

# Rapamycin ameliorates CCl<sub>4</sub>-induced liver fibrosis in mice through reciprocal regulation of the Th17/Treg cell balance

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**Abstract.** Previous investigations have suggested that the activation of Th17 cells and/or deficiency of regulatory T cells (Tregs) are involved in the pathogenesis of liver fibrosis. The aim of the present study was to investigate the effect of rapamycin on immune responses in a carbon tetrachloride (CCl<sub>4</sub>)-induced murine liver fibrosis model. Liver fibrosis was induced by intraperitoneal administration with CCl<sub>4</sub>. Following injection of CCl<sub>4</sub>, the mice were treated intraperitoneally with rapamycin (1.25 mg/kg/day) for 8 weeks. Hematoxylin and eosin staining and Masson's trichrome staining were used for histological examination. The protein levels of forkhead/winged helix transcription factor P3, retinoic-acid-related orphan receptor (ROR)- $\gamma$ t in liver tissue were determined by western blotting, the frequency of Th17 and Treg cells in the liver was evaluated by flow cytometry, and a suppression assay was measured by incorporating [3H]-thymidine. In addition, to explore the effect of Tregs expanded with rapamycin on hepatic stellate cells (HSC), HSCs were co-cultured with Tregs from rapamycin or phosphate-buffered saline-treated mice. It was found that rapamycin treatment led to a significant reduction in the number of Th17 cells and in the expression levels of ROR- $\gamma$ t in the liver tissues. Simultaneously, the results of the present study showed a significant increase in the frequency of Tregs and a marked enhancement in the expression of forkhead/winged helix transcription factor P3 in the rapamycin-treated mice. Furthermore, the Tregs in rapamycin-treated mice had significantly higher suppressive effects, compared with the cells from mice treated with phosphate-buffered saline. Consequently, rapamycin treatment prevented the development of CCl<sub>4</sub>-induced hepatic fibrosis, which was shown by its histological appearances. These results suggested that the immunosuppressive effect of rapamycin on liver fibrosis was

associated with the suppression of hepatic fibrogenesis and regulation of the Th17/Treg cell balance.

## Introduction

Liver fibrosis is the final pathological consequence of chronic liver diseases, and is characterized by the formation and accumulation of extracellular matrix, which leads to remodeling of the liver architecture. Following acute and chronic liver injury, inflammation is a hallmark pathological feature of chronic liver disease. The persistent inflammatory responses contribute to liver fibrosis and finally lead to cirrhosis, portal hypertension and hepatocellular carcinoma (HCC). During the process of chronic liver damage, adaptive immune cells are crucially involved in the pathogenesis of hepatic inflammation. The infiltrate contains CD4<sup>+</sup> T cells, which are important in liver injury, antiviral defenses to hepatitis viruses and autoimmunity (1,2).

Previous studies have suggested that Th17 cells and associated cytokines are critical factors in the pathogenesis of liver disease (3,4). Th17 cells expressing retinoic-acid-related orphan receptor (ROR)- $\gamma$ t, have been identified as a novel T cell subset, involved in the pathophysiology of inflammatory disease. Serial investigations have demonstrated that an increase in the number of Th17 cells contributes to disease progression in patients with liver fibrosis (5). By contrast, regulatory T cells (Tregs), also known as CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> T cells, are involved in the maintenance of immune tolerance and homeostasis via contact-dependent suppression or the release of anti-inflammatory cytokines (6,7). Of note, several studies have indicated that the imbalance in the development and function of Th17 cells and Tregs are of critical importance in liver diseases, including autoimmune hepatitis, primary biliary cirrhosis (PBC) and liver fibrosis (8-10). In our previous study, Th17/Treg cell imbalance was found to exist in mice with liver fibrosis, and hepatic stellate cell (HSC) activation was promoted by Th17 cells, but inhibited by Tregs, *in vitro* (11). Th17 cells and Tregs can be interconverted, and are reciprocally regulated during differentiation depending on the cytokine environment. Therefore, correcting Th17/Treg cell imbalance to suppress Th17 and enhance Treg cell numbers may be an attractive target for the treatment of liver fibrosis.

It has been demonstrated that the mammalian target of rapamycin (mTOR) inhibitor, rapamycin, is an

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immunosuppressive compound, which has been used in allograft rejection (12). It has been reported that rapamycin suppresses the transforming growth factor (TGF)- $\beta$  and interleukin (IL)-6-induced generation of IL-17-producing cells, and promotes the TGF- $\beta$ -mediated generation of Tregs (13). In addition, several studies have indicated that rapamycin attenuates inflammatory responses through promoting the differentiation of Tregs and inhibiting the generation of Th17 cells (14,15). Rapamycin markedly decreases numbers of lymphocytes, including CD4<sup>+</sup> T cell subsets, but selectively expands the Treg cell population, whilst maintaining cell function (16). Additional investigations have indicated that these expanded Tregs prevent allograft rejection *in vivo* and suppress the proliferation of T cells *in vitro* (17). However, whether rapamycin exerts immunoregulatory effects in liver fibrosis remain to be fully elucidated. Therefore, the present study aimed to further investigate the protective effects of rapamycin, and its function in regulating the Th17/Treg cell balance, in a carbon tetrachloride (CCl<sub>4</sub>)-induced murine liver fibrosis model.

## Materials and methods

**Animals.** Male C57BL/6 mice (aged 6-8 weeks) were obtained from the Shanghai SLAC Experimental Animal Centre (Shanghai, China) and maintained in specific pathogen-free conditions at 24°C with a 12 h light/dark cycle and unlimited access to food and water. The animal experiments were approved by the Research Ethics Committee of Renji Hospital (Shanghai, China; no. SYXY (hu) 2011-0121). The animals were cared for in accordance with protocols approved by the Animal Care and Use Committee of Renji Hospital.

**Induction of liver fibrosis and rapamycin treatment.** A total of 30 mice were randomly divided into three groups (n=10). To induce liver fibrosis, the mice were injected intraperitoneally, twice each week for 8 weeks, with 5  $\mu$ l/g of 20% CCl<sub>4</sub> (Shanghai Jiahe Biotechnology, Shanghai, China) dissolved in olive oil. Negative control mice were administered with the same volume of olive oil only, for for 8 weeks. Beginning on the day of the CCl<sub>4</sub> injection, the mice in the treatment group were treated intraperitoneally with rapamycin (1.25 mg/kg/day; Gene Operation, Ann Arbor, MI, USA) for the duration of the investigation. In the remaining group of mice, rather than rapamycin, the mice received equivalent volumes of phosphate-buffered saline (PBS) during the course of the experiment. All the mice were sacrificed 72 h following the final CCl<sub>4</sub> injection at 8 weeks, using CO<sub>2</sub> asphyxia.

**Blood biochemistry.** At the time of sacrifice, blood samples were collected from the mouse eyes (~1 ml) and immediately centrifuged for 10 min. Liver function tests were performed at Renji Hospital on serum samples to evaluate the levels of alanine aminotransferase, aspartate aminotransferase and total bilirubin.

**Histological examination.** At the time of sacrifice, the liver was dissected from 6-8 mice. The liver sections were fixed in 4% formaldehyde, embedded in paraffin, and sectioned

(5 mm). The liver sections were then stained with hematoxylin and eosin (H&E) for histopathological examination. Masson's trichrome staining was used for collagen determination. The content of collagen deposition was quantified using an image analyzer (Image-Pro Plus; Media Cybernetics, Inc., Rockville, MD, USA). Immunohistochemical examinations were performed to detect the expression of  $\alpha$ -smooth muscle actin (SMA). In brief, the paraffin sections were deparaffinized and rehydrated. The sections were exposed to fresh 3% hydrogen peroxide for 20 min, and then washed with PBS. Antigens were retrieved in 0.01 M citric acid. The samples were incubated for 30 min at room temperature in 5% normal blocking serum, and incubated with a 1:100 dilution of polyclonal rabbit anti- $\alpha$ -SMA (ab5694) or a 1:100 dilution of monoclonal mouse anti-TGF- $\beta$  (ab64715; Abcam, Cambridge, MA, USA) overnight at 4°C. The slides were then incubated with HRP-conjugated goat anti-rabbit secondary antibody (1:2,000; ab7090; Abcam) or HRP-conjugated goat anti-mouse (1:2,000; ab6789; Abcam) for 60 min at room temperature, and with 3,3'-diaminobenzidine as a substrate. Negative controls were obtained by omitting the primary antibodies. Finally, the sections were counterstained with haematoxylin, and mounted.

**Western blot analysis.** Frozen liver tissue (50 mg) was rapidly thawed and homogenized at 4°C in 0.5 ml lysis buffer (Beyotime Institute of Biotechnology) for 30 min. Homogenates were centrifuged at 12,000 x g for 10 min at 4°C to yield supernatants. A bicinchoninic acid protein assay kit was used to detect the protein concentrations. The samples were then heated at 95°C for 5 min. The proteins (30  $\mu$ g) were separated by 12% SDS-PAGE (Beyotime Institute of Biotechnology, Shanghai, China) and then transferred onto a polyvinylidene difluoride membrane (Beyotime Institute of Biotechnology). The membrane was blocked with 5% non-fat milk for 2 h at room temperature, and incubated with indicated antibodies, including anti- $\alpha$ -SMA (1:500 dilution), monoclonal mouse anti-forkhead/winged helix transcription factor P3 (FoxP3; 1:500 dilution; ab20034; Abcam) and polyclonal rabbit anti-ROR- $\gamma$ t (1:1,000 dilution; ab78007; Abcam) in Tris-buffered saline with 0.05% Tween overnight at 4°C. All antibodies were purchased from Abcam. The membranes were then rinsed and incubated at room temperature with HRP-conjugated secondary antibody (1:10,000 dilution; LI-COR Biosciences, Lincoln, NE, USA). Signals were detected using an Odyssey Infrared image system (LI-COR, Inc., Lincoln, NE, USA).

**Intracellular cytokine staining.** Intracellular cytokine staining was performed, as previously described (18). To investigate the frequency of Tregs, lymphocytes from the spleen and liver were prepared. To obtain single-cell suspensions from the spleen and liver, tissue was dissected into small pieces, ground and filtered through stainless steel meshes. Splenocytes were isolated from the cell suspensions by gradient centrifugation at 1,400 x g for 25 min at 4°C with Lymphoprep (Dakewei Biotechnology, Shenzhen, China). Following surface staining with anti-CD4-fluorescein isothiocyanate (FITC; 11-0041; eBioscience, San Diego, CA, USA) and anti-CD25-peridinin-chlorophyll-protein (45-0251;

Table I. Effects of rapamycin on serum levels of AST, ALT and TBIL in mice with liver fibrosis.

Group	ALT	AST	TBIL
Vehicle	43.32±1.98	105.78±6.53	1.42±0.30
CCl <sub>4</sub> +PBS	232.17±89.75	407.13±134.45	19.80±8.41
CCl <sub>4</sub> +rapamycin	91.30±8.45 <sup>a</sup>	136.92±6.87 <sup>a</sup>	9.08±0.89 <sup>a</sup>

Values are presented as the mean ± standard error of the mean. <sup>a</sup>P<0.05 compared with the CCl<sub>4</sub>+PBS-treated mice. ALT, alanine aminotransferase; AST, aspartate aminotransferase; TBIL, total bilirubin; CCl<sub>4</sub>, carbon tetrachloride; PBS, phosphate-buffered saline.

eBioscience), the cells were fixed, permeabilized and stained with anti-FoxP3-phycoerythrin (PE; 12-5773; eBioscience). For Th17 cell detection, the lymphocytes from the spleen and liver were stimulated for 5 h with phorbol-12-myristate-13-acetate (50 ng/ml), ionomycin (1 µg/ml) and 5 µg/ml brefeldin A (Sigma-Aldrich, St. Louis, MO, USA) in RPMI-1640 (GE Healthcare Life Sciences, Logan, UT, USA) containing 10% fetal bovine serum (FBS; GE Healthcare Life Sciences). The cells were harvested and stained with anti-CD4-FITC. The cells were fixed and permeabilized with Cytofix/Cytoperm (BD Pharmingen, San Diego, CA, USA) and stained intracellularly with anti-IL-17A-PE (12-7177; eBioscience). Cells stained with IgG isotype control were used as controls. All antibodies were purchased from eBioscience. Data were obtained using a FACSCalibur flow cytometer and analyzed using CXP analysis software, version 2.1 (Beckman Coulter, Inc., Brea, CA, USA).

**Cell isolation and culture.** To obtain single-cell suspensions from the spleen, tissue was dissected into small pieces, ground and filtered through stainless steel meshes. Splenocytes were isolated from the cell suspensions by gradient centrifugation at 1,400 x g for 25 min at 4°C with Lymphoprep (Dakewei Biotechnology). The splenocytes were separated into CD4<sup>+</sup>CD25<sup>+</sup> T cells and CD4<sup>+</sup>CD25<sup>-</sup> T cells using a CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer's protocol. These cells were cultured in RPMI-1640 medium supplemented with 10% FBS and penicillin/streptomycin. All cells were cultured at 37°C in a humidified 5% CO<sub>2</sub> incubator.

**Suppression assay.** The splenocytes from the rapamycin- or PBS-treated mice were separated into CD4<sup>+</sup>CD25<sup>+</sup> T cells and CD4<sup>+</sup>CD25<sup>-</sup> T cells, as described above. Subsequently, 2x10<sup>5</sup> CD4<sup>+</sup>CD25<sup>-</sup> T cells (responder cells) with titrated CD4<sup>+</sup>CD25<sup>+</sup> T cells (effector cells) were suspended in 200 µl RPMI-1640, seeded into 96-well plates, and stimulated with a combination of soluble anti-CD3 (1 µl/ml; 557306; BD Pharmingen) and soluble anti-CD28 (1 µl/ml; 553295; BD Pharmingen). In co-culture experiments, purified CD4<sup>+</sup>CD25<sup>+</sup> T cells and CD4<sup>+</sup>CD25<sup>-</sup> T cells were simultaneously added into the wells, and cultured at 37°C for 72 h. Following an 18 h pulse with [<sup>3</sup>H] thymidine (1 µCi/well; Shanghai Institute of Nuclear Research, Shanghai, China), proliferation was analyzed using a scintillation counter (Beckman LE 5000CE, Beckman Coulter, Inc.).

**Preparation of HSCs.** Mouse HSCs were isolated from the livers of the C57BL/6, as described previously (19). In brief, collagenase perfusion *in situ* and density gradient centrifugation were used for isolating the HSCs. The liver was perfused with collagenase at 37°C at a flow rate of 18, until the hepatic parenchyma beneath the capsule appeared liquefied. This was then subjected to gradient centrifugation using OptiPrep (Sigma-Aldrich), at 2,000 x g for 20 min at 4°C. The isolated HSCs were cultured in RPMI-1640 (GE Healthcare Life Sciences) containing 10% FBS and penicillin/streptomycin. The HSCs were plated at 3x10<sup>5</sup> cells/well into 6-well plates, and were cultured for 6 days, following which they were harvested for subsequent use. Cell viability was >90%, determined using a trypan blue exclusion assay. The purity of the HSCs was >92%, as determined by the typical appearance of the lipid droplets under a light microscope (IX50; Olympus Corporation, Tokyo, Japan) (20). All cells were cultured in a humidified incubator with 5% CO<sub>2</sub> at 37°C.

**Co-culture experiment.** A co-culture experiment was performed using the HSCs and splenic Tregs isolated from the rapamycin- and PBS-treated mice. The HSCs were plated at a density of 1x10<sup>5</sup> cells/well in a 24-well plate for 24 h, following which Treg cells (1x10<sup>5</sup> or 2x10<sup>5</sup> cells/well) were added to the culture system. The cells were co-cultured for 3 days and the expression levels of α-SMA were detected using immunofluorescence staining and western blot analysis.

**Immunofluorescence staining.** The cells were fixed in 4% formaldehyde and permeabilized in methanol. Following blocking, the cells were incubated overnight with rabbit anti-mouse α-SMA monoclonal antibody (1:100 dilution; Abcam) followed by CY3-conjugated goat anti-rabbit antibody (1:400 dilution; 112-165-143, Jackson ImmunoResearch, West Grove, PA, USA) at room temperature. The nuclei were stained with DAPI. The expression of α-SMA was observed under a fluorescent microscope (Nikon Eclipse Ti-S, Nikon Corporation, Tokyo, Japan) and fluorescence intensity was analyzed using Image-Pro Plus software, version 6.0.

**Statistical analysis.** Data are presented as the mean ± standard error of the mean. Statistical analyses were performed using SPSS version 17.0 software (SPSS, Inc., Chicago, IL, USA). Data were analyzed using Student's *t*-test or one way analysis of variance. P<0.05 was considered to indicate a statistically significant difference.



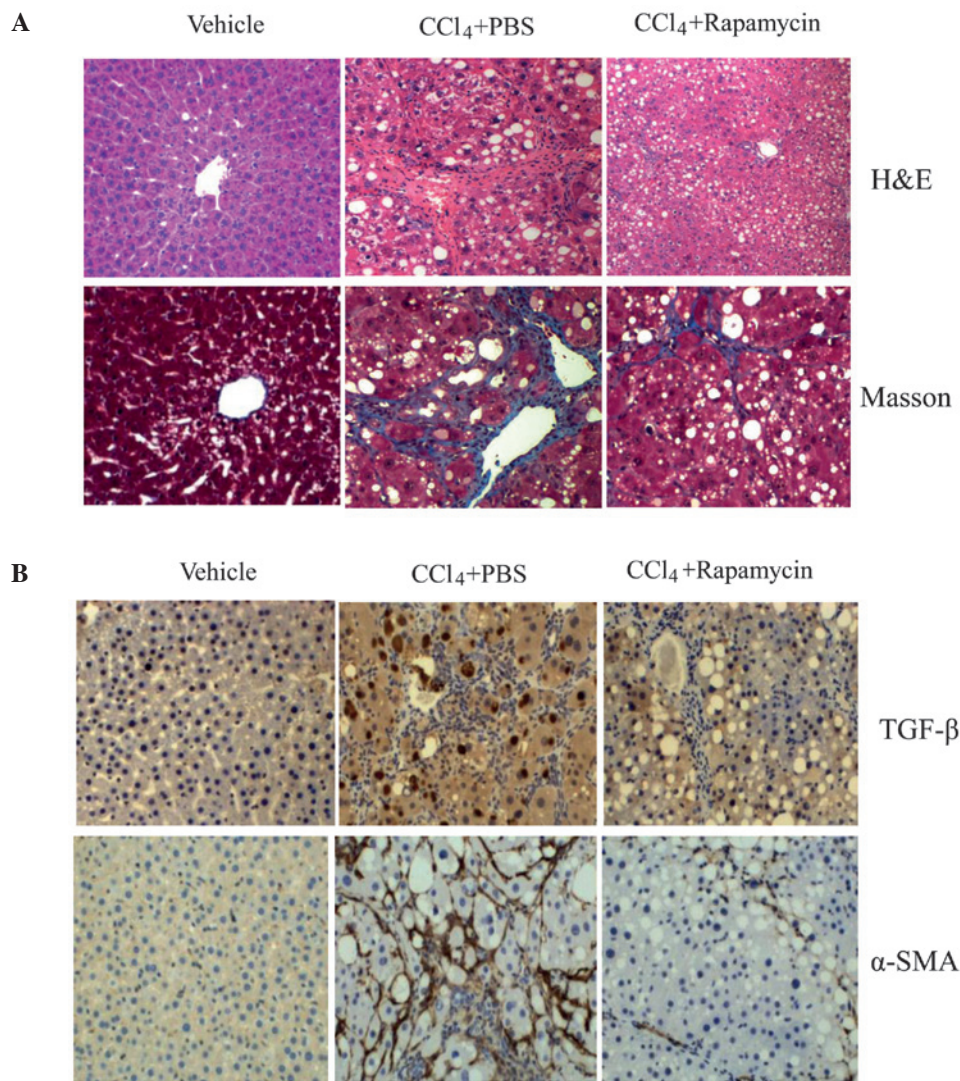


Figure 1. Rapamycin attenuates hepatic fibrosis induced by CCl<sub>4</sub> in mice. (A) Pathological changes of liver fibrosis in each group were determined using H&E or Masson's trichrome staining (magnification, x200). (B) Immunohistochemical analysis was used for detecting the protein expression of  $\alpha$ -SMA and TGF- $\beta$ 1 in the liver tissues of each group. The results show the protein expression of TGF- $\beta$ 1 (magnification, x200) and  $\alpha$ -SMA (magnification, x200) in the fibrotic livers of each group. CCl<sub>4</sub>, carbon tetrachloride; PBS, phosphate-buffered saline; H&E, hematoxylin and eosin; TGF- $\beta$ 1, transforming growth factor- $\beta$ 1;  $\alpha$ -SMA,  $\alpha$ -smooth muscle actin.

## Results

### *Rapamycin protects mice against CCl<sub>4</sub>-induced liver fibrosis.*

The present study aimed to assess the antifibrogenic effects of rapamycin via the expansion of Tregs and inhibition of Th17 cells in CCl<sub>4</sub>-induced hepatic fibrosis. To examine the effect of rapamycin on liver function, total bilirubin and serum aminotransferases were detected. The liver function tests showed that rapamycin significantly reduced the concentrations of total bilirubin and aminotransferases in the CCl<sub>4</sub>-induced liver fibrosis model, compared with the PBS-treated control group (Table I).

The liver fibrosis model was established by chronic CCl<sub>4</sub> injection, and the degree of hepatic fibrosis was detected using H&E and Masson's trichrome staining (Fig. 1A). Following CCl<sub>4</sub> administration, the liver tissues of the mice treated with PBS exhibited a distorted architecture, with extensive fibrosis combined with the development of micronodules throughout the liver parenchyma shown in the H&E staining. Liver

injury was attenuated in the rapamycin-treated mice. The deposition of collagen fibers as an indicator of liver fibrosis was determined using Masson's trichrome staining. The pathological progression in liver fibrosis was attenuated by rapamycin, with fewer and smaller fibrotic nodules observed. As a marker of HSC activation,  $\alpha$ -SMA is one of the sensitive indices of the rate of fibrogenesis (21). As shown in Fig. 1B, the expression of  $\alpha$ -SMA was significantly elevated in the mice with CCl<sub>4</sub>-induced liver fibrosis, and was reduced following rapamycin administration. As a key factor of fibrogenesis, TGF- $\beta$  in the rapamycin-treated mice showed reduced expression, compared with that in the PBS-treated mice.

*Rapamycin inhibits the generation of Th17 cells.* To analyze the immunoregulatory effect of rapamycin on Th17 cells in CCl<sub>4</sub>-induced liver fibrosis, the percentages of Th17 cells in the mouse spleen and liver were measured using flow cytometry (Fig. 2A). As shown in Fig. 2B, the cells from the PBS-treated mice showed markedly higher percentages of Th17 cells,

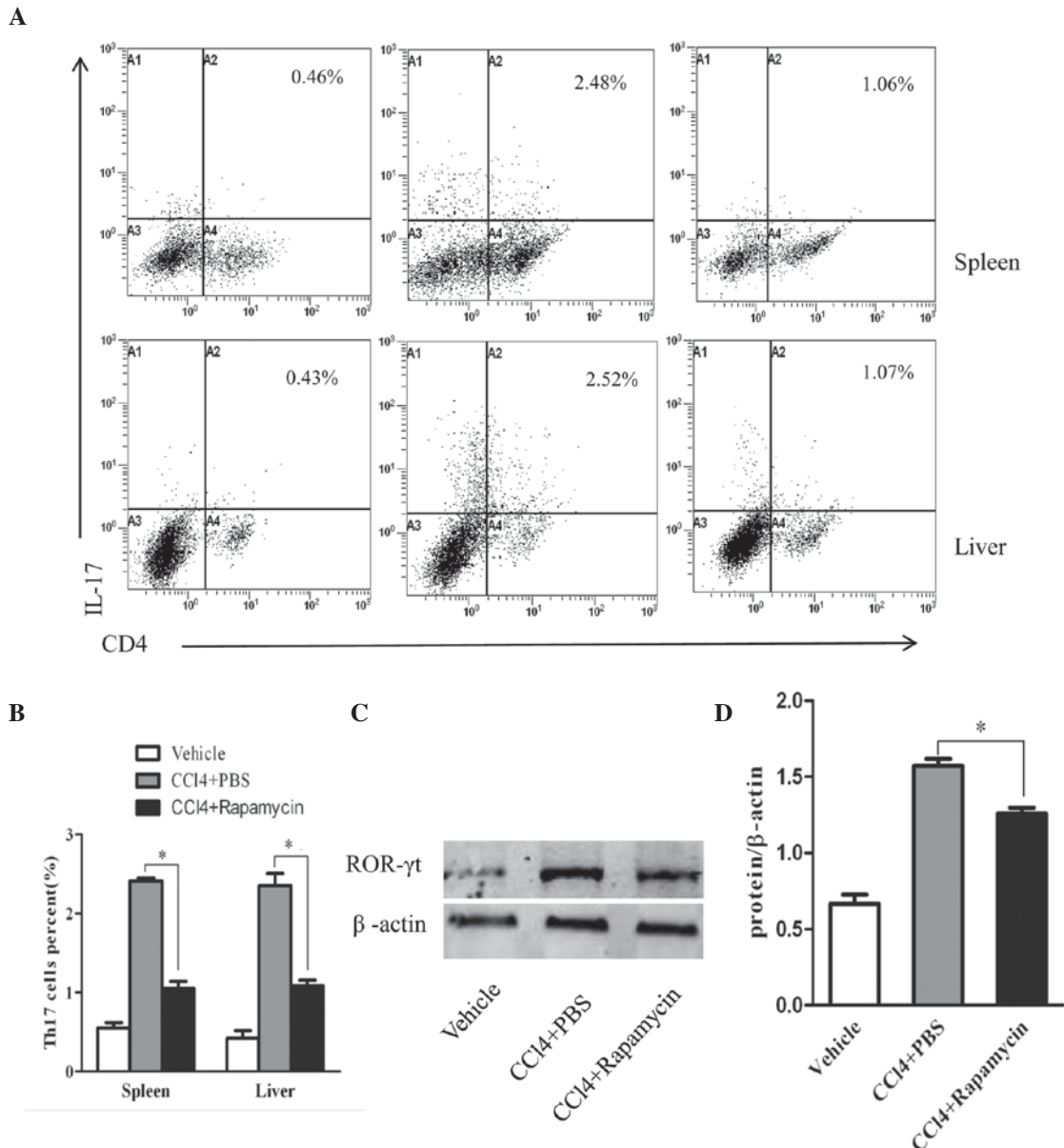


Figure 2. Rapamycin suppresses the generation of Th17 cells in liver tissues. (A) Cells were isolated from spleen and liver tissues in each group of mice and subjected to intracellular IL-17A staining. The percentages of Th17 cells were determined using flow cytometry and were (B) quantified. Data represent one of three separate experiments with similar results and values are expressed as the mean  $\pm$  standard error of the mean (n=5). \*P<0.01, vs. PBS-treated group. (C) Protein levels of ROR- $\gamma$ t in the liver tissues were determined using western blot analysis and (D) expressed as the relative change, compared with the control animals. Data are presented as the mean  $\pm$  standard error of the mean (n=6). \*P<0.05, vs. PBS-treated group. CCl<sub>4</sub>, carbon tetrachloride; PBS, phosphate-buffered saline; IL-17A, interleukin-17A; ROR-retinoic-acid-related orphan receptor- $\gamma$ t.

compared with the negative control group (oil without CCl<sub>4</sub>). Following rapamycin administration, the percentages of Th17 cells were significantly lower, compared with those in the PBS-treated mice, but were higher, compared with the negative control group (Fig. 2B). In addition, as a crucial transcription factor of Th17 cell differentiation, the expression of ROR- $\gamma$ t in the rapamycin-treated mice was decreased, compared with that in the PBS-treated mice (Fig. 2C). The relative protein levels are shown in Fig. 2D.

*Rapamycin expands the Treg cell population, with a suppressive effect in hepatic fibrosis.* The present study subsequently investigated whether the effect of rapamycin was associated with modulation of Treg cell function in CCl<sub>4</sub>-induced hepatic

fibrosis. The percentages of Tregs in the spleen and liver were analyzed using flow cytometry (Fig. 3A). The frequencies of Tregs in the spleen and liver from the rapamycin-treated mice were significantly higher, compared with those of the PBS-treated mice (Fig. 3B). The hepatic protein expression of FoxP3 was then examined using western blot analysis. Consistent with the results of the flow cytometric analysis, the expression of FoxP3 in the liver from the rapamycin-treated mice was markedly enhanced, compared with that from the PBS-treated mice (Fig. 3C). The relative protein levels are shown in Fig. 3D.

Furthermore, to clarify the effect of the mTOR inhibitor on the suppressive efficacy of Tregs, splenic CD4<sup>+</sup>CD25<sup>+</sup> responder T cells from the PBS-treated mice were stimulated with

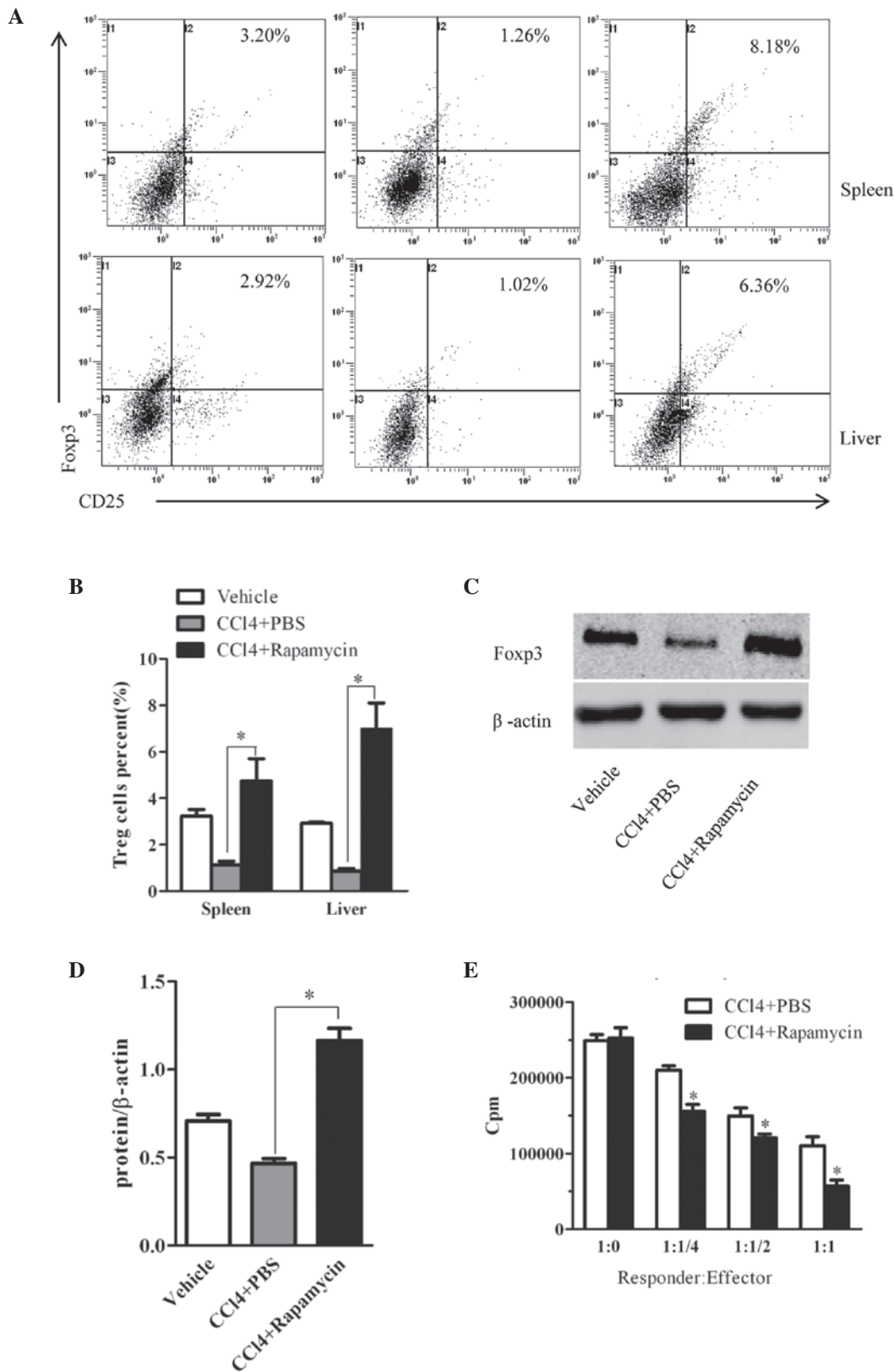


Figure 3. Rapamycin treatment upregulates the suppressive efficacy of Tregs in mice with liver fibrosis. (A) Cells were isolated from the spleen and liver tissues of mice in each group and subjected to intracellular FoxP3 staining. Treg cell frequency was analyzed using flow cytometry and (B) quantified. Data represent one experiment of three with similar results and values are expressed as the mean  $\pm$  standard error of the mean (n=5). \*P<0.01, vs. PBS-treated group. (C) Protein levels of FoxP3 in the liver were determined using western blot analysis and (D) expressed as relative change, compared with the control animals. Data are presented as the mean  $\pm$  standard error of the mean (n=6). \*P<0.01, vs. PBS-treated group. (E) CD4<sup>+</sup>CD25<sup>+</sup> T cells were purified from the spleen tissues of the rapamycin- or PBS-treated mice. The titrated CD4<sup>+</sup>CD25<sup>+</sup> T cells were co-cultured with  $2 \times 10^5$  CD4<sup>+</sup>CD25<sup>-</sup> T cells, as responder cells, which were obtained from PBS-treated mice in the presence of anti-CD3/CD28 antibody. The co-cultured cells were maintained for 72 h, and 1  $\mu$ Ci [<sup>3</sup>H]-thymidine was added to the culture 18 h prior to harvesting. The ratios of CD4<sup>+</sup>CD25<sup>+</sup> T cells: CD4<sup>+</sup>CD25<sup>-</sup> T cells are shown. Data are presented as the mean  $\pm$  standard error of the mean (n=3 in each group). \*P<0.05, vs. PBS-treated group. Tregs, regulatory T cells; CCl<sub>4</sub>, carbon tetrachloride; PBS, phosphate-buffered saline; FoxP3, forkhead/winged helix transcription factor P3.

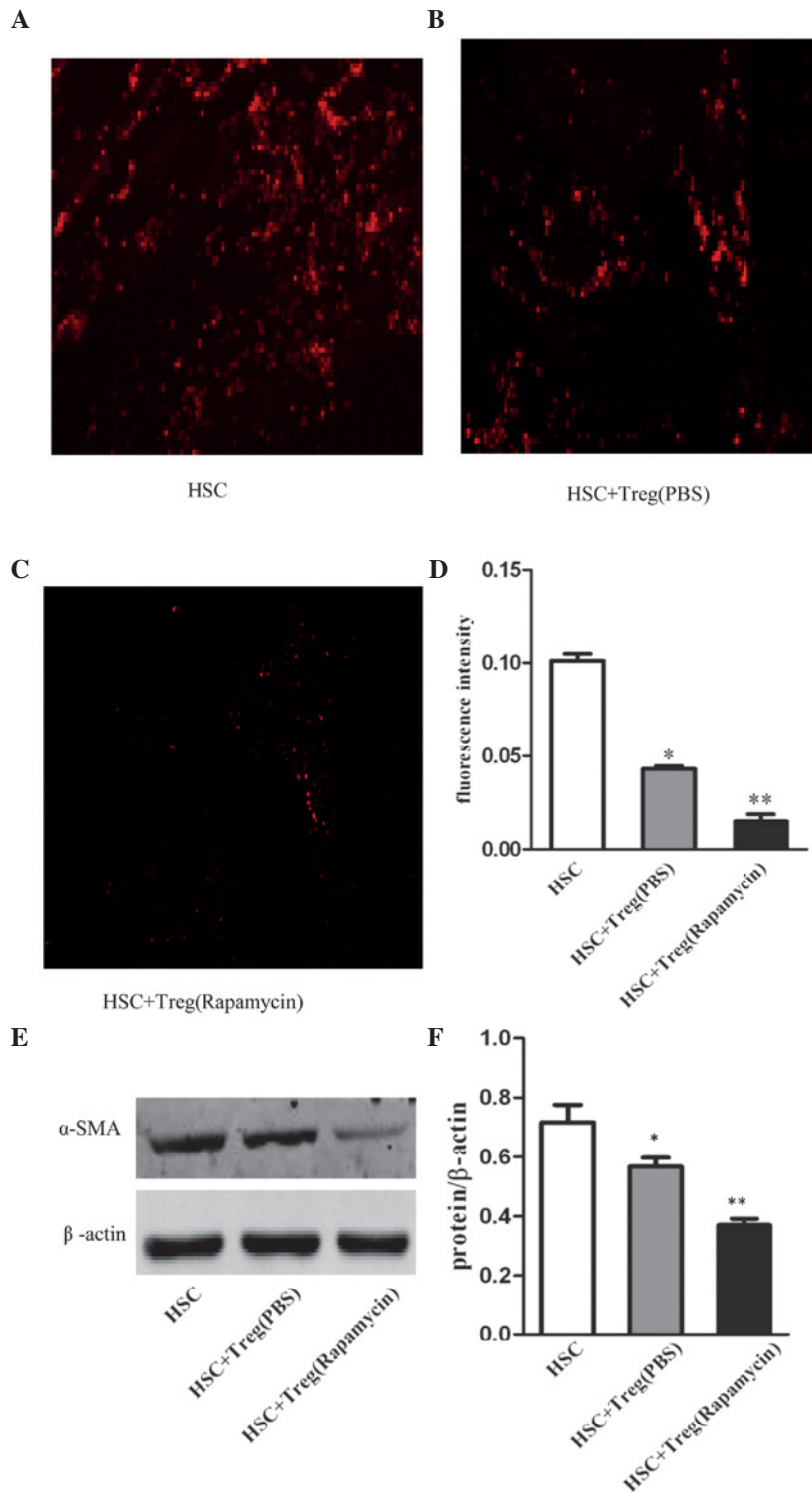


Figure 4. Treg expansion by rapamycin suppresses the activation of HSCs. According to previous methods, Tregs were isolated from rapamycin- or PBS-treated mice, and co-cultured with HSCs. (A-C) Expression levels of  $\alpha$ -SMA were detected using immunofluorescence staining (magnification, x200) and western blot analysis. (D) Fluorescence intensity reflecting the relative levels of intracellular  $\alpha$ -SMA. Values are expressed as the mean  $\pm$  standard error of the mean of triplicate experiments. \* $P < 0.01$ , vs. control; \*\* $P < 0.01$ , vs. HSC+Treg (PBS). (E) Western blot analysis and (F) quantification following treatment of HSCs with Tregs from different groups. Data are presented as the mean  $\pm$  standard error of the mean ( $n = 3$  in each group). \* $P > 0.05$ , vs. control; \*\* $P < 0.01$ , vs. HSC+Treg (PBS). HSCs, hepatocellular stellate cells; Treg, regulatory T cell;  $CCl_4$ , carbon tetrachloride; PBS, phosphate-buffered saline;  $\alpha$ -SMA,  $\alpha$ -smooth muscle actin.

anti-CD3/CD28 monoclonal antibody and co-cultured with  $CD4^+CD25^+$  Tregs from the rapamycin- or PBS-treated mice. As shown in Fig. 3E, the suppressive activities of the Tregs in the splenic tissues of the rapamycin-treated mice were markedly higher, compared with those of the PBS-treated mice.

*Effect of rapamycin-induced Treg cell expansion on HSCs.* It is known that regulatory immune cells, including  $CD4^+CD25^+$  T cells, are important in the pathogenesis of liver fibrosis and the activation of HSCs (9,11). To examine the effect of the Treg cell expansion by rapamycin on HSCs,



HSCs were isolated from the mouse liver, and then cultured with splenic Tregs from the rapamycin- or PBS-treated mice. Immunofluorescence staining and western blot analysis were performed to evaluate changes in the expression of  $\alpha$ -SMA. As shown in Fig. 4A-C, immunofluorescence staining showed that the expression of  $\alpha$ -SMA was significantly reduced following exposure to the Tregs from the rapamycin-treated mice, compared with those from the PBS-treated mice. Fig. 4D shows the fluorescence intensity of each group, which shows the trends described above. The results of the western blot analysis showed that, compared with the control group, the expression of  $\alpha$ -SMA was reduced following exposure to the Tregs, however, the difference was not significant. The protein expression of  $\alpha$ -SMA was also markedly decreased following exposure to Tregs from the rapamycin-treated mice, compared with the PBS-treated mice (Fig. 4E). The expression levels of  $\alpha$ -SMA are shown in Fig. 4F. These data showed that the expansion of the Treg cell population by rapamycin increased the capacity to inhibit the activation of HSCs.

## Discussion

In the present study, prominent decreases in the histological changes and the severity of liver fibrosis were observed in the mice treated with rapamycin, compared with those treated with PBS. The data from the present study showed that rapamycin effectively protected the liver in CCl<sub>4</sub>-induced hepatic fibrosis, which resulted from a significant increase in the functional activity of CD4<sup>+</sup>CD25<sup>+</sup> Tregs. Rapamycin was involved in the downregulation of the Th17 cell response in the development of liver fibrosis. Furthermore, rapamycin enhanced the suppressive capacities of the Tregs on the activation of HSCs.

Previous studies have demonstrated that Tregs positively restrict the inflammatory response, prevent the development of autoimmune diseases and inhibit a series of immune responses (22,23). By contrast, Th17 cells are involved in the induction of autoimmune diseases (24,25). Of note, Rong *et al* (26) found that the Th17 cell population and expression of ROR- $\gamma$ t were markedly increased in the peripheral blood of patients with primary biliary cirrhosis; whereas the Treg cell population and expression of FoxP3 were markedly decreased. Consequently, a lower Treg/Th17 ratio is likely to indicate increased liver injury and progression of fibrosis. Additional studies have reported that Tregs isolated from patients with chronic hepatitis B inhibited the proliferation and activation of HSCs, whereas recombinant IL-17 promoted the proliferation and activation of HSCs (27). In accordance, animal experiments have supported findings that Treg/Th17 imbalance in mice with liver fibrosis potentially promotes liver fibrosis via HSC activation (11). In addition, naïve CD4<sup>+</sup> T cell differentiation is manipulated by distinct expression patterns of transcription factors. For example, signal transducer and activator of transcription (STAT)5 and FoxP3 direct Treg cell differentiation and induce the production of regulatory cytokines, including TGF- $\beta$  and IL-10 (28), whereas STAT3 and ROR $\gamma$ t dominate Th17 cell polarization and IL-17 production (29). The mTOR inhibitor, rapamycin, has been reported to regulate FoxP3 and ROR- $\gamma$ t genes directly, which contribute to the induction of Treg cell differentiation and suppresses the

formation of Th17 cells (16,30). However, the role of mTOR inhibition in regulating Th17 and Treg cell differentiation has not been examined thoroughly in mice with liver fibrosis.

The present study found that, in the progression of CCl<sub>4</sub>-induced liver fibrosis, rapamycin treatment led to a significant decrease in the percentages of Th17 cells in the spleen and liver. Simultaneously, the numbers of Tregs in the spleen and liver were markedly increased, compared with the PBS-treated mice. In addition, the hepatic expression of ROR- $\gamma$ t was markedly suppressed, whereas the expression of FoxP3 in the liver was promoted following rapamycin treatment. These findings indicated that, in liver fibrosis, rapamycin was able to correct the imbalance between Th17 and Treg cells, thus inhibiting the inflammatory response. The mechanism underlying the rapamycin-induced downregulation of ROR- $\gamma$ t and upregulation of Foxp3 in hepatic fibrosis remains to be fully elucidated, indicating further *in vitro* experiments are required.

There has been increasing recognition that Tregs are important in suppressing autoimmune diseases. *In vitro*, Tregs with suppressive functions have been found in several diseases, including multiple sclerosis (31), type 1 diabetes (32), and acute hepatitis C virus infection (33). In addition, there is increasing evidence showing that rapamycin is able to promote the expansion of Tregs and suppress the proliferation of responder T cells (34,35). To determine whether rapamycin can expand the population of functional Tregs from mice with liver fibrosis, the present study co-cultured CD4<sup>+</sup>CD25<sup>+</sup> T cells and CD4<sup>+</sup>CD25<sup>-</sup> T cells to examine the suppressive function. The results revealed an enhanced immunosuppressive capacity of the Tregs in the rapamycin-treated group of mice, compared with the PBS-treated mice. Therefore, the present study also confirmed that the inhibitor of mTOR, rapamycin promoted the differentiation of Tregs, with a suppressive function in the development of liver fibrosis.

Saxena *et al* (36) reported that Tregs regulated the cardiac fibroblast phenotype and reduced the expression of  $\alpha$ -SMA *in vitro*. According to our previous studies (11,15), Tregs suppress immune responses, and inhibit the activation of HSCs. The activation of HSCs is the dominant event occurring in liver fibrogenesis, which is characterized by the transformation of HSCs into myofibroblasts. The expression level of  $\alpha$ -SMA is a sensitive indicator of the fibrogenic myofibroblasts (37). To investigate whether the expansion of Tregs by rapamycin *in vitro* increased the inhibitory activity on HSC activation, the HSCs were co-cultured with Tregs from rapamycin-treated mice or PBS-treated mice, respectively. It was found that Treg expansion due to rapamycin reduced the expression of  $\alpha$ -SMA and effectively inhibited HSC activation. *In vivo*, rapamycin treatment significantly reduced the expression levels of  $\alpha$ -SMA and TGF- $\beta$ 1 in the liver and reduced the progress of fibrogenesis. These results indicated that rapamycin had an inhibitory effect on HSC activation and hepatic fibrosis.

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