Protein S exacerbates chronic liver injury and fibrosis

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Conflict of Interest

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Abstract

Protein S is a vitamin K-dependent glycoprotein produced mainly in the liver with anticoagulant, anti-inflammatory, immune-modulatory and anti-apoptotic properties. Protein S exacerbates acute liver injury by prolonging the survival of liver immune cells. However, the effect of protein S on chronic liver injury and fibrosis is unknown. In the present study we investigated whether human protein S can affect chronic liver injury and fibrosis. Liver injury/fibrosis was induced by carbon tetrachloride injection in mice overexpressing human protein S and in wildtype mice. Human protein S transgenic mice receiving carbon tetrachloride showed significantly higher circulating levels of liver transaminases, increased liver expression of inflammatory cytokines, significantly much extended liver fibrosis and areas with DNA breakage after chronic injury compared to wild-type mice. Wild-type mice infused with exogenous human protein S exhibited exacerbated liver injury and increased number of hepatic stellate cells compared to untreated mice. Human protein S inhibited apoptosis and increased Akt pathway activation in hepatic stellate cells. The anti-apoptotic activity of protein S may play a role in chronic liver injury and subsequent liver fibrosis.

Key words:

Protein S; liver fibrosis; apoptosis; immune cells, hepatic stellate cells.

Introduction

Liver fibrosis is the final stage of chronic liver damage caused by viruses, toxins, excessive alcohol intake and autoimmunity.[1] It is characterized by abnormal deposition of collagen-rich extracellular matrix proteins associated with destruction of the liver parenchymal architecture.[2] Chronic inflammation may lead to apoptosis of hepatocytes releasing by-products that may in turn stimulate liver resident immune cells (natural killer cells, natural killer T cells, Kupffer cells) and recruited inflammatory leukocytes to secrete pro-inflammatory and pro-fibrotic cytokines.[1] During liver injury pro-fibrotic cytokines including transforming growth factor β1 (TGF-β1) activate hepatic stellate cells (HSCs) leading to excessive secretion and deposition of connective tissue components such as collagen I, tenascins, periostin that ultimately may result in liver fibrosis and cirrhosis.[1]

Protein S (PS) is a vitamin K dependent glycoprotein synthesized in the liver that functions as co-factor of activated protein C for inactivating coagulation factors Va and VIIIa to inhibit activation of the clotting cascade.[3] Independent of its anticoagulant activity, PS can suppress inflammation and apoptosis through its Tyro3, Axl and Mer (TAM) tyrosine kinase receptors.[4] After binding to TAM receptors, PS activates several molecules involved in intracellular signaling pathways such as NF-κB and Akt leading to inhibition of inflammatory cytokine secretion and suppression of cell apoptosis.[5-9] Inhibition of apoptosis of lung epithelial cells by PS attenuates pulmonary fibrosis and its inhibitory activity on apoptosis of islet β-cells ameliorates diabetes mellitus in mice.[10, 11] However, administration of exogenous PS has been shown to exert detrimental effects in a mouse model of acute alcoholic hepatitis by its ability to activate and prolong the survival of natural killer T cells.[12]

In the present study, we hypothesized that PS administration would worsen the pathological state in chronic liver injury and fibrosis. To demonstrate this hypothesis we compared the severity of liver injury/fibrosis between transgenic mice overexpressing human PS and wild type mice and between mice treated with human PS and untreated mice.

Materials and methods

Human PS transgenic (hPS-TG) mice were generated as previously described and backcrossed with mice with C57BL/6J backgrounds for more than ten generations.[12] Male hPS-TG (8-10 weeks old) mice were used in the experiments. Male littermates were used as controls mice. To evaluate the efficacy of exogenous hPS 8~12 weeks-old male wild type (WT) mice weighing 18~22 g were purchased from Nihon SLC (Hamamatsu, Japan). Mice were maintained in a specific pathogen-free environment and subjected to a 12-h light/dark cycle in the animal house of Mie University.

Ethical statement

The Committee for Animal Investigation of Mie University approved the experimental protocols (Approval No 24-50) and all procedures were performed in accordance with approved institutional guidelines.

Model of chronic liver injury and fibrosis

Liver fibrosis was induced by intraperitoneal injection of 600 μL/kg of carbon tetrachloride (CCl_4; Sigma-Aldrich, St Louis, MO) dissolved in corn oil at concentration of 20% and administered twice a week for 8 weeks.[13] Mice receiving intraperitoneal injection of the same volume of corn oil during the same period were used as controls.

There were four treatment groups in the experiments performed to induce liver fibrosis using hPS-TG mice: WT mice treated with corn oil (WT/OIL) or CCl_4 (WT/CCl_4), and hPS-TG mice treated with oil (hPS-TG/OIL) or CCl_4 (hPS-TG/CCl_4).

To investigate the effects of human PS (hPS) administration, WT mice were treated with exogenous hPS (0.23mg/kg-mouse-weight/day) through osmotic minipumps (model 2004; Alzet Corporation, Palo Alto, CA) for 8 weeks, and CCl_4 was administered intraperitoneally for 8 weeks using the same dose and protocol for CCl_4 as described above. The osmotic minipump of each mouse for hPS administration was replaced for a new minipump on the 5^{th} week of the experiment because the 2004 minipump model lasts only 4 weeks. Control mice received saline through osmotic minipumps and vehicle by intraperitoneal injection.

Blood and liver sampling

Euthanasia of mice was performed 3 days after the last CCl_4 injection by pentobarbital overdose, blood was collected in heparinized tubes, the liver was excised and one portion of it was fixed in 10% formalin for histological examination and another part was stored at -80°C for subsequent extraction of mRNA.

Liver collagen staining and quantification

Formalin-fixed liver tissue was embedded in paraffin, 3-mm-thick tissue sections were prepared and stained with hematoxylin and eosin (H&E). Tissue fibrotic areas were detected by staining with Masson's trichrome and quantified using the WinRoof image processing software (Mitani Corp., Fukui, Japan). Briefly, microscopic lung fields were randomly photographed in each mouse from all groups using an Olympus BX50 microscope and an Olympus DP70 digital camera (Tokyo, Japan). The lung area stained

with collagen was then measured using the WindRoof software and the values were averaged for each individual mouse.

Immunohistochemistry

Immunohistochemical staining with anti-mouse α-smooth muscle actin (α-SMA) (DAKO, Kyoto, Japan) was performed using the avidin-biotin-peroxidase complex technique. Positive cells were counted in at least 50 high-power fields and the results were expressed as a percentage of total cells. Double staining of terminal deoxynucleotidyl transferase dUTP Nick-End Labeling (TUNEL) and α-SMA was performed at the Biopathology institute Corporation using rabbit anti-α-SMA (abcam, ab5694), Alexa Fluor 594 goat anti-rabbit IgG and slowfade goldantifade reagent with 4',6-diamidino-2-phenylindole (DAPI).

Evaluation of apoptosis

DNA fragmentation in liver tissue was evaluated by the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) method using a commercially available kit (Chemicon International, Temecula, CA) following the manufacturer's instructions. The total area of cells with DNA breakage was measured using the WinRoof image processing software as described above.

Model of acute liver injury

To induce acute liver injury, WT and hPS-TG mice were separated into WT/OIL, WT/CCl4), hPS-TG/OIL and hPS-TG/CCl4 groups as described above but only one dose of CCl_4 (600 μL/kg) or similar volume of corn oil was intraperitoneally administered before sacrifice after 24h. The blood was collected for measuring liver transaminases and liver specimens were excised for H&E staining and for isolation of liver non-parenchymal cells. The methods for isolation and counting of

non-parenchymal cells by flow cytometry and the list of commercial antibodies have been previously reported.[12]

Cell culture

LX-2, the human hepatic stellate cell line was kindly provided by Dr Takato Ueno from the Research Center for Innovative Cancer Therapy of Kurume University, and primary human hepatic stellate cells were purchased from ScienceCell Research Laboratories (Carlsbad, CA). LX-2 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) and primary human hepatic stellate cells in stellate cell medium containing 10% heat-inactivated fetal bovine (FBS), 100 IU/ml penicillin and 100 μg/ml streptomycin.

Cell experimental procedures

Human hepatic stellate cell line, LX-2, or the primary human hepatic stellate cells were stimulated with 20 μg/ml hPS and cultured for further 16h in the presence or absence of 10%FBS. Total protein was extracted from cells with RIPA buffer, electrophoresed on SDS-PAGE and transferred to PVDF membrane (Millipore). Flow cytometry was used to assess apoptosis of primary human hepatic stellate cells after cell staining with annexin V and propidium iodide (PI) as previously described.[12]

Western blotting

Phosphorylated Akt, total Akt and cytochrome P450 2E1 (CYP2E1) were detected by Western blotting using anti-p-Akt (S473), antibody (rabbit mAb, clone D9E, Cell Signaling), anti-Akt (rabbit mAb, clone C6/E7, Cell Signaling), rabbit polyclonal anti-CYP2E1 (Bioworld Technology, St. Louis Park, MN), rabbit anti-caspase-3 (Cell Signaling Technology, Danvers, MA), and anti-rabbit IgG-HRP (goat polyclonal Ab, Santa Cruz) antibodies.

Biochemical analyses

The extent of liver injury was assessed by measuring the activity of plasma aspartate aminotransferase (AST) and alanine aminotransferase (ALT) using a Wako Transaminase CII-Test kit according to the manufacturer's protocol (Wako Pure Medical, Tokyo, Japan). Human PS was measured by enzyme immunoassays as described.[12]

RNA isolation and reverse-transcription polymerase chain reaction

Total RNA was extracted from liver tissues using TRIzol reagent, according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Reverse transcription was performed following standard methods. The expression levels of the mRNAs were determined by real-time PCR using SYBR Premix Ex Taq (Takara, Liaoning, China). The primers used in the experiments were as follows: mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH), forward: 5'-CCCTTATTGACCTCAACTACATGGT-3', reverse: 5'-GAGGGGCCATCCACAGTCTTCTG-3'; mouse transforming growth factor (TGF)β1, forward: 5'-ACTCCACGTGGAAATCAACGG-3', reverse: 5'-TAGTAGACGATGGGCAGTGG-3'; mouse chemokine (C-C motif) ligand 2 (CCL2), forward: 5'-ATGCAGGTCCCTGTCATGCTTC-3', reverse: 5'-ACTAGTTCACT GTCACACTGGTC-3'; mouse α-smooth muscle actin (SMA): forward: 5'-CAGGATGCAGAAGGAGATCAC-3', reverse: 5'-TGTTGCTAGGCCAGGGCTAC-3'; mouse collagen I (Col1a1), forward: 5'-TAAGGGTCCCCAATGGTGAGA-3', reverse: 5'-GGGTCCCTCGACTCCTACAT-3'; mouse CYP2E1, forward: 5'-ACAGAGACCACCAGCACAACT-3', reverse, 5'-AATGTCTCTGGATCTGGAAACT-3'; mouse tumor necrosis factor-(TNF)α, forward : 5'-ACGTGGAACTGGCAGAAGAG-3',

reverse : 5'-CTCCTCCACTTGGTGGTTTG-3'; mouse interleukin-(IL)-6, forward: 5'-CTGGTCTTCTGGAGTACCATAG-3', reverse: 5'-AAGTCAGATACCTGACAACAGG-3'; mouse IL-17, forward: 5'-GGCCCTCAGACTACCTCAAC-3', reverse: 5'-TCTCGACCCTGAAAGTGAAGG-3'; human GAPDH, forward: 5'-GGAGCGAGATCCCTCCAAAAT-3', reverse: 5'-GGCTGTTGTCATACTTCTCATGG-3'; human α-SMA, forward: 5'-GTGTTGCCCCTGAAGAGCAT-3', reverse: 5'-GCTGGGACATTGAAAGTCTCA-3'; human Col1a1, forward: 5'-AACCTGGATGCCATCAAACTC-3', reverse: 5'-TCCATGTAGGCCACGCTGTTC-3'; human fibronectin 1, forward: 5'-CGGTGGCTGTCAGTCAAAG-3', reverse, 5'-AAACCTCGGCTTCCTCCATAA-3'.

Statistical analysis

Results are presented as mean ± standard errors of the mean (SEM). Statistical difference between groups was calculated by analysis of variance with *post hoc* analysis using the Fisher's predicted least significant difference test. The StatView 4.5 package for Macintosh (Abacus Concepts, Berkeley, CA, USA) was used. Statistical significance was defined as $p < 0.05$.

Results

Protein and mRNA expression of CYP2E1

The protein expression of the cytochrome P450 enzyme CYP2E1 was significantly different between WT/OIL and WT/CCl_4 and between hPS-TG/OIL and hPS/CCl_4 mice but not significantly different between WT/CCL_4 and hPS-TG/CCL_4 mice. CYP2E1 mRNA expression was unchanged between all groups (**Supplementary Figure 1A,B**).

Increased plasma PS level in WT mice with liver injury

As expected, the plasma hPS level significantly increased in hPS-TG mice compared to WT mice (**Supplementary Figure 2**). The plasma PS levels significantly increased in the WT/CCL_4 group compared to their WT/OIL counterparts on the 3rd, 5th and 7th week after starting the experiment (**Supplementary Figure 2)**.

Mice overexpressing hPS have increased chronic liver injury and increased area of cells with DNA fragmentation

Compared to mice of the WT/CCl_4 group, mice of the hPS/CCl_4 group significantly showed more conspicuous cell infiltration and fatty degeneration in the liver, increased plasma concentrations of ALT and AST, enhanced liver tissue mRNA expression of TNFα and CCL2, and extensive areas of liver cells with DNA fragmentation (**Figure 1A,B,C**). However, hPS/CCl_4 mice showed a significant reduction of caspase-3 activation in the liver tissue compared to WT/CCl_4 mice (**Figure 1D)**.

Human PS decreases apoptosis of hepatic stellate cells *in vitro*

An *in vitro* experiment showed that hPS significantly inhibited starvation-mediated apoptosis of human primary hepatic stellate cells compared to untreated cells (**Figure 1E**). Analysis of the Akt signal pathway revealed that hPS significantly increases phosphorylation of Akt in LX-2 hepatic stellate cell lines (**Figure 1F**).

Human PS TG mice are prone to liver fibrosis

After chronic liver injury with CCl_4, hPS TG mice showed significantly enhanced collagen deposition and extensive number of α-SMA(+) cells in the liver compared to their WT counterparts (**Figure 2A,B**). The relative mRNA expressions of α-SMA and Col1a1 were also significantly enhanced in liver tissue from hPS TG/CCl_4 mice compared to WT/CCl_4 mice (**Figure 2C**).

Human PS exerts no effect on fibrogenic activity of primary human hepatic stellate cells

In *in vitro* study, hPS showed no effect on mRNA expression of Col1a1, α-SMA or fibronectin-1 in the presence or absence of transforming growth factor-β1 in primary human hepatic stellate cells (**Supplementary Figure 3**).

Administration of exogenous hPS is associated with exacerbation of chronic liver injury and increased hepatic stellate cells *in vivo*

WT mice received repeated injections of CCl4 and treated with or without hPS for 8 weeks. There were more cell infiltration and fatty degeneration in the liver and the plasma concentrations of AST and ALT significantly increased in WT mice treated with exogenous hPS compared to untreated WT mice (**Figure 3A**). There was also reduction of the number of TUNEL and α-SMA double-positive cells, but enhancement of the number of α-SMA (+) cells in mice treated with exogenous hPS compared to saline-treated mice (**Figure 3B**).

Increased percentage of immune cells and hepatic stellate cells in hPS-TG mice with acute liver injury

The percentage of activated NKT cells and hepatic stellate cells was significantly increased while the percentage of T cells and apoptotic NKT cells were significantly decreased in the liver tissue from hPS-TG/CCl_4 mice compared to WT/CCl_4 mice (**Supplementary Table 1**).

Overexpression of hPS worsens acute liver injury

WT and hPS TG mice received one injection of CCl_4 and sacrificed after 24 h. The plasma concentrations of AST and ALT and the tissue mRNA expressions of TNF-α,

IL-17, CCL-2 and IL-6 significantly increased in hPS TG mice compared to WT mice (**Figure 4**).

Discussion

PS can protect against excessive blood coagulation activation by increasing the anticoagulant activity of activated protein C, by directly inhibiting the prothrombinase complex and by accelerating fibrinolysis.[3, 4] PS is also protective against enhanced inflammatory responses by promoting phagocytes-mediated clearance of apoptotic cells, by abolishing activation of the complement system and by inhibiting TAM receptor-mediated expression of cytokines and cell apoptosis.[14-17] However, overprotection by PS may also be counterproductive under certain conditions including cancer by inhibiting apoptosis of malignant cells and acute alcoholic hepatitis by prolonging survival of activated NKT cells.[12, 18] In the current study, we assessed the effect of human PS overexpression on chronic injury and fibrosis induced in the liver by CCl_4, and found that hPS-TG mice receiving CCl_4 injection had significantly increased liver injury, inflammatory changes, enhanced liver mRNA expression of inflammatory cytokines and significantly enhanced area of liver cells with DNA fragmentation compared to their WT type counterparts. CCl_4-associated injury may lead to liver fibrosis by increasing the number and differentiation of hepatic stellate cells to myofibroblasts that secrete excessive extracellular matrix proteins.[19, 20] Here we demonstrated that mice overexpressing hPS have less caspase-3 activation during liver injury, and showed using an *in vitro* cell culture system that hPS can activate the anti-apoptotic Akt signal pathway and decrease apoptosis of hepatic stellate cells pointing a role for hPS in liver fibrosis. Consistent with this assumption, evaluation of tissue fibrosis markers revealed significantly enhanced collagen deposition, increased

number of α-SMA-positive myofibroblasts and significantly elevated mRNA expression of collagen I and α-SMA in the liver from PS-TG/CCl_4 compared to WT/CCl_4 mice. However, an *in vitro* study showed that protein S is unable to directly stimulate the secretion of fibrotic factors from primary human hepatic stellate cells. Overall, these observations suggest that high expression of hPS may promote chronic liver injury and fibrosis by prolonging the survival of activated hepatic stellate cells.

There are reports showing reduced circulating levels of protein S in patients with advanced liver fibrosis but there are also studies reporting no significant difference compared to healthy controls.[21-23] This discrepancy may be due to differences in disease stage of the patients. In this regard, a recent study performed in patients with different model for end-stage liver disease (MELD) scores showed that the activity of protein S relative to the international normalized ratio increases in parallel to the MELD scores.[24] To date no published work has shown the sequential changes of plasma protein S during the transition from hepatitis to liver fibrosis.[25] Here we evaluated sequential changes in the plasma PS levels after CCl4 injection and demonstrated that it increases in this model of chronic liver injury. Extrahepatic source of protein S may also explain the increased circulating level of protein S during liver injury. Lymphocytes, megakaryocytes, osteoblasts, cardiac muscles, vascular endothelial cells, lung and kidney epithelial cells can synthesize and secrete protein S.[15, 26-30]

To confirm the harmful effect of hPS, mice receiving several injections of CCl_4 were treated with exogenous hPS or saline alone for the same period of time and the results were compared. Liver damage and inflammation was significantly worse in mice treated with exogenous hPS than mice treated with saline, recapitulating the effect observed in mice with PS overexpression. In addition, we compared the number of hepatic stellate

cells in the liver between mice treated with exogenous PS and mice treated with saline and found that exogenous administration of hPS enhances the number of hepatic stellate cells in mice with liver injury. These results suggest that hPS can promote liver fibrogenesis by favoring the survival of hepatic stellate cells during liver injury.

In a previous study, we demonstrated that overexpression of PS exacerbates the inflammatory response in acute alcoholic hepatitis.[12] To clarify whether overexpression of hPS worsens the acute inflammatory response caused by CCl_4, we administered one intraperitoneal dose of CCl_4 to hPS-TG or WT and sacrificed them after 24h to make comparative analysis. hPS-TG mice receiving CCl_4 showed significantly higher plasma levels of liver enzymes and higher mRNA expression of inflammatory cytokines and chemokines than their WT counterparts, suggesting the injurious role of hPS in CCl_4-induced acute liver injury. Interestingly, there was significantly increased number of T cells and hepatic stellate cells and significantly increased activated natural killer (NK) T cells with significantly decreased apoptotic NKT cells in hPS-TG/CCl_4 mice compared to WT/CCl_4 mice, suggesting the participation of NKT cells and pro-fibrotic hepatic stellate cells in the chronic damage and fibrosis of the liver.

In summary, the results of this study suggest that hPS worsens chronic inflammation and tissue fibrosis in the liver.

References

[1] Marengo A, Rosso C, Bugianesi E: Liver Cancer: Connections with Obesity, Fatty Liver, and Cirrhosis. Annu Rev Med 2016, 67:103-17.

[2] Ge PS, Runyon BA: Treatment of Patients with Cirrhosis. N Engl J Med 2016, 375:2104-5.

[3] Griffin JH, Zlokovic BV, Mosnier LO: Protein C anticoagulant and cytoprotective pathways. Int J

Hematol 2012, 95:333-45.
[4] Hafizi S, Dahlback B: Gas6 and protein S. Vitamin K-dependent ligands for the Axl receptor tyrosine kinase subfamily. FEBS J 2006, 273:5231-44.
[5] Nguyen KQ, Tsou WI, Kotenko S, Birge RB: TAM receptors in apoptotic cell clearance, autoimmunity, and cancer. Autoimmunity 2013, 46:294-7.
[6] Rothlin CV, Ghosh S, Zuniga EI, Oldstone MB, Lemke G: TAM receptors are pleiotropic inhibitors of the innate immune response. Cell 2007, 131:1124-36.
[7] Suleiman L, Negrier C, Boukerche H: Protein S: A multifunctional anticoagulant vitamin K-dependent protein at the crossroads of coagulation, inflammation, angiogenesis, and cancer. Crit Rev Oncol Hematol 2013, 88:637-54.
[8] Takagi T, Taguchi O, Aoki S, Toda M, Yamaguchi A, Fujimoto H, Boveda-Ruiz D, Gil-Bernabe P, Ramirez AY, Naito M, Yano Y, D'Alessandro-Gabazza CN, Fujiwara A, Takei Y, Morser J, Gabazza EC: Direct effects of protein S in ameliorating acute lung injury. J Thromb Haemost 2009, 7:2053-63.
[9] Zhong Z, Wang Y, Guo H, Sagare A, Fernandez JA, Bell RD, Barrett TM, Griffin JH, Freeman RS, Zlokovic BV: Protein S protects neurons from excitotoxic injury by activating the TAM receptor Tyro3-phosphatidylinositol 3-kinase-Akt pathway through its sex hormone-binding globulin-like region. J Neurosci 2010, 30:15521-34.
[10] Urawa M, Kobayashi T, D'Alessandro-Gabazza CN, Fujimoto H, Toda M, Roeen Z, Hinneh JA, Yasuma T, Takei Y, Taguchi O, Gabazza EC: Protein S is protective in pulmonary fibrosis. J Thromb Haemost 2016, 14:1588-99.
[11] Yasuma T, Yano Y, D'Alessandro-Gabazza CN, Toda M, Gil-Bernabe P, Kobayashi T, Nishihama K, Hinneh JA, Mifuji-Moroka R, Roeen Z, Morser J, Cann I, Motoh I, Takei Y, Gabazza EC: Amelioration of Diabetes by Protein S. Diabetes 2016, 65:1940-51.
[12] Chelakkot-Govindalayathil AL, Mifuji-Moroka R, D'Alessandro-Gabazza CN, Toda M, Matsuda Y, Gil-Bernabe P, Roeen Z, Yasuma T, Yano Y, Gabazza EC, Iwasa M, Takei Y: Protein S exacerbates alcoholic hepatitis by stimulating liver natural killer T cells. J Thromb Haemost 2015, 13:142-54.
[13] Hammerich L, Bangen JM, Govaere O, Zimmermann HW, Gassler N, Huss S, Liedtke C, Prinz I, Lira SA, Luedde T, Roskams T, Trautwein C, Heymann F, Tacke F: Chemokine receptor CCR6-dependent accumulation of gammadelta T cells in injured liver restricts hepatic inflammation and fibrosis. Hepatology 2014, 59:630-42.
[14] Anderson HA, Maylock CA, Williams JA, Paweletz CP, Shu H, Shacter E: Serum-derived protein S binds to phosphatidylserine and stimulates the phagocytosis of apoptotic cells. Nat Immunol 2003, 4:87-91.
[15] Carrera Silva EA, Chan PY, Joannas L, Errasti AE, Gagliani N, Bosurgi L, Jabbour M, Perry A, Smith-Chakmakova F, Mucida D, Cheroutre H, Burstyn-Cohen T, Leighton JA, Lemke G, Ghosh S, Rothlin CV: T cell-derived protein S engages TAM receptor signaling in dendritic cells to control the

magnitude of the immune response. Immunity 2013, 39:160-70.
[16] Giri TK, Linse S, Garcia de Frutos P, Yamazaki T, Villoutreix BO, Dahlback B: Structural requirements of anticoagulant protein S for its binding to the complement regulator C4b-binding protein. J Biol Chem 2002, 277:15099-106.
[17] Webb JH, Blom AM, Dahlback B: Vitamin K-dependent protein S localizing complement regulator C4b-binding protein to the surface of apoptotic cells. J Immunol 2002, 169:2580-6.
[18] Linger RM, Keating AK, Earp HS, Graham DK: TAM receptor tyrosine kinases: biologic functions, signaling, and potential therapeutic targeting in human cancer. Adv Cancer Res 2008, 100:35-83.
[19] Hinz B, Phan SH, Thannickal VJ, Prunotto M, Desmouliere A, Varga J, De Wever O, Mareel M, Gabbiani G: Recent developments in myofibroblast biology: paradigms for connective tissue remodeling. Am J Pathol 2012, 180:1340-55.
[20] Vilaseca M, Garcia-Caldero H, Lafoz E, Garcia-Irigoyen O, Avila MA, Reverter JC, Bosch J, Hernandez-Gea V, Gracia-Sancho J, Garcia-Pagan JC: The anticoagulant rivaroxaban lowers portal hypertension in cirrhotic rats mainly by deactivating hepatic stellate cells. Hepatology 2017, 65:2031-44.
[21] Baccouche H, Labidi A, Fekih M, Mahjoub S, Kaabi H, Hmida S, Filali A, Romdhane NB: Haemostatic balance in cirrhosis. Blood Coagul Fibrinolysis 2017, 28:139-44.
[22] Krachmalnicoff A, Tombesi S, Valsecchi C, Albertini A, Mannucci PM: A monoclonal antibody to human protein S used as the capture antibody for measuring total protein S by enzyme immunoassay. Clin Chem 1990, 36:43-6.
[23] Lisman T, Caldwell SH, Burroughs AK, Northup PG, Senzolo M, Stravitz RT, Tripodi A, Trotter JF, Valla DC, Porte RJ, Coagulation in Liver Disease Study G: Hemostasis and thrombosis in patients with liver disease: the ups and downs. J Hepatol 2010, 53:362-71.
[24] Tischendorf M, Miesbach W, Chattah U, Chattah Z, Maier S, Welsch C, Zeuzem S, Lange CM: Differential Kinetics of Coagulation Factors and Natural Anticoagulants in Patients with Liver Cirrhosis: Potential Clinical Implications. PLoS One 2016, 11:e0155337.
[25] Kopec AK, Joshi N, Luyendyk JP: Role of hemostatic factors in hepatic injury and disease: animal models de-liver. J Thromb Haemost 2016, 14:1337-49.
[26] Hataji O, Taguchi O, Gabazza EC, Yuda H, Fujimoto H, Suzuki K, Adachi Y: Activation of protein C pathway in the airways. Lung 2002, 180:47-59.
[27] Jamison CS, McDowell SA, Marlar RA, Degen SJ: Developmental expression of protein C and protein S in the rat. Thromb Res 1995, 78:407-19.
[28] Kanthou C, Benzakour O: Cellular effects and signalling pathways activated by the anti-coagulant factor, protein S, in vascular cells protein S cellular effects. Adv Exp Med Biol 2000, 476:155-66.
[29] Maillard C, Berruyer M, Serre CM, Dechavanne M, Delmas PD: Protein-S, a vitamin K-dependent protein, is a bone matrix component synthesized and secreted by osteoblasts. Endocrinology 1992, 130:1599-604.

[30] Shimokawa T, Yamamoto K, Yamafuji E, Kojima T, Saito H: Expression of protein S in the murine heart and cultured mouse cardiomyocytes is down-regulated by cytokines. Thromb Haemost 2001, 86:623-9.

Figure legends

Figure 1. Human protein S (hPS) overexpression predisposes to chronic liver injury and attenuates apoptosis of hepatic stellate cells. Wild type (WT) and hPS transgenic (TG) mice received CCl_4 or oil injection twice a week for 8 weeks. (A) PS, alanine aminotransaminase (ALT) and aspartate aminotransferase (AST) enzyme activities were measured as described under Materials and methods. mRNA expression of tumor necrosis factor-(TNF)α and chemokine (C-C motif) ligand 2 (CCL2) was evaluated by RT-PCR. Significantly increased liver enzymes and mRNA relative levels of TNF-α and CCL2 in hPS-TG/CCl_4 compared to WT counterparts. (B) H&E staining of mouse livers. Scale bars indicate 50 μm. Increased fatty degeneration and cell infiltration (arrow) were observed in hPS-TG/CCl_4 mice compared WT/CCl_4 mice. N=4 in WT/OIL, n=5 in hPS/OIL, and n=7 in both WT/CCl_4 and hPS/CCl_4 groups. (C) Increased area of cells with DNA fragmentation assessed by TUNEL method in hPS-TG/CCl_4 mice and representative images of areas with DNA breakage (arrow). (D) Western blotting showed reduced caspase-3 activation in hPS-TG/CCl_4 mice. (E) Flow cytometry analysis shows hPS-mediated inhibition of apoptosis of primary human hepatic stellate cells. (F) Western blotting shows enhanced Akt activation by hPS in starved (FBS [-]) LX-2 hepatic stellate cell lines. Data as expressed as the mean ± SEM. FBS, fetal bovine serum. *$p<0.05$ versus WT/OIL; †$p<0.05$ versus WT/CCl_4; ‡$p<0.05$ versus hPS(-)/FBS(-).

Figure 2. Mice overexpressing human protein S (hPS) are prone to liver fibrosis. Wild type (WT) and hPS transgenic (TG) mice received CCl_4 or oil injection twice a week for 8 weeks. (A) Masson's trichrome stain of the liver and quantification by WindRoof. Scale bars indicate 50 μm. Increased staining of collagen (black arrows) in the liver from hPS-TG mice compared to wild type mice. (B) Immunohistochemical staining with antibody against α-smooth muscle actin (α-SMA) and quantification of cells per high power field (HPF). Increased staining of α-SMA (+) cells (white arrows) in the liver from hPS-TG mice compared to wild type mice. (C) mRNA expression of α-SMA and collagen I was measured by RT-PCR. Increased expression of α-SMA and Col1a1 in the liver from PS TG mice compared to wild type mice. Data as expressed as the mean ± SEM. N=3 in WT/OIL, n=3 in hPS/OIL, and n=5 in both WT/CCl_4 and hPS/CCl_4 groups. *$p<0.05$ versus WT/OIL; †$p<0.05$ versus WT/CCl_4.

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Figure 3. Administration of exogenous human protein S (hPS) is associated with exacerbation of chronic liver injury and increased number of hepatic stellate cells. Wild type (WT) mice received CCl_4 or saline injection twice a week and hPS or saline through subcutaneous minipumps for 8 weeks. (A) H&E staining of mouse livers. Scale bars indicate 50 μm. Alanine aminotransaminase (ALT) and asparate aminotransferase (AST) enzyme activities were measured using commercial kits. Increased fatty degeneration, cell infiltration (arrow) and enhanced plasma liver enzyme liver in mice receiving human PS. (B) Staining of α-smooth muscle actin (SMA), nuclei and of DNA fragments by TUNEL. Increased number of α-SMA (+) cells in mice treated with hPS.

N=6 in WT/OIL, n=5 in WT/CCl4 + SAL, and n=6 in WT/CCl4+Exo-hPS group. Data as expressed as the mean ± SEM. *p<0.05 versus WT/OIL; †p<0.05 versus WT/CCl_4.

Figure 4. Overexpression of hPS worsens acute liver injury. Wild type (WT) and human PS transgenic (TG) mice received one intraperitoneal injection of CCl_4 or oil and sacrificed after 24h. The plasma levels of liver enzymes, and the mRNA expression of tumor necrosis factor (TNF)-α, IL-17, chemokine (C-C motif) ligand 2 (CCL2) and IL-6 were significantly enhanced in mice overexpressing PS. Data as expressed as the mean ± SEM. N=3 in WT/OIL and hPS/OIL groups, n=4 in WT/CCl_4 and hPS/CCl_4 groups. *p<0.05 versus WT/OIL; †p<0.05 versus WT/CCl_4.

Figure 1

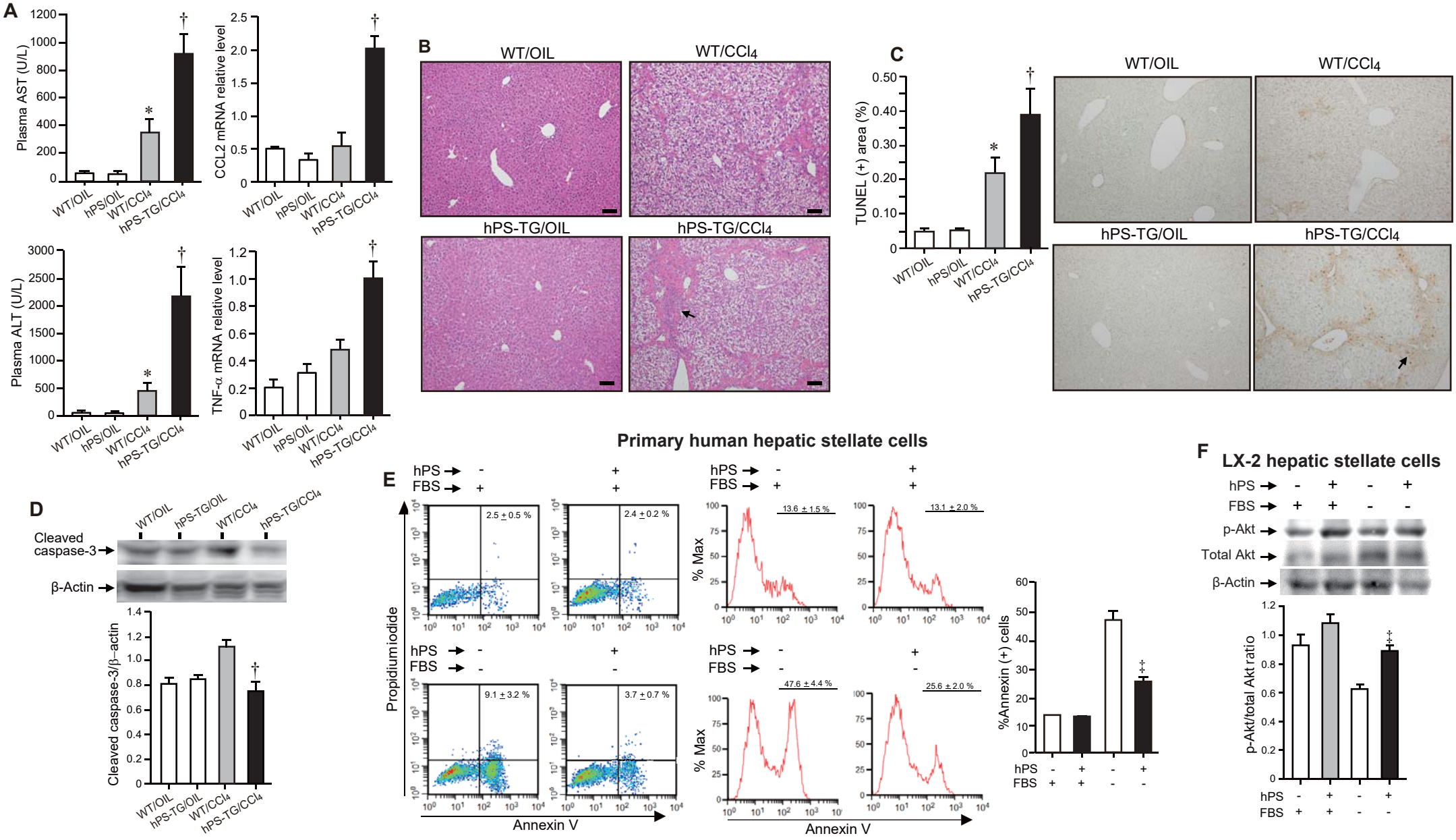

A
Plasma AST (U/L)
CCL2 mRNA relative level
Plasma ALT (U/L)
TNF-α mRNA relative level
WT/OIL
hPS/OIL
WT/CCl4
hPS-TG/CCl4
B
WT/OIL
WT/CCl4
hPS-TG/OIL
hPS-TG/CCl4
C
TUNEL (+) area (%)
WT/OIL
WT/CCl4
hPS-TG/OIL
hPS-TG/CCl4
D
Cleaved caspase-3
β-Actin
Cleaved caspase-3/β-actin
WT/OIL
hPS-TG/OIL
WT/CCl4
hPS-TG/CCl4
Primary human hepatic stellate cells
E
hPS
FBS
2.5 ± 0.5 %
2.4 ± 0.2 %
9.1 ± 3.2 %
3.7 ± 0.7 %
Propidium iodide
Annexin V
% Max
13.6 ± 1.5 %
13.1 ± 2.0 %
47.6 ± 4.4 %
25.6 ± 2.0 %
%Annexin (+) cells
F
LX-2 hepatic stellate cells
hPS
FBS
p-Akt
Total Akt
β-Actin
p-Akt/total Akt ratio

Figure 2

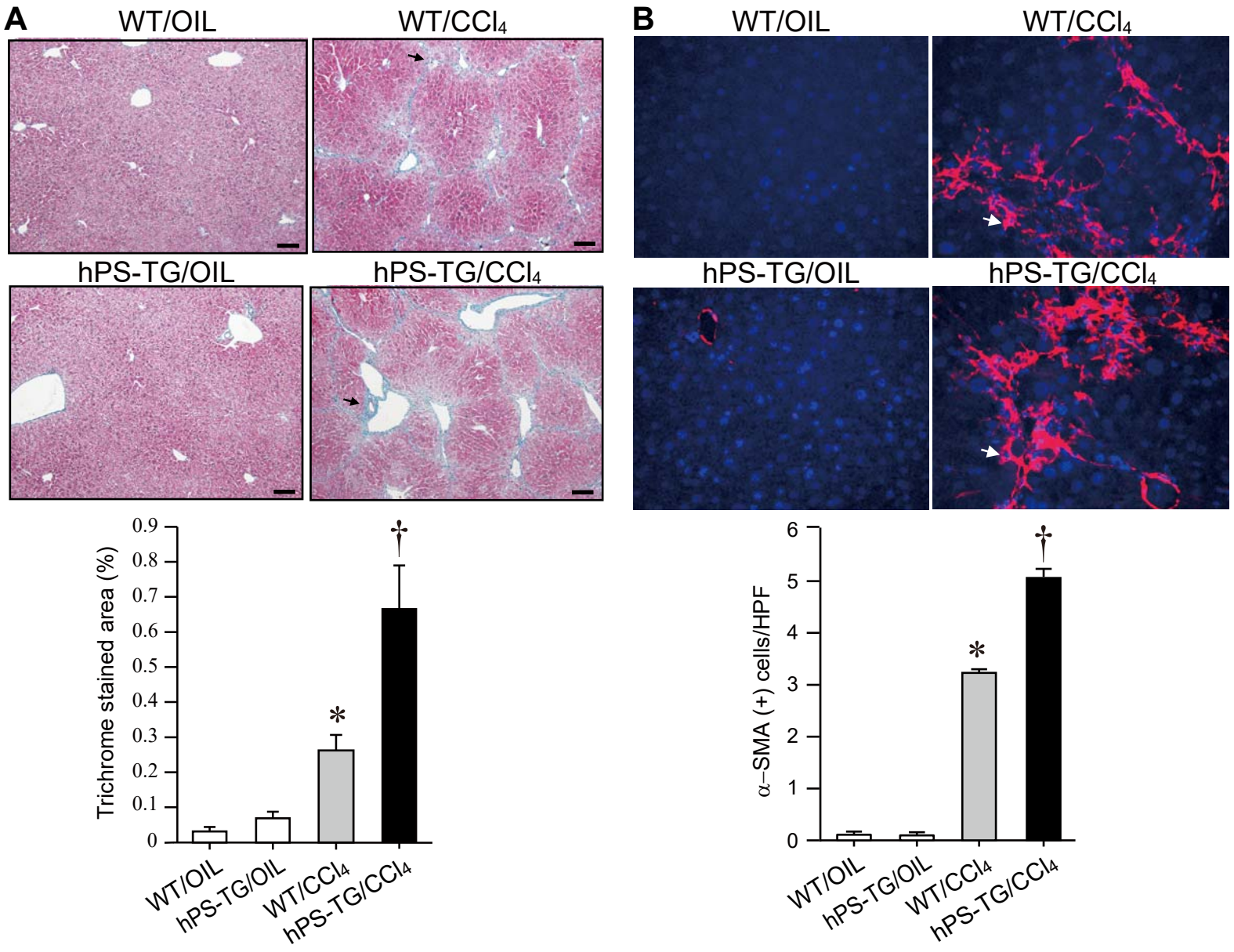

A
WT/OIL
WT/CCl4
hPS-TG/OIL
hPS-TG/CCl4
Trichrome stained area (%)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
*
†
WT/OIL
hPS-TG/OIL
WT/CCl4
hPS-TG/CCl4
B
WT/OIL
WT/CCl4
hPS-TG/OIL
hPS-TG/CCl4
α–SMA (+) cells/HPF
0
1
2
3
4
5
6
*
†
WT/OIL
hPS-TG/OIL
WT/CCl4
hPS-TG/CCl4
C
Col1a1 mRNA relative level
0
50
100
150
200
*
†
WT/OIL
hPS-TG/OIL
WT/CCl4
hPS-TG/CCl4
α–SMA mRNA relative level
0
0.2
0.4
0.6
0.8
1.0
*
†
WT/OIL
hPS-TG/OIL
WT/CCl4
hPS-TG/CCl4

Figure 3

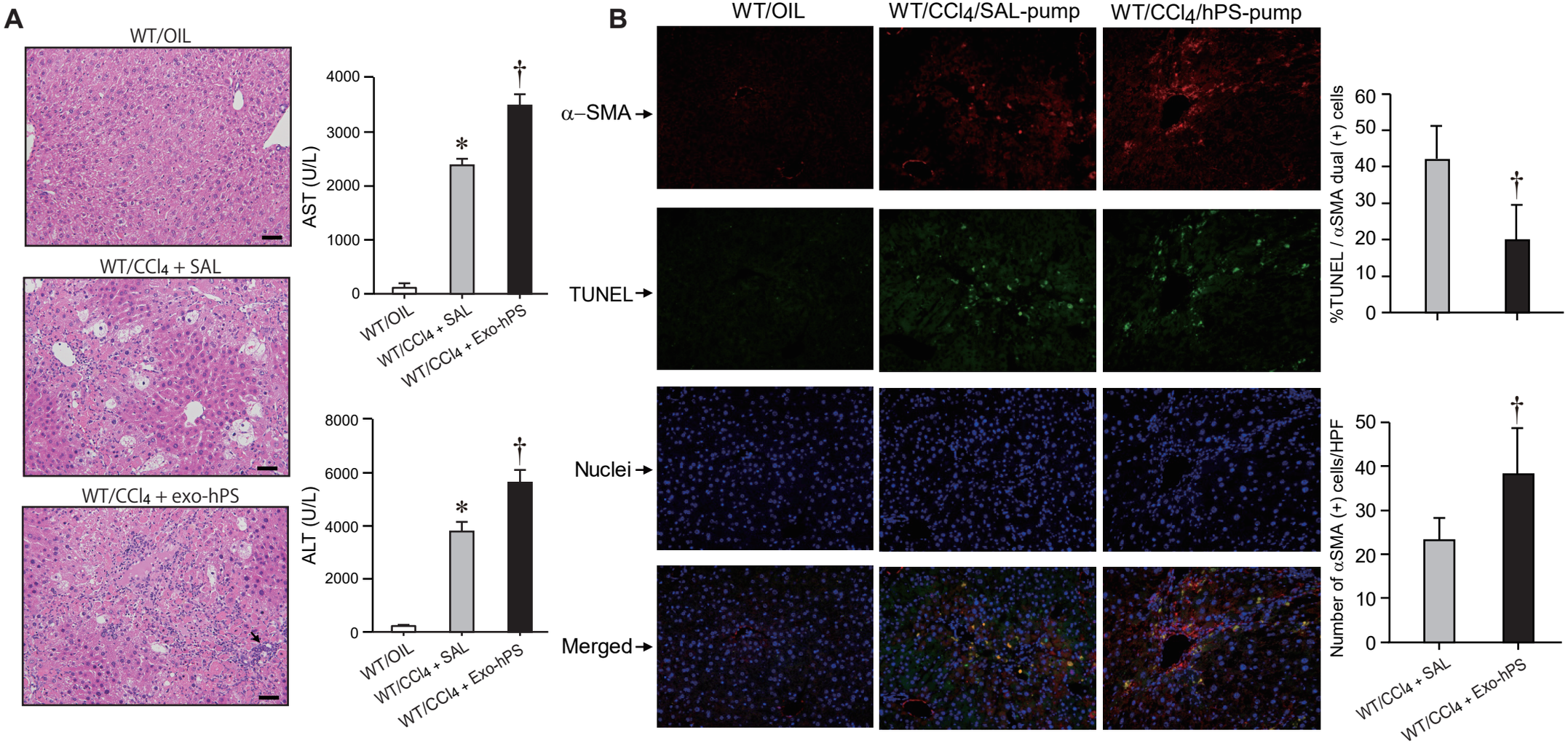
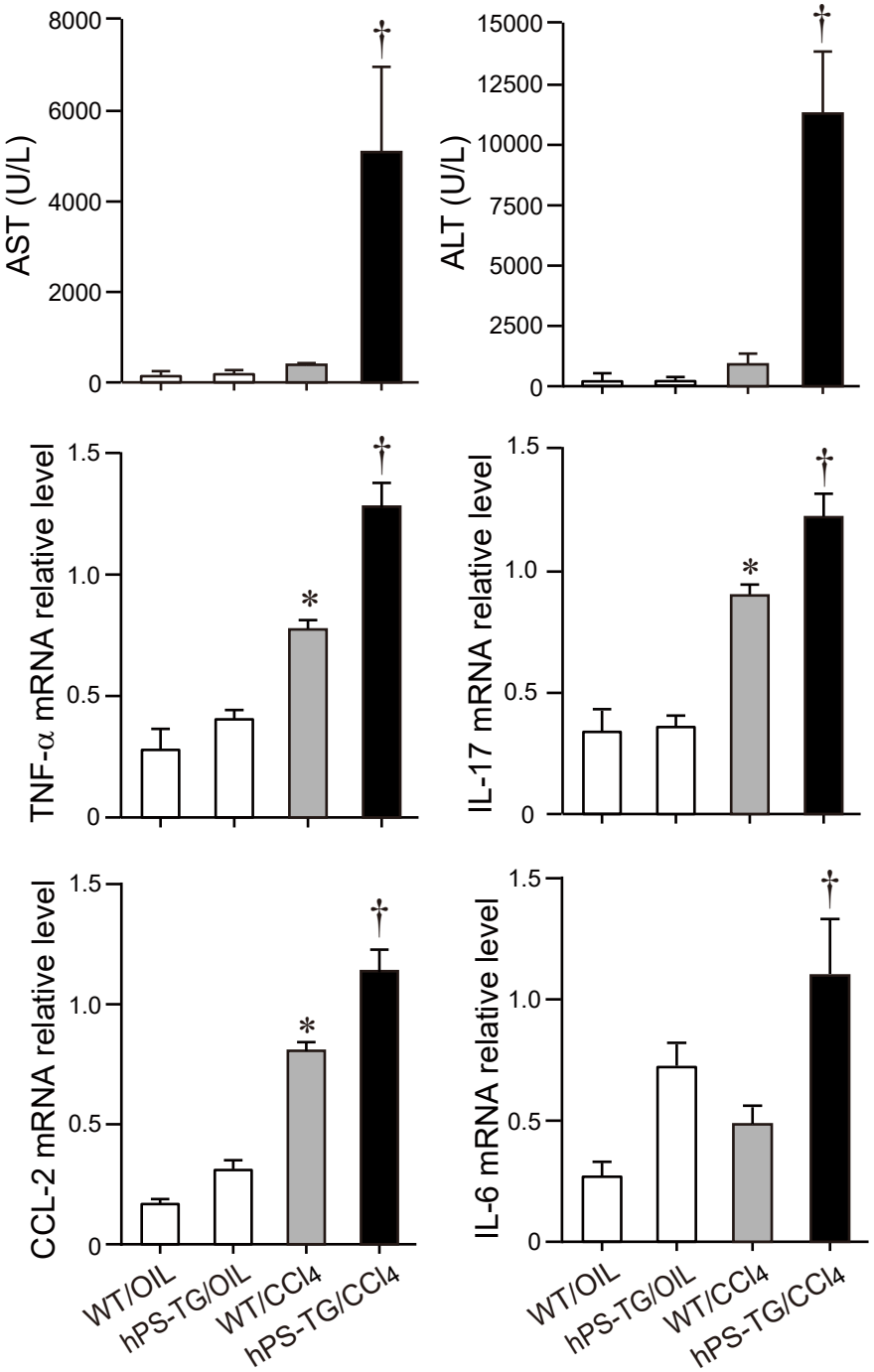
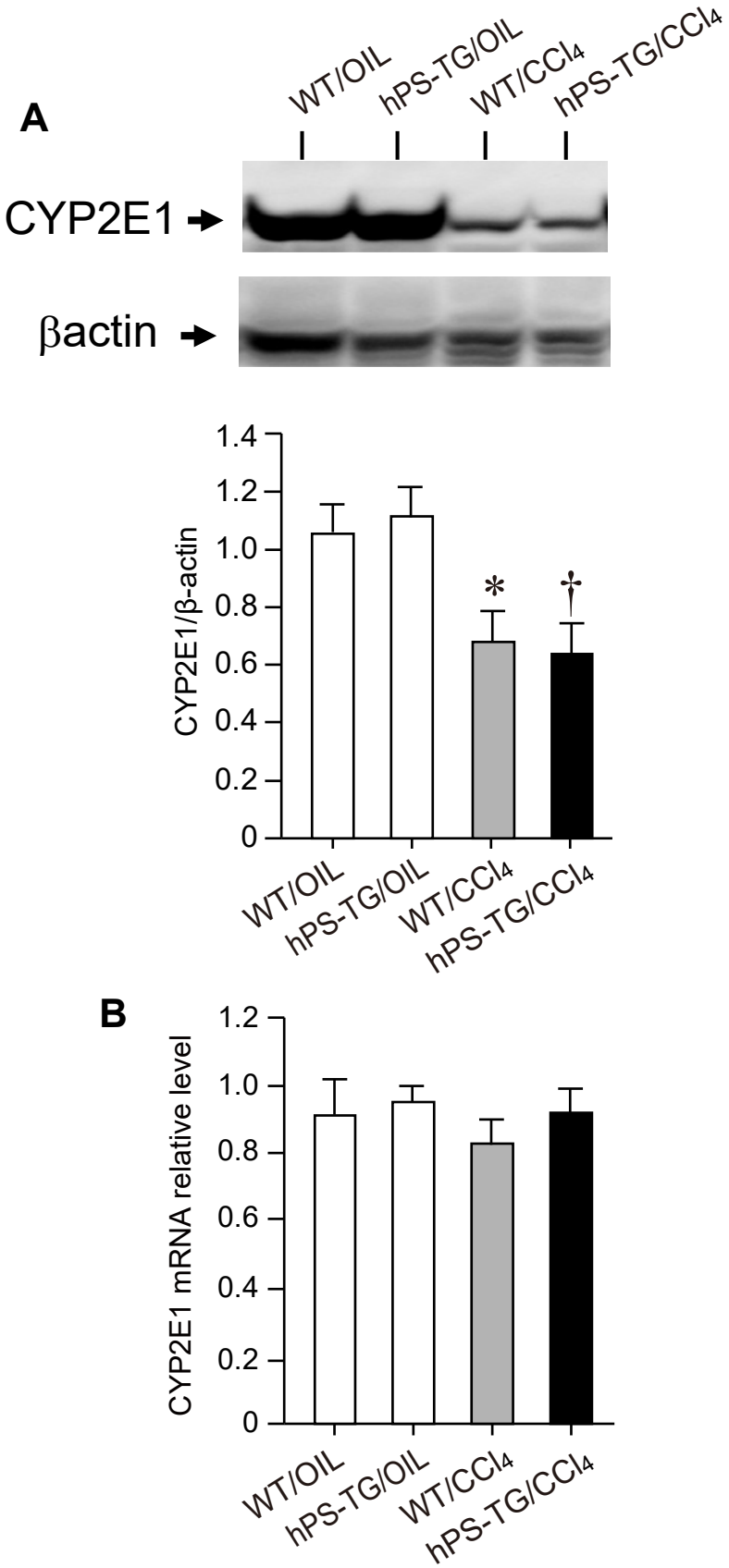

A
WT/OIL
WT/CCl4 + SAL
WT/CCl4 + exo-hPS
AST (U/L)
4000
3000
2000
1000
0
*
†
WT/OIL
WT/CCl4 + SAL
WT/CCl4 + Exo-hPS
ALT (U/L)
8000
6000
4000
2000
0
*
†
WT/OIL
WT/CCl4 + SAL
WT/CCl4 + Exo-hPS
B
WT/OIL
WT/CCl4/SAL-pump
WT/CCl4/hPS-pump
α–SMA
TUNEL
Nuclei
Merged
%TUNEL / αSMA dual (+) cells
60
50
40
30
20
10
0
†
Number of αSMA (+) cells/HPF
50
40
30
20
10
0
†
WT/CCl4 + SAL
WT/CCl4 + Exo-hPS

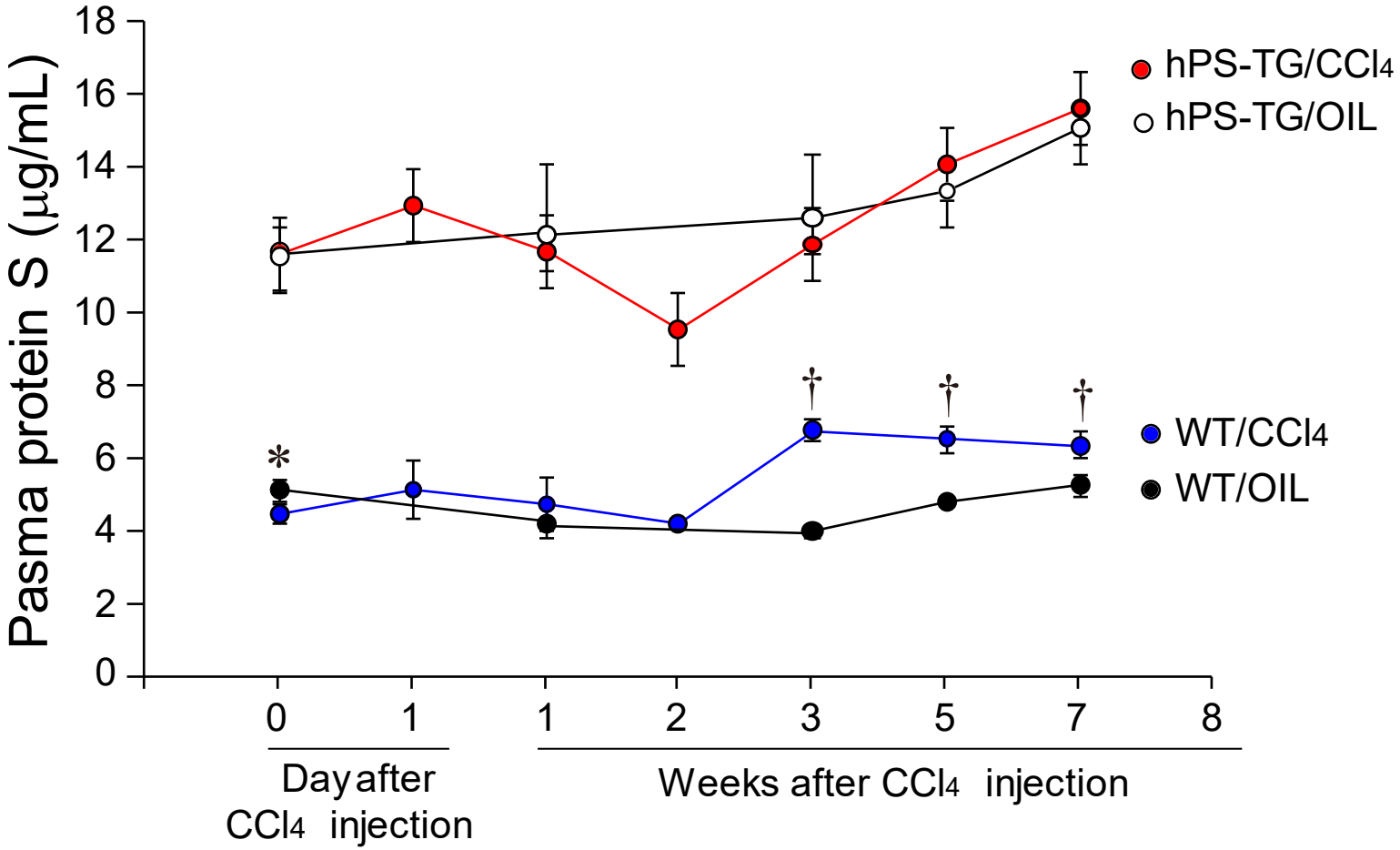
Figure 4


AST (U/L)
8000
6000
4000
2000
0
†
ALT (U/L)
15000
12500
10000
7500
5000
2500
0
†
TNF-α mRNA relative level
1.5
1.0
0.5
0
*
†
IL-17 mRNA relative level
1.5
1.0
0.5
0
*
†
CCL-2 mRNA relative level
1.5
1.0
0.5
0
*
†
IL-6 mRNA relative level
1.5
1.0
0.5
0
†
WT/OIL
hPS-TG/OIL
WT/CCl4
hPS-TG/CCl4

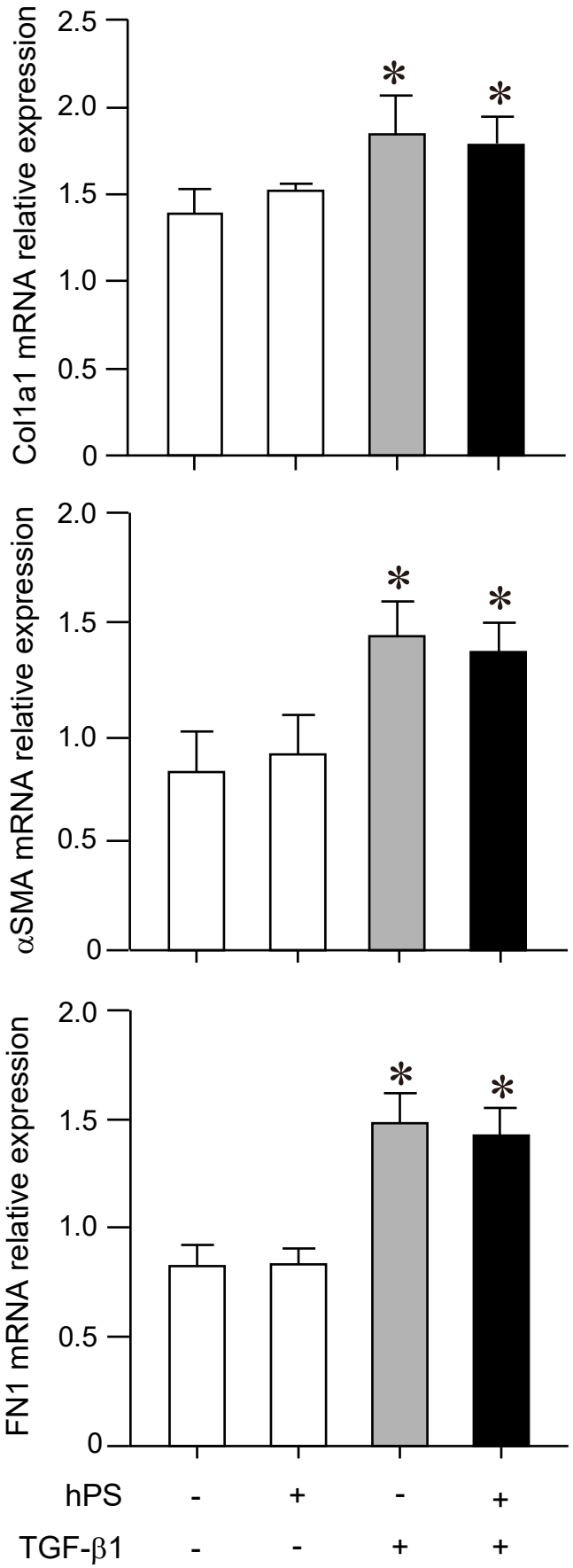
Supplementay Figure 1



Supplementary Figure 2



Supplementary Figure 3



Legends of Supplementary Figures

Supplementary Figure 1. Liver levels of CYP2E1 in wild type (WT) and hPS transgenic (TG) mice. (A) Protein expression of CYP2E1 was evaluated by Western blotting in liver tissues from WT/OIL, WT/CCl_4, hPS/OIL and hPS/CCl_4 groups. Each group with n=3. (B) CYP2E1 mRNA expression was evaluated by reverse-transcription polymerase chain reaction. Expression of CYP2E1 was comparable between WT/CCl_4 and hPS-TG/CCl_4 groups. Data as expressed as the mean ± SEM. *$p<0.05$ versus WT/OIL; †$p<0.01$ versus and hPS-TG/OIL.

Supplementary Figure 2. Sequential changes in the plasma levels of protein S during liver injury. Wild type (WT) and hPS transgenic (TG) mice received repeated intraperitoneal injections of oil or CCl_4 as described under materials and methods, allocated in WT/OIL (n=6), WT/CCl_4 (n=6), hPS/OIL (n=5) and hPS/CCl_4 (n=8) groups and blood sampling was periodically performed at the indicated times. Data as expressed as the mean ± SEM. †$p<0.05$ WT/OIL (black cycle) versus hPS-TG/OIL (white cycle); *$p<0.05$ WT/CCl_4 (blue cycles) versus hPS-TG/CCl_4 (red cycles).

Supplementary Figure 3. Human PS exerts no effect on fibrogenic activity of primary human hepatic stellate cells. Primary human hepatic stellate cells were cultured and stimulated with transforming growth factor (TGF)-β1 (10 ng/ml) in the presence or absence of hPS (20 μg/ml) and the mRNA relative expression of collagen I, α-smooth muscle actin (SMA) and fibronectin-1 was evaluated by reverse-transcription

polymerase chain reaction. Data as expressed as the mean ± SEM. *$p<0.05$ versus hPS (-)/TGF-β1 (-) and hPS (+)/TGF-β1 (-).

Supplementary Table 1. Number of non-parenchymal liver cells

Non-parenchymal liver cells	WT/OIL	hPS-TG/OIL	WT/CCl_4	hPS-TG/CCl_4
Macrophages (%)	3.61 ± 0.27	3.52 ± 0.37	13.89 ± 0.2.28 †	19.24 ± 2.11‡
Dendritic cells (%)	6.23 ± 1.08	6.20 ± 0.41	7.57 ± 1.18	4.86 ± 0.34§
B cells (%)	34.97 ± 1.38	38.45 ± 1.25	34.40 ± 1.31	35.60 ± 1.91
T cells (%)	21.89 ± 1.19	26.01 ± 1.21	19.31 ± 0.53	23.40 ± 1.45§
NK cells (%)	8.60 ± 0.73	7.60 ± 0.35	13.37 ± 0.69†	9.60 ± 0.97§
Total NKT cells (%)	11.90 ± 1.57	9.05 ± 0.89	6.86 ± 0.33†	8.09 ± 0.82
Activated NKT cells (%)*	11.13 ± 2.64	11.48 ± 1.70	16.84 ± 0.79	24.45 ± 2.79§‡
Apoptotic NKT cells (%)*	11.44 ± 5.01	13.36 ± 1.74	31.48 ±2.25†	24.94 ± 3.47§‡
Hepatic stellate cells (%)**	2.09 ± 0.31	1.86 ± 0.08	4.94 ± 0.40	5.72 ± 0.99§

Data are expressed as the mean percentage of the total number of non-parenchymal liver cells ± standard error of the means unless otherwise specified. *Data expressed as the percentage of total number of liver natural killer (NK) T cells. **Hepatic stellate cells were gated as α-smooth muscle actin (+) cells. N=3 in WT/OIL and hPS/OIL groups, and n=4 in WT/CCl4 and hPS/CCl4 groups. WT, wild type; hPS-TG, human protein S transgenic mouse; CCl_4, carbon tetrachloride. § $p<0.05$ versus WT/CCl_4; ‡$p<0.05$ versus hPS-TG/OIL; †$p<0.05$ versus WT/OIL