were elevated in BDL-challenged BL6 mice with or without IL-33 injection (Figure 4c). Similarly, pro-inflammatory cytokines, such as IL-1 β , granulocyte-macrophage colony stimulating factor, were significantly increased in both groups but not significantly different in the BDL-challenged two groups. (Figure 4d). Therefore, consistent with the microscopic data, exogenous IL-33 injection upregulates pro-inflammatory cytokine production but does not enhance BDL-induced liver injury and inflammation.

HSC activation via IL-33/ST2 signaling during liver fibrosis

As HSCs are the primary source of ECM and collagen, we asked whether HSC activation is dependent on IL-33/ST2 signaling. Therefore, HSCs were isolated from the livers of WT and ST2-KO mice, as previously described. First, the WT HSCs were restimulated with different concentrations of recombinant IL-33, resulting in HSC activation with increased IL-6 and TGF- β transcription (Figures 5a and b). Furthermore, α -SMA expression and collagen secretion were increased by IL-33 (Figures 5c and d). However, the IL-33 response was abrogated in ST2-deficient HSCs, even at the highest concentration of IL-33 (100 ng/ml). Therefore, the biological effect of IL-33 was dependent on ST2 signaling in HSCs *in vitro* and *in vivo*, suggesting that HSCs have a critical role in BDL-induced fibrosis.

We investigated the IL-33/ST2 signaling in HSC activation and focused on mitogen-activated protein kinase (MAPK)

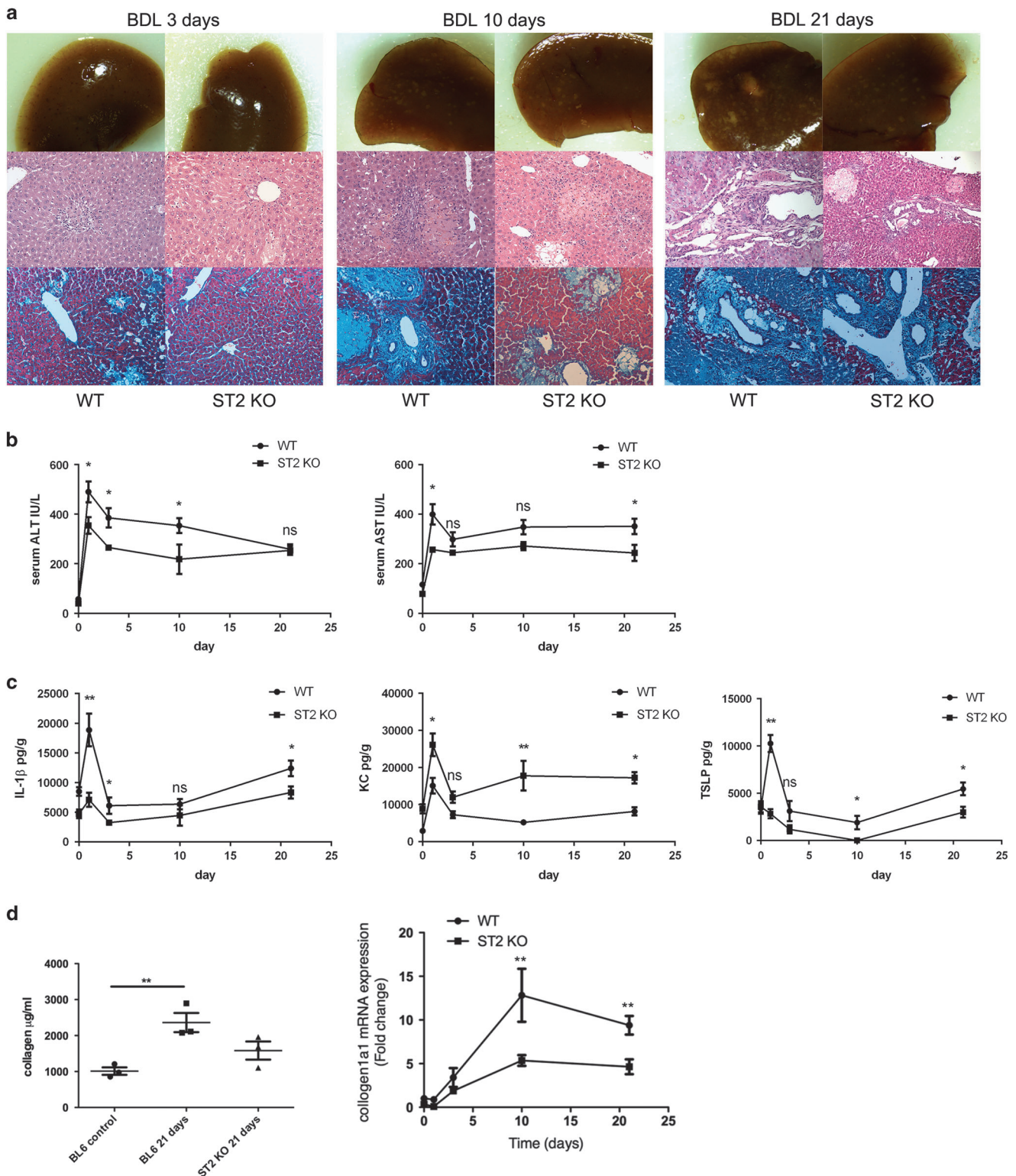


Figure 2 BDL-induced liver injury and fibrosis are reduced in the absence of ST2. Liver injury following bile-duct ligation (BDL) was analyzed in C57BL/6 and ST2-deficient (KO) mice. **(a)** Macro- and microphotographs demonstrate attenuated liver inflammation, necrosis and fibrosis in ST2-KO mice (H&E and Masson staining, original magnification $\times 200$). **(b)** Serum alanine aminotransferase and aspartate aminotransferase at 0, 1, 3, 10 and 21 days after BDL. **(c)** Inflammation was assessed in liver homogenates of C57BL/6 and IL-33/ST2-KO mice at 0, 1, 3, 10 and 21 days after BDL. IL-1 β , KC and thymic stromal lymphopoietin were reduced in ST2-KO mice compared with C57BL/6 mice. **(d)** Hepatic collagen expression was assessed with a Sircol assay and real-time PCR for collagen 1a1. The data are expressed as median values \pm s.e.m. ($n=6-8$ mice/group) and are representative of three independent experiments (* $P<0.05$, ** $P<0.01$, *** $P<0.001$).

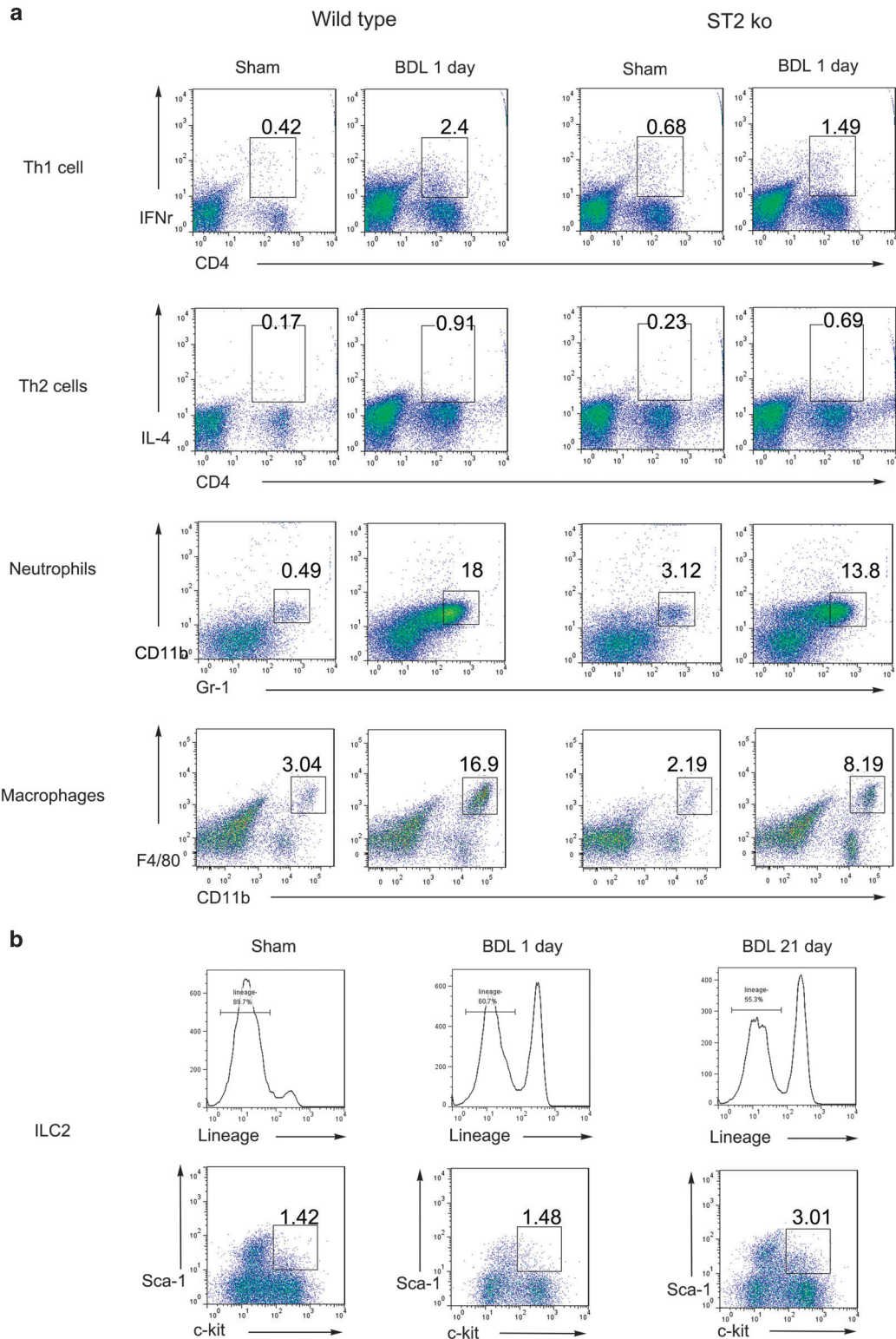


Figure 3 Attenuated inflammatory cell infiltration in ST2-KO mice in bile-duct ligation (BDL)-induced acute inflammation. Infiltrating mononuclear cells were isolated from the livers of WT or ST2-KO mice 1 day after BDL. Cells were stained by specific antibodies and analyzed by fluorescence activated cell sorting. **(a)** Proportion of Th1 (CD4⁺ IFN γ ⁺) cells, Th2 (CD4⁺ IL-4⁺) cells, neutrophils and myeloid cells (Gr-1^{hi} CD11b⁺) and macrophages (CD11b⁺ F4/80) were upregulated after BDL but significantly reduced in ST2-deficient mice. **(b)** ILC2s (group 2 innate lymphoid cells), identified as lineage⁻ Sca-1⁺ and c-kit⁺, were significantly increased 21 days after BDL. The data represent percentages and are from one representative experiment of three independent studies.

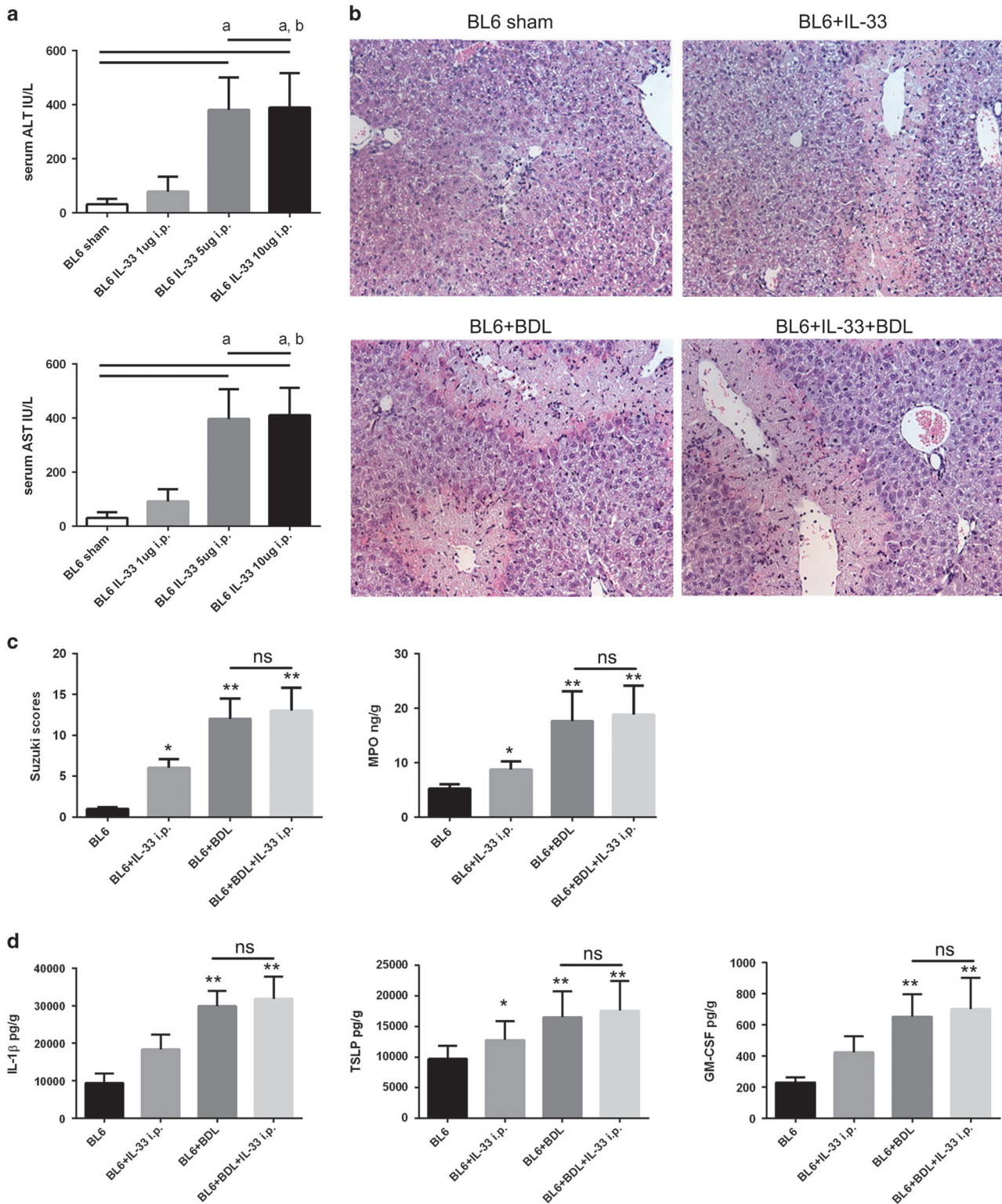


Figure 4 Recombinant IL-33 exacerbates inflammation in C57BL/6 mice. Recombinant mouse IL-33 was injected 3 days before BDL (intraperitoneally at 5 μ g per day) into C57BL/6 mice. **(a)** Serum alanine aminotransferase and aspartate aminotransferase levels from WT BL6 mice without or with rIL-33 (0 μ g, 1 μ g, 5 μ g and 10 μ g) at 1 day. The data are expressed as the mean values \pm s.e.m. ($n=6-8$ mice/group; **(a)** values are significantly different from the BL6 sham group; **(b)** not significantly different from the 5 μ g IL-33 group). **(b and c)** Increased liver injury and inflammation, as assessed by hematoxylin and eosin staining, myeloperoxidase and Suzuki score. **(d)** Hepatic IL-1 β , thymic stromal lymphopoietin and granulocyte-macrophage colony stimulating factor were increased. The data are expressed as the mean values \pm s.e.m. ($n=6-8$ mice/group) and are representative of three independent experiments (* $P<0.05$; ** $P<0.01$; ns, not significant).

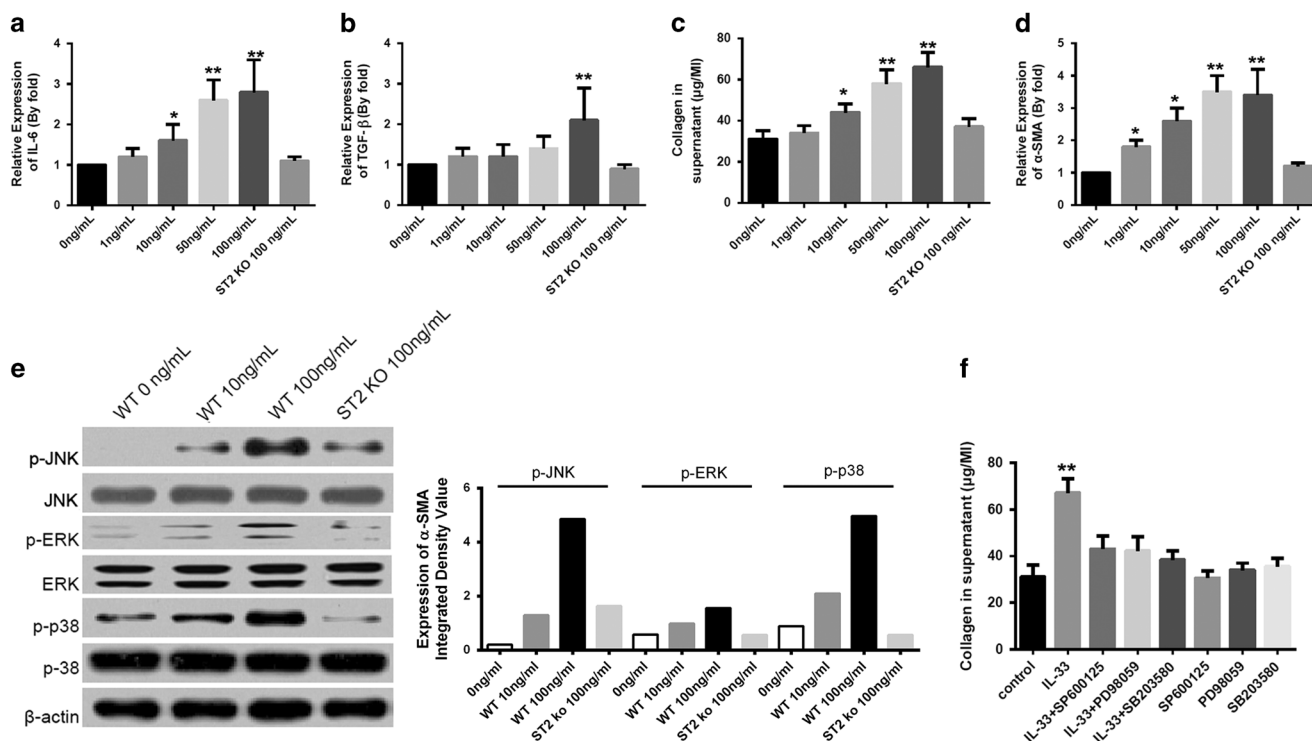


Figure 5 IL-33 activates hepatic stellate cells (HSCs) via mitogen-activated protein kinases signaling. Mouse HSCs were isolated from naive C57BL/6 and ST2-KO mice and activated with rmlL-33 *in vitro* (0, 1, 10, 50 or 100 ng/ml). IL-6, TGF-β, α-SMA RNA transcription and soluble collagen in the supernatant were increased at 24 h (a–d). HSCs expressed increased phosphorylated JNK, ERK or p38 on activation by rmlL-33 at 24 h, which was ST2-dependent. Levels of JNK, ERK or p38 phosphorylation were quantified by ImageJ software (e). The JNK inhibitor SP600125, ERK/MEK1 inhibitor PD98059, or p38 inhibitor SB203580 were given 1 h before rmlL-33 at 100 ng/ml and inhibited collagen production, as measured by Sircol assay (f). Mean values ± s.e.m. of a representative study of three independent experiments are shown; comparisons between subgroups were performed by one-way ANOVA followed by a Newman–Keuls posttest: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (compared with cells cultured in medium).

pathway activation, which is critical in HSC activation and liver fibrogenesis. Therefore, 10 ng/ml and 100 ng/ml IL-33 was used to investigate whether HSC activation is dependent on IL-33/ST2-associated MAPK phosphorylation. By using western blotting and ImageJ software, we demonstrated that JNK, ERK and p38 were dose-dependently activated by rmlL-33 stimulation, whereas the activation of the JNK/ERK/p38 pathway was abrogated in ST2-deficient HSCs, which indicates that MAPK is a vital pathway downstream of IL-33/ST2 signaling in HSCs (Figure 5e). To investigate whether IL-33-induced HSC activation is MAPK-dependent, nontoxic concentrations of the JNK inhibitor SP600125 (25 μM), ERK/MEK1 inhibitor PD98059 (20 μM), or p38 inhibitor SB203580 (10 μM) were used to treat the WT HSCs for 1 h. After 24 h of stimulation with 100 ng/ml IL-33, the secretion of collagen was determined by Sircol assay (Figure 5f). We found that inhibition of either JNK, ERK or p38 dramatically decreased the production of collagens. Therefore, the IL-33/ST2 signaling through the JNK/ERK/p38-MAPK pathway is essential for activation and function of HSCs.

DISCUSSION

Liver fibrosis is the replacement of necrotic hepatocytes with deposition of ECM and collagen. Bile acid accumulation

induced by BDL has been implicated in liver cytoplasmic membrane disintegration and oxidative stress, which might affect liver microenvironment and immune regulation.¹⁹

HSC are pericytes found in the perisinusoidal space of the liver, and they are the principle source of ECM and collagen.^{20,21} Abundant collagen production following liver damage and repair is the hallmark of liver fibrosis and cirrhosis, which may lead to portal hypertension and hepatic failure.²² HSCs are quiescent at steady state, but on injury, HSCs are activated and produce ECM proteins.²¹

Interleukin-33 (IL-33), a newly described member of the IL-1 family, is expressed by many cell types following pro-inflammatory stimulation and is released following cell necrosis.^{23,24} On binding to ST2 and IL-1 receptor accessory protein to form a trimeric complex, IL-33 activates T helper 2 (Th2) cell differentiation.¹⁹ Further investigations revealed that IL-33 is host-protective against helminth infection and reduces atherosclerosis by promoting Th2-related immune responses.²⁵ However, IL-33 also exacerbates antigen-induced arthritis, and blockade of IL-33/ST2 signaling by soluble ST2 significantly attenuated allergic airway inflammation through the reduction of Th2-type cytokines, such as IL-5, IL-13 and IL-4.^{26,27} The dual role of IL-33 in inflammation is not fully understood.

Recent research suggested that IL-33/ST2 signaling and Th2-type cytokines have an important role in fibrogenesis. Li *et al.* found that IL-33 promotes the initiation and progression of pulmonary fibrosis by recruiting inflammatory cells and enhancing pro-fibrogenic cytokine production that is dependent on ST2.^{28,29} Marvie *et al.* reported that IL-33 is strongly associated with fibrosis in chronic liver injury, and activated HSCs are a source of IL-33.³⁰ In our present work, we demonstrate that IL-33 is elevated in cirrhotic livers, and ILC2s are upregulated, along with hepatic fibrogenesis.³¹ Furthermore, ST2-deficient mice reveal that IL-33/ST2 is both required and sufficient for BDL-induced liver inflammation and fibrosis. Intraperitoneal injection of rmIL-33 induces liver inflammation in naive BL6 mice, as reported.³² No significant difference was observed between the 5 or 10 µg groups. Interestingly, rmIL-33 did not augment BDL-induced acute liver injury. The data may be interpreted that injury-induced endogenous IL-33 is sufficient to cause inflammation and fibrosis in the BDL model, which is not further enhanced by rmIL-33.

IL-33 in the normal liver in both mice and humans is expressed by sinusoidal endothelial cells.^{30,33} Here, we demonstrate that hepatocytes are the primary sources of IL-33 in fibrotic liver. We assumed that on hepatocyte damage, released IL-33 might have a direct effect on HSCs, increasing secretion of both cytokines and collagen, as verified *in vitro* with cultured HSCs stimulated with rmIL-33. We found ST2 expression on the membrane of HSCs, which respond to IL-33, as reported for pancreatic stellate cells.³⁴ Furthermore, ST2 signals via the JNK/ERK/p38-MAPK pathway, thereby regulating inflammation within innate lymphoid cells, macrophages and HSCs.^{17,35,36}

Because IL-33 has an important role in immune regulation, we aimed to verify whether the population of liver-infiltrating mononuclear cells is dependent on IL-33/ST2 signaling. We observed decreased Th1 cells, neutrophils and macrophages in ST2-KO mice, which could be due to the reduced hepatic injury and inflammation. Moreover, although previous reports demonstrated that Th2 cell differentiation is partially IL-33-dependent, in this study, we found a slight decrease of Th2 cells in ST2-deficient mice. We assume that the increased liver-infiltrating T helper cells on day 1 on BDL initially migrated from the peripheral blood. Then, as the inflammation persisted, the hepatic microenvironment finally differentiated T helper cells. Our findings also indicated that IL-33-related ILC2s were significantly upregulated during BDL-induced liver pathology, which might confirm our hypothesis. Mchedlidze *et al.*¹¹ found that activation and expansion of liver-resident innate lymphoid cells (ILC2s) is IL-33 dependent, and ILC2-derived IL-13, acting through the type-II IL-4Ra receptor, activated HSCs via the transcription factor STAT6. In this study, we demonstrate that IL-33 activates HSCs directly, through MAPK phosphorylation.

MAPKs are a class of mitogen-activated protein kinases, including p38, ERK and JNK, that are responsive to stress stimuli, such as cytokines, ultraviolet irradiation, heat shock and osmotic shock, and are involved in cell differentiation,

apoptosis and autophagy.³⁷ Iikura *et al.*³⁸ reported that IL-33-mediated IL-8 production by human umbilical cord blood-derived mast cells was markedly reduced by the p38-MAPK inhibitor, SB203580, which might imply that IL-33 can activate p38-MAPK. Sakai *et al.*³⁹ reported that IL-33 is an important endogenous regulator of hepatic ischemia/reperfusion injury through activation of NF-κB, MAPK, cyclin D1 and Bcl-2 expression in hepatocytes, which limits liver injury and reduces the stimulus for inflammation. In the present study, we found that pro-inflammatory cytokines and collagen production were significantly reduced when JNK/ERK/p38-MAPK signaling was blocked by each inhibitor. Thus, IL-33 not only induces an inflammatory response but also activates HSCs directly via a MAPK-dependent pathway.

In conclusion, IL-33/ST2 signaling is associated with liver inflammation and fibrosis, and absence of ST2 prevented liver inflammation both in the acute and chronic phases, with attenuated activation of MEK/ERK/p38-MAPK signaling.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

Qianghui Liu and Zhongming Tan performed the study and wrote the paper; Runqiu Jiang and Long Lv contributed to clinical sample collection and analysis; Siamak S. Shoto, Junwei Tang and Wenjie Zhang contributed to HSC isolation; Isabelle Mailliet conducted FACS analysis; Valerie Quensiaux contributed to the revision of the paper; Bernhard Ryffel and Beicheng Sun designed and supervised the study.

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