

Migration of splenic lymphocytes promotes liver fibrosis through modification of T helper cytokine balance in mice

Kazutaka Tanabe · Kojiro Taura · Yukinori Koyama · Gen Yamamoto · Takahiro Nishio · Yukihiro Okuda · Kojiro Nakamura · Kan Toriguchi · Kenji Takemoto · Kenya Yamanaka · Keiko Iwaisako · Satoru Seo · Masataka Asagiri · Etsuro Hatano · Shinji Uemoto

Received: 13 July 2014 / Accepted: 7 February 2015
© Springer Japan 2015

Abstract

Background Sustained liver injury causes liver fibrosis and eventually cirrhosis. Understanding the pathophysiological mechanisms of liver fibrosis and interventions in the fibrotic process is crucial for improving the prognosis of patients with chronic liver diseases. Although studies have shown that splenectomy suppresses liver fibrosis, the mechanism by which this occurs is poorly understood. The present study focuses on the immunological functions of the spleen to investigate its role in liver fibrosis.

Methods BALB/c and severe combined immunodeficiency (SCID) mice underwent splenectomies or sham operations prior to induction of liver fibrosis with carbon tetrachloride or thioacetamide.

Electronic supplementary material The online version of this article (doi:10.1007/s00535-015-1054-3) contains supplementary material, which is available to authorized users.

K. Tanabe (✉) · K. Taura (✉) · Y. Koyama · G. Yamamoto · T. Nishio · Y. Okuda · K. Nakamura · K. Toriguchi · K. Takemoto · K. Yamanaka · S. Seo · E. Hatano · S. Uemoto
Department of Surgery, Graduate School of Medicine, Kyoto University, 54 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto 6068507, Japan
e-mail: tanabe75@kuhp.kyoto-u.ac.jp

K. Taura
e-mail: ktaura@kuhp.kyoto-u.ac.jp

K. Iwaisako
Department of Target Therapy Oncology, Graduate School of Medicine, Kyoto University, 54 Kawaharacho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan

M. Asagiri
Innovation Center for Immunoregulation and Therapeutics, Graduate School of Medicine, Kyoto University, Yoshida Konoe, Sakyo-ku, Kyoto 606-8501, Japan

Results Sirius red staining and hydroxyproline assays showed that splenectomy suppressed liver fibrogenesis in BALB/c mice. Reverse transcription PCR analysis of T helper type 1 (Th1) and T helper type 2 (Th2) cytokines demonstrated that splenectomy shifted the Th1/Th2 balance in the liver towards Th1 dominance. In SCID mice, the inhibitory effect on liver fibrosis was abrogated. The number of CD4⁺ T helper lymphocytes in the spleen decreased after liver injury. Green fluorescent protein positive (GFP⁺) splenocytes were transplanted into the spleens of syngeneic wild-type mice to trace their destination after fibrosis induction. GFP⁺CD4⁺ lymphocytes appeared in the liver after induction of fibrosis, and flow cytometry revealed the vast majority of them were Th2 lymphocytes. Transfer of splenocytes via the portal vein into syngeneic splenectomized mice cancelled the suppressive effect of splenectomy on liver fibrosis.

Conclusions The present study demonstrated that Th2-dominant splenic lymphocytes migrate into the liver and promote liver fibrosis by shifting the cytokine balance towards Th2 dominance. Splenectomy suppresses the progression of fibrosis at least partly by restoring the Th1/Th2 balance.

Keywords Splenectomy · Liver fibrosis · T helper lymphocyte

Abbreviations

BDL	Bile duct ligation
GFP	Green fluorescent protein
IL	Interleukin
PBS	Phosphate-buffered saline
qRT-PCR	Quantitative reverse transcription PCR
SCID	Severe combined immunodeficiency
α-SMA	α-Smooth muscle actin

TAA	Thioacetamide
Th1	T helper type 1
Th2	T helper type 2

Introduction

Sustained liver injury results in liver fibrosis regardless of the cause. Hepatic fibrosis is characterized by a progressive accumulation of fibrillar extracellular matrix proteins in the liver that distorts the hepatic architecture and may eventually result in liver cirrhosis [1]. Since the progression of liver fibrosis is directly associated with complications in chronic liver diseases, understanding the pathophysiological mechanisms of liver fibrosis and intervention in the fibrotic process is crucial for improving the prognosis of patients with chronic liver diseases. Hypersplenism is a major complication in patients with advanced liver fibrosis. For those patients, splenectomy is beneficial and often indicated as it increases the platelet count and makes antiviral therapy or invasive treatment feasible [2]. Moreover, clinical observations have revealed that splenectomy not only increases the platelet count, but also improves liver function [3, 4]. In addition, some experimental studies have shown that splenectomy suppresses the progression of liver fibrosis. The underlying mechanisms are largely unknown, although a few studies have proposed explanations for this phenomenon [5–7].

The spleen plays important roles in the immune system. Specifically, B cells and plasma cells mature in the spleen, and have key functions in humoral immunity. Moreover, T cells and the cytokines they produce play important roles in modifying the maturation process of these lymphocytes. Importantly, the immune system can affect the progression of liver fibrosis; for instance, strain-specific differences in murine hepatic fibrotic responses are mediated by divergent T helper cytokine responses [8]. In addition, immunosuppressants differently modulate hepatic fibrotic responses in mice and humans [9, 10]. These observations have led us to the hypothesis that the spleen modulates liver fibrosis through its immunological functions. The present study aimed to investigate the role of the spleen in liver fibrosis, with particular focus on its immunological functions.

Materials and methods

Animals

BALB/c, C57BL/6, severe combined immunodeficiency (SCID), and C57BL/6-Tg(CAG-EGFP)C14-Y01-FM131Osb (“green mice”) male mice were purchased at 10 weeks of age from Japan SLC (Shizuoka, Japan) and

CLEA Japan (Tokyo, Japan). After acclimation for 7 days, the mice were divided into sham-operation and splenectomy groups. Splenectomies were performed under pentobarbital anaesthesia (50 mg/kg) as previously described [5]. Splenic arteries and veins were ligated twice with 6-0 nylon sutures with a minimal incision on the upper abdominal midline. Sham-operation mice underwent a laparotomy without splenectomy. The protocol for animal handling was reviewed and approved by the Animal Care and Use Committee of Kyoto University.

Experimental liver fibrosis models

Liver fibrosis was induced by repetitive injections of carbon tetrachloride (CCl₄) or thioacetamide (TAA). In the CCl₄-induced liver fibrosis model, each mouse was subjected to intraperitoneal injections of CCl₄ (0.5 µL/g body weight; 1:4 dilution with corn oil) or corn oil as a control twice a week for 6 weeks [11]. Livers were harvested 7 days after the last injection. TAA-induced liver fibrosis was induced by intraperitoneal injections with escalating doses of TAA or normal saline as a control three times a week for 6 weeks (first dose, 100 mg/kg, weeks 1–2, 200 mg/kg; weeks 3–4, 300 mg/kg; weeks 4–6, 400 mg/kg), as previously described [12]. Livers were harvested 3 days after the last injection. Bile duct ligation (BDL) was performed by surgical ligation of the common bile duct, as previously described [13]. The common bile duct was ligated three times with 6-0 nylon sutures under pentobarbital anaesthesia (50 mg/kg). Sham-operation mice underwent laparotomy without ligation of the common bile duct. Mice were killed for analysis at 10 days and 3 weeks after the operation.

Sirius red staining

Formalin-fixed specimens were embedded in paraffin, sliced with a thickness of 4 µm, and mounted on silanized glass slides. Sirius red staining was performed as previously described [13]. Ten nonoverlapping fields per mouse at a final magnification of ×100 were randomly selected and photographed. The Sirius red positive area was quantified using an image analysis software package (Image J; National Institutes of Health).

Immunofluorescence staining

The specimens were fixed in 4 % paraformaldehyde, embedded in OCT compound, and sliced with a thickness of 5 µm. Antigen retrieval was performed by incubation in citric acid buffer at 90 °C, for 20 min. After blocking, the sections were incubated with a primary antibody against α -smooth muscle actin (α -SMA; ab5694; Abcam,

Cambridge, UK), CD4 (IH95-0042; eBioscience, San Diego, CA, USA), CD45RC (ab3135; Abcam), or green fluorescent protein (GFP; A1122; Invitrogen, Carlsbad, CA, USA) at 1:100 dilution overnight at 4 °C, and were then incubated with a corresponding secondary antibody at room temperature for 1 h. The CD4-positive area was evaluated as a percentage using Image J.

Hydroxyproline content measurement

Liver tissues were homogenized in ice-cold distilled water (1 mL). Subsequently, 125 μ L of 50 % trichloroacetic acid was added, and the homogenates were incubated on ice for 30 min. The precipitated pellets were hydrolysed for 24 h at 110 °C in 6 N HCl. After hydrolysis, the samples were

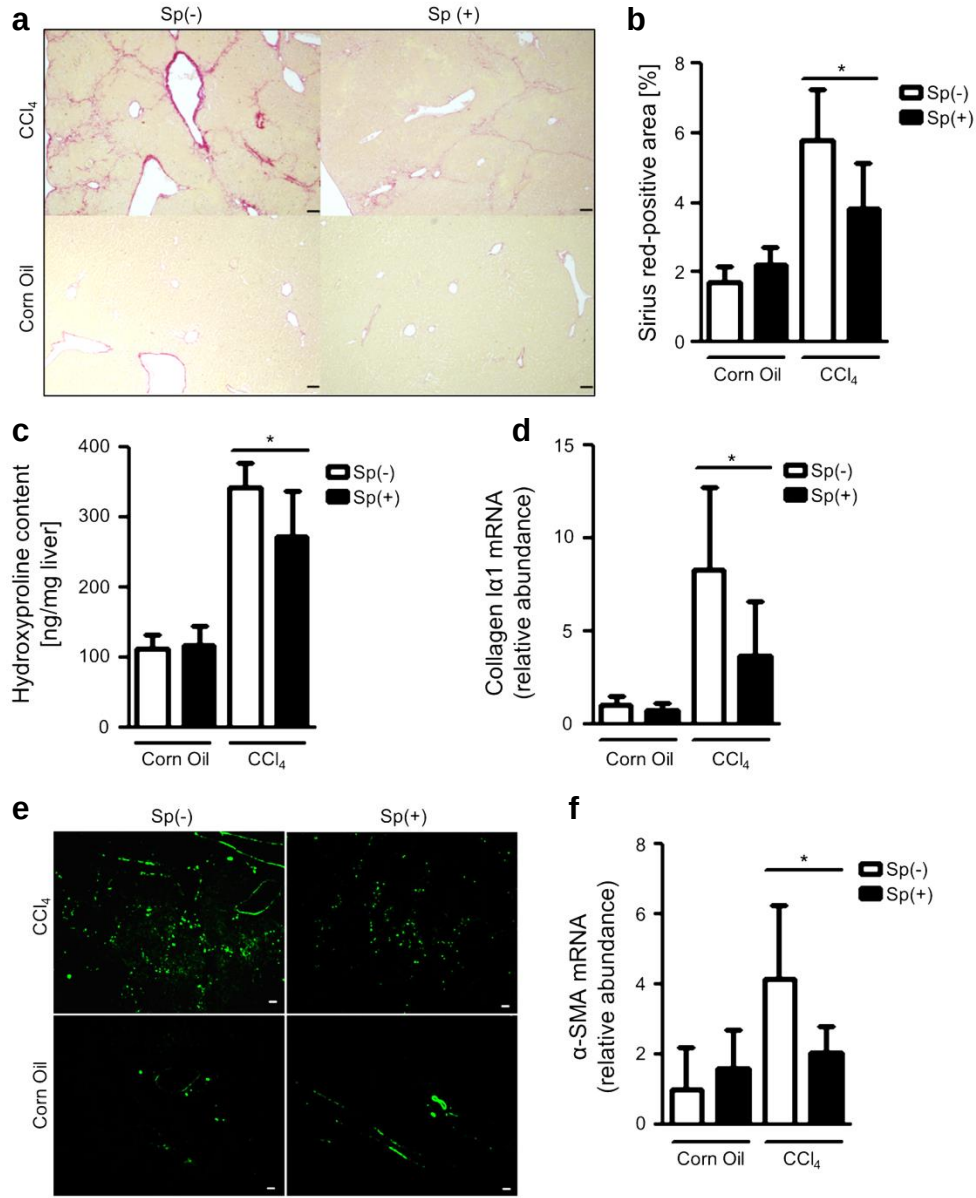


Fig. 1 Splenectomy suppresses the progression of liver fibrosis in a CCl₄-induced liver injury mouse model. BALB/c mice were divided into sham-operation and splenectomy groups and received injections of CCl₄ ($n = 7$ mice per group) or corn oil ($n = 6$ mice per group). **a** Liver fibrosis was evaluated by Sirius red staining. Original magnification $\times 40$. **b** The Sirius red positive area was quantified as a percentage using Image J. **c** Collagen deposition was quantified by measuring the hydroxyproline content in the liver. **d** Quantitative reverse transcription PCR (qRT-PCR) was performed to measure messenger RNA (mRNA) expression levels of *Coll1a1* (which encodes

type I collagen α_1). *Gapdh* (which encodes glyceraldehyde 3-phosphate dehydrogenase) was used as an internal control. **e** Activation of hepatic stellate cells was evaluated by immunofluorescence staining for α -smooth muscle actin (α -SMA; which is encoded by *Acta2*). Original magnification $\times 100$. **f** Hepatic expression levels of *Acta2* mRNA were measured using qRT-PCR. *Gapdh* was used as an internal control. The mean $\pm$ the standard deviation is shown. Asterisks indicate statistically significant differences ($P < 0.05$) determined by the Mann–Whitney U test. *Sp(-)* sham-operation group, *Sp(+)* splenectomy group

filtered, neutralized with 10 N NaOH, and oxidized with chloramine-T (Sigma-Aldrich, St Louis, MO, USA) for 25 min at room temperature. The reaction mixture was incubated in Ehrlich's perchloric acid solution at 65 °C for 20 min and then cooled to room temperature. Sample absorbance was measured at 560 nm in duplicate. Purified hydroxyproline (Sigma-Aldrich) was used as a standard. The hydroxyproline content was expressed as nanograms of hydroxyproline per milligram of liver.

Quantitative reverse transcription PCR analysis

Total RNA was extracted from liver samples using TRIzol (Invitrogen, Tokyo, Japan) and RNeasy Mini kits with on-column DNA digestion (Qiagen, Tokyo, Japan). Total RNA was reverse transcribed to complementary DNA using an Omniscript reverse transcription kit (Qiagen, Valencia, CA, USA). We performed quantitative reverse transcription PCR (qRT-PCR) with gene-specific PCR primers (Table S1) and SYBR Green I master reaction mix using a LightCycler 480 II instrument (Roche Diagnostics, Basel, Switzerland). The relative abundance of the target genes was determined by comparison with a standard curve and was normalized to the abundance of *Gapdh* as an internal control using the LightCycler 480 software (version 1.5).

Isolation of hepatic lymphoid cells

Hepatic lymphoid cells were isolated from murine livers by a method described previously [13, 14]. Livers were minced into small fragments and washed free of blood in cold phosphate-buffered saline (PBS). Fragments were passed through a 100- μ m steel mesh. The cell suspension was filtered through a nylon mesh (BD Falcon cell strainer; BD Biosciences) to remove tissue debris. Filtered cells were centrifuged at 50g for 1 min to remove the hepatocyte fraction. The remaining nonparenchymal cell fraction was collected, washed, and resuspended in PBS. Leukocytes were isolated by density-gradient centrifugation (Histopaque-1077; Sigma-Aldrich). Cell viability was assessed by trypan blue staining.

Isolation of lymphocytes from spleen

Spleens were minced through a nylon mesh (BD Falcon cell strainer; BD Biosciences) to obtain single-cell suspensions in PBS as previously described [15]. Erythrocytes were lysed by incubation in lysis buffer (00-4333-57; eBioscience, San Diego, CA, USA) for 4 min at room temperature. Following these procedures, lymphocytes were washed twice in PBS by centrifugation at 300g at 4 °C and were resuspended in PBS.

Fig. 2 Splenectomy modifies the T helper type 1 (*Th1*)/T helper type 1 (*Th2*) balance in the liver in a CCl₄-induced liver injury model. Quantitative reverse transcription PCR (qRT-PCR) was performed to measure hepatic messenger RNA (*mRNA*) expression levels of **a** *Ifng*, *Il2*, and *Il12a/Il12b* (which encode Th1 cytokines) and **b** *Il4* and *Il13* (which encode Th2 cytokines). *Gapdh* was used as an internal control. **c** The ratio of the mRNA expression levels of *Ifng* (Th1) to *Il4* (Th2) in each mouse was compared between the CCl₄ sham-operation group and the CCl₄ splenectomy group. **d** The mRNA expression levels of *Tgfb1* (which encodes a potent profibrogenic cytokine) were measured by qRT-PCR. **e** Intrahepatic lymphocytes were isolated from CCl₄ splenectomy and sham-operation mice and were subjected to flow-cytometric analysis for expression of CD4 and CD45RC. The statistical result found in at least seven mice is shown. **f** Double immunofluorescence staining for CD45RC (green) and CD4 (red). The number of CD4⁺ cells and CD45RC⁺CD4⁺ cells were counted under a magnification of $\times 100$, and the proportion of CD45RC⁺CD4⁺ cells among CD4⁺ cells in the liver is shown. ($n = 7$ for CCl₄ groups, $n = 6$ for control groups). The mean $\pm$ the standard deviation is shown. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, DAPI 4',6-diamidino-2-phenylindole, IFN interferon, IL interleukin, ns not significant ($P > 0.05$), Sp(-) sham-operation group, Sp(+) splenectomy group, TGF transforming growth factor

Flow-cytometric analysis

Hepatic lymphoid cell isolation from liver tissues and staining for flow cytometry were performed as described in [14]. In brief, lymphoid cells were stained with anti-CD4 (BD Pharmingen 553051; BD Biosciences, San Diego, CA, USA) and anti-CD45RC (BD Pharmingen 557357; BD Biosciences San Diego, CA, USA) mouse antibodies labelled with allophycocyanin and phycoerythrin, respectively. Three-colour flow-cytometric analyses were performed using a BD Accuri C6 flow cytometer (BD Biosciences, San Jose, CA, USA).

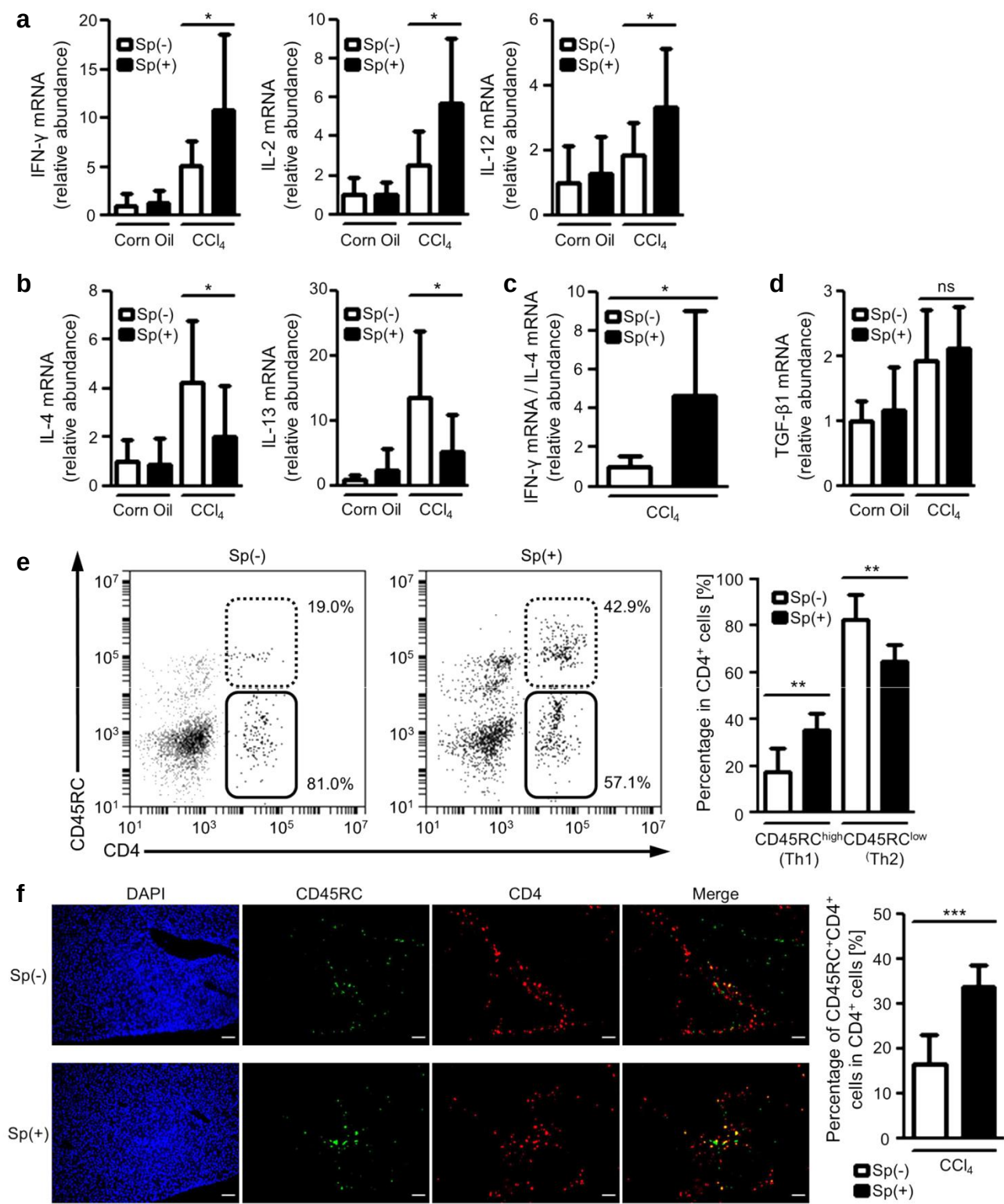
Statistical analysis

Quantitative data are expressed as the mean $\pm$ the standard deviation. For statistical analysis, we used the Mann–Whitney *U* test. *P* values less than 0.05 were regarded as statistically significant.

Results

Splenectomy suppresses progression of liver fibrosis induced by CCl₄ and TAA in mice

Liver fibrosis was induced by repetitive administration of CCl₄ for 6 weeks, and hepatic collagen deposition was evaluated by Sirius red staining of the liver sections. In the corn oil control groups, Sirius red staining demonstrated no fibrous regions, except in the portal areas. Collagen deposition was observed in the livers of mice that received CCl₄ for 6 weeks. The area of fibrous changes in the CCl₄



splenectomy group was markedly attenuated compared with that of the CCl₄ sham-operation group (Fig. 1a). The Sirius red positive area quantified by Image J was significantly

reduced in the CCl₄ splenectomy group compared with the CCl₄ sham-operation group ($3.82 \pm 1.29\%$ vs $5.79 \pm 1.43\%$; $P = 0.018$; Fig. 1b). The hepatic content of

hydroxyproline, a biochemical marker of collagen accumulation, was elevated to 344 ± 34.2 ng/mg liver in the CCl₄ sham-operation group (Fig. 1c). Comparatively, it was reduced to 274 ± 64.7 ng/mg liver in the CCl₄ splenectomy group, resulting in a statistically significant difference between the two groups ($P = 0.018$). Hepatic expression of *Colla1* (which encodes type I collagen α_1) evaluated by qRT-PCR was increased by 8.1-fold in the CCl₄ sham-operation group as compared with the corn oil group (Fig. 1d). By comparison, *Colla1* expression was suppressed by 4.5-fold in the CCl₄ splenectomy group ($P = 0.026$). Activation of hepatic stellate cells was evaluated by immunostaining for α -SMA. Immunostaining for α -SMA in the CCl₄ sham-operation group demonstrated a significant increase in the number of α -SMA-positive cells. The number of α -SMA-positive cells in the CCl₄ splenectomy group was lower than in the CCl₄ sham-operation group (Fig. 1e). Similarly, hepatic expression of *Acta2* (which encodes α -SMA) was significantly lower in the CCl₄ splenectomy group than in the CCl₄ sham-operation group ($P = 0.038$; Fig. 1f).

The effect of splenectomy on liver fibrosis was also investigated using the TAA-induced liver injury model (Fig. S1). Sirius red staining demonstrated that collagen deposition in the TAA splenectomy group was significantly decreased compared with that in the TAA sham-operation group. The ratio of the fibrous area in the TAA splenectomy group was reduced compared with that in the TAA sham-operation group (6.62 ± 1.65 % vs 8.84 ± 0.82 %; $P = 0.007$). The hydroxyproline content was elevated to 277 ± 59.7 ng/mg liver in the TAA sham-operation group and was reduced to 221 ± 24.3 ng/mg liver in the TAA splenectomy group ($P = 0.038$). Hepatic expression of *Colla1* was suppressed in the TAA splenectomy group compared with the TAA sham-operation group ($P = 0.038$). Immunostaining for α -SMA demonstrated that the number of α -SMA-positive cells in the TAA splenectomy group was lower than that in the TAA sham-operation group. Hepatic expression of *Acta2* was also suppressed in the TAA splenectomy group compared with the TAA sham-operation group ($P = 0.026$).

These results indicate that splenectomy suppressed collagen synthesis in the liver and alleviated liver fibrosis in the CCl₄-induced and TAA-induced liver injury models.

We also investigated the effect of splenectomy in the BDL-induced liver injury model (Fig. S2). Sirius red staining demonstrated remarkable fibrotic changes in the BDL-induced liver injury model. In contrast to the toxin-induced liver fibrosis model, the fibrotic area was not different between the BDL sham-operation group and the BDL splenectomy group at both 10 days and 3 weeks after BDL (10 days, 7.97 ± 1.82 % vs 7.47 ± 2.28 %, $P = 0.779$; 3 weeks, 9.51 ± 1.67 % vs 8.25 ± 2.23 %, $P = 0.456$). The hydroxyproline content was also similar

between the two groups (10 days, 181 ± 32.9 ng/mg liver vs 165 ± 42.8 ng/mg liver, $P = 0.463$; 3 weeks, 418 ± 83.2 ng/mg liver vs 451 ± 200 ng/mg liver, $P = 0.209$). In addition, hepatic expression of *Colla1* and *Acta2* was comparable between the two groups (10 days, $P = 0.902$ and $P = 0.165$, respectively; 3 weeks, $P = 0.902$ and $P = 0.383$; respectively).

Splenectomy modifies the T helper type 1/T helper type 2 balance in toxin-induced liver fibrosis models

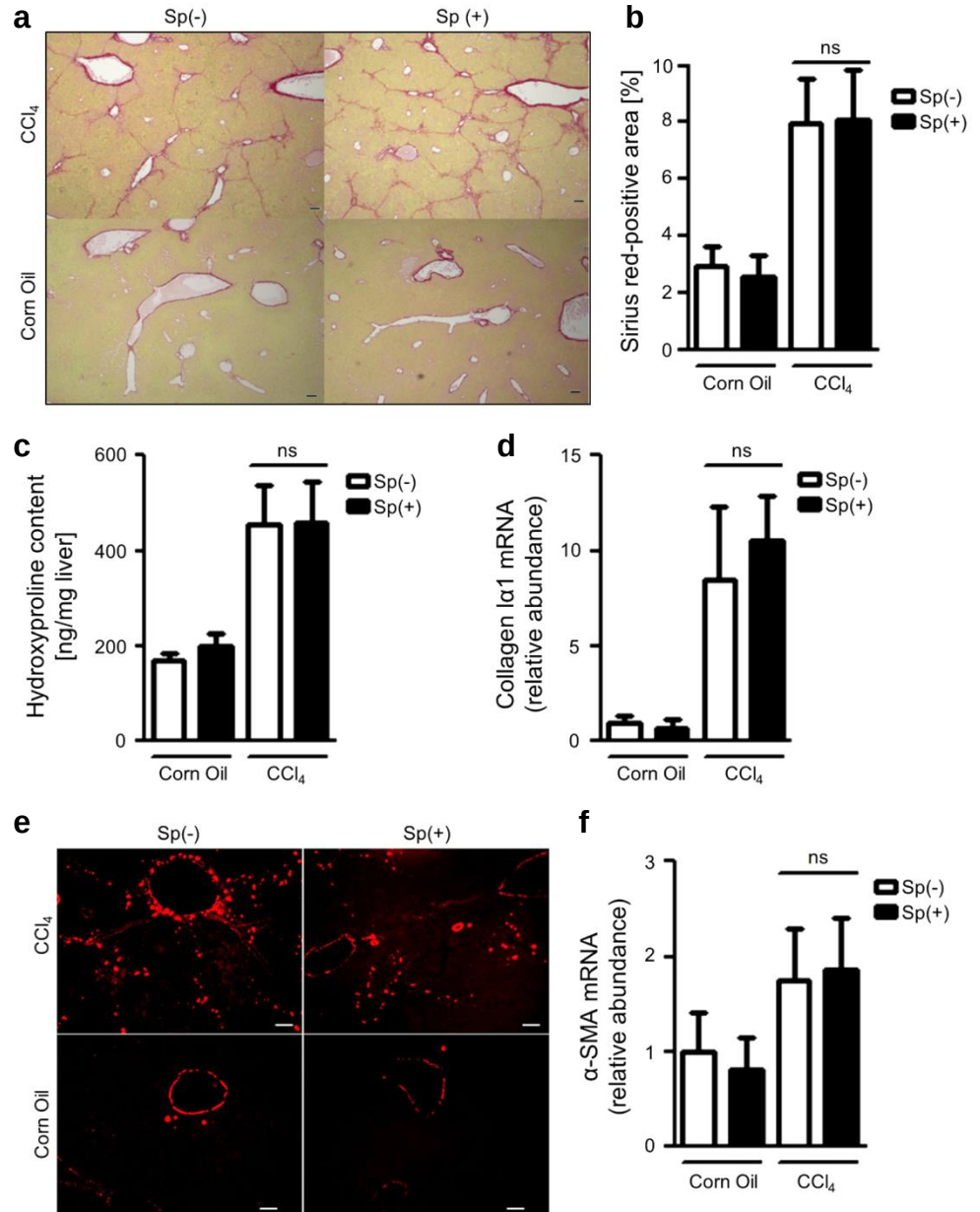
We analysed the effect of splenectomy on the inhibition of fibrotic responses induced in toxin-induced liver injury models, especially focusing on the T helper type 1 (Th1)/T helper type 2 (Th2) balance. Liver fibrosis was induced by repetitive administration of CCl₄ for 6 weeks, and livers were harvested 7 days after the last injection. Expression of *Ifng* mRNA (*Ifng* encodes interferon- γ , a typical Th1 cytokine) was increased by 5.0-fold in the CCl₄ sham-operation group in comparison with the corn oil control group. Expression of *Ifng* mRNA was further enhanced by 8.2-fold in the CCl₄ splenectomy group ($P = 0.038$; Fig. 2a). Similarly, expression of *Il2* [which encodes interleukin (IL)-2] and *Il12a/Il12b* (which encode IL-12) mRNA was increased by 2.6-fold and 1.8-fold in the CCl₄ sham-operation group, and this increase was further enhanced by 5.4-fold and 2.6-fold, respectively, in the CCl₄ splenectomy group (*Il2*, $P = 0.038$; *Il12a/Il12b*, $P = 0.038$; Fig. 2a). In the CCl₄ sham-operation group in comparison with the corn oil group, expression of *Il4* mRNA (*Il4* encodes IL-4, a representative Th2 cytokine) was increased by 4.2-fold. In contrast to the Th1 cytokines, *Il4* mRNA expression was suppressed by 2.4-fold in the CCl₄ splenectomy group ($P = 0.038$; Fig. 2b). Similarly, *Il13* mRNA expression was increased by 13-fold in the CCl₄ sham-operation group, and this increase was inhibited by 2.2-fold after splenectomy ($P = 0.038$; Fig. 2b). The ratio of *Ifng* (Th1) to *Il4* (Th2) mRNA expression in each mouse was increased by 4.7-fold in the CCl₄ splenectomy group compared with the CCl₄ sham-operation group ($P = 0.038$; Fig. 2c). The expression levels of *Tgfb1* (which encodes transforming growth factor β_1 , one of the most potent profibrogenic cytokines) did not differ between the CCl₄ splenectomy and CCl₄ sham-operation groups [16] ($P = 0.383$; Fig. 2d). We also evaluated Th1 and Th2 cytokine expression from lymphocytes isolated from livers (Fig. S3). Similarly to the results obtained from the whole liver tissue, Th1 cytokines were upregulated whereas Th2 cytokines were downregulated in the CCl₄ splenectomy group in comparison with the CCl₄ sham-operation group. The ratio of *Ifng* to *Il4* mRNA expression was increased by 2.5-fold in the CCl₄ splenectomy group compared with the CCl₄ sham-operation group ($P = 0.0082$). The Th1/Th2

balance in the liver was also evaluated, on the basis of different CD45RC expression profiles between Th1 and Th2 lymphocytes [17, 18]. Flow-cytometric analysis revealed a significant increase in the proportion of Th1 (CD45RC^{high}CD4⁺) lymphocytes among CD4⁺ lymphocytes in the CCl₄ splenectomy group compared with the CCl₄ sham-operation group ($35.6 \pm 7.2\%$ vs $17.3 \pm 10.2\%$; Fig. 2e). Double immunostaining for CD45RC and CD4 in the CCl₄ splenectomy group demonstrated a significant increase in the number of CD45RC⁺CD4⁺ cells (Th1) after CCl₄ treatment compared with the CCl₄ sham-operation group. The ratio of CD45RC⁺CD4⁺ cells to CD4⁺ cells was $33.8 \pm 4.63\%$ in

the CCl₄ splenectomy group, whereas it was $16.7 \pm 6.50\%$ in the CCl₄ sham-operation group ($P = 0.0003$; Fig. 2f).

We also investigated the change in the liver Th1/Th2 balance resulting from splenectomy in the TAA-induced liver injury model (Fig. S4). The expression levels of *Ifng*, *Il2*, and *Il12a/Il12b* mRNA were increased by 3.8-, 3.0-, and 2.8-fold, respectively, in the TAA sham-operation group in comparison with the control saline group. The expression of *Ifng*, *Il2*, and *Il12a/Il12b* mRNA was enhanced by 5.5-, 7.5-, and 5.3-fold, respectively, in the TAA splenectomy group (*Ifng*, $P = 0.038$; *Il2*, $P = 0.038$; *Il12a/Il12b*, $P = 0.038$). In contrast, the expression levels

Fig. 3 Suppressive effect of splenectomy on liver fibrosis is abrogated in immunodeficient severe combined immunodeficiency (SCID) mice. SCID mice were divided into sham-operation and splenectomy groups, and received injections of CCl₄ ($n = 7$ mice per group) or corn oil ($n = 6$ mice per group). **a** Liver fibrosis was evaluated by Sirius red staining. Original magnification $\times 40$. **b** The Sirius red positive area was quantified as a percentage using Image J. **c** Collagen deposition was quantified by measuring the hydroxyproline content in the liver. **d** Quantitative reverse transcription PCR (qRT-PCR) was performed to measure messenger RNA (mRNA) expression levels of *Col1a1*. *Gapdh* was used as an internal control. **e** Activation of hepatic stellate cells was evaluated by immunofluorescence staining for α -smooth muscle actin (α -SMA). Original magnification $\times 100$. **f** Hepatic expression levels of *Acta2* mRNA were measured by qRT-PCR. *Gapdh* was used as an internal control. The mean $\pm$ the standard deviation is shown. *ns* not significant, *Sp*(-) sham-operation group, *Sp*(+) splenectomy group



of *Il4* and *Il13* mRNA were increased by 5.2- and 7.7-fold in the TAA sham-operation group compared with the saline group. Moreover, their expression levels were inhibited by 4.2- and 3.3-fold, respectively, in the TAA splenectomy group (*Il4*, $P = 0.038$; *Il13*, $P = 0.026$). The ratio of *Ifng* to *Il4* mRNA expression was increased by 3.0-fold in the TAA splenectomy group compared with the TAA sham-operation group ($P = 0.007$). The expression level of *Tgfb1* was not altered by splenectomy in the TAA-induced liver injury model ($P = 0.805$). The proportion of CD45RC^{high}CD4⁺ lymphocytes among CD4⁺ lymphocytes in the TAA splenectomy group measured by flow cytometry was increased compared with that in the TAA sham-operation group ($39.8 \pm 2.9\%$ vs $21.2 \pm 7.1\%$). The ratio of CD45RC⁺CD4⁺ cells to CD4⁺ cells was also increased in the TAA splenectomy group compared with the TAA sham-operation group, as demonstrated by immunostaining ($35.2 \pm 4.84\%$ vs $19.6 \pm 3.58\%$; $P = 0.0002$). Taken together, these results showed that splenectomy shifted the Th1/Th2 balance in the liver towards Th1 dominance in the toxin-induced liver fibrosis models.

In the BDL-induced liver injury model, there was no statistical difference in the expression levels of *Ifng* and *Il4* mRNA between splenectomy and sham-operation groups at both 10 days and 3 weeks after BDL (Fig. S5).

Effect of splenectomy is abrogated in immunodeficient SCID mice

Immunodeficient SCID mice were used to determine if immunological mechanisms were involved in the effect of splenectomy on liver fibrosis observed in wild-type mice. Although liver fibrosis was similarly induced with CCl₄ in SCID mice as in wild-type mice, the inhibitory effect of splenectomy on liver fibrosis was not observed in SCID mice (Fig. 3a). In addition, the fibrotic area did not differ between CCl₄ splenectomy and sham-operation groups ($8.12 \pm 1.71\%$ vs $7.97 \pm 1.57\%$, $P = 0.902$; Fig. 3b). The hepatic hydroxyproline content was similarly elevated in the CCl₄ splenectomy and sham-operation groups (457 ± 92.8 ng/mg liver vs 454 ± 81.4 ng/mg liver, $P = 0.902$; Fig. 3c). The hepatic expression level of *Colla1* was not affected by splenectomy ($P = 0.165$; Fig. 3d). Furthermore, splenectomy had no effect on hepatic expression of α -SMA, as demonstrated by immunostaining (Fig. 3e) and qRT-PCR ($P = 0.535$; Fig. 3f).

Next, we evaluated the influence of splenectomy in the immunodeficient SCID mice on cytokine expression in our CCl₄-induced liver fibrosis model. As expected, the expression levels of *Ifng* and *Il4* mRNA were not influenced by splenectomy in SCID mice (Fig. 4a, b). The ratio of *Ifng* to *Il4* mRNA in each SCID mouse was comparable

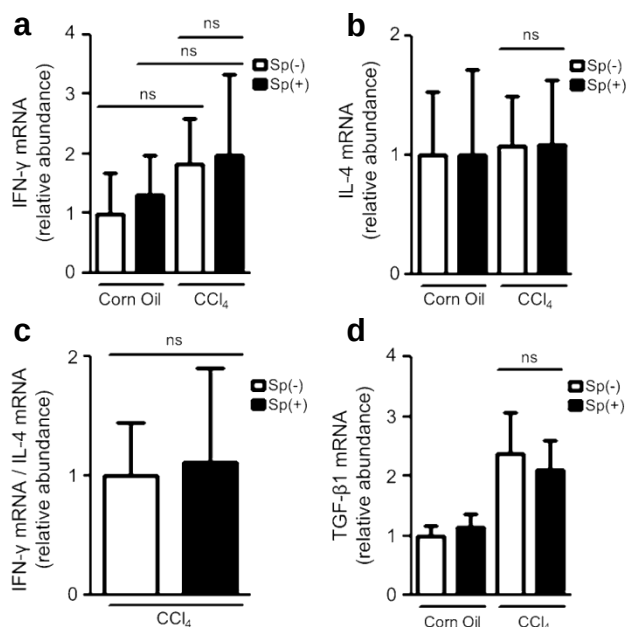


Fig. 4 Hepatic T helper type 1 (Th1)/T helper type 2 (Th2) cytokine expression levels in a CCl₄-induced liver injury model are not modified by splenectomy in immunodeficient severe combined immunodeficiency mice. Quantitative reverse transcription PCR (qRT-PCR) was performed to measure messenger RNA (mRNA) expression of *Ifng* (Th1) (a) and *Il4* (Th2) (b). *Gapdh* was used as an internal control. c The ratios of the mRNA expression levels of *Ifng* (Th1) to *Il4* (Th2) in each mouse were compared between the CCl₄ splenectomy and CCl₄ sham-operation groups. d The mRNA expression levels of *Tgfb1* (which encodes a potent profibrogenic cytokine) were measured by qRT-PCR. ($n = 7$ for CCl₄ groups, $n = 6$ for control groups). The mean $\pm$ the standard deviation is shown. IFN interferon, IL interleukin, ns not significant, Sp(-) sham-operation group, Sp(+) splenectomy group, TGF transforming growth factor

between the CCl₄ splenectomy and sham-operation groups ($P = 0.902$; Fig. 4c). *Tgfb1* mRNA expression levels showed no differences between the CCl₄ splenectomy and sham-operation groups ($P = 0.383$; Fig. 4d).

Number of CD4⁺ cells in spleen is reduced in toxin-induced liver fibrosis models

Pathophysiological changes in the spleen of toxin-induced liver fibrosis model mice were evaluated to elucidate the mechanisms by which splenectomy suppressed liver fibrosis and modified the Th1/Th2 balance in the liver. Histological examination of spleens by haematoxylin and eosin staining revealed no structural changes between the control group and toxin-induced liver injury groups (Fig. 5a). In the CCl₄-induced liver injury model, we evaluated the serial CD4⁺ cell counts in the spleen. Immunostaining for the T helper lymphocyte marker CD4 demonstrated a time-dependent decrease in CD4⁺ cell numbers (Fig. 5b). The CD4-positive area was $8.43 \pm 0.82\%$ before CCl₄ treatment, and it was

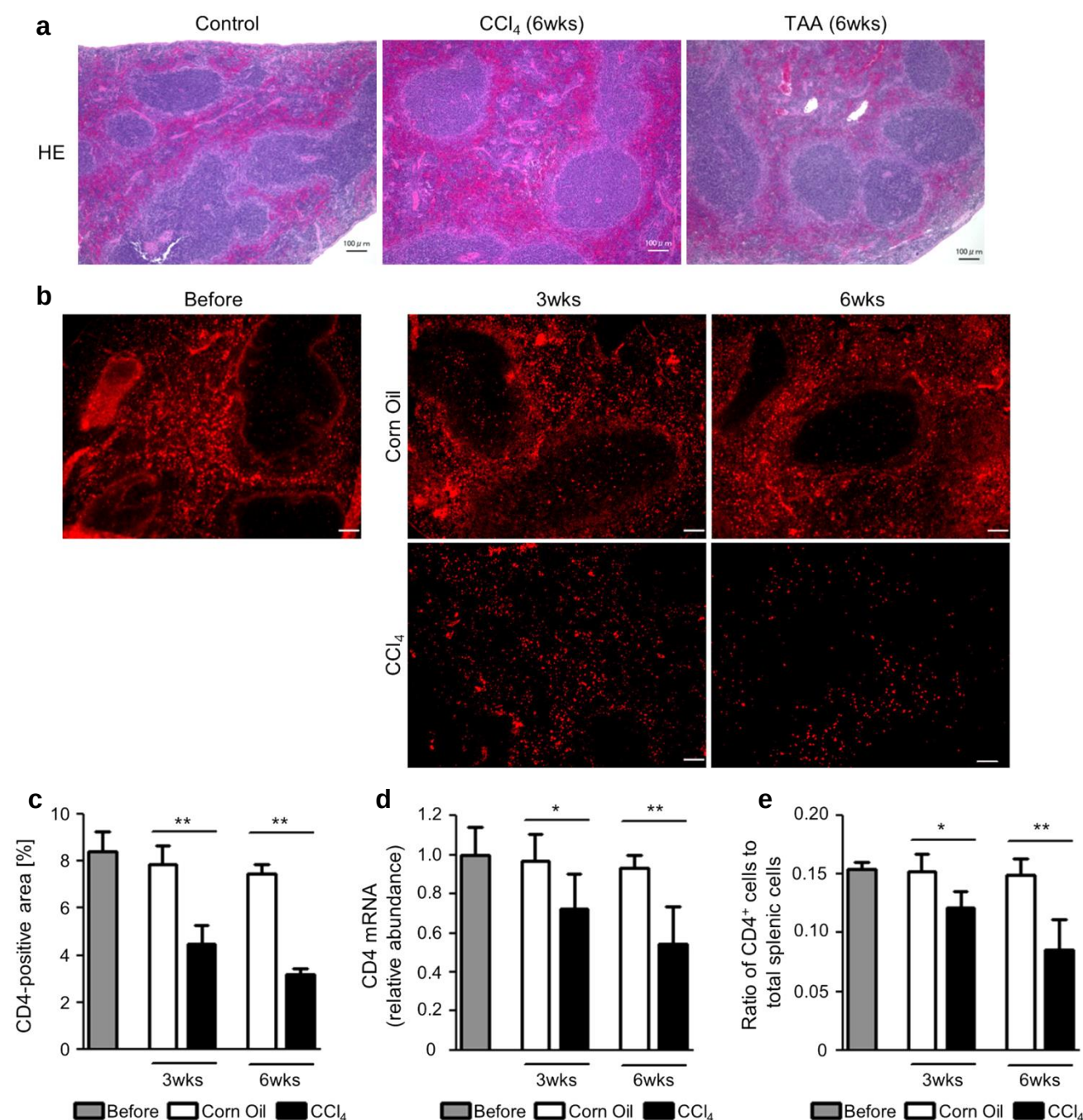


Fig. 5 The number of CD4⁺ T helper lymphocytes in spleen decreases time-dependently in response to liver injuries. Liver fibrosis was induced by injection of CCl₄ or thioacetamide (TAA) as described in “Materials and methods”. **a** Haematoxylin and eosin (HE) staining of the spleen. Original magnification $\times 40$. **b** The expression of CD4, a T helper lymphocyte marker, in the spleen was evaluated by immunofluorescence staining after CCl₄ treatment for 3 and 6 weeks. Original magnification $\times 40$. **c** The CD4-positive area was quantified

as a percentage using Image J. **d** Quantitative reverse transcription PCR was performed to measure messenger RNA (mRNA) expression levels of *Cd4* in the spleen. *Gapdh* was used as an internal control. **e** Splenic lymphocytes of the control and CCl₄ treatment group mice were analysed for CD4 expression by flow cytometry. The proportion of CD4⁺ cells among total splenic cells is shown. The mean $\pm$ the standard deviation of values found in at least six mice is shown. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

reduced to 4.49 ± 0.80 % and 3.18 ± 0.26 % after CCl₄ treatment for 3 and 6 weeks, respectively (3 weeks, $P = 0.0043$; 6 weeks, $P = 0.0040$; Fig. 5c). *Cd4* mRNA

expression was also decreased by 0.75- and 0.58-fold after CCl₄ treatment for 3 and 6 weeks, respectively (3 weeks, $P = 0.030$; 6 weeks, $P = 0.0039$; Fig. 5d). The proportion

of CD4⁺ lymphocytes to total splenic cells showed a time-dependent decrease after CCl₄ treatment (15.4 ± 0.67 % before CCl₄ treatment, 12.2 ± 1.39 % at 3 weeks after CCl₄ treatment, and 8.54 ± 2.65 % at 6 weeks after CCl₄ treatment; Fig. 5e). Similar findings were obtained in the TAA-induced liver injury model (Fig. S6).

The CD4⁺ cells accumulated in the liver after toxin-induced liver injuries as demonstrated by the increase of the CD4⁺ area and *Cd4* mRNA expression in the liver (Fig. S7). Splenectomy slightly reduced the accumulation of CD4⁺ cells, although the difference did not reach statistical significance.

Splenic CD4⁺ cells migrate to the liver in the CCl₄-induced liver injury model

The decrease in the number of T helper cells in the spleen prompted us to explore their fate after CCl₄-induced liver injury. For this purpose, we isolated splenic cells from C57BL/6-Tg mice ubiquitously expressing an enhanced green fluorescent protein (GFP) [19]. The GFP⁺ splenic cells were transplanted into the spleen of syngeneic wild-type mice, and liver injury was induced by CCl₄. Livers were examined on the days following the injection of the first, third, and fifth doses of CCl₄. The migration of spleen-derived T helper cells in the liver was evaluated by GFP and CD4 immunostaining.

In the control groups, GFP⁺ cells were scarcely seen in the liver, and they were almost negative for CD4 (Fig. S8a). In the CCl₄-induced liver injury model, we observed a time-dependent increase in the number of GFP⁺ cells in the liver (Fig. 6a). Immunostaining for CD4 revealed that the CD4⁺ cell count was also increased in a time-dependent manner (Fig. S8b). Furthermore, the number of GFP⁺CD4⁺ cells (spleen-derived T helper lymphocytes) was time-dependently increased (three doses, $6.9 \pm 0.4/\times 100$ high-power field, five doses, $11.1 \pm 1.1/\times 100$ high-power field). The proportion of GFP⁺ cells among the CD4⁺ cells in the liver was 24.5 ± 1.72 % and 22.4 ± 1.62 % after three and five injections of CCl₄, respectively (Fig. S8c). To exclude the possibility that circulating GFP⁺ lymphocytes, not splenic GFP⁺ lymphocytes, were the source of GFP⁺ lymphocytes in the liver, we splenectomized mice 20 min after inoculation of the spleen with GFP⁺ splenocytes, and determined whether GFP⁺ lymphocytes appeared in the liver after fibrosis induction. Immunostaining showed that there were few GFP⁺CD4⁺ cells in the liver (Fig. 6a), indicating that the major source of GFP⁺ lymphocytes appearing in the liver was not circulating GFP⁺ lymphocytes.

GFP⁺CD4⁺ cells (spleen-derived T helper lymphocytes) in the liver were analysed for CD45RC expression by flow cytometry. The percentage of Th2 (CD45RC^{low})

Fig. 6 T helper type 2 (Th2)-predominant spleen-derived T helper lymphocytes migrate from the spleen to the liver in response to liver injury. Splenic lymphocytes isolated from transgenic mice ubiquitously expressing green fluorescent protein (GFP) were transplanted into the spleens of syngeneic wild-type mice. Liver fibrosis was induced by injection of CCl₄. The mice were killed at 24 h after the first, third, and fifth injection. **a** Double immunofluorescence staining for GFP (green) and CD4 (red) with or without splenectomy. Original magnification $\times 100$. **b** CD4⁺ cells in the liver after the fifth injection were analysed for CD45RC expression to determine their T helper type 1 (Th1; CD45RC^{high}) and Th2 (CD45RC^{low}) phenotype by flow cytometry. The relative proportion of Th1 and Th2 lymphocytes among intrahepatic GFP⁺CD4⁺ lymphocytes or GFP⁻CD4⁺ lymphocytes is shown. The mean $\pm$ the standard deviation of values obtained in seven mice is shown. DAPI 4',6-diamidino-2-phenylindole

lymphocytes was almost 100 % in GFP⁺CD4⁺ cells, whereas it was 88.0 ± 5.2 % in GFP⁻CD4⁺ cells, indicating that T helper lymphocytes migrating from the spleen were Th2 dominant compared with the resident T lymphocytes in the liver (Fig. 6b).

Transfer of splenocytes cancels the suppressive effect of splenectomy on liver fibrosis

The contribution of Th2-dominant splenic lymphocytes to liver fibrosis was investigated by adoptive transfer of isolated splenocytes to splenectomized mice. In this experiment, we evaluated liver fibrosis in the 3-week CCl₄ model (not the 6-week model) considering a short life span of injected splenic lymphocytes. Sirius red staining demonstrated that collagen deposition in the splenocyte-transferred CCl₄ splenectomy group was significantly enhanced compared with that in the PBS-injected CCl₄ splenectomy group, and it was comparable to that in the CCl₄ sham-operation group (Fig. 7a, b). The hydroxyproline content was also elevated in the splenocyte-transferred CCl₄ splenectomy group compared with the PBS-injected CCl₄ splenectomy group (282 ± 74.1 ng/mg vs 234 ± 13.5 ng/mg, $P = 0.026$; Fig. 7c). The expression level of *Colla1* was enhanced in the splenocyte-transferred CCl₄ splenectomy group compared with the PBS-injected CCl₄ splenectomy group ($P = 0.026$; Fig. 7d). Hepatic expression of α -SMA was also enhanced in the splenocyte-transferred CCl₄ splenectomy group compared with the PBS-injected CCl₄ splenectomy group, as demonstrated by immunostaining (Fig. 7e) and qRT-PCR ($P = 0.030$; Fig. 7f). These results demonstrated that transfer of splenocytes cancelled the suppressive effect of splenectomy on liver fibrosis.

Discussion

This study demonstrates that splenectomy inhibited toxin-induced liver fibrosis in mice. A few experimental studies have shown that splenectomy suppressed liver fibrosis;

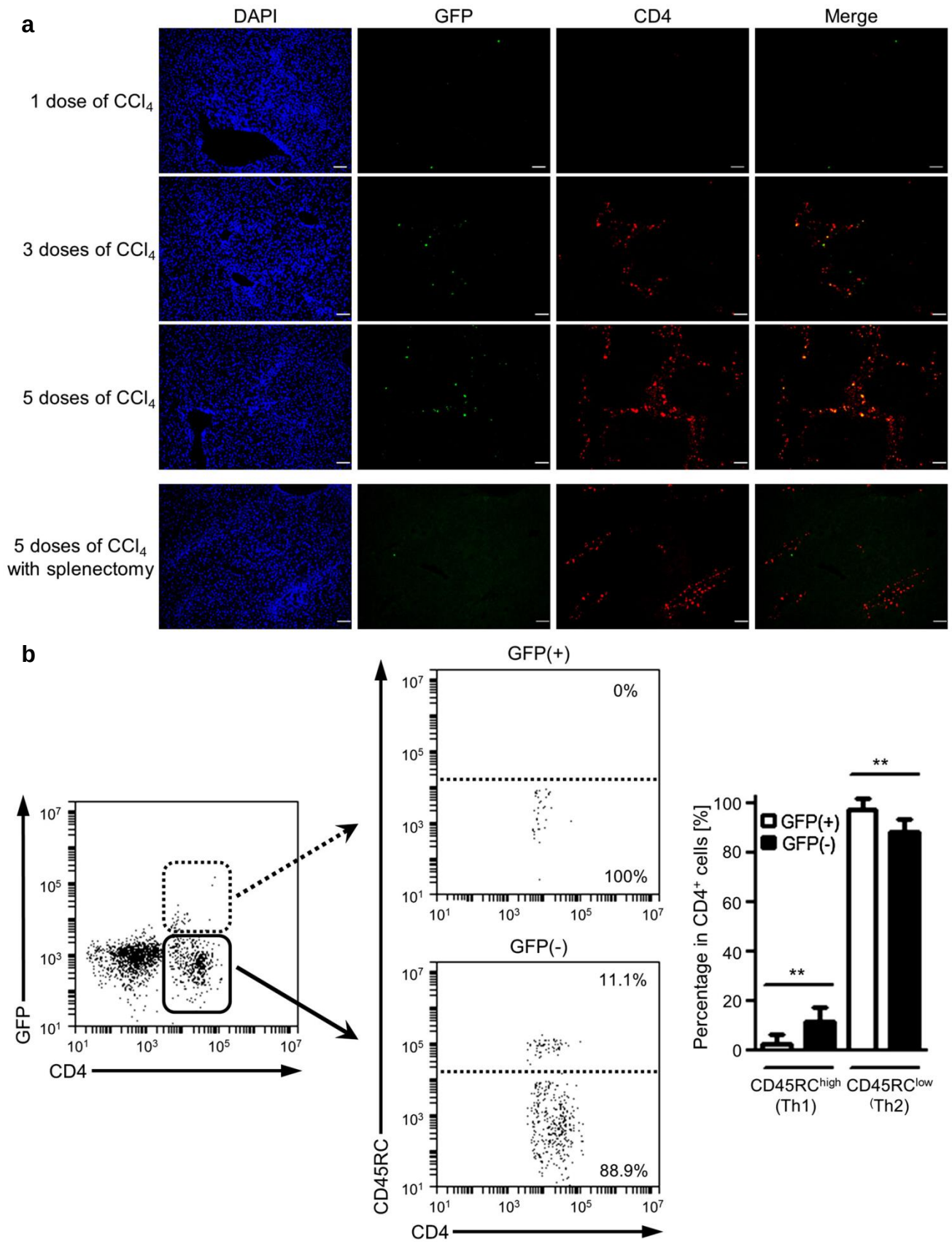
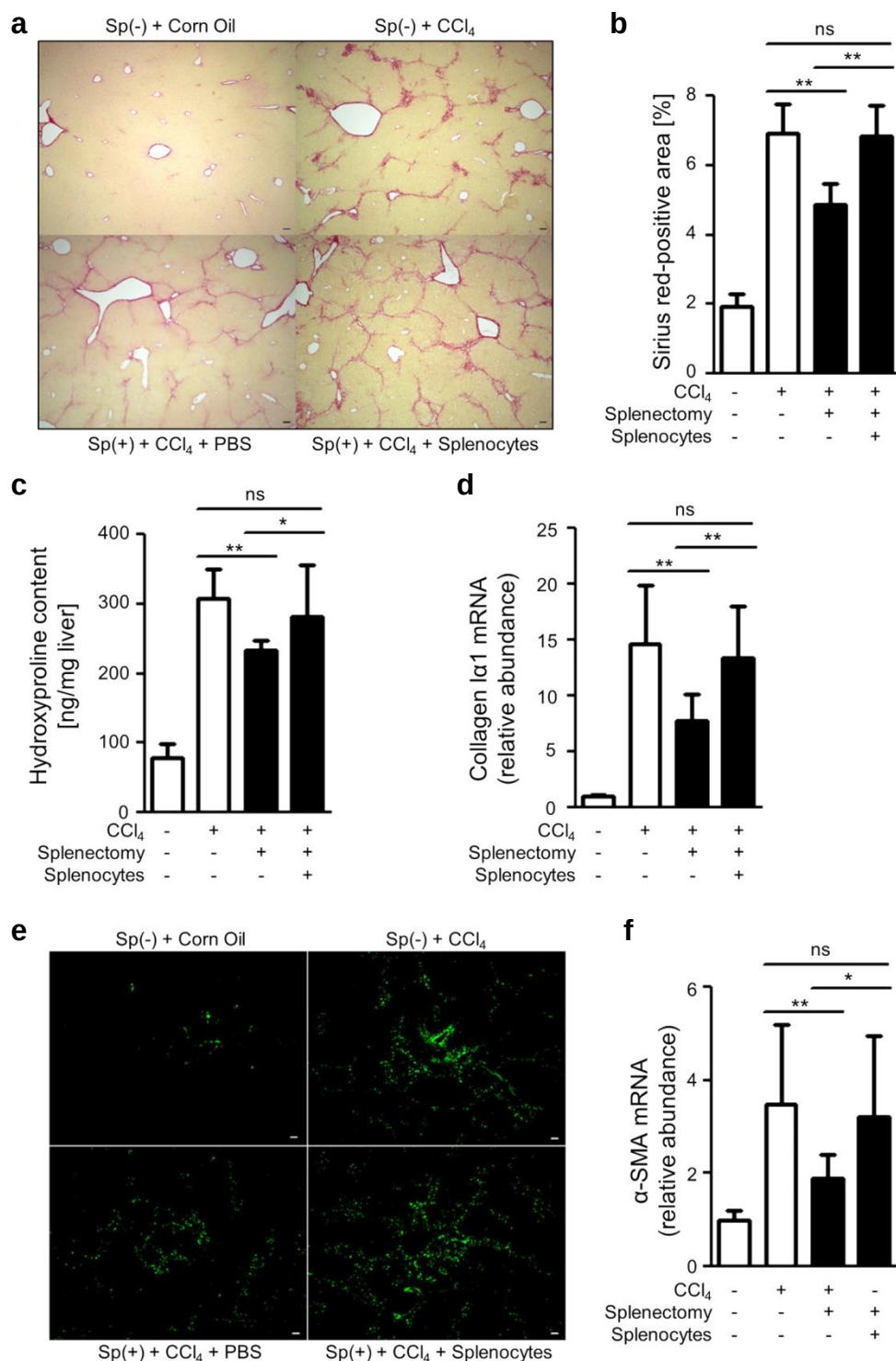


Fig. 7 Transfer of splenocytes cancels the suppressive effect of splenectomy on liver fibrosis. Liver fibrosis was induced by repetitive administration of CCl_4 after adoptive transfer of the isolated splenocytes or phosphate-buffered saline (PBS) as a control via the portal vein into syngeneic splenectomized mice. Liver fibrosis was evaluated after 3 weeks of CCl_4 treatment. **a** Liver fibrosis was evaluated by Sirius red staining. Original magnification $\times 40$. **b** The Sirius red positive area was quantified as a percentage using Image J. **c** Collagen deposition was quantified by measuring the hydroxyproline content in the liver. **d** Quantitative reverse transcription PCR (qRT-PCR) was performed to measure messenger RNA (mRNA) expression levels of *Colla1*. *Gapdh* was used as an internal control. **e** Activation of hepatic stellate cells was evaluated by immunofluorescence staining for α -smooth muscle actin (α -SMA). Original magnification $\times 40$. **f** Hepatic expression levels of *Acta2* mRNA were measured using qRT-PCR. *Gapdh* was used as an internal control. The mean $\pm$ the standard deviation of values obtained in seven mice is shown. * $P < 0.05$, ** $P < 0.01$, ns not significant, Sp(-) sham-operation group, Sp(+) splenectomy group



however, the mechanisms underlying this phenomenon are largely unknown [5–7]. In the present study, we focused on the immunological implication of the spleen. The spleen is a lymphoid organ where lymphocytes mature. In addition, the spleen is directly connected to the liver via the splenic and portal veins and is an important constituent of the

portal system. Therefore, it is plausible that the spleen influences the immunological status of the liver.

In this study, splenectomy modified the balance of Th1/Th2 cytokine expression towards Th1 dominance in the liver in CCl_4 -induced and TAA-induced liver fibrosis models. Previous studies have revealed that Th1 cytokines

have a suppressive effect on liver fibrosis. In particular, interferon- γ is a potent inhibitor of the activation of hepatic stellate cells [20, 21]. By comparison, Th2 cytokines such as IL-4 and IL-13 promote activation of hepatic stellate cells and progression of liver fibrosis [16, 22, 23]. Therefore, our results suggest that the suppressive effect of splenectomy on liver fibrosis is mediated by an altered Th1/Th2 balance. To verify our hypothesis, we used immunodeficient SCID mice lacking functional lymphocytes [24]. In SCID mice, the suppressive effect on liver fibrosis, as well as the influence on the Th1/Th2 balance following splenectomy, was abrogated. These results strongly suggest that the modified Th1/Th2 cytokine balance following splenectomy observed in wild-type mice plays a causative role in the inhibition of liver fibrosis.

Interestingly, SCID mice lacking profibrotic Th2 lymphocytes showed liver fibrosis in response to CCl₄ to the same extent as or to an even higher extent than immunocompetent wild-type mice. Although the significance of lymphocyte proinflammatory and profibrotic properties has been well recognized, recent studies have demonstrated that there are lymphocyte subsets that regulate and control inflammatory response, such as regulatory T cells or $\gamma\delta$ T cells. For example, deficiency in regulatory T cells aggravates the progression of liver fibrosis [25]. We suppose that the lack of an immunomodulatory response by these lymphocytes in SCID mice might have contributed to enhancement of the fibrogenic response, although this is only speculation and needs to be investigated further.

Previous studies have implied other antifibrotic mechanisms mediated by splenectomy. For instance, it was suggested that platelets themselves could inhibit the development of liver fibrosis and that splenectomy exerted an antifibrotic effect via thrombocytosis [5, 6]. Comparatively, Akahoshi et al. [7] proposed that the loss of spleen-derived transforming growth factor β_1 mediated the inhibitory effect of splenectomy on liver fibrosis. Decreases in portal flow and portal pressure are other possible mechanisms of the antifibrotic effect of splenectomy. Although our study did not preclude the aforementioned mechanisms, our observation that the splenectomy-mediated antifibrotic effect was abrogated in SCID mice strongly supports the proposal that immunological mechanisms are involved in this phenomenon.

We explored the mechanisms by which splenectomy modifies the Th1/Th2 balance in the liver. We observed that the number of CD4⁺ cells (T helper lymphocytes) in the spleen was dramatically decreased after liver injury. In contrast, the number of CD4⁺ cells was increased in the liver. These observations led us to speculate that T helper lymphocytes in the spleen migrated to the liver in response to liver injury. To examine our hypothesis, we isolated GFP⁺ splenic cells from C57BL/6-Tg(CAG-EGFP)C14-

Y01-FM131Osb mice and transplanted them into the spleen of syngeneic wild-type mice. After CCl₄-induced liver injury, we observed time-dependent increases in the number of cells that were double positive for GFP and CD4 (spleen-derived T helper lymphocytes) in the injured liver, thereby indicating that T helper lymphocytes migrated from the spleen to the liver. GFP⁺ T helper lymphocytes were rarely observed in the control (corn oil) mice or splenectomized CCl₄-induced liver injury mice inoculated with GFP⁺ splenocytes. These results precluded the possibility that spleen-derived T helper lymphocytes observed after CCl₄-induced liver injury were merely due to the leakage of GFP⁺ lymphocytes at the time of injection, and confirmed that they migrated from the spleen in response to liver injury. Swirski et al. [26] reported that splenic monocytes migrated to the injured site in response to ischaemic myocardial injury, and discovered a novel role of the spleen as a site for storage and rapid deployment of monocytes. Similarly to their findings, the present study demonstrates that the spleen functions as a site for storage and mobilization of T helper lymphocytes in response to liver injury.

Th2 lymphocytes are the main constituent of the immune environment in the spleen [27, 28]. Furthermore, in this study, we observed that most of the spleen-derived T helper cells in the liver were Th2 lymphocytes after liver injury. From our findings taken together, we propose that following liver injury, Th2-predominant splenic T lymphocytes migrate to the liver and promote liver fibrosis by modifying the Th1/Th2 balance in the liver towards Th2 dominance. Splenectomy decreases the absolute number of Th2 lymphocytes in the liver and reverses this Th1/Th2 imbalance, resulting in inhibition of liver fibrosis.

The origin of Th1 lymphocytes on liver injuries is another important issue. Lymphoid organs other than the spleen, such as marrow and thymus, may be possible sources of Th1 lymphocytes, although this is speculation. Further studies are needed to elucidate this.

Comparatively, splenectomy did not influence the progression of liver fibrosis and the liver Th1/Th2 cytokine balance in the BDL-induced liver injury model. The reason for the discrepancy between toxin-induced and cholestasis-induced liver injury models is of interest. Clinical observations have shown that the immune function is disturbed in patients with obstructive jaundice. For instance, inhibition of cellular immunity and suppression of the T lymphocyte proliferative response in jaundice were suggested [29, 30]. Animal studies have also shown that obstructive jaundice strongly influences the immune status. Katz et al. [31] demonstrated that obstructive jaundice expanded the number of regulatory T lymphocytes, which in turn suppressed bulk T cell functions in the liver. In addition, other studies have shown that BDL influenced splenocyte

activity such as mitogenic response [32]. Thus, we speculate that the functional suppression of T helper cells in the liver and/or the selective depression of the lymphocytes derived from the spleen in obstructive jaundice may be the reasons why the effect of splenectomy on the development of liver fibrosis and the Th1/Th2 balance in the liver was not seen in the BDL model. However, further studies are needed to investigate this hypothesis.

In conclusion, the present study unveiled a novel role of the spleen in the progression of liver fibrosis as a site for storage and mobilization of T helper lymphocytes in response to liver injury. Further clinical studies are required to verify the beneficial effect of splenectomy on liver fibrosis in clinical situations. Our findings provide new insights into the use of immunomodulation as an effective strategy for treatment of liver fibrosis. This conclusion might be particularly important in the setting of liver transplantation to treat hepatitis C, as various immunosuppressive regimens may affect the progression of liver fibrosis differently, if the eradication of the virus is unsuccessful. A more detailed understanding of the mechanisms underlying these observations may allow the development of clinically useful immunosuppressive regimens.

Acknowledgment This work was supported by a grant from the Japan Society for the Promotion of Science: Grant-in-Aid for Scientific Research (B), no. 23390322.

Conflict of interest The authors declare that they have no conflict of interest.

References

- Friedman SL. Mechanisms of hepatic fibrogenesis. *Gastroenterology*. 2008;134:1655–69.
- Murata K, Ito K, Yoneda K, et al. Splenectomy improves liver function in patients with liver cirrhosis. *Hepatogastroenterology*. 2008;55:1407–11.
- Imura S, Shimada M, Utsunomiya T, et al. Impact of splenectomy in patients with liver cirrhosis: results from 18 patients in a single center experience. *Hepatol Res*. 2010;40:894–900.
- Sugawara Y, Yamamoto J, Shimada K, et al. Splenectomy in patients with hepatocellular carcinoma and hypersplenism. *J Am Coll Surg*. 2000;190:446–50.
- Watanabe M, Murata S, Hashimoto I, et al. Platelets contribute to the reduction of liver fibrosis in mice. *J Gastroenterol Hepatol*. 2009;24:78–89.
- Kodama T, Takehara T, Hikita H, et al. Thrombocytopenia exacerbates cholestasis-induced liver fibrosis in mice. *Gastroenterology*. 2010;138:2487–98.
- Akahoshi T, Hashizume M, Tanoue K, et al. Role of the spleen in liver fibrosis in rats may be mediated by transforming growth factor β -1. *J Gastroenterol Hepatol*. 2002;17:59–65.
- Shi Z, Wakil AE, Rockey DC. Strain-specific differences in mouse hepatic wound healing are mediated by divergent T helper cytokine responses. *Proc Natl Acad Sci U S A*. 1997;30(94):10663–8.
- Patsenker E, Schneider V, Ledermann M, et al. Potent antifibrotic activity of mTOR inhibitors sirolimus and everolimus but not of cyclosporine A and tacrolimus in experimental liver fibrosis. *J Hepatol*. 2011;55:388–98.
- van der Laan LJ, Hudson M, McPherson S, et al. Results of a two-center study comparing hepatic fibrosis progression in HCV-positive liver transplant patients receiving cyclosporine or tacrolimus. *Transplant Proc*. 2010;42:4573–7.
- Taura K, Miura K, Iwaisako K, et al. Hepatocytes do not undergo epithelial-mesenchymal transition in liver fibrosis in mice. *Hepatology*. 2010;51:1027–36.
- Popov Y, Sverdlov DY, Sharma AK, et al. Tissue transglutaminase does not affect fibrotic matrix stability or regression of liver fibrosis in mice. *Gastroenterology*. 2011;140:1642–52.
- Taura K, De Minicis S, Seki E, et al. Hepatic stellate cells secrete angiopoietin 1 that induces angiogenesis in liver fibrosis. *Gastroenterology*. 2008;135:1729–38.
- Arias-Diaz J, Ildefonso JA, Muñoz JJ, et al. Both tacrolimus and sirolimus decrease Th1/Th2 ratio, and increase regulatory T lymphocytes in the liver after ischemia/reperfusion. *Lab Invest*. 2009;89:433–5.
- Novobrantseva TI, Majeau GR, Amatucci A, et al. Attenuated liver fibrosis in the absence of B cells. *J Clin Invest*. 2005;115:3072–82.
- Farah IO, Mola PW, Kariuki TM, et al. Repeated exposure induces periportal fibrosis in *Schistosoma mansoni*-infected baboons: role of TGF- β and IL-4. *J Immunol*. 2000;164:5337–43.
- Subra JF, Cautain B, Xystrakis E, et al. The balance between CD45RC^{high} and CD45RC^{low} CD4 T cells in rats is intrinsic to bone marrow-derived cells and is genetically controlled. *J Immunol*. 2001;166:2944–52.
- Nishimura H, Hattori S, Abe M, et al. Differential expression of three CD45 alternative structures on murine T cells: exon 6-dependent epitope as a marker for functional heterogeneity of CD4+ T cells. *Int Immunol*. 1992;4:923–30.
- Okabe M, Ikawa M, Kominami K, et al. ‘Green mice’ as a source of ubiquitous green cells. *FEBS Lett*. 1997;407:313–9.
- Czaja MJ, Weiner FR, Takahashi S, et al. γ -Interferon treatment inhibits collagen deposition in murine schistosomiasis. *Hepatology*. 1989;10:795–800.
- Baroni GS, D’Ambrosio L, Curto P, et al. Interferon gamma decreases hepatic stellate cell activation and extracellular matrix deposition in rat liver fibrosis. *Hepatology*. 1996;23:1189–99.
- Wynn TA. Fibrotic disease and the Th1/Th2 paradigm. *Nat Rev Immunol*. 2004;4:583–94.
- Cheever AW, Williams ME, Wynn TA, et al. Anti-IL-4 treatment of *Schistosoma mansoni*-infected mice inhibits development of T cells and non-B, non-T cells expressing Th2 cytokines while decreasing egg-induced hepatic fibrosis. *J Immunol*. 1994;153:753–9.
- Bosma GC, Custer RP, Bosma MJ. A severe combined immunodeficiency mutation in the mouse. *Nature*. 1983;301:527–30.
- Roh YS, Park S, Lim CW, et al. Depletion of Foxp3+ regulatory T cells promotes profibrogenic milieu of cholestasis-induced liver injury. *Dig Dis Sci*. 2014. doi:10.1007/s10620-014-3438-2.
- Swirski FK, Nahrendorf M, Etzrodt M, et al. Identification of splenic reservoir monocytes and their deployment to inflammatory sites. *Science*. 2009;325:612–6.
- Vitetta ES, Brooks K, Chen YW, et al. T-cell-derived lymphokines that induce IgM and IgG secretion in activated murine B cells. *Immunol Rev*. 1984;78:137–57.
- Coffman RL, Seymour BW, Lebman DA, et al. The role of helper T cell products in mouse B cell differentiation and isotype regulation. *Immunol Rev*. 1988;102:5–28.

29. Vierucci A, De Martino M, Novembre E, et al. Effect of the serum of patients with cholestasis on lymphocyte E rosette formation. *Ann Sclavo*. 1977;19:1119–29.
30. Li H, Xiong ST, Zhang SX, et al. Interleukin 2 production and its relationship with T lymphocyte subsets in patients with obstructive jaundice. *J Tongji Med Univ*. 1992;12:164–8.
31. Katz SC, Ryan K, Ahmed N, et al. Obstructive jaundice expands intrahepatic regulatory T cells, which impair liver T lymphocyte function but modulate liver cholestasis and fibrosis. *J Immunol*. 2011;187:1150–6.
32. Chaignaud BE, White JG, Nie CH, et al. Splenocytes from bile duct ligated rats do not elicit a normal immune response in the intact host. *Am Surg*. 1994;60:7–11.

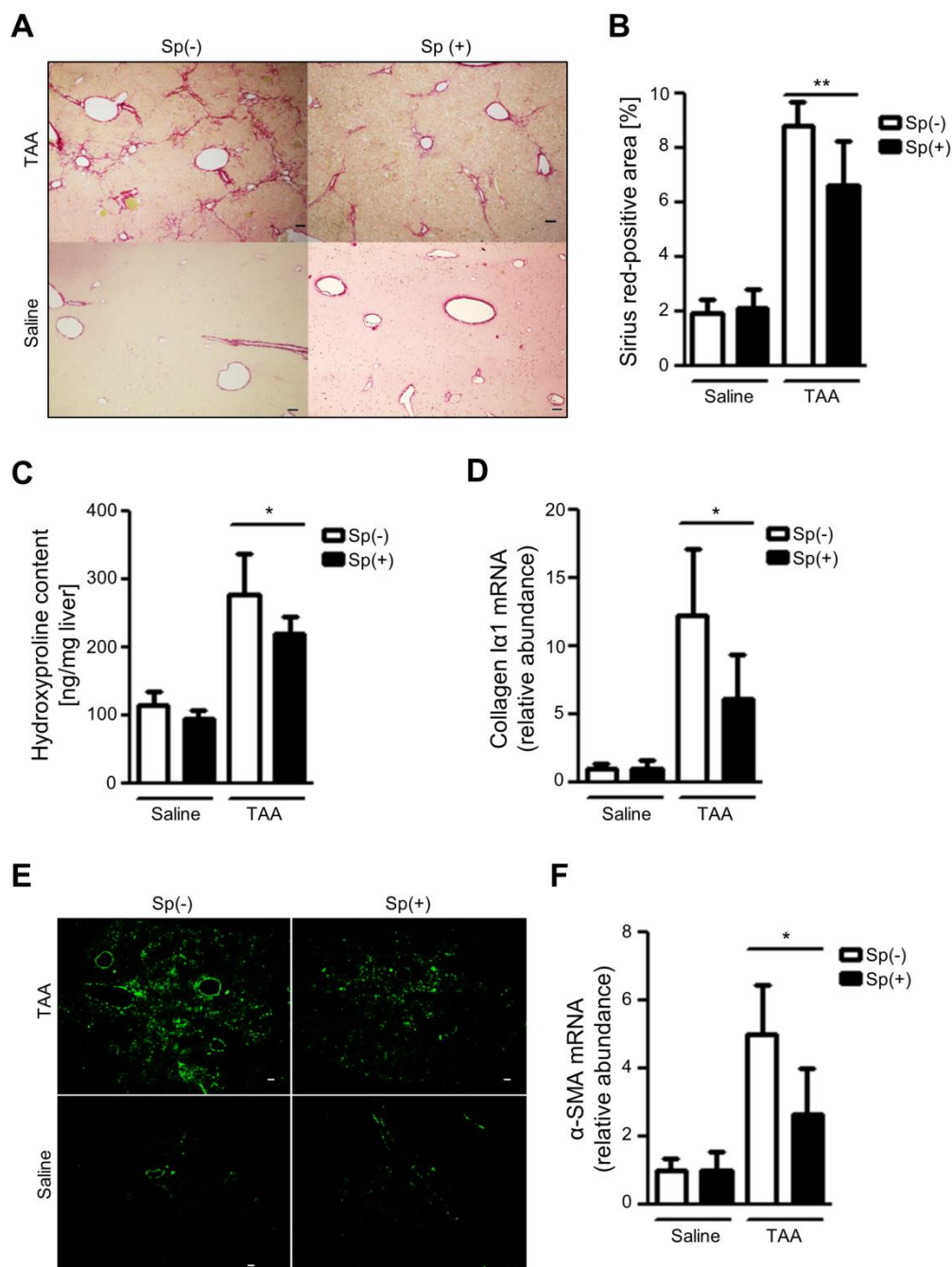


Fig. S1 Splenectomy suppresses the progression of liver fibrosis in a TAA-induced liver injury mouse model. BALB/c mice were divided into sham-operation (Sp(-)) and splenectomy (Sp(+)) groups, and liver fibrosis was induced by escalating injection of TAA for 6 weeks (n = 7 mice/group). Control mice received saline injections (n = 6 mice/group). (A) Liver fibrosis was evaluated by Sirius red staining. Original magnification: $\times 40$. (B) The percentage of the Sirius red-positive area was quantified using Image J. (C) Collagen deposition was quantified by measuring the hydroxyproline content in the liver. (D) Quantitative RT-PCR was performed to measure the mRNA expression levels of type I collagen $\alpha 1$. GAPDH was used as an internal control. (E) Activation of the HSCs was evaluated by immunofluorescence staining for α -SMA. Original magnification: $\times 100$. (F) Hepatic expression levels of α -SMA mRNA were measured using qRT-PCR. GAPDH was used as an internal control. The mean $\pm$ SD is shown. * P < 0.05, ** P < 0.01.

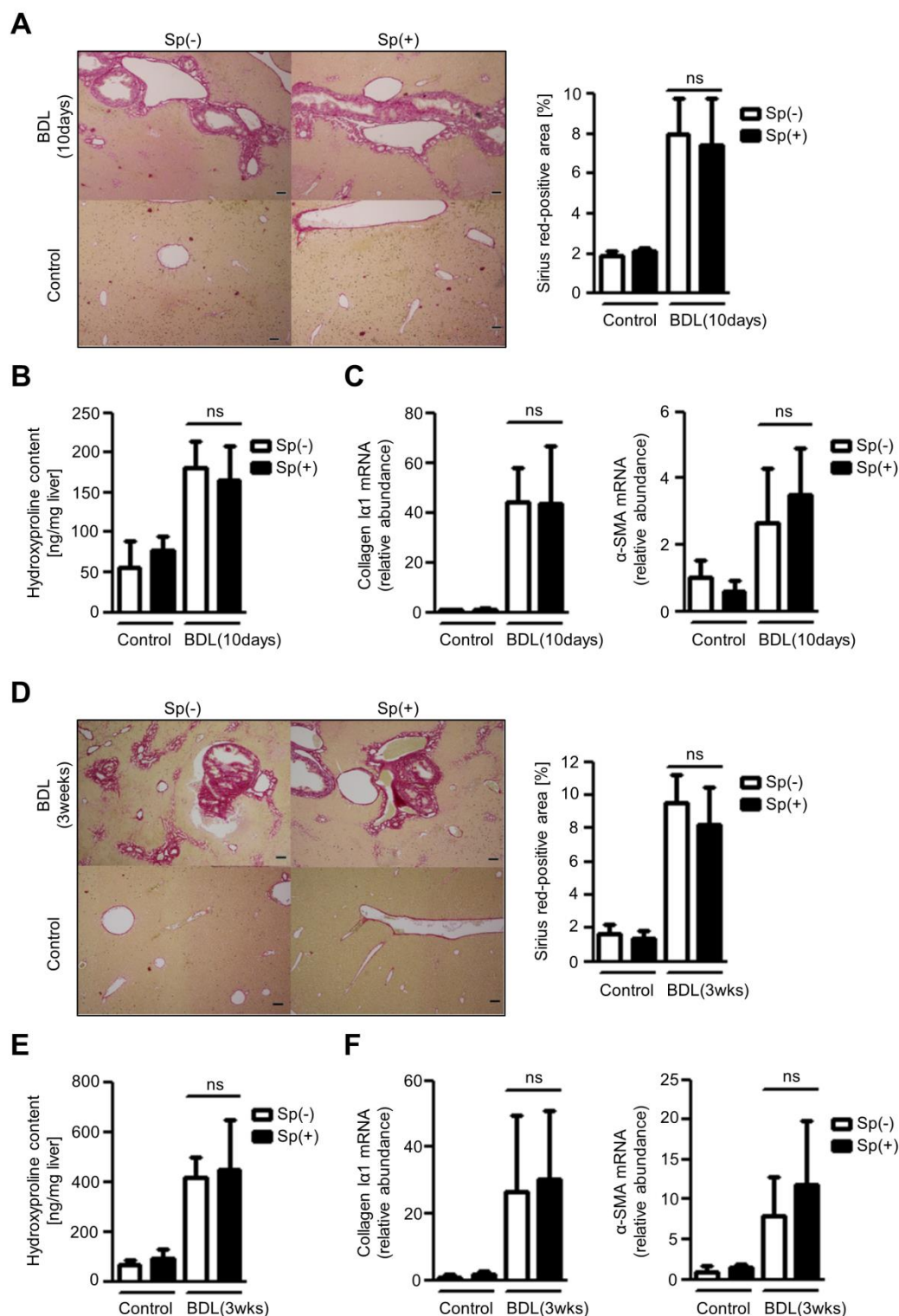


Fig. S2 Splenectomy does not influence the progression of liver fibrosis in a BDL-induced liver injury model. BALB/c mice were divided into sham-operation, splenectomy, BDL and BDL + splenectomy groups. The mice were killed at 10 days and 3 weeks after BDL ($n = 6$ or 7 mice/group). Liver fibrosis was evaluated by Sirius red staining, and the percentage of the Sirius red-positive area was quantified by Image J at 10 days (A) and 3 weeks (D) after BDL. Original magnification: $\times 40$. (B, E) Collagen deposition was quantified by measuring the hydroxyproline content in the liver. (C, F) Quantitative RT-PCR was performed to measure mRNA expression levels of type I collagen $\alpha 1$ and α -SMA. GAPDH was used as an internal control. The mean $\pm$ SD is shown. ns = not significant.

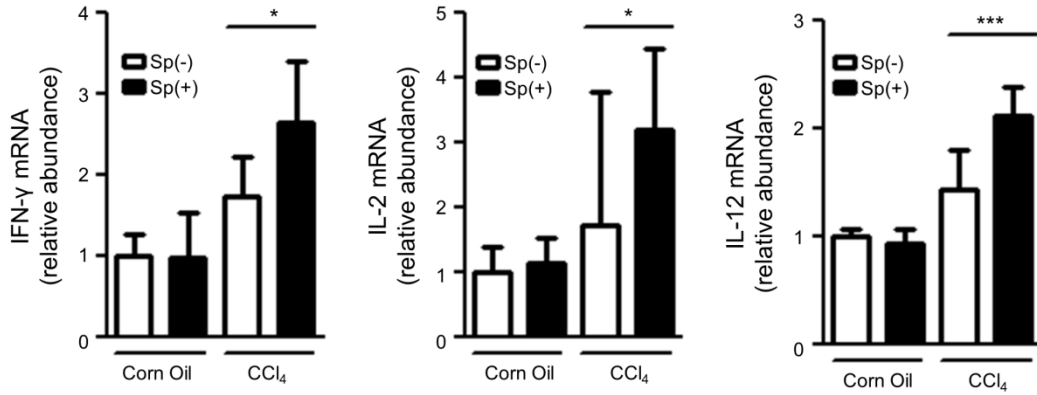
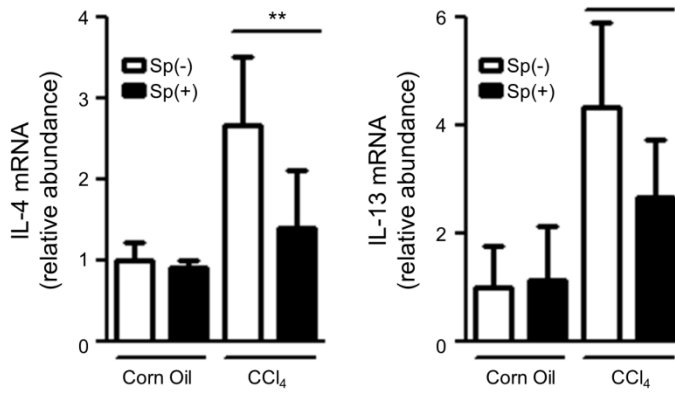
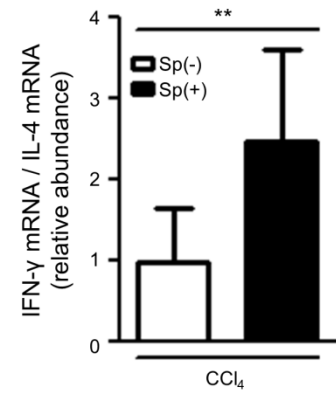
A**B****C**

Fig. S3 Splenectomy modifies Th1/Th2 balance in the liver lymphocytes in a CCl₄-induced liver injury model. Liver fibrosis was induced by CCl₄ for 6 weeks and lymphocytes were isolated from the liver. Quantitative RT-PCR was performed to measure hepatic mRNA expression levels of (A) IFN- γ , IL-2, and IL-12 (Th1 cytokines) and (B) IL-4 and IL-13 (Th2 cytokines). CD4 was used as an internal control. (C) The ratio of the mRNA expression levels of IFN- γ (Th1) to IL-4 (Th2) in each mouse was compared between the CCl₄ Sp(-) group and the CCl₄ Sp(+) group. (n = 8 for CCl₄ groups, n = 5 for control groups). The mean $\pm$ SD is shown. * P < 0.05; ** P < 0.01; *** P < 0.001.

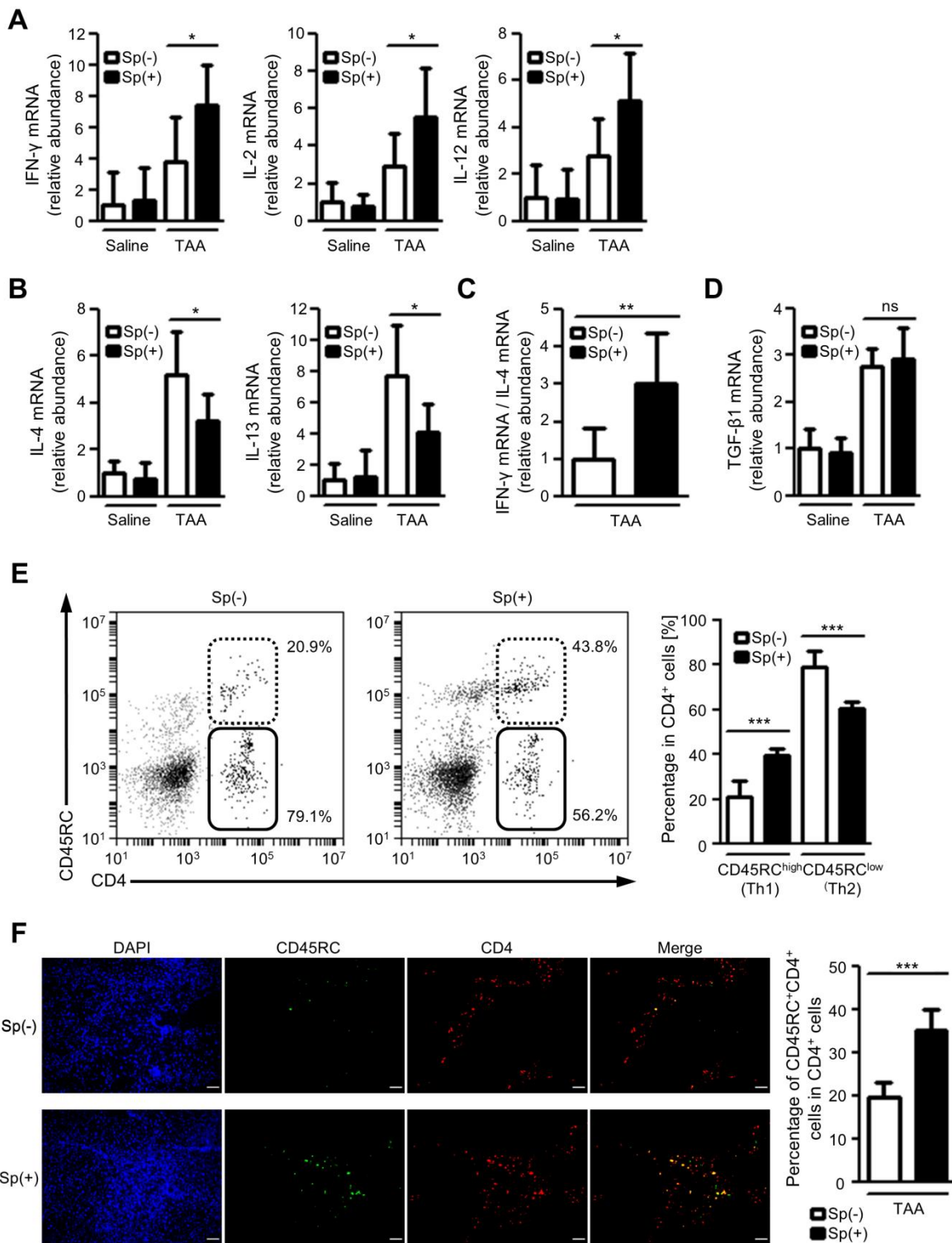


Fig. S4 Splenectomy modifies Th1/Th2 balance in the liver in a TAA-induced liver injury model. Quantitative RT-PCR was performed to measure hepatic mRNA expression levels of (A) IFN- γ , IL-2, and IL-12 (Th1 cytokines) and (B) IL-4 and IL-13 (Th2 cytokines). GAPDH was used as an internal control. (C) The ratio of the mRNA expression levels of IFN- γ (Th1) to IL-4 (Th2) in each mouse was compared between the TAA Sp(-) group and TAA Sp(+) group. (D) The expression levels of TGF- β 1 mRNA, a potent profibrogenic cytokine, were measured by qRT-PCR. (E) Intrahepatic lymphocytes were isolated from Sp(+) and Sp(-) TAA mice and were subjected to flow cytometric analysis for CD4 and CD45RC expressions. Statistical result found in at least seven animals is shown. (F) Double immunofluorescence staining for CD45RC (green) and CD4 (red). The number of CD4⁺ cells and CD45RC⁺CD4⁺ cells were counted under a magnification of 100 $\times$ and the proportion of CD45RC⁺CD4⁺ cells among CD4⁺ cells in the liver is shown. (n=7 for CCl₄ groups, n=6 for control groups). The mean $\pm$ SD is shown. * P < 0.05; ** P < 0.01; *** P < 0.001; ns = not significant.

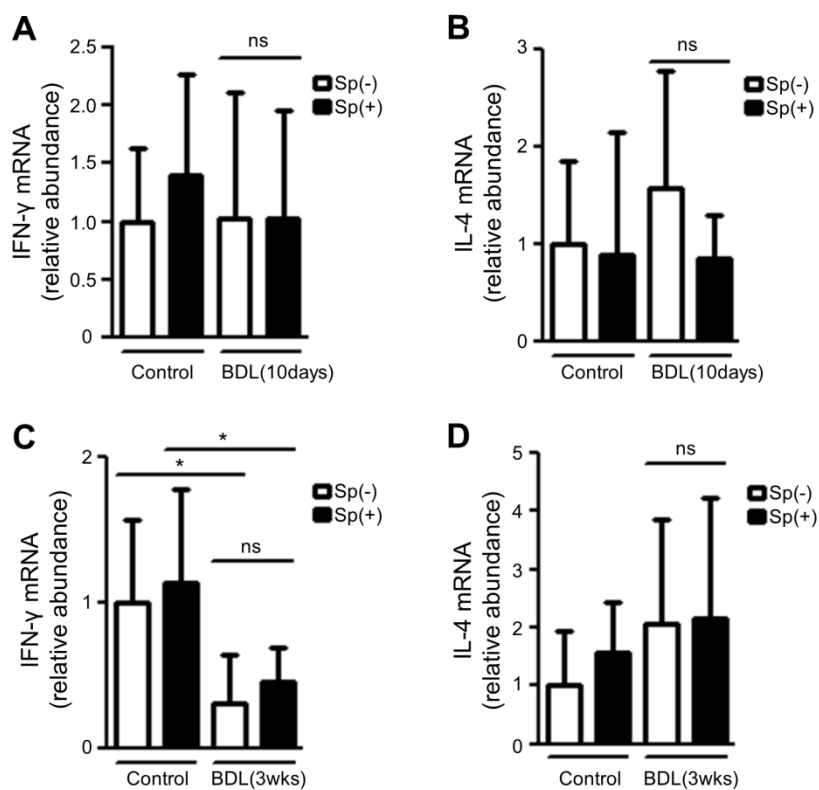


Fig. S5 Splenectomy does not influence Th1 and Th2 cytokine expression in the liver in BDL-induced liver injury. Quantitative RT-PCR was performed to measure mRNA expression levels of IFN- γ (Th1) (A, C) and IL-4 (Th2) (B, D) at 10 days and 3 weeks after BDL. GAPDH was used as an internal control. The mean $\pm$ SD is shown (n = 7 for BDL groups, n = 6 for control groups). * P < 0.05; ns = not significant.

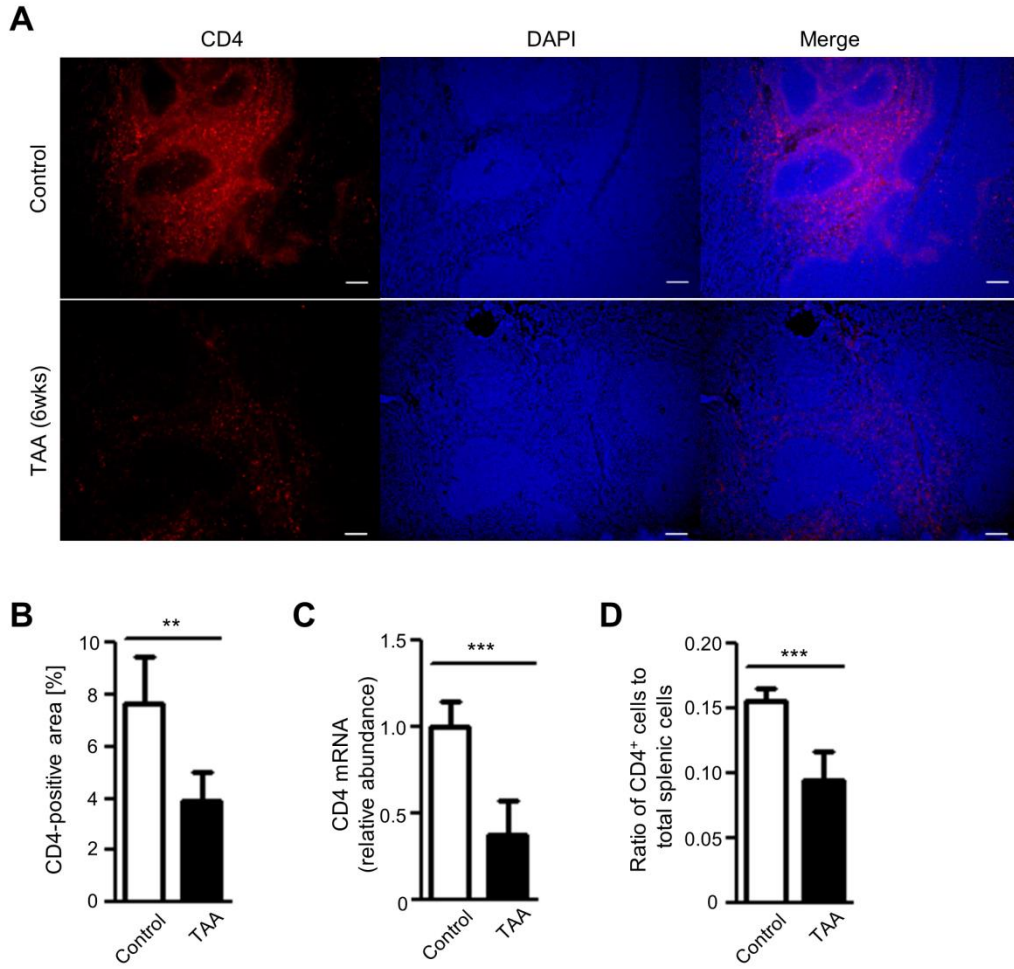


Fig. S6 The number of CD4⁺ T helper lymphocytes in spleen decreases in response to liver injury induced by TAA. Liver fibrosis was induced by injection of TAA as described in Materials and Methods. (A) The expression of CD4, a T helper lymphocyte marker, in the spleen was evaluated by immunofluorescence staining. Original magnification: $\times 40$. (B) The percentage of the CD4-positive area was quantified using Image J. (C) qRT-PCR was performed to measure mRNA expression levels of CD4 in the spleen. GAPDH was used as an internal control. (D) Splenic lymphocytes of the non-treatment control and TAA treatment group mice were analyzed for CD4 expression by flow cytometry. The proportion of CD4⁺ cells among total splenic cells is shown. The mean $\pm$ SD of values found in at least six animals is shown. ** $P < 0.01$, *** $P < 0.001$.

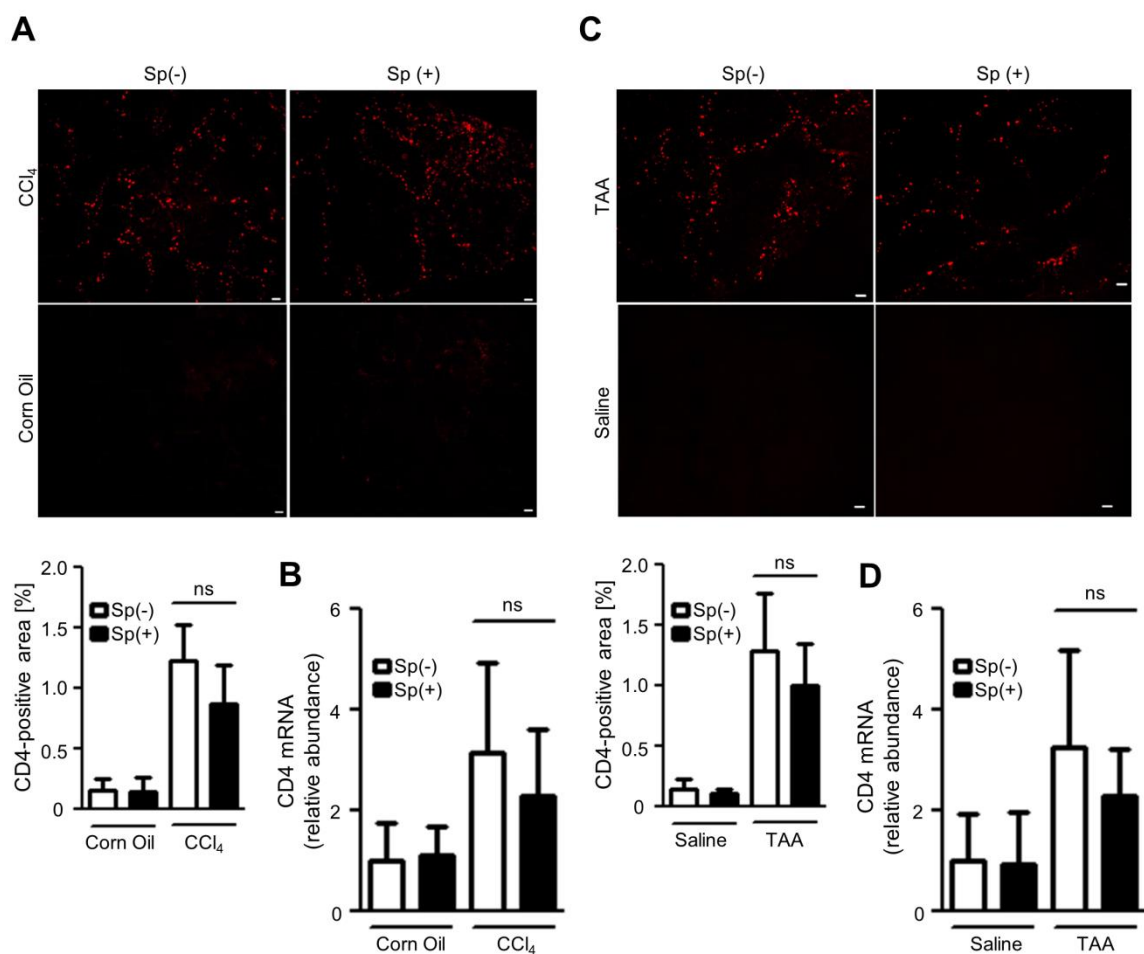


Fig. S7 The number of CD4+ T helper lymphocytes in the liver increases in response to liver injuries Liver fibrosis was induced by injection of CCl₄ or TAA as described in Materials and Methods. (A, C) The expression of CD4, a T helper lymphocyte marker, in the liver was evaluated by immunofluorescence staining. Original magnification: $\times 40$. The percentage of the CD4-positive area was quantified using Image J. (B, D) qRT-PCR was performed to measure mRNA expression levels of CD4 in the spleen. GAPDH was used as an internal control. The mean $\pm$ SD of values found in at least six animals is shown. ns = not significant.

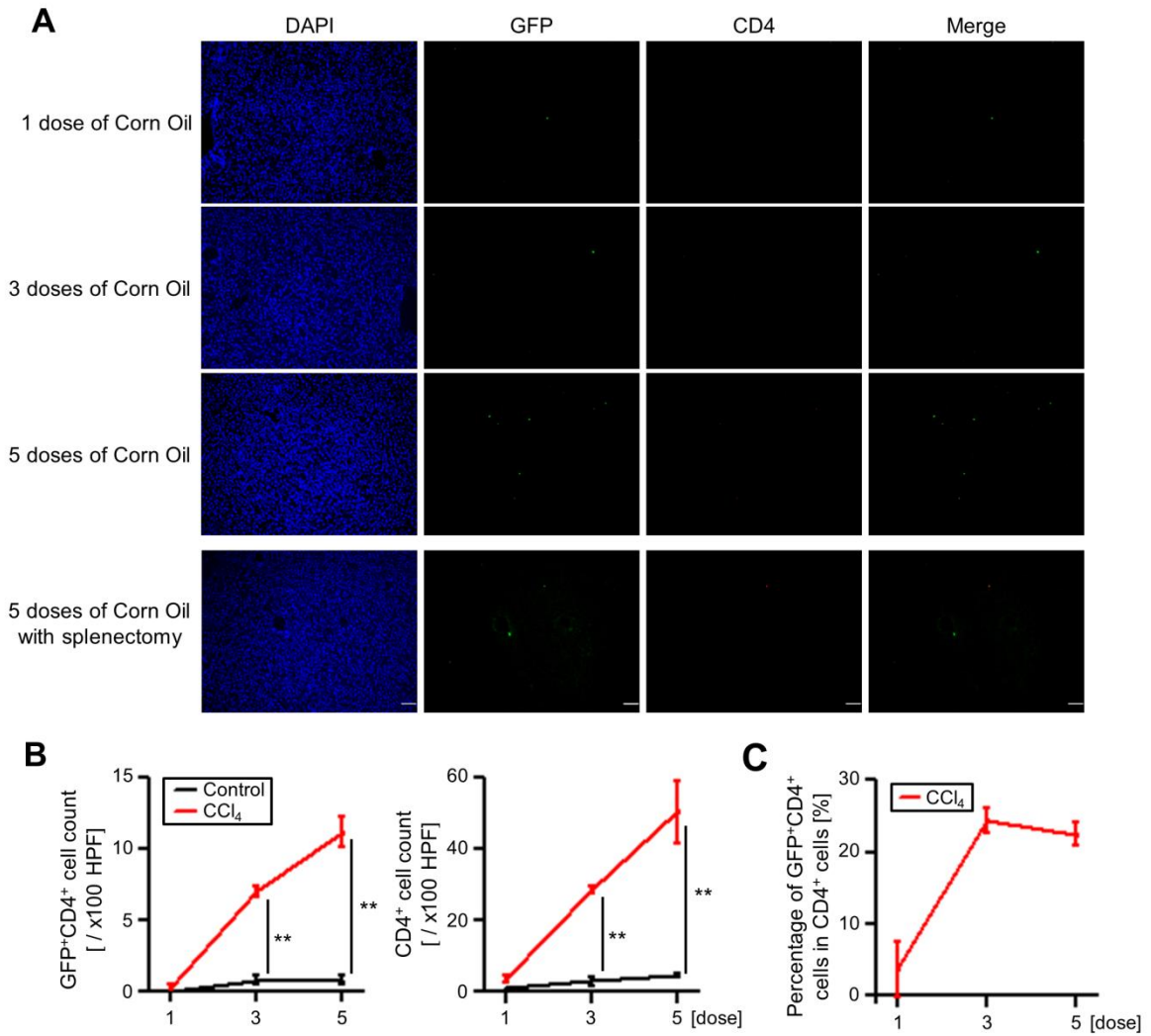


Fig. S8 Spleen-derived T helper lymphocytes migrate from the spleen to the liver in response to liver injury. Splenic lymphocytes isolated from transgenic mice ubiquitously expressing GFP were transplanted into the spleens of syngeneic wild type mice. Liver fibrosis was induced by injection of CCl₄ and control mice received injections of corn oil. The mice were killed at 24 h after the first, third, and fifth injection. (A) Double immunofluorescence staining for GFP (green) and CD4 (red) with or without splenectomy. Original magnification: $\times 100$. (B) The number of CD4⁺ cells and the number of GFP⁺CD4⁺ cells (spleen-derived T helper lymphocytes) were counted under a magnification of $100\times$. (C) The proportion of GFP⁺CD4⁺ cells among CD4⁺ cells in the liver is shown. The mean $\pm$ SD of values obtained in seven animals is shown. ** $P < 0.01$.

Table S1

Primers used quantitative reverse transcription polymerase chain reaction.

Primer	Forward	Reverse
<i>Col-1α1</i>	GCTCCTCTTAGGGGCCACT	CCACGTCTCACCATTGGGG
<i>α-SMA</i>	GTCCCAGACATCAGGGAGTAA	TCGGATACTTCAGCGTCAGGA
<i>IFN-γ</i>	ATGAACGCTACACACTGCATC	TCTAGGCTTTCAATGACTGTGC
<i>IL-2</i>	TGAGCAGGATGGAGAATTACAGG	GTCCAAGTTCATCTTCTAGGCAC
<i>IL-12</i>	TGGTTTGCCATCGTTTTGCTG	ACAGGTGAGGTTCACTGTTTCT
<i>IL-4</i>	GGTCTCAACCCCCAGCTAGT	GCCGATGATCTCTCTCAAGTGAT
<i>IL-13</i>	CCTGGCTCTTGCTTGCCTT	GGTCTTGTGTGATGTTGCTCA
<i>TGF-β1</i>	CCGCAACAACGCCATCTATG	CCCGAATGTCTGACGTATTGAAG
<i>CD4</i>	AAGTGACCTTCAGTCCGGGTA	GGGTTAGAGACCTTAGAGTTGCT
<i>GAPDH</i>	AGGTCGGTGTGAACGGATTTG	GGGGTCGTTGATGGCAACA

The final publication is available at Springer via
<http://dx.doi.org/10.1007/s00535-015-1054-3>.