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Immune Regulation of Intrahepatic Regulatory T Cells in Fibrotic Livers of Mice

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Statistical Analysis C
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Manuscript Preparation E
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Background: Liver fibrosis is the result of chronic inflammation and repair, and many immune cells contribute to the process. Regulatory T cells (Tregs) mediate immune tolerance and are highly expressed in liver fibrosis. However, few reports have studied the specific effects of Tregs on regulating immune cells in liver fibrosis. The present study aimed to investigate the regulation of Tregs on intrahepatic immune cells in liver fibrosis by depleting Tregs in mice.

Material/Methods: Liver fibrosis was induced by carbon tetrachloride, and an anti-CD25 mAb (PC61) was used to deplete Tregs. Liver fibrosis and injury were reflected by immunofluorescence staining and alanine aminotransferase level. The expressions of immune cell Tregs and cytokines were detected by flow cytometry and/or real-time PCR. Interferon-γ (IFN-γ) concentration was measured by ELISA.

Results: Tregs were rich in fibrotic livers; after Tregs depletion, the intrahepatic $CD4^+$ T cell and Kupffer cells (KC) populations did not change compared with liver fibrosis, but $CD8^+$ T cells were slightly elevated. However, natural killer (NK) cells and IFN-γ levels were significantly decreased in fibrosis and increased after Tregs depletion. Interesting, we found Tregs promoted KC M1/M2 balance to M2, because inducible nitric oxide synthase (M1) was increased but arginase-1 (M2) was reduced after depleting Tregs. Furthermore, in isolated KCs from livers, IL-12 (M1) was increased, but TGF-β (M2) was reduced after depleting Tregs, compared with fibrotic livers.

Conclusions: Tregs are involved in the immune regulation of liver fibrosis, primarily by suppressing NK cells and M1 KCs, and mildly suppressing $CD8^+$ T cells.

MeSH Keywords: Immunity, Cellular • Liver Cirrhosis • T-Lymphocytes, Regulatory

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Background

The liver is known to induce immune tolerance and immunity, such as the tolerization of food antigens in the liver and acceptance of liver allografts across full major histocompatibility complex barriers, which serves as a shield between the gut and systemic circulation [1]. The liver is rich in abundant immunocytes that perform immune functions. Regulatory T cells (Tregs) are important mediators of immune suppression, and they prevent reactions against self by inducing regulatory signals to antigen-presenting cells and/or effector T cells [2,3]. Tregs represent a unique lineage of $CD4^+$ T cells characterized by a constitutively high expression of CD25 ($CD4^+CD25^+$ Tregs), in which the transcription factor forkhead box P3 (Foxp3) is critically important for their development and function [4,5]. Tregs have been shown to suppress effector T cells, which are inherent immunocytes [6], and are often found increased in chronically infected tissues, fibrosis, and tumors [7–9].

Hepatic fibrosis is a wound-healing response to liver injury from several etiologies, and many immune cells, such as $CD8^+$ and $CD4^+$ T cells, natural killer (NK) cells, and cytokines, are involved in fibrosis [10]. $CD4^+CD25^+$ Tregs are highly expressed in the peripheral blood of post-hepatitis cirrhosis patients, suggesting that they may be involved in cirrhosis [7]. However, few studies have reported the specific effects of Tregs on regulating immune cells in liver fibrosis. The present study aimed to investigate the regulation of Tregs on some immune cells in liver fibrosis by depleting Tregs, including $CD4^+$ T cells and $CD8^+$ T cells, NK cells, and Kupffer cells (KCs), which may help us better understand the net immune regulation of Tregs in liver fibrosis.

Material and Methods

Animals

Male C57BL/6 mice (6-8 weeks of age) were purchased from the Laboratory Animal Center, Academy of Military Medical Science (Beijing, China). Experimental procedures were approved by the Institutional Animal Care and Use Committee at Capital Medical University, which complies with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

Liver fibrosis was induced in mice by intraperitoneal administration of carbon tetrachloride (CCl_4) (30% in mineral oil) at a dose of 10 ml/g twice a week, total 7-8 weeks. The same volume of saline was injected in mice as controls.

$CD4^+CD25^+$ regulatory T cell depletion

Anti-CD25 mAbs were purified from the culture supernatant of hybridoma PC61 cells. The details of the process were previously described in our published paper [11].

Material sampling

Mice were anesthetized by 10% chloral hydrate at 0.1 ml/10 g body weight. The liver was perfused with saline from the inferior vena cava to rinse out intrahepatic blood and was then isolated. Some liver samples were frozen at –80°C for RNA isolation, and some fresh samples were prepared for flow cytometry analysis.

Flow cytometry analysis

Fresh liver tissue was minced through a cell strainer with Dulbecco's modified Eagle's medium (DMEM) and centrifuged at 2000 rpm for 10 min, and the supernatant was discarded. The pellets were resuspended with 2 ml DMEM, containing type-IV collagenase (200 U/ml) and DNase (50 U/ml) (Sigma-Aldrich, St. Louis, MO). The suspension was rocked for 40 min at 37°C. After washing, the cells underwent gradient centrifugation on 40% Percoll and 70% Percoll (GE Healthcare Bio-Sciences, Sweden), 2000 rpm for 20 min at 4°C. Then the middle layers were collected, washed, and resuspended in phosphate-buffered saline (PBS). Cells were incubated with anti-mouse CD25-PE-Cy5, and anti-mouse CD4-FITC, or anti-mouse F4/80- PE-Cy5, anti-mouse CD8-FITC, and anti-mouse NK 1.1- PE. Intracellular Foxp3 staining was performed after cellular surface staining, as detailed in our published paper [11]. Stained cells were analyzed on a Cytomics FC500 flow cytometer (Beckman-Coulter, USA). Data were analyzed by CXP software (Beckman-Coulter, USA). All antibodies were purchased from eBioscience (San Diego, CA, USA).

Isolation of RNA and real-time PCR

Total RNA was prepared from liver tissue or isolated KCs using TRIZOL Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA was then transcribed using RNA PCR Kit (AMV) (TaKaRa, Dalian, Liaoning, China). Quantitative real-time PCR was conducted according to the manufacturer's instructions using SYBR Premix EXTaq™ (TaKaRa, Dalian, Liaoning, China). The specific gene primers are shown in Table 1. The reactions were performed on ABI 7000 (Applied Biosystems) using the following program: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. All transcript copy numbers per target gene were calculated by normalization to GAPDH. Analysis was performed using StepOne software 2.0 (Applied Biosystems).

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Table 1. The primer used in Real-time PCR.

Gene	Sequence
Foxp3	F: 5'- CCC AGG AAA GAC AGC AAC CTT -3'
	R: 5'- TTC TCA CAA CCA GGC CAC TTG -3'
iNOS	F: 5'- CGG AGC CTT TAG ACC TCA ACA -3'
	R: 5'- CCC TCG AAG GTG AGC TGA AC-3'
Arginase-1	F: 5'- CTG GCA GTT GGA AGC ATC TCT-3'
	R: 5'- GTG AGC ATC CAC CCA AAT GAC -3'
IL-1β	F: 5'- ACT CCT TAG TCC TCG GCC A -3'
	R: 5'- TGG TTT CTT GTG ACC CTG AGC -3'
IL-12P40	F: 5'- CAG CTT CTT CAT CAG GGA CAT -3'
	R: 5'- CTT GAG GGA GAA GTA GGA ATG G-3'
TNF-α	F: 5'- TCT CTT CAA GGG ACA AGG CTG -3'
	R: 5'- ATA GCA AAT CGG CTG ACG GT -3'
IL-10	F: 5'- GGA CAA CAT ACT GCT AAC CG -3'
	R: 5'- TTC ATG GCC TTG TAG ACA CC -3'
TGF-β	F: 5'- GTG CGG CAG CTG TAC ATT GAC TTT -3'
	R: 5'- TGT ACT GTG TGT CCA GGC TCC AAA -3'
GAPDH	F: 5'- AAC TTT GGC ATT GTG GAA GG -3'
	R: 5'- ACA CAT TGG GGG TAG GAA CA -3'

Histological and Immunofluorescence staining

Liver tissue samples embedded in Optimum Cutting Temperature Medium (Sakura Finetek USA, Inc, Torrance, Calif) were cut into 5-μm sections, and dried overnight. The details of Masson's trichrome and immunofluorescence staining can be found in our previous report [11]. Anti-collagen-I-PE was from Southern Biotech and anti-Foxp3-FITC was from eBioscience.

Analysis of plasma aminotransaminase

The serum alanine aminotransferase (ALT) activities were determined by the automated clinic biochemical system, which was quantified liver injury.

Isolation of Kupffer cells

KCs were isolated from mouse livers and we detected the cytokines of different subtypes of KCs. In brief, fresh livers were digested in the presence of Pronase (Roche, Mannheim, Germany) and collagenase IV, put through a cell strainer (70-μm) in a 50-ml tube and then rocked for 40 min at 37°C. After short centrifugation and washing, the cells underwent gradient centrifugation in 40% Percoll and were loaded on 70% Percoll at 1100 g for 20 min. The middle white layer was harvested and gradient centrifugation in 25% Percoll and loaded on 50% Percoll at 1800 g for 30 min. The middle white layer (KCs) was washed and plated in culture dishes for RNA extracts.

ELISA analysis

The interferon-γ (IFN-γ) concentration in mouse serum was measured by use of an ELISA kit (Quantikine® ELISA, R&D Systems, Inc) according to the manufacturer's instructions. The optical density was read at 450 nm on a microplate reader (Bio-Rad).

Statistical analyses

The analysis and graphs were completed using GraphPad Prism, 5.0 (GraphPad Software Inc., San Diego, CA, USA). The differences between values were evaluated with an unpaired two-tailed Student's *t* test or one-way ANOVA, followed by Newman-Keuls test. All results are expressed as the mean ±SEM, and $P<0.05$ was considered statistically significant.

Results

Tregs expression in the fibrotic liver with or without CD25 antibody injection

CCl_4 is extensively used to study hepatic fibrosis in animal models [10], which we also used to induce liver fibrosis in this study. To study the effects of Tregs on intrahepatic immune cells, we depleted Tregs using anti-CD25 mAb in mice with liver fibrosis. In the fibrotic liver, type-I collagen and $Foxp3^+$ cells were distributed largely (Figure 1A), and $CD4^+CD25^+Foxp3^+$ Tregs (Tregs) were significantly increased in the livers of CCl_4-treated mice compared with control mice (n=7, $P<0.001$) (Figure 1B, 1C). After anti-CD25 mAb treatment, Tregs decreased by 51.3% compared with CCl_4-treated mice (n=7–8, $P<0.001$) (Figure 1B, 1C). We also measured Foxp3 mRNA levels by real-time PCR and found that Foxp3 levels increased in fibrotic livers and decreased after Tregs depletion (n=7) (Figure 1D). An IgG_1 isotype control for anti-CD25 mAb (PC61) did not affect Foxp3 expression in the fibrotic liver (n=4) (Figure 1E). These results suggest that PC61 effectively depleted Tregs in our experiments. In addition, we found that liver fibrotic status was improved after Tregs depletion (Figure 1F), which agrees with our pervious results [11]. The level of ALT in serum was not different between liver fibrosis with or without Tregs depletion (Figure 1G), indicating that Tregs depletion did not further aggravate liver injury on the basis of liver fibrosis.

The regulation of Tregs on hepatic T lymph cells in the fibrotic liver

Cell immunity is mediated by T lymphocytes, which can be divided into $CD4^+$ T lymphocytes and $CD8^+$ T lymphocytes. We detected these 2 populations of T lymphocytes in fibrotic livers of mice with or without Tregs depletion. $CD4^+$ T cells were reduced in fibrotic livers compared with control livers (n=6, $P<0.001$)

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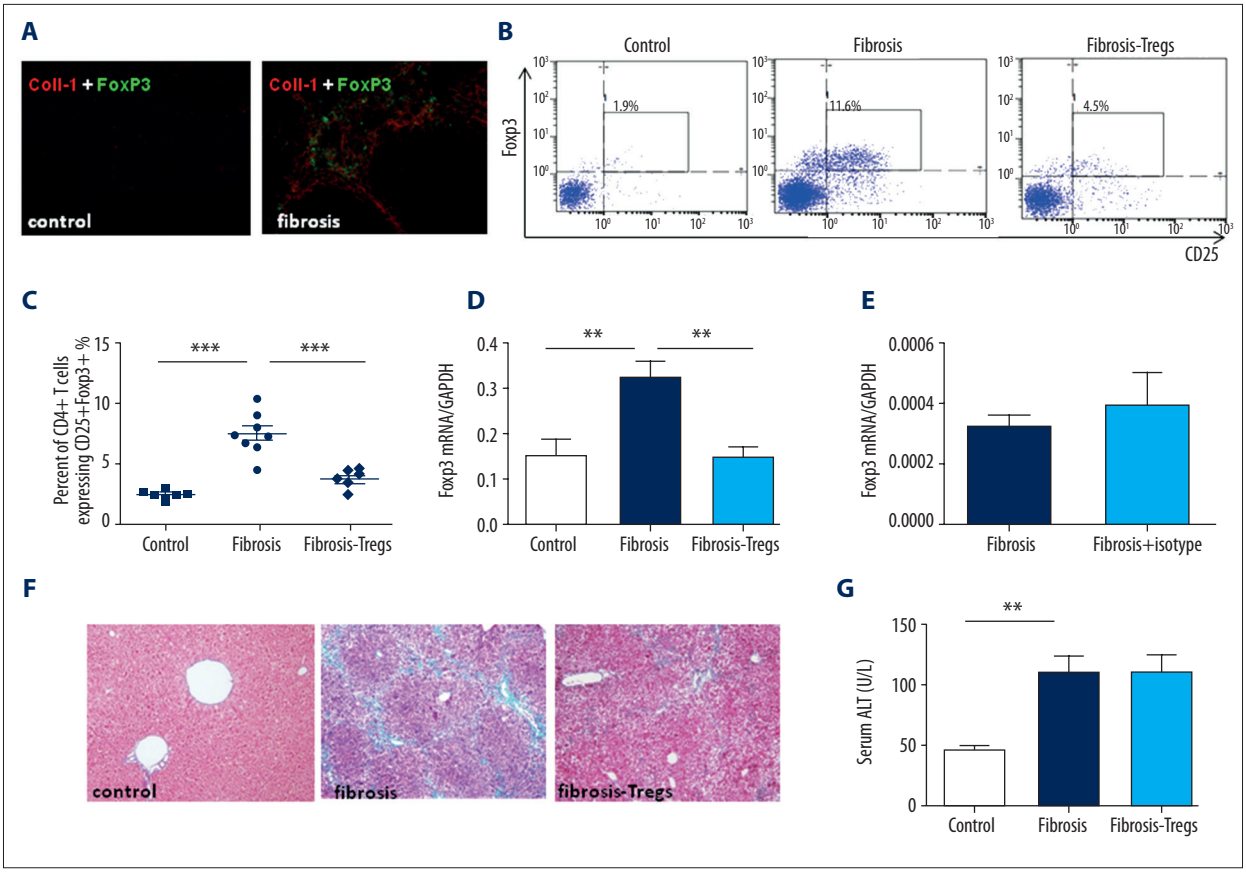

Figure 1. Anti-CD25 mAb injection depletes Tregs *in vivo*. (**A**) The intimate interaction of Tregs (Foxp3$^+$) with type-I collagen was determined by immunofluorescent staining. (**B, C**) Tregs expression in control and fibrosis-induced mice with or without Tregs depletion by flow cytometry. (**D**) Foxp3 mRNA levels in liver fibrosis of mice with or without Tregs depletion. (**E**) Foxp3 mRNA levels in fibrosis-induced mice treated with rat IgG_1 as the isotype control for PC61. (**F**) The fibrotic status was improved after Tregs depletion by Masson trichrome staining. (**G**) The serum ALT of mice was measured. Data are presented as the mean ±SEM. ** P<0.01, *** P<0.001.

and did not change after Tregs depletion (n=6) (Figure 2A, 2C). CD8$^+$ T cells were significantly increased by 2.6-fold in fibrotic livers (n=7–8, P<0.01) compared with controls. However, Tregs depletion resulted in an 39% increase in CD8$^+$ T cells compared with fibrosis, but did not reach statistical significance (n=7–8, Figure 2B, 2D). These results suggest that Tregs do not significantly affect CD4$^+$ T lymphocytes, but might partly reduce the CD8$^+$T lymphocyte population in liver fibrosis.

The regulation of Tregs on hepatic innate immune cells in liver fibrosis

Normal livers and injured livers are enriched in innate immune cells, which have a significant impact on hepatic fibrogenesis. Among them, KCs and NK cells have been shown to play an important role in regulating liver fibrosis, whereas other innate immune cells, such as mast cells, neutrophils, and NKT cells, have less of effect on experimental liver fibrosis [12]. Therefore, we measured the expression of KCs (F4/80) and NK cells (NK1.1). KCs were enhanced by 1-fold in fibrotic livers compared with control mice (n=8, P<0.01). However, there was no difference between fibrotic livers and those depleted of Tregs (Figure 3A, 3B). Furthermore, we examined changes in KCs subtype "pro-inflammatory" M1 (inducible nitric oxide synthase [iNOS]) and "immunoregulatory" M2 (arginase-1). M1 KCs were slightly reduced in the fibrotic livers, but significantly increased after Tregs depletion (n=5, P<0.05, Figure 3C). M2 KCs were reduced in the liver after Tregs depletion compared with fibrotic livers (n=6, P<0.05, Figure 3C). To further confirm the KC subtype, we isolated KCs from mouse livers and detected the representative cytokines of M1 KCs such as tumor necrosis factor-alpha (TNF-α), IL-1β, and IL-12, as well as M2 KCs such as TGF-β and IL-10. In KCs of fibrotic livers, the expression of IL-1β, IL-12P40, IL-10, and TGF-β was higher than those in control livers (n=9, P<0.05 in IL-1β; P<0.05 in IL-12P40; P<0.05 in IL-10; P<0.01 in TGF-β) (Figure 3D). After Tregs depletion, IL-12P40 was significantly higher than that in fibrosis (n=9, P<0.05), but TGF-β was lower than that in fibrosis (n=9, P<0.01) (Figure 3D).

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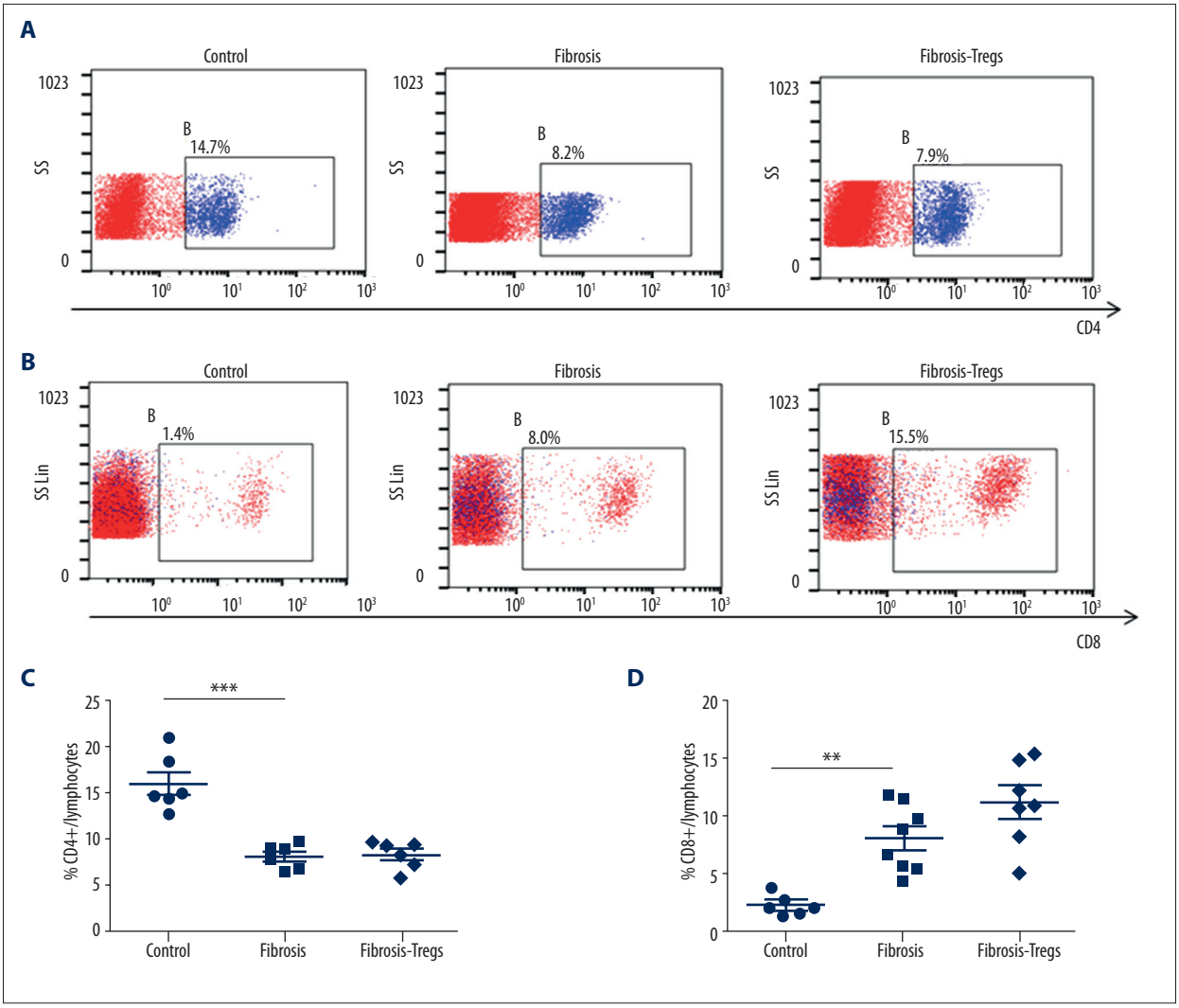

Figure 2. The regulation of Tregs on intrahepatic CD4⁺ T cells and CD8⁺ T cells. (**A**) CD4⁺ T cell and CD8⁺ T cell (**B**) in control and fibrosis mice with or without Tregs depletion. (**C**) Statistical results of CD4⁺ T cells and CD8⁺ T cells (**D**) by flow cytometry are shown in the graph. Data are presented as the mean ±SEM. ** *P*<0.01, *** *P*<0.001.

NK cells were significantly decreased in fibrotic livers (*n*=8, *P*<0.01) but increased after Tregs depletion (*n*=8, *P*<0.01) (Figure 4A, 4B). IFN-γ was the primary cytokine secreted by NK cells. IFN-γ levels in serum were 2.5±0.3 pg/ml in control mice, which decreased by 44% in fibrosis (1.4±0.3 pg/ml, *n*=5, *P*<0.05) but increased to 2.6±0.3 pg/ml after Tregs depletion (fibrosis *vs.* Tregs depletion, *n*=5, *P*<0.05) (Figure 4C). We further found that the changes of IFN-γ mRNA in mouse livers were consistent with the results in serum (*n*=6, fibrosis *vs.* control, *P*<0.05; *n*=6, fibrosis *vs.* Tregs depletion, *P*<0.05) (Figure 4D).

Discussion

The liver had an immune-privileged state, featuring by oral tolerance, acceptance of allografts, hepatic viral persistence, and high incidence of tumor metastasis from other organs [13]. Due to its function, the liver is frequently exposed to various insults that cause cell death and hepatic dysfunction. The occurrence and development of hepatic fibrosis is primarily caused by immune injury and fiber repair. Tregs are important immune inhibitory cells that play important roles in controlling immune balance. Several studies have suggested that Tregs are closely linked to many liver diseases, such as chronic hepatitis B virus or hepatitis C virus infection, non-alcoholic fatty liver disease, autoimmune hepatitis, and alcoholic hepatitis [7,14,15]. The present study found that Tregs are involved in the maintenance of liver fibrosis via regulating the balance of matrix metalloproteinase (MMP) and tissue inhibitors of metalloproteinase (TIMP) [11]. Although Tregs are not the primary cause of liver fibrosis, their suppression of some immune cells affects disease development to a certain extent. The present

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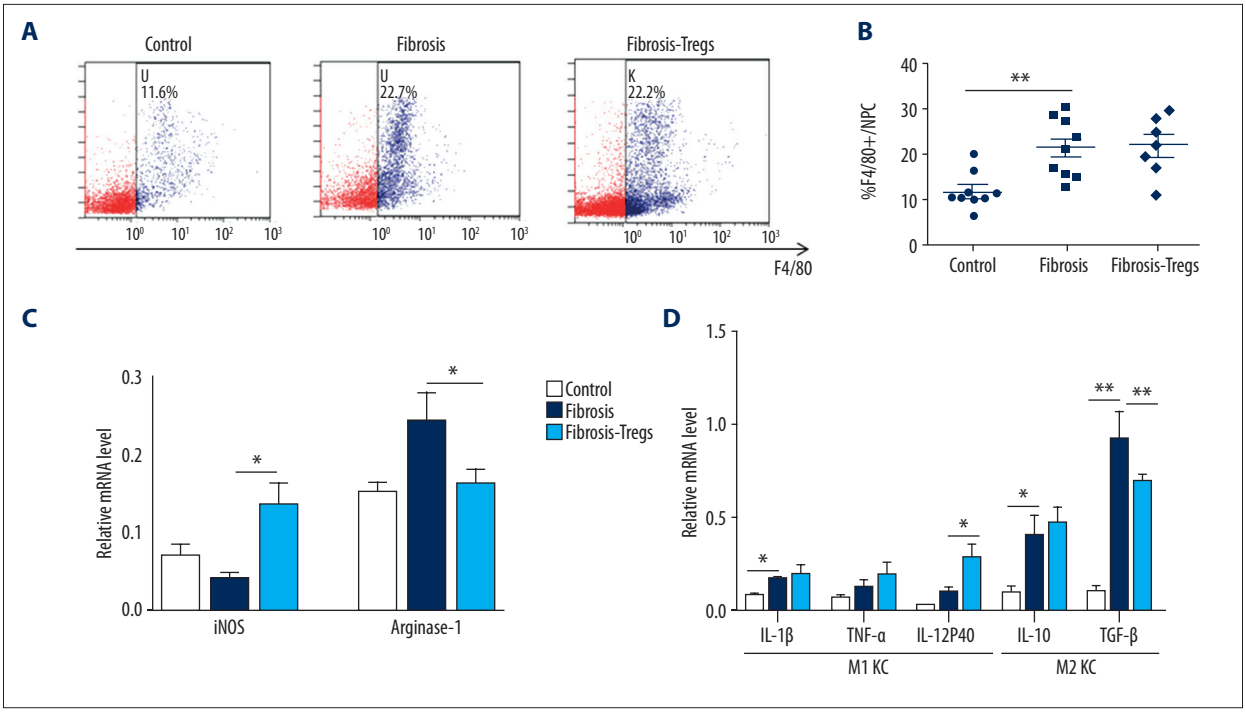

Figure 3. The regulation of Tregs on intrahepatic KCs. (**A, B**) KCs expression in control and fibrosis mice with or without Tregs depletion. (**C**) iNOS (M1 KCs) mRNA and arginase-1 (M2 KCs) mRNA expression in the livers of different groups of mice. (**D**) The mRNA expression of cytokines in the isolated KCs of mice livers. Data are presented as the mean ±SEM. * *P*<0.05, ** *P*<0.01.

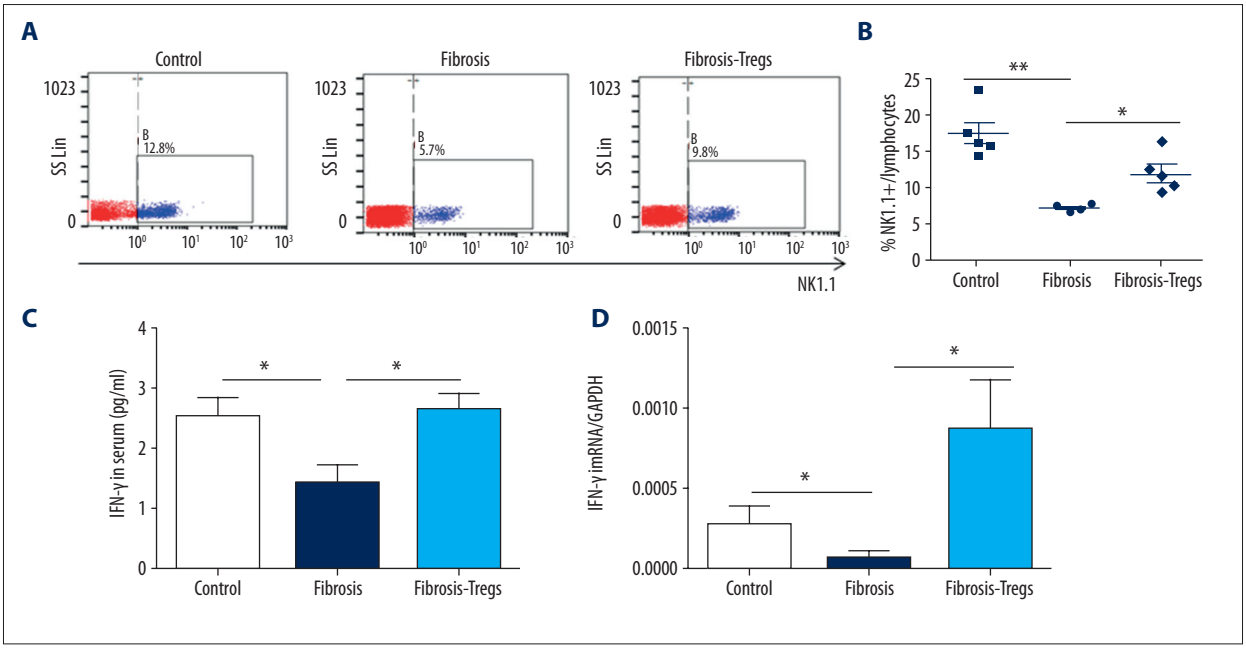

Figure 4. The regulation of Tregs on intrahepatic NK cells. (**A, B**) NK cells expression in control and fibrosis mice with or without Tregs depletion. (**C**) The concentration of IFN-γ was measured by ELISA in mice serum. (**D**) The expression of IFN-γ mRNA in the livers of mice. Data are presented as the mean ±SEM. * *P*<0.05, ** *P*<0.01.

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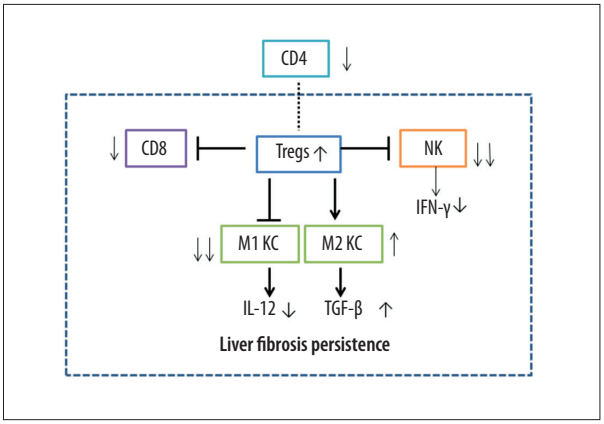

Figure 5. The regulation of Tregs on immune cells in liver fibrosis contributes to the persistence of liver fibrosis. The accumulation of abundant Tregs in a fibrotic liver predominately suppress M1 KCs and NK cells, accompanied by decreased IL-12 and IFN-γ, and mild suppression of CD8⁺ T cells, but not CD4⁺ T cells. KCs, NK cells, and CD8⁺ T cells have the function of removing infected cells or target cells. The inhibitory regulation of Tregs favors the formation of chronic inflammation and contributes to the persistence of liver fibrosis.

study revealed the effects of Tregs on some important intrahepatic immune cells in liver fibrosis, including CD4⁺ T lymphocytes and CD8⁺ T lymphocytes, and the predominant innate immune cells, KCs and NK cells. Their relationship is summarized in Figure 5.

CD4⁺ T cells and CD8⁺ T cells are the primary T lymphocytes that mediate cell immunity. CD4⁺ T lymphocytes are the primary activated immune cells that recognize antigen-presenting cell antigens and secrete cytokines (IL-2, IL-4, IFN), promoting the activation and proliferation of subsets of B cells to help produce antibodies. It has been demonstrated that Tregs suppress CD4⁺ T cell function [16]. We found that the CD4⁺ T cells population significantly decreased in the fibrotic livers but did not change after Tregs depletion, suggesting that Tregs were not the main down-regulator of CD4⁺ T cells. CD8⁺ T cells are a class of T lymphocytes with targeted killing function, and they play important roles in the removal of cells and tumor cells [17]. It has been reported that the local tissue of CD8⁺ T cells were increased in the CCl_4-induced mouse model of liver fibrosis; implanting CD8⁺ T cells into immunodeficiency mice aggravated the degree of hepatic fibrosis, suggesting that CD8⁺ T cells play an important role in liver fibrosis formation [18]. We also found that CD8⁺ T cell frequency was significantly higher in liver fibrosis compared with controls. Tregs were found to have an inhibitory effect on CD8⁺ T cell proliferation and function in hepatitis C [19]. In the present study, the CD8⁺ T cells showed an increasing trend after Tregs depletion compared with hepatic fibrosis, suggesting that Tregs have an inhibitory effect on intrahepatic CD8⁺ T cells in liver fibrosis, but the latter is not the main target.

It is reported that KCs play distinct and opposing roles in liver fibrosis, both ongoing liver injury and recovery [12]. Tregs have been demonstrated to influence KCs function. Together with KCs, Tregs create a local suppressive microenvironment to prevent the establishment of a cytotoxic lymphocyte (CTL) response [1]; Tregs regulate the KC-mediated inflammatory response to reduce ischemia-induced liver injury [20]. Our results show that Tregs regulate KCs to influence the balance of MMP and TIMP, and are involved in the maintenance of liver fibrosis [11]. All the above suggest the close relationship between Tregs and KCs. Here, we further found Tregs did not obviously affect KCs frequency in liver fibrosis, because Tregs depletion did not change the high expression levels of KCs, but it selectively influenced KCs subtypes. KCs can be classed as M1 or M2 macrophages by their polarization. M1 KCs are 'classical macrophages', have functions of bacterial clearance, antiviral activity, and release of pro-inflammatory cytokines such as TNF, IL-1β, IL-12; while M2 KCs are 'alternatively activated macrophages', promote defense against parasitic infections, are involved in tissue remodeling and secrete immune-modulatory mediators such as IL-10, TGF-β, IL-13 [21,22]. In the present study, total KCs population was increased in fibrotic livers, but M2 KCs seem to be the predominant macrophages, whatever KC marker (arginase-1) or cytokines from isolated KCs in mouse livers. After Tregs depletion, M1 KCs were increased, but M2 KCs were reduced. It is suggested that Tregs might primarily suppress M1 KCs (iNOS), and that the M1/M2 balance is skewed towards M2, protecting against exacerbated inflammation and limiting tissue injury [23,24].

In the healthy liver, NK cells play an important role in the first-line innate defense and immune surveillance of the liver by removing invading pathogens, toxins, and circulating tumor cells from circulation [25], but they also contribute to liver disease pathogenesis [12]. These processes are predominantly via IFN-γ and TNF-α secretion or directing cytotoxic activity [26]. Numerous clinical studies have demonstrated that NK activity and IFN-γ secretion are reduced in liver fibrosis and cirrhosis [7,27,28]. Moreover, Tregs have been found to suppress NK cells reactivity [29]. In the current study, we found that NK cells and IFN-γ levels were decreased in liver fibrosis but significantly increased after Tregs depletion, further suggesting that Tregs inhibit NK cells in liver fibrosis. NK cells play an important role in suppressing liver fibrosis in mice and humans by killing activated hepatic stellate cells (HSCs) and producing IFN-γ [28,30]. Therefore, in the fibrotic liver, the suppression of Tregs on NK cells limits the development of inflammation and injury, and also limits the clearance of HSCs and other pathogenic factors. All these are involved in maintaining fibrosis.

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Notably, continuous injection of anti-CD25 mAb has been shown to effectively down-regulate Tregs in published papers by our team and others [11,31,32]. Although anti-CD25 mAb can activate effector T cells, a study has demonstrated that the altered intensity of the immune response with anti-CD25 mAb was more consistent with the depletion of Tregs than that of effector T cells [33]. To investigate the immune regulation of Tregs on other immune cells in liver fibrosis, we depleted Tregs levels by 50%. The main reason is Tregs are present at certain percentage in physiological conditions to maintain the immune balance. Therefore, in this fibrosis model, we did not completely down-regulate Tregs, but decreased them to close to normal level to avoid excessive inflammation response. In addition, although we examined the predominant immune cells, the other immune cells, such as dendritic cells and NKT cells, are also the targets of Tregs. Whether they are also influenced by Tregs in liver fibrosis requires further investigation.

We believe that with the increase of Tregs in liver diseases, their suppression on hepatic immune cells will be stronger and stronger, and the types of immune cells and extent of inhibition may be increased.

Conclusions

Tregs are involved in immune regulation in liver fibrosis, predominately through suppression of M1 KCs and NK cells, and through mild suppression of $CD8^+$ T cells, but not $CD4^+$ T cells. The regulation of Tregs favors the formation of chronic inflammation, and contributes to the persistence of liver fibrosis.

Disclosures

The authors have no financial conflicts of interest.

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