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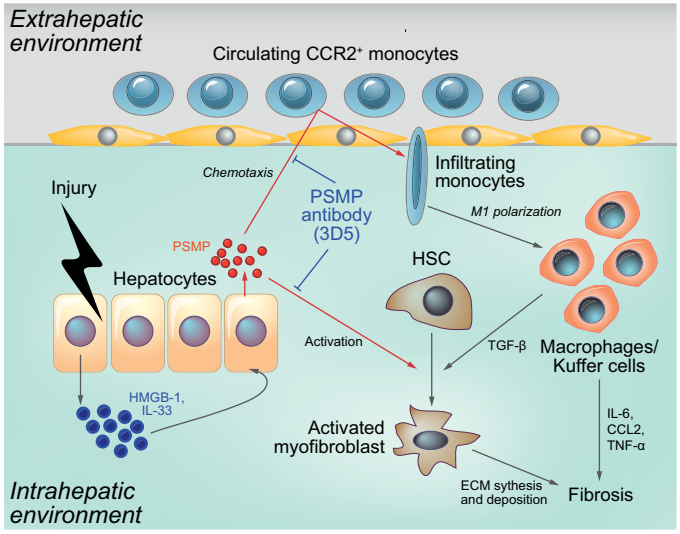
Graphical abstract



Highlights

- PSMP is highly expressed in fibrotic/cirrhotic livers associated with different liver disease etiologies.
- *Psmp* knockout in mice resulted in a marked amelioration of hepatic fibrosis.
- A PSMP-neutralizing antibody significantly alleviates liver fibrosis in mice.
- PSMP promotes liver fibrosis through inflammatory macrophages and HSCs *via* CCR2.
- DAMP molecules, HMGB-1 and IL-33, induce mouse hepatocytes to produce PSMP.

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Lay summary

Our present study identifies the essential role of the protein PSMP for the development and progression of liver fibrosis in humans and mice. PSMP promotes liver fibrosis through inflammatory macrophage infiltration, polarization and production of proinflammatory cytokines, as well as direct activation of hepatic stellate cells via its receptor CCR2. A PSMP antibody can significantly reduce liver fibrosis development *in vivo*. These findings indicate that PSMP is a potential therapeutic target and its antibody is a potential therapeutic agent for the treatment of liver fibrosis.

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PSMP/MSMP promotes hepatic fibrosis through CCR2 and represents a novel therapeutic target

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Background & Aims: C-C motif chemokine receptor 2 (CCR2) has been recognized as a promising target for the treatment of liver fibrosis. PC3-secreted microprotein (PSMP)/micro-seminoprotein (MSMP) is a novel chemotactic cytokine and its receptor is CCR2. In the present study we investigated the expression and role of PSMP in liver fibrosis/cirrhosis.

Methods: PSMP expression was studied in patients with fibrosis/cirrhosis and in 3 murine models of liver fibrosis, including mice treated with carbon tetrachloride (CCl_4), bile-duct ligation, or a 5-diethoxycarbonyl-1,4-dihydrocollidine diet. The role of PSMP was evaluated in *Psmp*$^{-/-}$ mice and after treatment with a PSMP antibody in wild-type mice. The direct effects of PSMP on macrophages and hepatic stellate cells were studied *in vitro*.

Results: In this study, we found that PSMP was highly expressed in fibrotic/cirrhotic tissues from patients with different etiologies of liver disease and in the 3 experimental mouse models of fibrosis. Damage-associated molecular pattern molecules HMGB-1 and IL-33 induced hepatocytes to produce PSMP. PSMP deficiency resulted in a marked amelioration of hepatic injury and fibrosis. In CCl_4-induced hepatic injury, the infiltration of macrophages and CCR2$^+$ monocytes into the liver was significantly decreased in *Psmp*$^{-/-}$ mice. Consistent with the decreased levels of intrahepatic macrophages, proinflammatory cytokines were significantly reduced. Moreover, adeno-associated virus-8 vectors successfully overexpressing human *PSMP* in *Psmp*$^{-/-}$ mouse livers could reverse the attenuation of liver injury and fibrosis induced by CCl_4 in a CCR2-dependent manner. Treatment with a specific PSMP-neutralizing antibody, 3D5, prevented liver injury and fibrosis induced by CCl_4 in mice. At the cellular level, PSMP directly promoted M1 polarization of macrophages and activation of LX-2 cells.

Conclusion: PSMP enhances liver fibrosis through its receptor, CCR2. PSMP is a potentially attractive therapeutic target for the treatment of patients with liver fibrosis.

Lay summary: Our present study identifies the essential role of the protein PSMP for the development and progression of liver fibrosis in humans and mice. PSMP promotes liver fibrosis through inflammatory macrophage infiltration, polarization and production of proinflammatory cytokines, as well as direct activation of hepatic stellate cells via its receptor CCR2. A PSMP antibody can significantly reduce liver fibrosis development *in vivo*. These findings indicate that PSMP is a potential therapeutic target and its antibody is a potential therapeutic agent for the treatment of liver fibrosis.

Keywords: Chemokine; PSMP; CCR2; Liver fibrosis; Liver cirrhosis; Anti-PSMP antibody; Macrophages; Hepatic stellate cells.
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Introduction

Liver fibrosis, a wound-healing response to chronic liver injury, is characterized by excessive deposition of extracellular matrix (ECM) in the liver and is triggered by a variety of causes, including hepatitis virus infection, alcohol abuse, cholestasis, autoimmune, drug/toxin and non-alcoholic steatohepatitis (NASH), which eventually lead to loss of liver function and disruption of the liver structure.[1,2] The initiation of fibrosis crucially depends on an inflammatory phase in which liver resident macrophages, Kupffer cells, are activated and release transforming growth factor-β (TGF-β), as well as other proinflammatory cytokines that activate hepatic stellate cells (HSCs).[3–7] HSCs are responsible for producing most of the ECM

and play a central role in liver fibrogenesis.[8,9] HSCs are quiescent and located in the space between hepatocytes and sinusoidal endothelium (space of Disse) as retinoid storage cells.[10] Upon liver injury, HSCs, the major collagen-synthesizing cells in the liver, are activated and transdifferentiate into myofibroblast-like cells, which show enhanced proliferation, chemotaxis, survival and collagen production.[8,9,11,12] HSC activation is driven by multiple mediators, such as chemokines, reactive oxygen species, growth factors, matrix stiffness, matricellular proteins and damage-associated molecular patterns (DAMPs).[5,8,13] Currently, there are no approved drugs that can effectively reverse liver fibrosis, further highlighting the urgent clinical need for novel antifibrotic therapies.[14]

Extensive *in vitro* and *in vivo* investigations have elucidated the pivotal role played by the chemokine-chemokine receptor system in the pathogenesis of liver diseases.[15,16] Among these mediators, the C-C motif chemokine receptor 2 (CCR2)/C-C motif chemokine ligand 2 (CCL2) axis was shown to have a predominant role in liver inflammation and fibrosis.[17–26] Disruption of CCR2 signaling impedes liver fibrosis, as shown by the altered chemotaxis and transdifferentiation of HSCs.[17–21] In human liver diseases, increased CCL2 is associated with macrophage recruitment and liver fibrosis progression.[22] In addition, CCL2 inhibition attenuated CCl_4-induced liver injury and fibrosis by inhibiting macrophage recruitment.[23–26] Cenicriviroc (CVC) is a novel oral dual CCR2/CCR5 antagonist with nanomolar potency against both receptors.[27] Both preclinical and clinical data have indicated that CVC is a safe and potent antifibrotic agent for the treatment of alcohol-induced steatohepatitis and NASH with fibrosis, and this drug is currently being tested in a phase III trial.[27–29]

PSMP, namely, PC3-secreted microprotein, or microseminoprotein (MSMP), was initially found in PC3 cells, benign and malignant prostate tissues.[30] Our previous study using omics strategies revealed that PSMP is a novel chemotactic cytokine acting as a CCR2 ligand to recruit peripheral blood monocytes and lymphocytes that may influence inflammation and cancer development.[31] The affinity between PSMP and CCR2 was found to be comparable to that between CCL2 and CCR2.[31] PSMP was expressed in human colitis tissues and significantly upregulated in dextran sulfate sodium (DSS)-induced mouse colitis.[32] PSMP plays a vital role in promoting DSS-induced colitis by chemoattraction of $Ly6C^{hi}$ monocytes in a CCR2-dependent manner.[32] Another study found that PSMP expression is induced with prolonged anti-VEGF therapy, specifically under hypoxia, and has an important proangiogenic role in treatment-resistant ovarian tumors.[33]

In this study, we first discovered that PSMP expression was significantly upregulated in patients with fibrosis/cirrhosis compared to that of normal human liver tissues by screening PSMP expression in the tissues of patients with liver disease. However, the physiological and pathological functions of PSMP in the liver have not yet been reported. Our study showed that knocking out *Psmp* or neutralizing PSMP activity attenuated liver injury and fibrosis induced by carbon tetrachloride (CCl_4), bile-duct ligation (BDL), and 5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) diet[34] in mice. We addressed the profibrotic roles of PSMP by combining *Psmp* knockout mice and adeno-associated virus (AAV)-8 vectors expressing the human *PSMP* (h*PSMP*). We also demonstrated that PSMP promoted M1 macrophage polarization and HSC activation *in vitro*. Together, these results are the first to show that PSMP is a potential therapeutic target for liver fibrosis.

Materials and methods

Human samples

A hepatocellular carcinoma (HCC) progression tissue array (HLivH060CD03) was purchased from Shanghai Outdo Biotech Co., Ltd (Shanghai, China). Human liver samples were collected from Beijing Friendship Hospital, Capital Medical University. This study protocol conformed to the ethical guidelines of the Declaration of Helsinki Principles and was approved by the Ethics Committee of Beijing Friendship Hospital, Capital Medical University (2019-P2-088-01).

Murine models

For toxic liver fibrosis, 6- to 8-week-old male wild-type (WT), $Psmp^{-/-}$ and $Ccr2^{-/-}$ mice were given intraperitoneal (i.p.) injections of CCl_4 (1.0 ml/kg body weight, dissolved in corn oil at a ratio of 1:9) (Aladdin, Shanghai, China) or vehicle (corn oil) twice a week for 4 or 6 weeks (n = 5/group). The mice were sacrificed 2 days after the final CCl_4 injection.

For the induction of cholestatic liver fibrosis, 6- to 8-week-old male WT and $Psmp^{-/-}$ mice underwent ligation of the common bile duct (n = 5/group). Sham-operated mice underwent the same procedure but without ligation. BDL mice developed cholestasis and associated fibrosis over a 14-day period.

For the DDC diet, 8-week-old male WT and $Psmp^{-/-}$ mice were fed a diet supplemented with 0.1% DDC (Sigma-Aldrich, St Louis, MO) for 4 weeks (n = 5/group). Control mice received a standard mouse diet.

For the induction of acute liver injury, 6- to 8-week-old male WT and $Psmp^{-/-}$ mice were given a single i.p. injection of CCl_4 (1.0 ml/kg body weight) or acetaminophen (APAP, 300 mg/kg body weight), dissolved in warm PBS (55°C) (Sigma-Aldrich) (n = 5/group). Control mice received a similar volume of vehicle. Mice were sacrificed 24 h after injection.

Antibody treatments

For protective treatment, 6- to 8-week-old male WT mice were injected (i.p.) with 5 mg/kg of 3D5 or mIgG twice a week after each CCl_4 (1.0 ml/kg body weight, twice a week) injection for 4 weeks (n = 5/group). For therapeutic treatment, 6- to 8-week-old male WT mice were injected with (1, 5, and 10) mg/kg of 3D5 or mIgG twice a week after each CCl_4 (1.0 ml/kg body weight, twice a week) injection from the 4th week to the 6th week (n = 5/group).

Mouse AAV8 construction and injection

The AAV8 delivery system that overexpresses the h*PSMP* gene in mouse livers was constructed by Vigene Bioscience (Shangdong, China). The empty associated adenovirus (AAV8-null) served as a control. Titers of the vector genome were measured by quantitative reverse-transcription PCR (qRT-PCR) with vector-specific primers. The 6- to 8-week-old male $Psmp^{-/-}$ and $Psmp^{-/-}Ccr2^{-/-}$ mice were injected with 100 μl of virus containing 2 × 10^11 AAV8 vector genomes via the tail vein for 4 weeks and then induced by CCl_4 (1.0 ml/kg body weight, twice a week) for another 4 weeks (n = 5/group).

For further details regarding the materials and methods used, please refer to supplementary information and the CTAT table.

Results

PSMP expression is upregulated in human and murine liver fibrosis

We used a variety of cancer and paracancerous tissue arrays and found that PSMP showed significantly higher expression in the

liver cancer-adjacent tissues. To determine whether PSMP expression is associated with liver disease, we initially examined PSMP levels in tissue arrays of different liver diseases by immunohistochemistry. PSMP was significantly upregulated in cirrhotic and HCC-adjacent liver tissues, which showed diffuse cytoplasmic staining in hepatocytes (Fig. 1A). Then, we confirmed this finding in human fibrotic/cirrhotic liver tissues from cases with different etiologies of liver disease, including chronic hepatitis B (n = 20), chronic hepatitis C (n = 8), primary biliary cholangitis (n = 13), autoimmune hepatitis (n = 5), drug-induced liver disease (n = 7), alcohol-related liver disease (n = 6) and Buddi-Chiari syndrome (n = 3). Normal control samples were obtained from 7 liver donors to transplantation with normal liver function and no appearance of hepatic steatosis, inflammation or fibrosis in histology. Demographics and clinical characteristics of human samples were presented in Table S1. Immunohistochemical analysis revealed that hepatic PSMP expression was significantly increased in fibrotic and cirrhotic tissues compared to normal human liver tissues, regardless of etiology (Fig. 1B). Similar to the human data, PSMP expression was also markedly increased in mouse models of liver fibrosis induced by CCl_4, BDL or DDC diet compared with the livers from control mice (Fig. 1C, D and Fig. S1). These data indicated that PSMP signaling might be involved in the pathogenesis of liver fibrosis. To determine when and where PSMP is upregulated before fibrosis, we established 2 mouse acute liver injury models by CCl_4 or APAP treatment. We found that PSMP was already upregulated in the early stage of liver injury and predominantly expressed in the nonapoptotic area by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining (Fig. 1C, E and Fig. S1B). Furthermore, the increased PSMP expression in the liver was localized mainly to albumin-expressing hepatocytes but not the α-smooth muscle actin (α-SMA)-expressing HSCs by double immunolabeling, indicating that hepatocytes are major producers of PSMP (Fig. 1E). Accordingly, fractionated mouse primary hepatocytes were found to produce PSMP upon CCl_4, APAP, high-mobility group box 1 (HMGB-1) protein, interleukin (IL)-33 and IL-1β stimulation (Fig. 1F, G, H).

PSMP deficiency protects against liver fibrosis in mice

To further examine the role of PSMP signaling in liver fibrosis, we first generated *Psmp*$^{-/-}$ mice using TALEN technology to delete 7 bases and mutate 1 base in exon 1 of the *Psmp* gene (Fig. S2). PSMP was undetectable in the livers of *Psmp*$^{-/-}$ mice, indicating successful knockout (Fig. S1). We then investigated fibrogenesis in *Psmp*$^{-/-}$ mice used to generate a toxic fibrosis mouse model induced by CCl_4 treatment. Mice were repetitively exposed to CCl_4 for 4 weeks (2 times/week); similar to *Ccr2*$^{-/-}$ mice, *Psmp*$^{-/-}$ mice displayed significantly attenuated liver injury and fibrosis, as assessed by H&E and Sirius red staining, compared with oil controls (Fig. 2A, 2B). Consistently, the upregulation of α-SMA, a marker of HSC activation, and hepatic hydroxyproline content was notably decreased in *Psmp*$^{-/-}$ mice (Fig. 2C, 2D). The serum levels of alanine aminotransferase (ALT) showed a significant decrease in *Psmp*$^{-/-}$ mice, indicating improvement of liver damage (Fig. 2E). Like *Ccr2*$^{-/-}$ mice, the hepatic mRNA expression of prototypical profibrotic genes (*Acta2*, *Col1a1*, *Tgfb1*, *Timp1* and *Pdgfr*) were also reduced in CCl_4-induced *Psmp*$^{-/-}$ mice (Fig. 2F).

To further analyze the role of PSMP in liver fibrosis due to other causes, we employed the BDL mouse model and DDC-diet model, recapitulating clinical features of human biliary fibrosis. Following BDL, *Psmp*$^{-/-}$ mice displayed a significant reduction of the Sirius red-positive area, hydroxyproline content, and α-SMA expression (Fig. 3A-D). Serum ALT levels were lowered in *Psmp*$^{-/-}$ mice after BDL (Fig. 3E). We observed a significant reduction in mRNA levels of *Acta2*, *Col1a1* and *Tgfb1* in *Psmp*$^{-/-}$ mice (Fig. 3F). After 4 weeks of DDC feeding, *Psmp*$^{-/-}$ mice also displayed a significant reduction of the Sirius red-positive area and α-SMA expression (Fig. 3G-I). However, serum ALT levels were compatible between WT and *Psmp*$^{-/-}$ mice (Fig. 3J), suggesting similar liver injury occurred. We also observed a significant reduction in mRNA levels of *Acta2*, *Col1a1*, *Tgfb1*, *Timp1* and *Pdgfr* in *Psmp*$^{-/-}$ mice (Fig. 3K).

Taken together, these results suggest that activation of PSMP signaling is involved in the pathogenesis of hepatic fibrosis.

AAV8-h*PSMP* restores hepatic PSMP expression and promotes liver fibrosis in a CCR2-dependent manner

Our results showed that the human and mouse PSMP proteins have common chemotactic activities and exhibit mouse monocyte chemotaxis in a CCR2-dependent manner *in vitro* (Fig. S3). Therefore, we investigated whether human PSMP protein also affects liver fibrosis in mice. Thus, we used AAV8 (a gene vector isolated from rhesus monkeys, which is used to transduce hepatocytes because of its high affinity for liver cells)[35] to overexpress human PSMP in *Psmp*$^{-/-}$ mice (Fig. 4A). As expected, *Psmp*$^{-/-}$ mice that received AAV8-h*PSMP* had strongly increased levels of hPSMP compared with those receiving a control AAV8-null (Fig. 4B). Using this approach, we next examined the consequences of PSMP overexpression under conditions of chronic liver damage. 4 weeks after the AAV8 injection, the mice were treated with CCl_4 for another 4 weeks to induce liver fibrosis (Fig. 4A). We found a significant increase in liver injury and fibrosis elicited by PSMP overexpression compared to the effects observed in mice that were injected with AAV8-null (Fig. 4C-H). To evaluate whether CCR2 was required for PSMP-promoted fibrosis, we generated *PSMP*- and *CCR2*-double-knockout mice by crossed *Psmp*$^{-/-}$ mice with *Ccr2*$^{-/-}$ mice. The results showed that the double knockout of *Psmp* and *Ccr2* did not reverse the attenuation of liver injury and fibrosis found in *Psmp*$^{-/-}$ mice (Fig. 4C-H).

The data showed that PSMP overexpression promotes liver fibrosis development in a CCR2-dependent manner.

Neutralization of PSMP signaling alleviates murine liver fibrosis

Given that PSMP deficiency significantly attenuated the development of liver fibrosis in mice, we examined the effect of a specific PSMP-neutralizing antibody, 3D5, on liver fibrosis induced by CCl_4. We first investigated the protective effects of 3D5 on mice within 4 weeks of CCl_4-induced liver fibrosis; mice were treated with 3D5 after each CCl_4 injection (Fig. 5A). H&E and Sirius red staining assays showed reduced liver injury and fibrosis after 3D5 treatment compared with that of the CCl_4-treated group (Fig. 5B, C). Accordingly, 3D5 notably reduced α-SMA expression and liver hydroxyproline content in the fibrotic livers (Fig. 5D, E). Serum levels of ALT were significantly lower in the 3D5-treated group than in the CCl_4-treated group, indicating an improvement in liver function

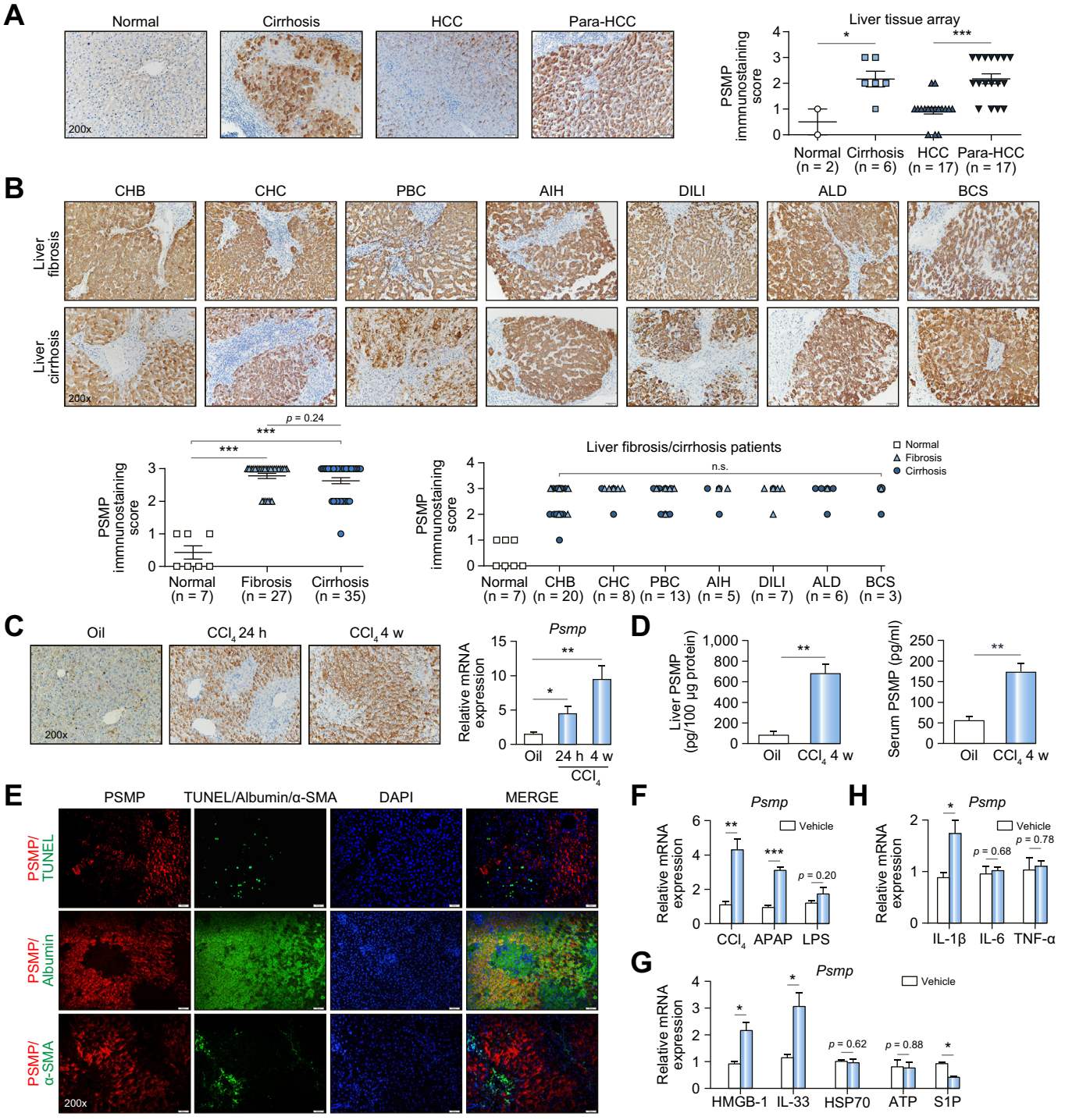

Fig. 1. PSMP is highly expressed in livers from patients and mice with fibrosis or cirrhosis. (A) Representative immunohistochemical staining of PSMP in a human hepatocellular carcinoma (HCC) progression tissue array (HLivH060CD03) and its statistical summary. (B) Representative immunohistochemical staining of PSMP in normal and fibrotic/cirrhotic human livers, and its statistical summary. (C) Representative liver immunohistochemical staining of PSMP and its mRNA levels were measured by qRT-PCR. (D) PSMP protein levels in the liver and serum were measured by cytokine bead assay. (E) Upper: Representative immunofluorescence images of PSMP (red) and TUNEL (green) from the 24 h of CCl_4 treated mouse liver. Middle and Below: Representative immunofluorescence images of PSMP (red) and albumin or α-SMA (green) from the 4 weeks of CCl_4 treated mouse liver. (F-H) qRT-PCR analysis for *PSMP* mRNA levels in mouse primary hepatocytes treated with different liver pathogenic stimuli *in vitro*. (Scale bars, 50 μm. n.s. = not significant. *p <0.05; **p <0.01; ***p <0.001 by the Mann-Whitney *U* test for human and one-way ANOVA or Student's *t* test for mice; n = 5/group). AIH, autoimmune hepatitis; ALD, alcohol-related liver disease; APAP, acetaminophen; BCS, Budd-Chiari syndrome; CCl_4, carbon tetrachloride; CHB, chronic hepatitis B; CHC, chronic hepatitis C; DILI, drug-induced liver injury; HMGB1, high-mobility group box 1; HSP, heat shock protein; LPS, lipopolysaccharide; PBC, primary biliary cholangitis; qRT-PCR, quantitative reverse-transcription PCR; S1P, sphingosine-1-phosphate; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; WT, wild-type.

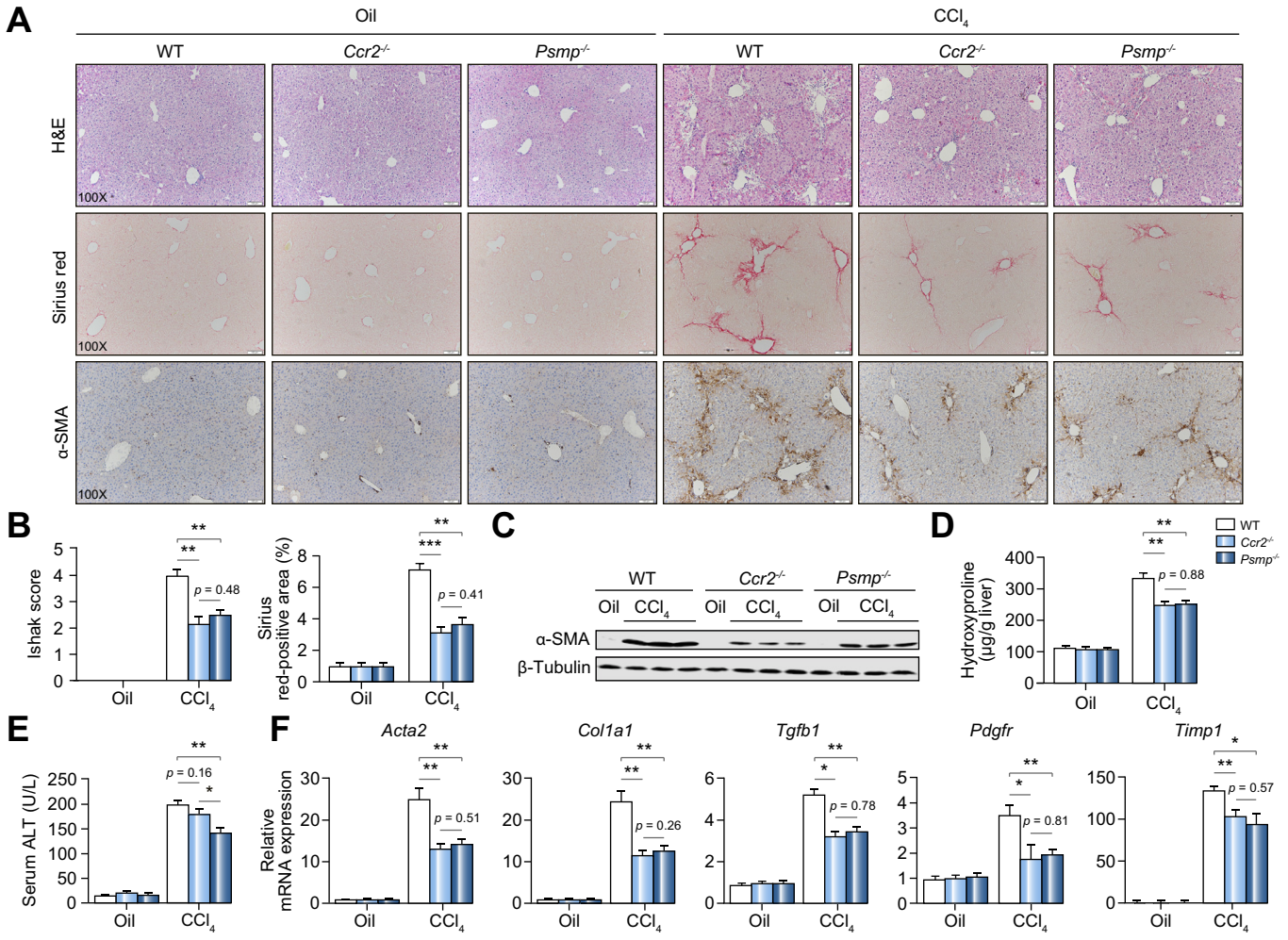

Fig. 2. PSMP deficiency inhibits CCl_4-induced liver fibrosis in mice. WT, *Psmp*$^{-/-}$ and *Ccr2*$^{-/-}$ mice were treated with 4 weeks of CCl_4. (A, B) Representative liver histology of H&E and Sirius Red staining (A) and quantification of collagen deposition as measured by the Ishak score and ImageJ software (B). (A, C) Expression of α-SMA was determined by immunohistochemistry (A) and western blotting (C). (D, E) Hepatic hydroxyproline content (D) and serum levels of ALT (E) were measured. (F) Hepatic mRNA levels of fibrogenic genes were measured by qRT-PCR. (Scale bars, 100 μm. *p <0.05; **p <0.01; ***p <0.001 by one-way ANOVA or the Mann-Whitney *U* test for non-normal distributions; n = 5/group). ALT, alanine aminotransferase; CCl_4, carbon tetrachloride; qRT-PCR, quantitative reverse-transcription PCR; WT, wild-type.

(Fig. 5F). qRT-PCR assays showed that 3D5 also significantly reduced the mRNA levels of fibrogenic genes (*Acta2*, *Col1a1*, *Tgfb1*, *Timp1* and *Pdgfr*) (Fig. 5G).

Moreover, to evaluate the therapeutic potential of 3D5, we used 3D5 treatment in mice from the 4th week to the 6th week with CCl_4 treatment (Fig. 6A). Mice treated with 3D5 displayed a significant reduction in the Sirius red-positive area, hydroxyproline content, α-SMA expression, and serum ALT compared with the CCl_4-treated mice (Fig. 6B-F). Consistently, we also found a significant reduction in *Acta2*, *Col1a1*, *Tgfb1* and *Pdgfr* mRNA levels in mice treated with 3D5 (Fig. 6G).

Different doses of 3D5 (1 mg/kg, 5 mg/kg, and 10 mg/kg) were used to treat mice with CCl_4-induced liver fibrosis (Fig. S4A). H&E staining and Sirius red staining assays showed that mice with liver fibrosis that were treated with 3D5 had milder liver fibrosis (dose-dependent) than the CCl_4-treated mice (Fig. S4B). The levels of a-SMA, liver hydroxyproline content, serum ALT, and mRNA expression of fibrogenic genes (*Acta2*, *Col1a1*, *Tgfb1*, *Timp1* and *Pdgfr*) in CCl_4-treated mice were also dose-dependently decreased after 3D5 treatment (Fig. S4B-G).

These results showed that blockade of PSMP signaling significantly ameliorated the pathogenesis of liver fibrosis, consistent with the *Psmp*$^{-/-}$ results in mice, suggesting that the PSMP antibody could be a potential drug for liver fibrosis treatment.

PSMP deficiency inhibits hepatic macrophage infiltration, M1 polarization and proinflammatory cytokine production induced by CCl_4 challenge

The infiltration of macrophages into the liver upon chronic injury has been convincingly linked to the progression of liver inflammation and fibrosis in mice and humans.[3–5] Therefore, we investigated the composition of immune cells in the liver after CCl_4 treatment by flow cytometric analysis. Infiltrating hepatic macrophage (iMΦ, F4/80$^+$ CD11b^{hi}) and CD11b$^+$CCR2$^+$ cell accumulation were significantly reduced in *Psmp*$^{-/-}$ mice (Fig. 7A, B). Parallel to the intrahepatic macrophages, we also investigated other immune cells, such as neutrophils (CD11b$^+$ Ly6G$^+$), B cells

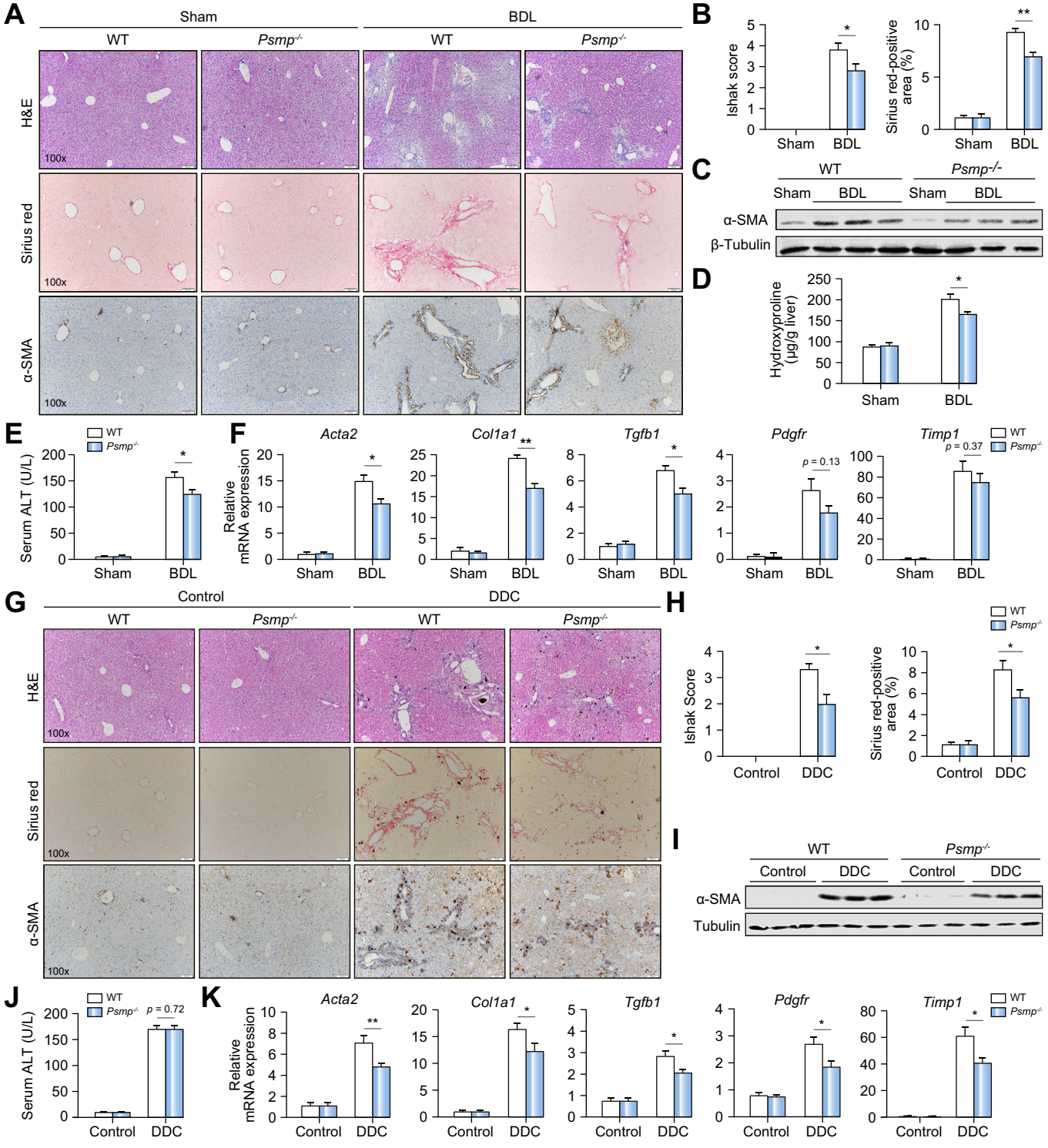

Fig. 3. PSMP deficiency inhibits BDL- and DDC-diet-induced liver fibrosis in mice. (A-F) WT and *Psmp*$^{-/-}$ mice underwent BDL for 14 days. (G-K) WT and *Psmp*$^{-/-}$ mice underwent DDC feeding for 4 weeks. Representative liver histology of H&E and Sirius Red staining (A, G) and its quantification (B, H). Expression of α-SMA was determined by immunohistochemistry (A, G) and western blotting (C, I). Hepatic hydroxyproline content (D) and serum levels of ALT (E, J) were measured. (F, K) Hepatic mRNA levels of fibrogenic genes were measured by qRT-PCR. (Scale bars, 100 μm. $^{*}p$ <0.05; $^{**}p$ <0.01 by one-way ANOVA or the Mann-Whitney *U* test for non-normal distributions; n = 5/group). ALT, alanine aminotransferase; BDL, bile-duct ligation; CCl_4, carbon tetrachloride; DDC, 5-diethoxycarbonyl-1,4-dihydrocollidine; qRT-PCR, quantitative reverse-transcription PCR; WT, wild-type.

(B220^{+}) and T cells (CD3^{+}, CD4^{+}, CD8^{+}), which were not significantly affected except for some elevation or decrease of T cells in the *Psmp*$^{-/-}$ mice (Fig. S5).

We next investigated the consequences of reduced hepatic macrophage accumulation due to PSMP deficiency in the liver. An important function of hepatic macrophages

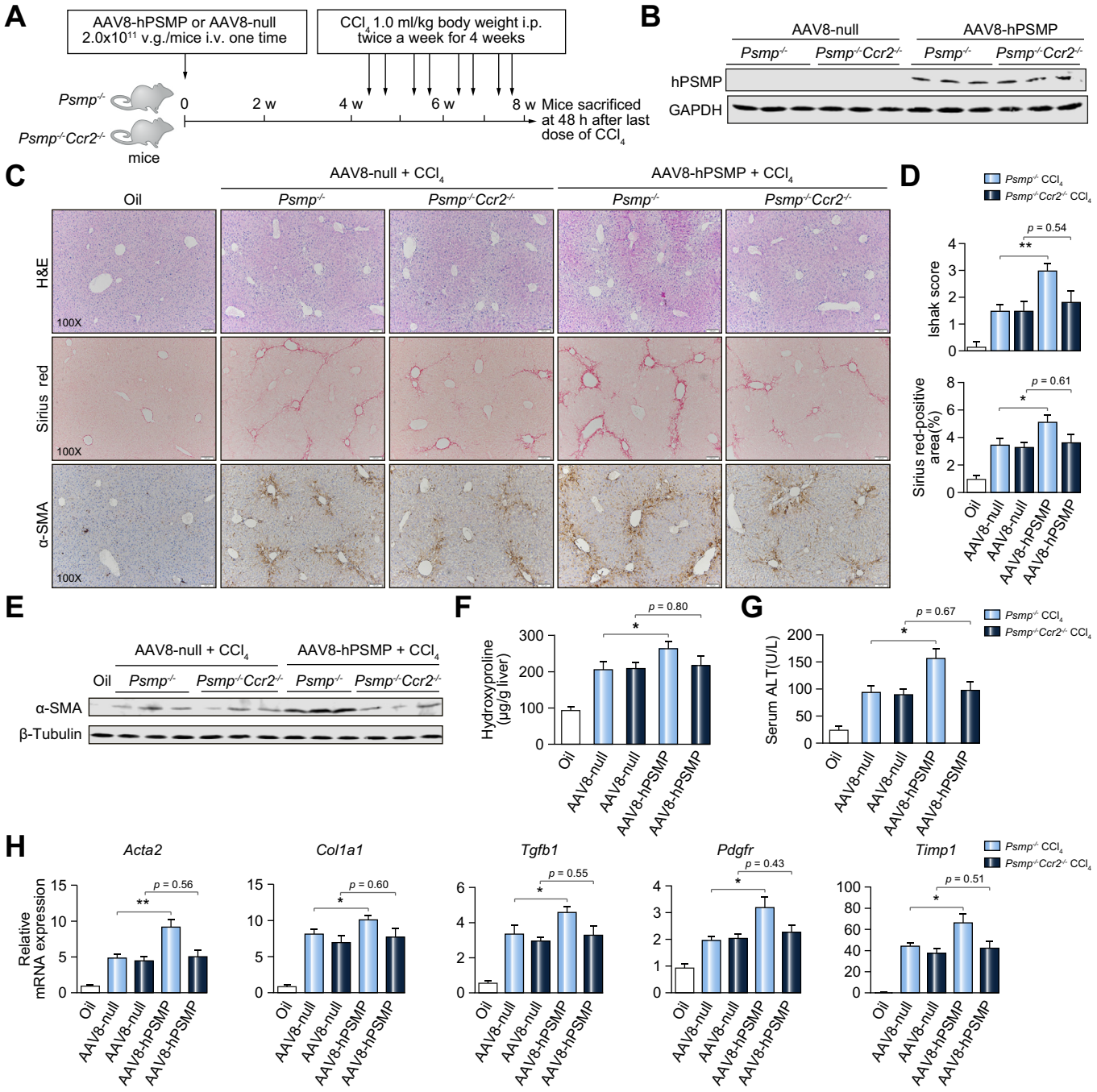

Fig. 4. Effect of PSMP overexpression on CCl_4-induced liver fibrosis in mice by AAV8 vectors. (A) Schematic of the experimental design of AAV8 overexpression treatment in *Psmp*$^{-/-}$ and *Psmp*$^{-/-}$*Ccr2*$^{-/-}$ mice. (B) Liver PSMP protein levels was detected by western blotting from mice that were injected with AAV8 for 4 weeks. (C, D) Representative liver histology of H&E and Sirius Red staining (C) and its quantification (D). (C, E) Expression of α-SMA was detected by immunohistochemistry (C) and western blotting (E). (F, G) Hepatic hydroxyproline content (F) and serum levels of ALT (G) were measured. (H) Hepatic mRNA levels of fibrogenic genes were measured by qRT-PCR. (Scale bars, 100 μm. **p* <0.05; ***p* <0.01 by one-way ANOVA or the Mann-Whitney *U* test for non-normal distributions; n = 5/group). AAV, adeno-associated virus; ALT, alanine aminotransferase; CCl_4, carbon tetrachloride; qRT-PCR, quantitative reverse-transcription PCR.

during disease progression is the secretion of proinflammatory cytokines.[3,4] *Psmp*$^{-/-}$ mice had decreased serum concentrations of CCL2, tumor necrosis factor-α (TNF-α), IL-6 and TGF-β upon CCl_4 treatment, which is consistent with the reduced numbers of intrahepatic macrophages (Fig. 7C).

Further, to investigate whether PSMP deficiency influenced macrophage polarization, we measured expression of macrophage polarization markers in the fibrotic liver by qRT-PCR. Compared with the WT mice, *Psmp*$^{-/-}$ mice expressed lower mRNA levels of *Il-12* and *iNOS* (M1 marker) but higher mRNA levels of *Cd206*, *Il-10* (M2 marker) (Fig. 7D).

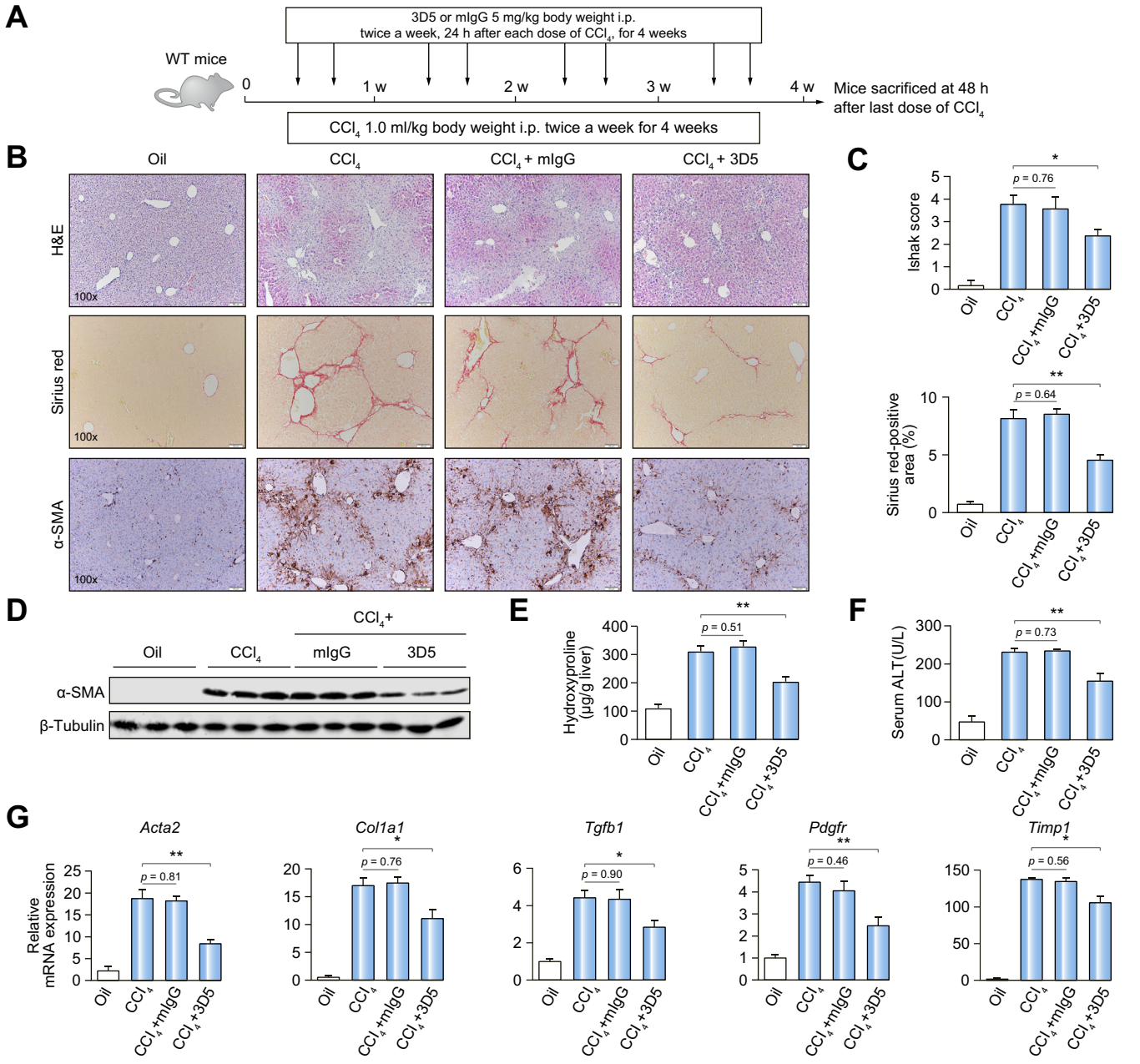

Fig. 5. Protective effects of PSMP-neutralizing antibody treatment on liver fibrosis induced by CCl_4. (A) Schematic of the experimental design of the PSMP-neutralizing antibody 3D5 or mIgG treatment in WT mice. (B, C) Representative liver histology of H&E and Sirius Red staining (B) and its quantification (C). (B, D) Expression of α-SMA was determined by immunohistochemistry (B) and western blotting (D). (E, F) Hepatic hydroxyproline content (E) and serum levels of ALT (F) were measured. (G) Hepatic mRNA levels of fibrogenic genes were measured by qRT-PCR. (Scale bars, 100 μm. *p <0.05; **p <0.01 by one-way ANOVA or the Mann-Whitney U test for non-normal distributions; n = 5/group). ALT, alanine aminotransferase; CCl_4, carbon tetrachloride; qRT-PCR, quantitative reverse-transcription PCR; WT, wild-type.

Treatment of mouse BMDMs with PSMP increases expression of M1 markers, while decreasing M2 markers expression

Based on the results above, we investigated the direct effect of PSMP treatment on macrophage polarization *in vitro*, using WT bone marrow-derived macrophages (BMDMs). We measured the gene expression of M1/M2 polarization markers in macrophages by qRT-PCR. Significantly increased expression of the M1 markers (*Il-1β, Il-12*) were observed following cell incubation with lipopolysaccharide (LPS) + interferon-γ (IFN-γ) and recombinant PSMP protein. In contrast, PSMP downregulated BMDM expression of the M2 markers (*Cd206, Il-10* and *Arg-1*) following incubation with IL-4 (Fig. 7E). Taken together these results suggest that PSMP promotes macrophage polarization to the proinflammatory M1-like phenotype.

These data indicate that PSMP may promote fibrosis by affecting the infiltration and polarization of macrophages and then the production of proinflammatory cytokines.

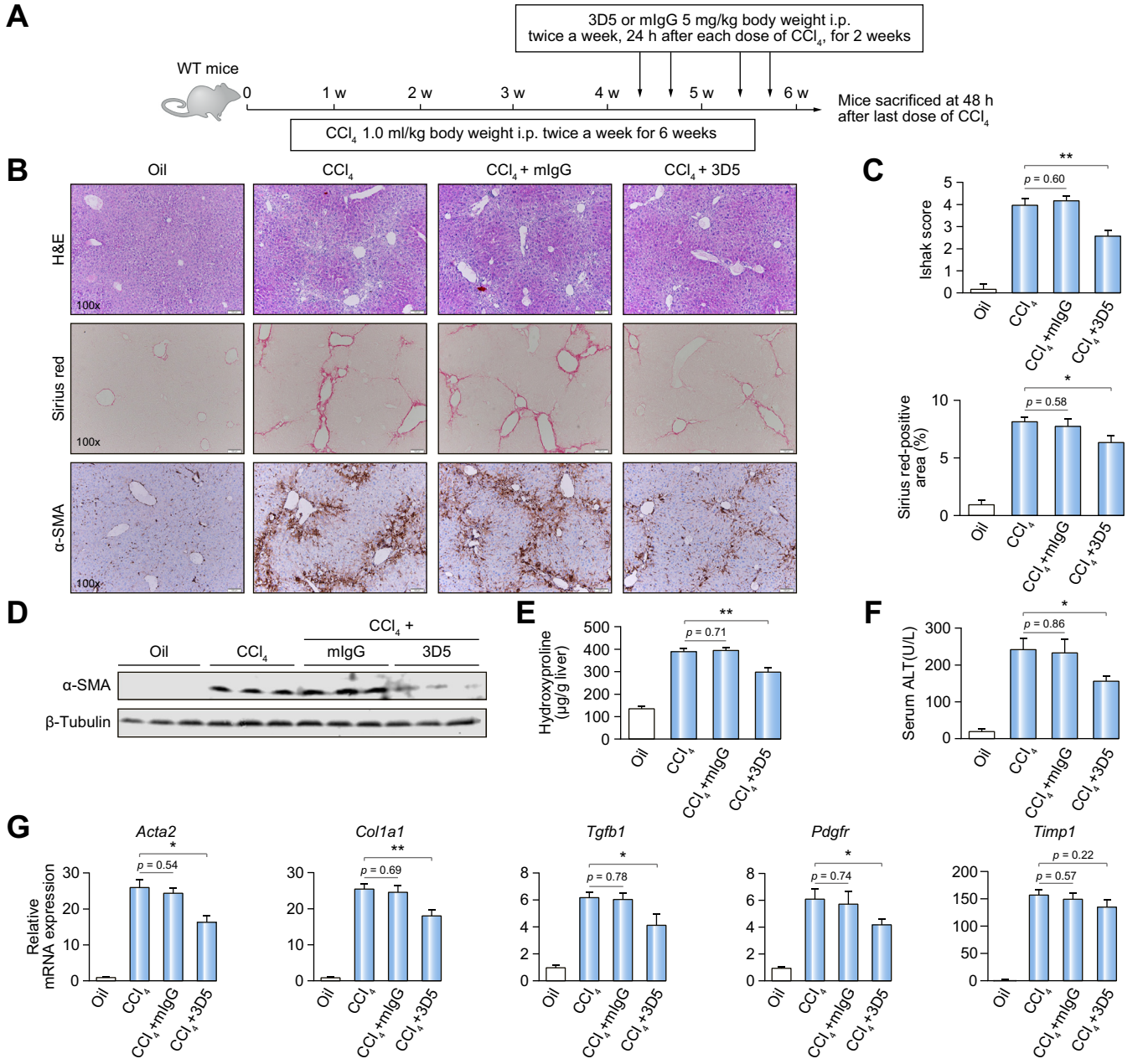

Fig. 6. Therapeutic effects of PSMP-neutralizing antibody treatment on established liver fibrosis induced by CCl_4. (A) Schematic of the experimental design of the PSMP-neutralizing antibody 3D5 or mIgG treatment in WT mice. (B, C) Representative liver histology of H&E, Sirius Red staining (B) and its quantification (C). (B, D) Expression of α-SMA was detected by immunohistochemistry (B) and western blotting (D). (E, F) Hepatic hydroxyproline content (E) and serum levels of ALT (F) were measured. (G) Hepatic mRNA levels of fibrogenic genes were measured by qRT-PCR. (Scale bars, 100 μm. $^{*}p$ <0.05; $^{**}p$ <0.01 by one-way ANOVA or the Mann-Whitney *U* test for non-normal distributions; n = 5/group). ALT, alanine aminotransferase; CCl_4, carbon tetrachloride; qRT-PCR, quantitative reverse-transcription PCR; WT, wild-type.

PSMP promotes HSC activation and survival

CCR2 was shown to promote HSC activation and the development of hepatic fibrosis.[17] We hypothesized that increased PSMP may regulate HSC activation. In the current study, LX-2 (a human HSC line with key features of activated HSCs despite limitations due to immortalization)[36] cells were treated with recombinant PSMP protein. qRT-PCR analysis demonstrated that PSMP significantly increased the mRNA levels of profibrogenic genes (*ACTA2*, *COL1A1*, *TGFB*) in LX-2 cells (Fig. 8A). Additionally, the PSMP upregulated group pretreated with RS504393, which is an antagonist of CCR2, displayed reduced expression of the HSC activation markers in LX-2 cells, which indicated that PSMP directly promotes HSC activation through CCR2 (Fig. 8A). The α-SMA positive HSCs are activated HSCs, which are the major fibrogenic populations during liver fibrosis development. Compared with the CCl_4-induced WT mice, TUNEL and immunohistochemical double immunolabeling demonstrated that the apoptosis of α-SMA positive HSCs

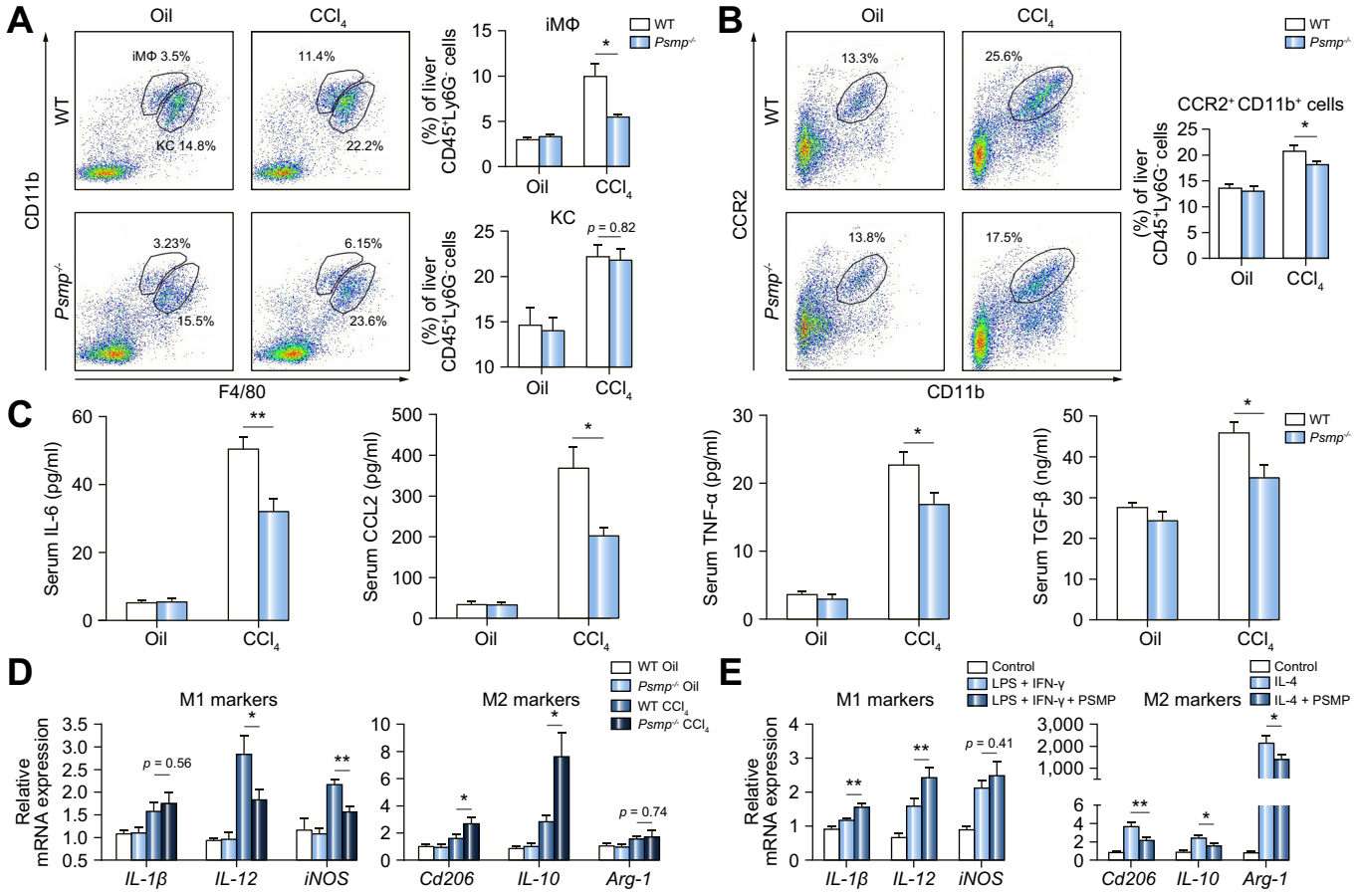

Fig. 7. Effects of PSMP on macrophage infiltration and polarization. (A-D) WT and *Psmp*$^{-/-}$ mice were treated with 4 weeks of CCl_4. Hepatic non-parenchymal cells were stained and analyzed by flow cytometry. (A, B) Infiltrating macrophages (iMΦ, $CD45^+$ $Ly6G^-$ $F4/80^+$ $CD11b^{high}$), Kupffer cells (KC, $CD45^+$ $Ly6G^-$ $F4/80^{hi}$ $CD11b^+$) (A) and $CD45^+$ $CCR2^+$ $CD11b^+$ (B) cells were quantified by flow cytometric analysis. Left, representative flow cytometry plot; Right, quantitative analysis. (C) The serum cytokines IL-6, TNF-α, and CCL2 were detected by cytokine bead assay, and TGF-β was detected by ELISA. (D) The mRNA levels of M1 and M2 macrophage markers in the liver were measured by qRT-PCR. (E) BMDMs from WT mice were either untreated (control) or stimulated with LPS + IFN-γ or IL-4 or recombinant PSMP protein. The mRNA levels of M1 and M2 macrophage markers were analyzed by qRT-PCR. (*p <0.05; **p <0.01 by one-way ANOVA; n = 5/group). BMDMs, bone marrow-derived macrophages; CCl_4, carbon tetrachloride; IFN, interferon; LPS, lipopolysaccharide; qRT-PCR, quantitative reverse-transcription PCR; WT, wild-type.

significantly increased in the livers of CCl_4-induced *Psmp*$^{-/-}$ mice (Fig. 8B).

These results suggested that PSMP also promotes liver fibrosis through regulation of HSC activation and survival.

Discussion

Liver fibrosis/cirrhosis is a major health problem worldwide, and there are currently no approved therapies.[14] The CCR2-CCL2 pathway has been extensively confirmed in various experimental models of liver fibrosis using genetic deletion of *CCR2* or pharmacological inhibition of CCL2, resulting in attenuation of liver fibrosis in mice.[17–26] The dual CCR2/CCR5 antagonist CVC, as a antifibrotic agent for the treatment of alcohol-induced steatohepatitis and NASH with fibrosis, is currently being tested in a phase III trial.[27–29] PSMP is a new ligand for CCR2, with a high affinity comparable to that of CCL2.[31] In this study, we first found significantly enhanced PSMP expression in the livers of patients with fibrosis/cirrhosis. We hypothesize that PSMP may play an important role in the development and progression of liver fibrosis.

Early fibrosis becomes problematic and clinically relevant when dysregulated and excessive scarring occurs in response to persistent injury and leads to altered tissue function, eventually becoming cirrhotic.[37] We found that PSMP was upregulated in the early stage of liver injury and continuously expressed in 3 experimental murine models: CCl_4-, BDL- or DDC-diet-induced liver fibrosis. To confirm the effect of upregulation of PSMP during liver fibrosis, we first used *Psmp*$^{-/-}$ mice to verify that genetic inactivation of *Psmp* attenuated chronic liver injury and fibrosis, leading to a recovery of liver function in mice. Despite the possible redundancy of other CCR2 ligands in *Psmp*$^{-/-}$ mice, some of our data showed a lighter trend in liver fibrosis in *Ccr2*$^{-/-}$ mice. *Psmp*$^{-/-}$ mice still displayed significantly attenuated liver injury and fibrosis similar to *Ccr2*$^{-/-}$ mice, indicating that PSMP, as a CCR2 key ligand, plays an essential role in liver injury and fibrosis. Furthermore, overexpressing h*PSMP* in *Psmp*$^{-/-}$ mice by AAV8 vectors could reverse the attenuation of liver injury and fibrosis found in *Psmp*$^{-/-}$ mice, indicating that h*PSMP* could also promote liver fibrosis. However, this effect was not observed in *Psmp*- and *Ccr2*-double-knockout mice, indicating that PSMP promotes liver fibrosis development in a CCR2-dependent

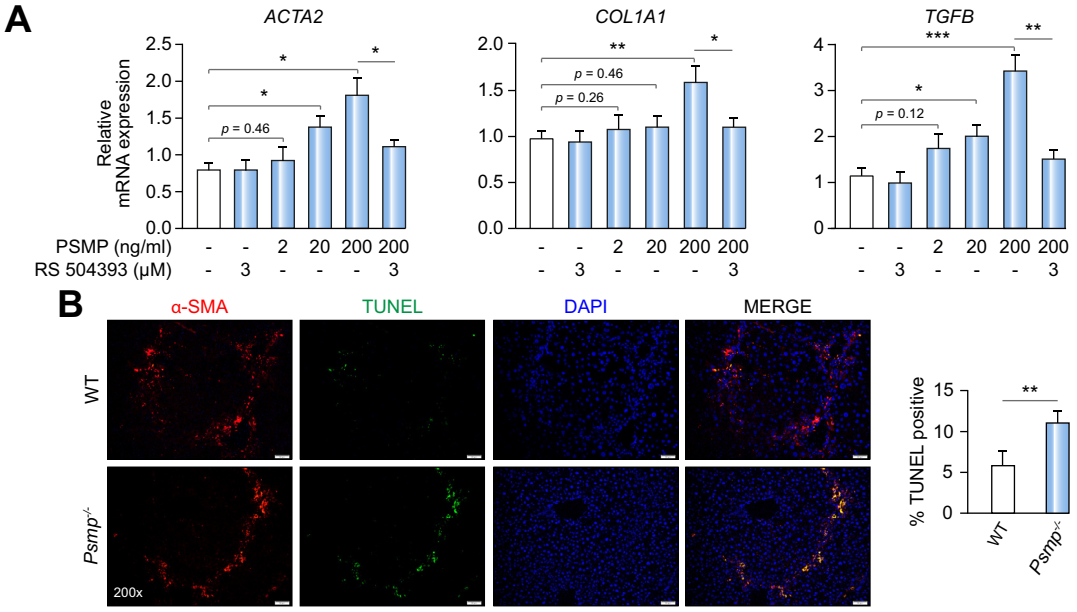

Fig. 8. PSMP promotes HSC activation and survival. (A) LX-2 cells were incubated with or without recombinant PSMP protein at the indicated dose, and pretreated with or without RS504393 (CCR2 antagonist). Hepatic mRNA levels of fibrogenic genes were measured by qRT-PCR. (B) Representative immunofluorescence images of α-SMA (red) and TUNEL (green) from WT and *Psmp*$^{-/-}$ mice treated with 4 weeks of CCl_4. Right, quantitative analysis. (Scale bars, 50 μm. **p* <0.05; ***p* <0.01; ****p* <0.001 by one-way ANOVA or Student's *t* test; n = 5/group). CCl_4, carbon tetrachloride; HSCs, hepatic stellate cells; qRT-PCR, quantitative reverse-transcription PCR; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; WT, wild-type.

manner. These results confirmed that upregulation of PSMP indeed contributes to the development and progression of liver fibrosis.

At the same time, we found that a PSMP-specific neutralizing antibody, 3D5, had significant *in vivo* antifibrotic activity along the same line, with fibrosis alleviation in *Psmp*$^{-/-}$ mice. Our data showed that 3D5 significantly reduced liver fibrosis and rescued hepatic function. We used 2 treatment protocols: the protective treatment and the therapeutic treatment, both treatment models showed reduced liver injury and fibrosis after 3D5 treatment. To further assess the therapeutic effect of PSMP antibody on liver fibrosis, we used different doses of 3D5 to treat mice with CCl_4-induced liver fibrosis. The results showed that the degree of liver fibrosis was reduced by 3D5 in a dose-dependent manner. These findings indicated that PSMP could be a new therapeutic target for liver fibrosis and that the PSMP antibody is a potential treatment option of liver fibrosis.

Based on the results that PSMP was already upregulated in the early stage of liver injury and predominantly expressed in the nonapoptotic area by TUNEL staining, we further explore the mechanism of PSMP overexpression. Excessive cell death and the release of DAMPs has been identified as a central mechanism of liver damage in both acute and chronic liver diseases.[38,39] We then found that the increased PSMP expression in the liver was localized mainly to hepatocytes by double immunolabeling. Fractionated primary hepatocytes were found to produce PSMP upon the stimulation of CCl_4, APAP and DAMP molecules HMGB-1 or IL-33, but not HSP70, ATP and S1P. Therefore, our results suggest that PSMP is a damage-induced chemotactic cytokine.

To further explore the mechanism of PSMP in liver fibrosis, we characterized and identified PSMP-mediated effects on macrophages and HSCs. During liver injury, PSMP could chemoattract $CCR2^+$ monocytes from blood to the injured liver and then differentiate into macrophages to promote liver inflammation. PSMP deficiency inhibits hepatic macrophage infiltration, M1 polarization and proinflammatory cytokine production. We next investigated the direct effect of PSMP treatment on macrophage polarization *in vitro*. The results suggest that PSMP promotes macrophage polarization to the proinflammatory M1-like phenotype. Despite the critical role of proinflammatory monocytes in the pathogenesis of liver fibrosis, HSCs are the most relevant profibrogenic cells. Previous studies have shown that macrophage infiltration and proinflammatory cytokines protected against the death of activated HSCs.[5,13] Consistent with these findings, our data demonstrated that the apoptosis of activated HSCs significantly increased in the livers of CCl_4-induced *Psmp*$^{-/-}$ mice. In addition, CCR2 was shown to be expressed on HSCs, and CCL2 directly activated HSCs.[17,20] We then tested the effects of recombinant PSMP protein in human LX-2 cells. The results showed that PSMP can directly activate LX-2 cells in a CCR2-dependent manner. Taken together, these data indicate that PSMP as a chemotactic cytokine not only promotes inflammation and survival of activated HSCs but also directly activates HSCs.

Thus, we developed the following working model for the role of PSMP in the progression of liver injury and fibrosis. When hepatocytes are injured, DAMP molecules such as HMGB-1 and IL-33 are released. These molecules induce the production of PSMP by hepatocytes. Sustained cell damage signaling and increased PSMP expression promote inflammatory macrophage infiltration, M1 polarization and proinflammatory cytokine secretion. PSMP also directly activates quiescent HSCs *via* CCR2. Moreover, activated HSCs become myofibroblasts that are responsible for the excess deposition of ECM. Eventually, all of these fibrogenesis-promoting effects contribute to liver fibrosis and cirrhosis. In therapeutic applications, combining the characteristics that PSMP is lowly expressed in multiple normal tissues[31] and damage-induced in liver injury and fibrosis tissues may provide long-term therapeutic safety and tolerability for targeted PSMP.

In conclusion, PSMP was highly expressed in fibrotic/cirrhotic liver tissues from patients with different etiologies of liver disease. The upregulation of PSMP contributes to the development and progression of liver fibrosis in a CCR2-dependent manner. PSMP promotes liver fibrosis through inflammatory macrophage infiltration, M1 polarization and proinflammatory cytokines, as well as direct activation of HSCs via CCR2. A PSMP antibody reduced liver fibrosis development *in vivo*. These findings indicate that PSMP may be a potential therapeutic target and its antibody may be a potential therapeutic agent for liver fibrosis.

Abbreviations

α-SMA, α-smooth muscle actin; AAV, adeno-associated virus; ALT, alanine aminotransferase; APAP, acetaminophen; BDL, bile-duct ligation; BMDMs, bone marrow-derived macrophages; CCL2, C-C motif chemokine ligand 2; CCl_4, carbon tetrachloride; CCR2, C-C motif chemokine receptor 2; CVC, cenicriviroc; DAMP, damage-associated molecular pattern; DSS, dextran sulfate sodium; ECM, extracellular matrix; HCC, hepatocellular carcinoma; HSP, heat shock protein; HMGB1, high-mobility group box 1; HSCs; hepatic stellate cells; IL, interleukin; LPS, lipopolysaccharide; NASH, non-alcoholic steatohepatitis; PSMP, PC3-secreted microprotein; qRT-PCR, quantitative reverse-transcription PCR; S1P, sphingosine-1-phosphate; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; WT, wild-type.

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Conflict of interest

The authors declare no conflict of interest with respect to this manuscript.

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

Authors' contributions

Y.W., H.Y., S.P.S. and X.N.W. contributed to the experimental design, data interpretation and manuscript preparation. Y.W. conceived the project. S.P.S. performed most of the experiments. J.Z.X. constructed and provided the *Psmp* knockout mice. X.N.W and H.Y. provided human specimens and data collection. D.F.Z., X.L.P, J.M., P.L., Q.S.S., C.Z., W.W.L., S.Y.H., Q.Q.L., Z.T.L., Z.M.S and Y.Z.L. contributed to specific experiments. Y.M.S. and J.L.Z. contributed to the human specimen management and data collection. L.N. and C.Y.G. contributed to the score of all slides. Y.Z. and W.K. provided intellectual input.

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Supplementary data

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Author names in bold designate shared co-first authorship

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