Supplemental Materials

Platelet TGF-β1 deficiency decreases liver fibrosis in a mouse model of liver injury

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Supplemental Methods:

Materials

Hamster anti-mouse GP1bα mAb (R300) and an isotype-matched hamster IgG (R301) were obtained from Emfret Analytics, Germany. Sources and dilutions of all immunofluorescence staining antibodies are listed in Supplemental Table 1. All other reagents related to TGF-β1 assays were purchased from sources previously reported.[1, 2] Carbon tetrachloride (CCl_4) and mineral oil (oil) and other chemicals were obtained from Sigma-Aldrich and Thermo Fisher Scientific.

Preparation of mouse plasma and liver exudates

Plasma preparation. Mouse platelets and plasma were prepared from blood drawn using our previously described method to minimize in vitro platelet activation.[2] Briefly, blood was drawn from anesthetized mice by retrobulbar (RB) puncture and placed in a polypropylene tube containing a 0.1 volume of 3.8% sodium citrate, pH 7.4. Plasma was prepared by centrifuging the samples at 12,000*g* for 5 minutes at room temperature) immediately after the blood was drawn. ProtaglandinE_1 (PGE_1; 1 μM final concentration; Cayman Chemical) was added to the blood

collection tubes to prevent the release of platelet granule contents during blood drawing or plasma preparation.

Liver exudate preparation. Acute liver injury was induced by injecting CCl_4, as described above. Mice were sacrificed before, 6 hours, 1 day, and 3 days after the 1st CCl_4 challenge, and whole livers were harvested immediately, washed in cold PBS, and placed on a plastic weighing tray on ice. A total of 200 mg of liver tissue was minced with a surgical blade; incubated in 200 µl of HBMT buffer (10 mM HEPES, 0.137 M NaCl, 12 mM $NaHCO_3$, 2.7 mM KCl, 0.36 mM $Na_2H_2PO_4$) containing 0.35% BSA, pH 7.4, on ice for 1 hour; and then centrifuged at 14,000*g* for 20 minutes at 4°C. The supernatants were separated as liver exudates and stored at -80°C until analysis. Total and active TGF-β1 levels were measured in liver exudates using the ELISA protocols described below.

Induction of thrombocytopenia in mice. On day 0 or day 18, C57Bl/6 mice were injected i.p. with 0.25 mg/kg BW of anti-GP1bα mAb (R300) or an isotype-matched IgG (R301) 6 hours before the subsequent CCl_4 injection (as shown in Figure 3A and Supplemental Figure 4C). Blood samples were obtained 1 day after the antibody injection using the RB method, and platelet numbers were counted with a Hemavet system.

Measurement of TGF-β1

Total TGF-β1 in platelets, liver exudates, and plasma was measured after conversion of latent TGF-β1 to active TGF-β1 by acidification (20-minute incubation at room temperature with a 0.5 volume of 1 N HCl for plasma and serum and a 0.2 volume of 1 N HCl for platelet lysates and exudates, followed by neutralization via addition of the same volume of 1.2 N NaOH in 0.5 M HEPES). Measurements were made using a 2-antibody ELISA Duo-assay system specific for the activated form of TGF-β1 (#DY240; R&D Systems). For active TGF-β1 measurements, samples were assayed without the acidification step.

Flow cytometry of liver cells

Liver sections obtained before, 6 hours after, and 1 day after the first CCl_4 challenge were washed in cold PBS and then placed on a 70-mm mesh filter in 200 µl of HBMT buffer. Cells were gently dissociated using a syringe plunger and washed with HBMT buffer containing PGE1. Cells were fixed with BD-Fixation buffer and stained with fluorescent-labelled anti-CD41 (48-0411-80, Affymetrix Inc.) and CD62P (46-0626-80, Affymetrix Inc.), as well as Jon/A-PE (D200, Emfret Analytics). Cells were counted using the LSR-II flow cytometer (BD Biosciences) and data analysis was performed using FlowJo software.

Tissue and section preparation

Animals were sacrificed before (Control), 6 hours, 1 day, 3 days, and 36 days after CCl_4 or oil challenges, perfused, and liver lobes (the left lateral lobe was primarily used to achieve consistent results, but other lobes were also compared) were excised and divided into 4 parts, 1 of which was fixed in 4% paraformaldehyde and embedded in OCT for cryosections immunostaining. A second part was also fixed in 4% paraformaldehyde but was embedded in paraffin for evaluation of gene expression by *in situ* hybridization staining. The two remaining parts (without fixative) were quickly washed with ice-cold PBS, immediately snap-frozen in separate tubes, and kept at -80°C for various analyses, including hydroxyproline, qRT-PCR, and liver exudate preparation. Paraffin- or OCT-embedded liver lobes were coded and organized from different genotype animals in the same block for blinded study. Liver fibrosis was evaluated histologically by staining with picrosirius red or Masson's trichrome, according to the manufacturer's instructions (IHCWORLD).

Quantification of liver fibrosis

Fibrotic areas in the livers were determined by picrosirius staining, which stains collagen bundles red; under polarized light, however, the bundles appear as a mixture of green, red and yellow fluorescent colors, which are more clearly differentiated from the background and are therefore easy to quantify. Whole liver sections (4-µm thick) were stained with picrosirius red, and whole slide images containing multiple liver sections from different animals were scanned using

an Aperio Digital Pathology Slide Scanner (Leica Biosystems) at 20x magnification to cover whole sections of liver. Whole liver sections were also imaged under polarized light at high resolution with 20 and 40x magnification, and the images were scanned/tiled using the Zen Blue program of a Zeiss 710 microscope. Because large blood vessels contain collagen fiber, they were removed in Photoshop or the Paint program (tiled images are shown in Supplemental Figure 1A). Because areas of fibrosis were heterogeneous, scanned/tiled images of whole liver lobes were used to quantify fibrosis. (Representative scanned/tiled pictures are shown in Supplemental Figure 1A.). Picrosirius red-stained liver lobes without large vessels were then quantified using ImageJ software (NIH) with a background setting threshold, such that fibrotic areas were a clearly visible yellow color (Supplemental Figure 1B). The percentage of fibrosis was calculated by dividing the fibrotic area (x) by the total area (y), then multiplying by 100 (Supplemental Figure 1B).

Immunohistochemistry (IHC)

Dual immunohistochemical staining was performed on paraffin- and OCT-embedded liver sections according to standard deparaffinization, hydration, and antigen retrieval procedures (for paraffin-embedded sections). Sections were washed and blocked with PBS containing 0.05% Triton-X-100 and 1% BSA. Slides were then incubated overnight at 4°C with specific primary antibodies against the specific markers in blocking buffer. (Sources, species, and catalog numbers, as well as dilutions of all antibodies, including secondary-conjugated antibodies are listed in Supplemental Table 1.) After washing, the respective species-matched secondary antibodies were tagged with Alexa 488 (for the green channel), Alexa 594 (for the red channel), or both and incubated at room temperature for 2 hours. Sections were washed and mounted in medium containing DAPI to stain the nuclei. Fluorescence images were obtained with a Zeiss 710 confocal or Nikon Eclipse 80i fluorescence microscope.

Hydroxyproline assay

Hydroxyproline proline content in the liver tissues was measured using a commercial hydroxyproline assay kit (Sigma-Aldrich), according to the manufacturer's instructions.

In situ hybridization

In situ hybridization was used to assess gene expression in specific areas of the liver at the indicated time points before and after CCl_4 challenge. The detailed method for *in situ* hybridization was described previously[3]. Primers are listed in Supplemental Table 2.

The following steps were used for *in situ* hybridization:

1) Total RNA was isolated from mouse fibroblast cells using an RNA isolation kit (Macherey-Nagel, Germany). Template cDNA was synthesized using a reverse transcriptase cDNA synthesis kit (Roche, Mannheim).

2) cDNA-specific primers containing a short terminal 5' sequence followed by the T7-RNA-polymerase promoter were designed to synthesize antisense cRNA probes containing digoxigenin- and fluorescein-labeled nucleotides. Briefly, 1 µg of purified cDNA for each gene was mixed with 1X digoxigenin- and fluorescein-labeled nucleotide mix (10X stock, Roche, Mannheim), which was followed by the addition of 1 µl of T7 RNA polymerase (20 unit/µl, Thermo Fisher Scientific) in a 10 µl total volume. The reaction was incubated at 37°C for 2 hours, then the labeled cRNA probes were precipitated and purified on a 1.2% agarose gel with a gel extraction kit (Qiagen).

3) Liver sections were deparaffinized and hydrated according to standard procedures. Sections were permeabilized with proteinase-K (10 µg/ml) for 10 minutes and then incubated with a prehybridization mix (5 ml of formamide; 2.5 ml of 20x SSC, pH 4.5; 100 mg of Boehringer Block; 2 ml of Millipore water; 100 µl of 0.5 M EDTA, pH 8.0; 100 µl of Tween 20 (10%); 100 µl of 10% CHAPS; 4 µl of heparin (50 mg/ml); and 200 µl of tRNA (50 mg/ml)) for 1 hour at 70°C. The cRNA probes were denatured at 95°C for 5 minutes and chilled on ice immediately before being added to tissue sections. Double-staining hybridization was performed simultaneously with a mixture of digoxigenin- and fluorescein-labeled probes, as described previously.[4]

Real-time PCR

Total RNA was extracted from liver tissues before (0 hour), 6 hours, 1 day, 3 days, and 36 days after CCl_4 challenge using a multi-DNA, RNA, and protein purification kit (Macherey-Nagel, Germany). cDNA was prepared from RNA using a reverse transcriptase cDNA synthesis kit (Roche, Mannheim). Real-time PCR was performed with primer sets for mouse *Col1a1*, *PAI-1, Fn1, Alb,* and *Gapdh* with a BIO-RAD real-time PCR system. Thermocycler conditions were 90°C for 5 minutes, followed by 95°C for 15 seconds, 62°C for 30 seconds, and 72°C for 30 seconds. A total of 40 cycles were run. Data were normalized to levels of the endogenous control gene *Gapdh* or to *Alb* and were analyzed using CFX96 Real-time software (BIO-RAD).**Supplemental Tables:**

Supplemental Table 1.

Antibody sources, dilutions, and hosts:

Antibodies	Vendors	Type	Catalog number	Host (sp)
Anti p-Smad2 (phospho-specific)	Millipore	Primary Ab	aB3849	Rabbit
Anti-mouse Vimentin	abcam	Primary Ab	[EPR3776]	Rabbit
Anti-mouse Desmin	abcam	Primary Ab	ab15200	Rabbit
Anti-COL1A1	Sigma-Aldrich	Primary Ab	SAB1402151	Mouse
Anti-Smooth Muscle Actin	Biolegend	Primary Ab	904601	Mouse
$F(ab')_2$ Anti-Rabbit IgG	Jackson Immuno Research	Alexa Fluor 594	711-586-152	Donkey
Anti-Armenian Hamster IgG	Jackson Immuno Research	Alexa Fluor 488	127-545-099	Goat
$F(ab')_2$ Anti-Rat IgG	Jackson Immuno Research	Cy3	712-166-150	Donkey
$F(ab')_2$ Anti-Mouse IgG	Jackson Immuno Research	Alexa Fluor 594	715-586-150	Donkey
$F(ab')_2$ Anti-Mouse IgG	Jackson Immuno Research	Alexa Fluor 488	715-546-150	Donkey
$F(ab')_2$ Anti-goat gG	Jackson Immuno Research	Alexa Fluor 594	705-586-147	Donkey
$F(ab')_2$ Anti-goat gG	Jackson Immuno Research	Alexa Fluor 488	705-546-147	Donkey

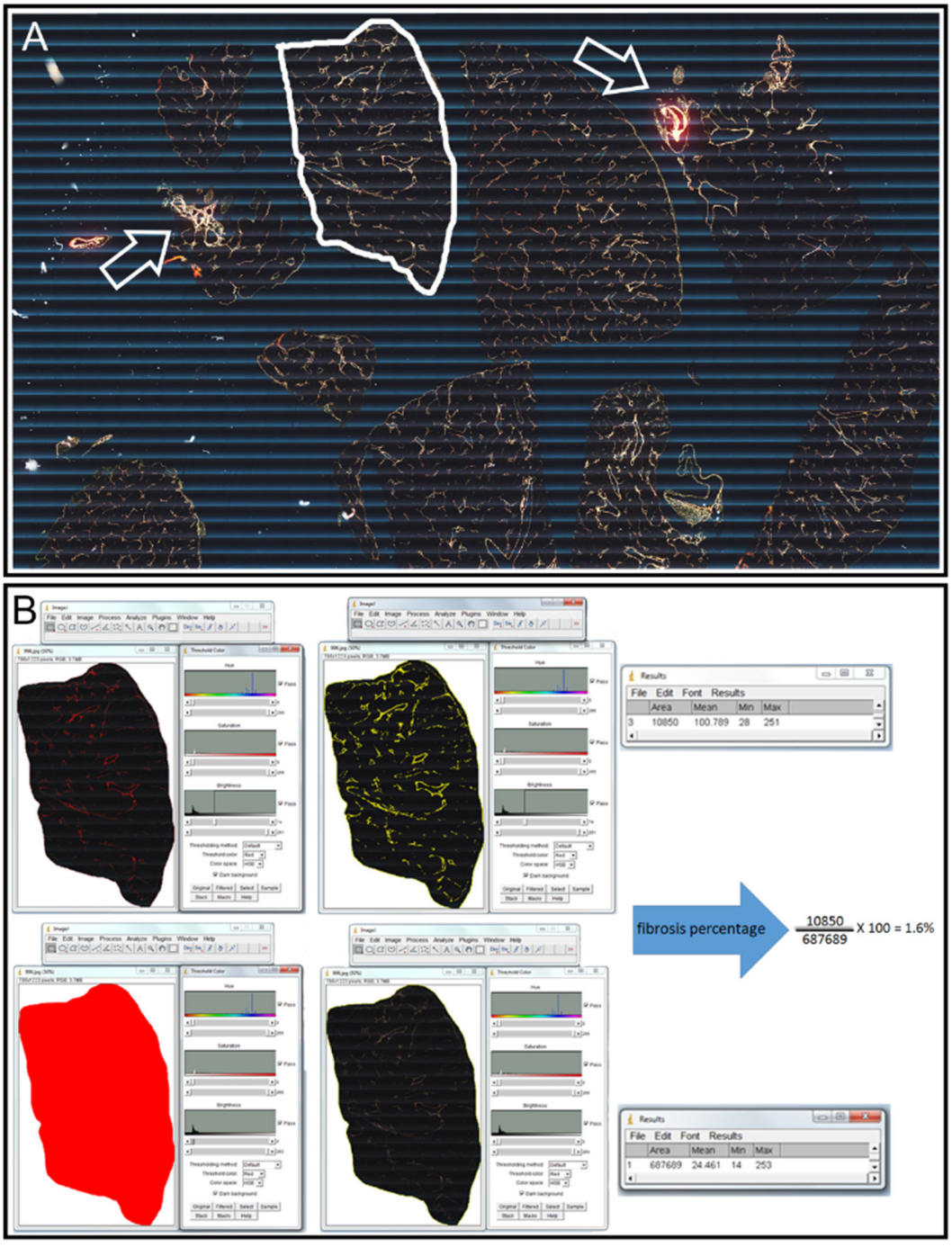
All primary and secondary antibodies were diluted (1:300) in blocking buffer.

Supplemental Table 2.

Primer sequences for RT-PCR and *in situ* hybridization probes

RT-PCR primers			
Genes	NCBI Reference	Primer sequence forward	Primer sequence reverse
Alb	NM_009654.3	GTCTTAGTGAGGTGGAGCATGACAC	GCAAGTCTCAGCAACAGGGATACAG
Gapdh	NM_008084.2	CTTCAACAGCAACTCCCACTCTTCC	GGTTTCTTACTCCTTGGAGGCCATG
Fn1	NM_001276408.1	GCCCTTACAGTTCCAAGTTCCTGGA	CCGTGTAAGGGTCAAAGCATGAGTC
Col1a1	NM_007742.4	AAGGGTAACAGTGGTGAACCTGGTG	AACACCATCAGCACCAGGGAAACCA
***In situ* hybridization primers**			
Genes	NCBI Reference	Primer sequence forward	Primer sequence reverse
Col1a2	NM_007743.2	CTCAGGTACTACTGGAGAAGTTGGC	CCAGATGCACCTGTTTCTCCAGTTC
Alb	NM_009654.3	CCTGCAACACAAAGATGACAACCCC	GGGATCCACTACAGCACTTGGTAAC
Cyp2e1	NM_021282.2	CAAGGAGGTGCTACTGAACCACAAG	GATGACATATCCTCGGAACACGGTG
Gpx4	NM_008162.2	GCTTACTTAAGCCAGCACTGCTGTG	GCTGGTTTTCAGGCAGACCTTCATG

Supplemental Figure-1

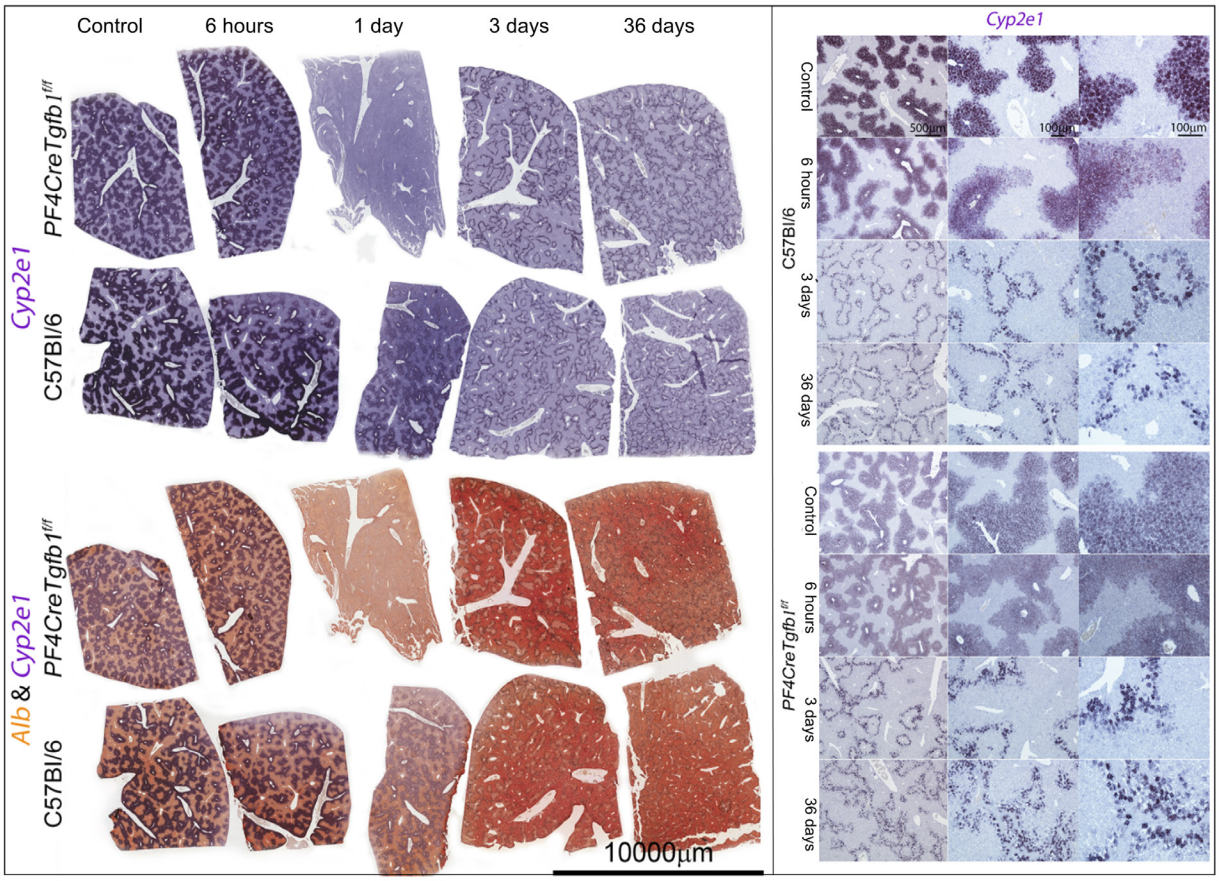


Supplemental Figure 1.

(A) Representative whole liver sections from mice after staining with picrosirius red were scanned using an Apria scanner at 20x magnification. Picrosirius-stained collagen fluorescent images (tiled pictures at 10x that covered whole sections of the liver) were taken with a confocal microscope

under polarized light and showed fluorescent collagen areas with fibrosis. Arrows show basal collagen associated with blood vessels. (B) For quantification of fibrosis, each liver section was carefully examined to identify and remove large vessels associated with collagen staining (examples shown with arrows in A), and set images in the ImageJ program (NIH) showing adjustments were used to calculate the percentage of fibrotic area within the total area of each liver section.

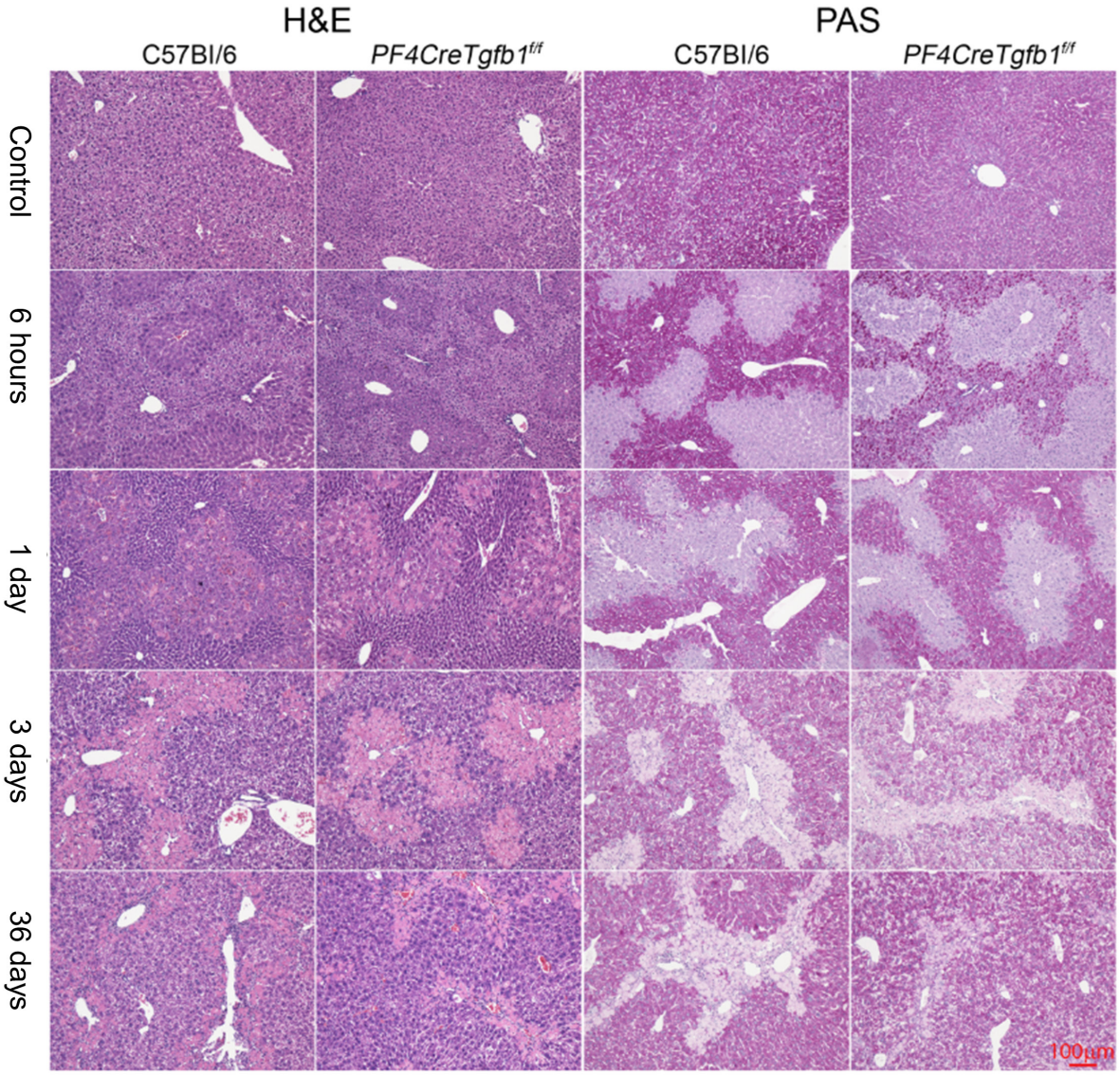
Supplemental Figure-2



Supplemental Figure 2.

In situ hybridization of liver sections from wt and *PF4CreTgfb1*$^{f/f}$ mice at different time points, showing the patterns of *Cyp2e1* and *Alb* gene expression before and at the indicated times after CCl_4 challenge. Tiled representative pictures of dual (*Cyp2e1+Alb*) and single (*Cyp2e1*) staining images are shown.

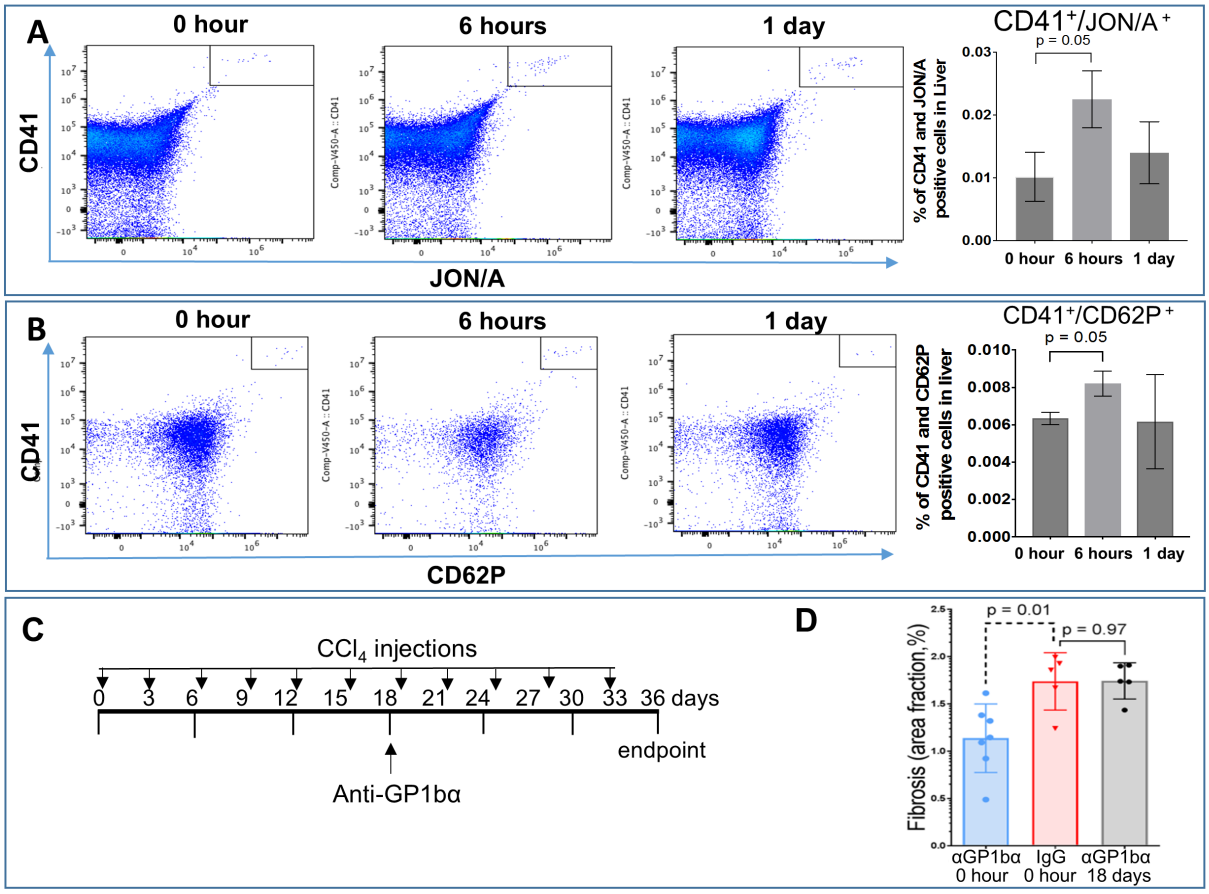
Supplemental Figure-3



Supplemental Figure 3.

Hematoxylin & eosin (H&E) and periodic acid-schiff (PAS) staining in wt and *PF4CreTgfb1$^{f/f}$* mice liver at 0 h (control/no injection), 6 h, 1 day, 3 days, and 36 days after CCl_4 challenge.

Supplemental Figure-4



Supplemental Figure 4.

Flow cytometry results showing (A) CD41 plus Jon/A and (B) CD41 plus P-selectin (CD62P) double-positive populations (small box in the upper right corner) in wt mice liver sections before (Control), 6 hours after, or 1 day after a single CCl_4 challenge. The numbers of double-positive cells are shown in the right panels (n=3). (C) Depiction of the experimental protocol showing the α-GP1bα injection time point, as well as the CCl_4 injection time points (indicated by arrows). (D) Anti-GP1bα antibody injection at 18 days of CCl_4 challenge did not reduce fibrosis compared to IgG injection (p=0,97), but mice injected with anti-GP1bα on day 0 exhibited significantly less fibrosis than IgG-injected mice (shown also in Figure 3A and 3D).

References:

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2. Meyer A, Wang W, Qu J, Croft L, Degen JL, Coller BS and Ahamed J. Platelet TGF-beta1 contributions to plasma TGF-beta1, cardiac fibrosis, and systolic dysfunction in a mouse model of pressure overload. *Blood*. 2012;119:1064-1074.

3. Ghafoory S, Breitkopf-Heinlein K, Li Q, Dzieran J, Scholl C, Dooley S and Wolfl S. A fast and efficient polymerase chain reaction-based method for the preparation of in situ hybridization probes. *Histopathology*. 2012;61:306-13.

4. Ghafoory S, Breitkopf-Heinlein K, Li Q, Scholl C, Dooley S and Wolfl S. Zonation of nitrogen and glucose metabolism gene expression upon acute liver damage in mouse. *PLoS One*. 2013;8:e78262.