Original Paper

Galectin-1 Restores Immune Tolerance to Liver Transplantation Through Activation of Hepatic Stellate Cells

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Key Words

Galectin-1 • Hepatic stellate cells • Immune tolerance • Liver transplantation

Abstract

Background/Aims: Immune tolerance is considered the only way to manage liver transplantation (LT). The current study hypothesized that galectin-1 via the activation of hepatic stellate cells (HSCs) is capable of inducing immune tolerance in LT. **Methods:** Lentiviral-mediated gene knockdown and overexpression of galectin-1 were conducted in HSC-T6 cells. Reverse transcription quantitative polymerase chain reaction and western blot analysis were used to determine galectin-1 expression. LT was performed in 20 C57BL/J6 mice and 20 C3H mice. T-cells were assigned into control, Galectin-1 shRNA, Galectin-1 OE, Galectin-1 OE SB431542, Galectin-1 OE Sulforaphane, Galectin-1 OE Y27632, and Galectin-1 OE UO126 groups. CFSE, flow cytometry, and ELISA were respectively employed to detect T-cell proliferation, CD4⁺/CD8⁺ ratio and IL-2, IL-10 and TGF- β levels. After establishing mouse models of immune tolerance and acute rejection, immunohistochemistry, TUNEL, and immunofluorescence assay were performed to determine CD3⁺ expression, apoptosis, α -SMA, and desmin. Mouse models of CCl₄-induced liver fibrosis were established, followed by assigning the control₁ and CCl₄ groups. ELISA was used to determine ALT, AST, TBIL and Hyp levels. A total of 3 C57BL/J6 mice (donor) and 6 C3H mice (recipient) were grouped into the control₂ and UO126 groups, followed by ELISA detection for IL-2, IL-10 and TGF- β . **Results:** In T-cells, galectin-1 shRNA increased cell proliferation and IL-2 levels with reduced IL-10 and TGF- β levels, while the Galectin-1 OE and Galectin-1 OE UO126 groups revealed the opposite results. Galectin-1 overexpression elevated the ratio of the CD4⁺ to CD8⁺ T-cells. The acute rejection group exhibited enhanced desmin expression and reduced α -SMA expression. Compared with the immune tolerance group, the acute rejection group displayed higher galectin-1 expression, a positive expression rate of CD3⁺ T-cells, and an increased apoptosis rate. Compared with the control₁ group, the CCl₄ group exhibited higher galectin-1 expression, ALT, AST, TBIL, and Hyp

levels, α -SMA expression and CD4⁺/CD8⁺ T-cell ratio, in addition to decreased expression of desmin. Compared with the control₂ group, UO126 increased galectin-1 expressions, IL-10 and TGF- β levels and reduced IL-2 levels with inactivated HSCs. **Conclusions:** The findings of the current study indicated that the overexpression of galectin-1 promoted the activation of HSCs, which reduced the inflammatory response by exerting immunosuppressive effects and accordingly contributed to immune tolerance in LT.

Introduction

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Liver transplantation (LT) is regarded as the ideal treatment for acute and chronic end-stage liver diseases and early hepatocellular carcinoma; however, the side effects of immunosuppression, transplantation of functionally impaired grafts and recurrence of original disease cannot be ignored over the long term [1]. Additionally, the preparative regimens are important for treatment of the indicated disease and for prevention of graft rejection [2]. It was previously reported that acute rejection following LT must be overcome to achieve better prognoses in liver transplant recipients [3]. Similarly, another study investigated the balance between the regulatory mechanisms of the induction of immune tolerance and the pathogenic factors associated with immune effects involving rejection to overcome acute rejection following LT [4]. Recently, spontaneous immune tolerance in LT of mice has attracted the attention of various researchers in transplantation-immunity studies [5]. Moreover, non-parenchymal liver cells, including Kupffer cells (KCs) and hepatic stellate cells (HSCs), have been demonstrated to contribute to the tolerogenic properties of the liver, suggesting that they play potential roles in immune tolerance [6]. In line with the aforementioned data, it was reported that multiple mechanisms are involved in immune tolerance via induction of HSCs, such as attenuation of effector T lymphocyte functions and augmentation of regulatory T lymphocytes [7]. Interestingly, galectin-1 has been reported to negatively mediate the activation and survival of T-cells in human tumors and to regulate immune tolerance in the spleen and thymus via apoptosis by binding to CD45⁺ T-cells [8, 9]. Furthermore, a study revealed that HSCs serve as important immune regulators in LT by inducing T-cells [10], the prolonged depletion of which after LT leads to life-threatening infections [11]. Thus, the current study speculated that galectin-1 might influence immune tolerance in LT by regulating HSCs.

Galectin-1 belongs to a family of B-galactoside binding proteins and is widely expressed in a variety of normal and pathological tissues and organs, such as the thymus, liver, and smooth muscles [4, 12]. Furthermore, galectin-1 is known to play significant roles in multiple biological processes, including inflammatory response, tumorigenesis, and immune cell homeostasis [9]. In addition, galectin-1 has been reported to be involved in transplantation tolerance due to its effects on the reduction of morbidity and mortality [13]. Moreover, the upregulation of galectin-1 has been suggested to enhance apoptosis of T-cells and cytokine production induced by HSCs [14]. HSCs, as fat-storing cells, store approximately 80% of the body's vitamin A in lipid droplets, [15] and as antigen-presenting cells (APCs), which are involved in hepatic fibrosis [16]. Furthermore, it has been previously demonstrated that co-transplanted HSCs exert effects on islet allografts protecting them from rejection and alleviating the severity of graft-versus-host disease, suggesting that HSCs possess immunosuppressive functions [17]. In addition, a previous study revealed that activated HSCs combined with B7-H1 suppressed T-cell responses via mediation of T-cell apoptosis [18]. Therefore, the current study aimed to explore the molecular mechanisms of galectin-1 in immune tolerance to LT by regulating HSCs.

Materials and Methods

Experimental animals

First, the mouse HSC line HSC-T6 (0089, Shanghai Fuxiang Biotech Co., Ltd., Shanghai, China) was cultured in Iscoves modified Dulbecco medium (IMDM, LM-11092/500, Shanghai Biosun Sci & Tech Co., Ltd., Shanghai, China) containing 10% fetal bovine serum (FBS) at 37°C with 5% CO₂ in air. The cell line was subcultured once every 2-3 days. A total of 20 C3H/HeJ male mice (aged: 3-5 weeks; Jinan Biobase Biotech Co., Ltd., Jinan, Shandong, China), and 20 C57BL/6 male mice (aged: 7-8 weeks old; Beijing HFK Bioscience Co., Ltd., Beijing, China) were raised in separate cages according to their types (C3H or C57BL/6 mice). All of the mice were housed under controlled conditions of temperature (18-22°C), relative humidity (40-70%), and noise levels < 50 dB and with ad libitum access to food and water, in addition to a 12-h light/dark cycle.

Construction of lentiviral vectors

A total of 3 short hairpin RNAs (shRNAs) were designed and synthesized with galectin-1 as the target with cleavage sites of Bgl II and Hind III on both sides and a 9-nt hairpin structure. The nonsense shRNA sequence was regarded as the negative control (NC) group. The expression of galectin-1 in the Galectin-1-sh1, Galectin-1-sh2, and Galectin-1-sh3 groups was observed. The sequences are shown in Table 1 [19]. Next, 1 µl of single-stranded shRNA (3 µg/µl) was added to 48 µl of annealing buffer (100 mmol/l NaCl and 50 mmol/l N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid [HEPES], pH 7.4), followed by immersion in a water bath at 95°C for 4 min, 70°C for 10 min, 37°C for 20 min, and 10°C for 10 min and then preservation at 4°C for further use. Simultaneously, a lentiviral vector with galectin-1 OE was constructed. Then, the lentivirus vector plasmid PNL-IRES₂-EGFP was co-transfected using helper plasmids in 293T-cells. The lentivirus plasmid pLVshRNA-eGFP (VL3101, Invogen Tech. Co., Ltd., Beijing, China) system was adopted to transfect 293T-cells. The collected lentivirus was centrifuged at 50,000 g for 2 h at 4°C, and the supernatant was discarded. The condensed lentivirus was collected and preserved at 4°C for further use. After 24 h, the culture medium in the wells was replaced with 500 µl of fresh medium and added to 5 µg/ml polybrene (0832-050, Shanghai Sidansai Biotechnology Co., Ltd., Shanghai, China), followed by incubation for 8 h. A total of 3 wells were set to determine lentiviral titers as follows: the 1st well had 25 µl of lentivirus added, the 2nd had 250 µl of lentiviral particles added, and the 3rd had polybrene in the same concentration added. The culture medium was replaced with 1 ml of fresh culture medium without polybrene within 12 h, followed by further incubation for 24-48 h. Next, the HSC-T6 cells were infected with the condensed lentivirus another time. Puromycin (60210ES25, Shanghai YEASEN Biotechnology Co., Ltd., Shanghai, China) was employed to screen for positive transfection of HSC-T6 cells. After 72 h of culture, the shRNA exhibiting better interference effects was selected by reverse transcription quantitative polymerase chain reaction (RT-qPCR) for subsequent experiments, when 90% transfection efficiency was verified using a fluorescence microscope.

RT-qPCR

RT-qPCR was performed to determine expression of galectin-1 in each group, and the group of cells exhibiting the best interfering effect was selected for subsequent experiments. HSC-T6 cells in logarithmic growth phase were added to 1 ml of TRIzol[®] reagent (Invitrogen Inc., Carlsbad, CA, USA). Next, the total RNA content in the cells was extracted according to the manufacturer's protocol for TRIzol[®] Reagent. The purity and concentration of RNA were measured using ultraviolet (UV) spectrophotometry (UV1901, Shanghai Aucy Technology Instrument Co., Ltd., Shanghai, China). RNA (A260/A280) ranging from 1.8 to 2.0 was adjusted to a concentration of 50 ng/µl and was used as a template for reverse transcription reaction using the PrimeScript[™] RT Reagent Kit (RR047A, Takara, Beijing Think-Far Technology Co., Ltd., Beijing, China). The reaction conditions were as follows: denaturation at 65°C for 10 min, annealing at 25°C for 10 min, extension at 37°C for 60 min, and 70°C for 10 min. The collected cDNA (50 ng/µl) was preserved at -80°C for further use. The primers (Table 2) were designed using Gene Tool software and were synthesized

Table 1. ShRNA sequences with Galectin-1 as the target gene. Note: ShRNA, short hairpin RNA

Gene	Sequence
Galectin-1-sh1	Sense strand: 5'-GATCCCGACAGCAGCAACACTGTGCTTCAGCAGAGTTGTGTCTCTTTTAA-3' Antisense strand: 5'-AGCTTAAAAAGACAGCAGCAACACTGTGCTCTCTTTGAAGCAGAGTTGTGTCTCTGGG-3'
Galectin-1-sh2	Sense strand: 5'-GATCCCGCTCCAGATGGATGAGATTCAGAGATTCATGATCTGTCAGC-3' Antisense strand: 5'-AGCTTAAAAAGCTCCAGATGGATGAGATTCATGAGATTCATGATCTGTCAGC-3'
Galectin-1-sh3	Sense strand: 5'-GATCCGACCTGTGCTACACCTCAATTCAAGAGATTGAAGTGTAGGCAGAGTTTCTTGGAAA-3' Antisense strand: 5'-GGTGGACAGGATGTGAAGTTAAGTTCTCTAACTTCACATCCGTGTCCAAAAAACCTTTTCGA-3'

by Beijing TsingKe Biological Technology Co., Ltd. (Beijing, China) [20]. Subsequently, RT-qPCR was performed with the ABI PRISM® 7900HT apparatus using the two-step method, with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) serving as the internal reference. The reaction conditions were as follows: pre-denaturation at 95°C for 30 s, followed by 40 cycles of denaturation at 95°C for 5 s and annealing at 58°C for 30 s. The relative mRNA expression of galectin-1 was calculated using the $2^{-\Delta\Delta C_t}$ method. Triplicate parallel wells were set for each target gene of each sample. The experiment was conducted three times.

Western blot analysis

HSC-T6 cells in logarithmic growth phase were centrifuged at 3000 r/min at 4°C for 20 min, and the supernatant was discarded. After packed cell volume (PCV, condensed cell volume after centrifugation) was estimated, per 20 µl of PCV cells were lysed with 100 µL of lysate and 1 µl of phosphatase inhibitor (Roche, Beijing Jiamay Biotechnology Co., Ltd., Beijing, China) on ice for 30 min and were centrifuged at 12000 r/min at low temperature for 10 min. The protein samples (50 µg in each group) were dissolved in 2× sodium dodecyl sulphate (SDS) sample buffer and boiled for 5 min for degeneration. Next, the proteins were separated using 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and then were transferred to a poly-vinylidene fluoride (PVDF) membrane and blocked with 5% skimmed milk at room temperature for 1 h. After rinsing with phosphate-buffered saline (PBS) for 2 min, the membrane was incubated overnight at 4°C with diluted rabbit anti-mouse primary antibody of galectin-1 (dilution ratio of 1: 1000, ab58085, Abcam, London, UK) and then rinsed with Tris-buffered saline Tween-20 (TBST) three times. Later, the samples were cultured with the secondary antibody of horseradish peroxidase (HRP)-labeled goat anti-rabbit immunoglobulin G (IgG) (dilution ratio of 1:5000) for 1 h, followed by rinsing with TBST for 3 × 5 min. Next, the protein samples were developed using an enhanced chemiluminescence (ECL) kit (10001, Beijing Keyushenlan Technology Co., Ltd., Beijing, China), exposed to X-rays. A gel imaging analysis system (GelDoc XR+, Bio-Rad Laboratories, Hercules, CA, USA) was employed to analyze the absorbance values of the bands. The average absorbance ratio of the target protein band to the internal reference band was regarded as the relative protein expression. The experiment was conducted three times.

T-cell isolation

C3H mice were anesthetized with 2% sodium pentobarbital, fixed in the supine position on operating tables, and sterilized. The skin overlying the abdomen was incised along the linea alba to expose the abdominal cavity and spleen. Then, a 5-ml syringe was used for multiple pricking into the collected spleen. After spleen samples were rinsed with PBS, the samples were centrifuged, and the supernatant was removed. Next, the samples were lysed by erythrocyte lysate and centrifuged, and the supernatant was discarded. The cell samples were re-suspended in Dulbecco's minimum essential medium (DMEM) medium (SH30022, Beijing North TZ-Biotech Develop. Co. Ltd., Beijing, China) containing 10% FBS and were incubated for 3 h. Subsequently, the suspended cells were extracted. T-cells were isolated from the cell suspension using a Nylon wool column, labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE, 5 µmol/L, C375, Dojindo China Co., Ltd., Shanghai, China), and incubated while avoiding exposure to light at 37°C for 10 min. The centrifuge tube was shaken gently at two min intervals for uniform CFSE labeling. Then, the samples were centrifuged at 3000 r/min for 5 min to remove the remnant CFSE.

Cell grouping and culture

HSC-T6 cells in logarithmic growth phase were randomly divided into the control group (normal HSC-T6 cell line), the Galectin-1 shRNA group (HSC-T6 cell line with galectin-1 shRNA), the Galectin-1 OE group (HSC-T6 cell line with galectin-1 overexpression), the Galectin-1 OE SB431542 group (HSC-T6 cell line with galectin-1 overexpression cultured with transforming growth factor beta (TGF-β)/Smads inhibitor), the Galectin-1 OE Sulforaphane group (HSC-T6 cell line with galectin-1 overexpression cultured with phosphoinositide 3-kinase (PI3K)/Akt inhibitor), the Galectin-1 OE Y27632 group (HSC-T6 cell line with galectin-1 overexpression cultured with Rho/Rho-associated protein kinase (ROCK) inhibitor), and

Table 2. Primer sequence for reverse transcription quantitative polymerase chain reaction. Note: GAPDH, glyceraldehyde-3-phosphate dehydrogenase

Gene	Primer sequence
Galectin-1	Forward: 5'-GCATCAGAGGT-3'
	Reverse: 5'-AGGCCACGCACCTTAATCTTG-3'
GAPDH	Forward: 5'-TGCACCACCAACTGCTTAGC-3'
	Reverse: 5'-GGCATGGACTGTGGTCATGAG-3'

the Galectin-1 OE UO126 group (HSC-T6 cell line with galectin-1 overexpression cultured with mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) inhibitor). The HSC-T6 cells in the control, Galectin-1 shRNA, and Galectin-1 OE groups were cultured with T-cells, while the HSC-T6 cells with galectin-1 overexpression in the Galectin-1 OE SB431542, Galectin-1 OE Sulforaphane, Galectin-1 OE Y27632, and Galectin-1 OE UO126 groups were cultured with T-cells *in vitro* and 10 μ mol/L signaling pathway inhibitors (according to the grouping, TGF- β /Smads inhibitor SB431542, PI3K/Akt inhibitor sulforaphane, Rho/ROCK inhibitor Y27632, MAPK/ERK inhibitor UO126) for 3 h in 96-well plates. CFSE labeled T-cells were suspended at a density of 1×10^7 cells/ml using DMEM complete medium. Triplicate parallel wells were set for each group. Then, T-cells were seeded in wells (at a density of 2×10^5 cells/well) and were subcultured in an incubator at 37°C with 5% CO₂ in air.

CFSE staining and flow cytometry detecting T lymphocyte proliferation

At days 3, 5, and 7 after culture, the T-cells labeled with CFSE in each group were selected and allowed to attain room temperature. The CellTrace™ CFSE Proliferation Kit (KGA318, Shanghai Yuanxiang Medical Instrument Co., Ltd., Shanghai, China) was employed to detect the proliferation of T-cells according to the kit instructions. Flow cytometry (Becton Dickinson Bio-sciences, San Jose, CA, USA) was used to detect the split-peak, and Modfit software was used to calculate the number of cells split per passage and the proliferation rate of T lymphocytes. The experiment was conducted three times.

Flow cytometry detecting the ratio of CD4⁺ to CD8⁺ T-cells

At days 3, 5, and 7 after culturing, the T-cells labeled with CFSE in each group were selected and added to PBS (pH 7.2, 0.01 mol/L) to a total volume of 1 ml. Glacial acetic acid at a concentration of 2% was used for cell counting. Next, the cells were cultured with fluorescein isothiocyanate/phyco-erythrina (FITC/PE)-conjugated rat anti-mouse CD4⁺/CD8⁺ monoclonal antibody (SZB0003, dilution ratio of 1:100, Shanghai Boyao Biotech Co., Ltd., Shanghai, China) at 4°C for 50 min. Then, the cells were rinsed with PBS two times (pH 7.2, 0.01 mol/L), and immune parameters were detected using FACScan flow cytometry. Splenocytes were collected using CellQuest software, and the ratio of CD4⁺ to CD8⁺ T-cells was analyzed by ModFit software. The experiment was conducted three times.

Enzyme-linked immunosorbent assay (ELISA)

At days 3, 5, and 7 after culture, T-cells in each group were selected and digested with pancreatin. After centrifugation at 1500 r/min for 10 min, the T-cells were diluted with serum-free medium to a density of 10⁶ cells/ml and were accordingly counted. The diluted cell suspension was added to a 96-well plate (100 μ l of suspension for each well) and was cultured at 37°C overnight for cell adherence. The following day, the levels of IL-2, IL-10, and TGF- β were detected with ELISA kits purchased from Wuhan Moshake Biological Technology Co., Ltd. (Wuhan, Hubei, China). The samples and the ELISA kits for IL-2 (kit No: 69-99852), IL-10 (kit No: 69-99847), and TGF- β (kit No: 69-50020) were placed outside for 30 min prior to the experiment and allowed to reach room temperature. A microplate reader (Multiskan GO, Thermo Fisher Scientific, CA, USA) was employed to analyze the optical density (OD) value. The procedures were in accordance with respective kit instructions. The experiment was conducted three times.

Establishment of mouse models of immune tolerance and acute rejection in allogeneic LT

A total of 4 C57BL/6 mice (donor) and 6 C3H mice (receptor) were randomly selected and assigned to the sham operation group (2 C3H mice), the immune tolerance group (1 C57BL/6 mouse and 2 C3H mice) and the acute rejection group (1 C57BL/6 mouse and 2 C3H mice). The donor mice received pretreatment with Flt3L (bs-5905R, Beijing Boosen Biotechnology Co., Ltd., Beijing, China). At day 7 before LT, the donor mice were administered intraperitoneal injections of 10 μ g of Flt3L dissolved in 100 μ l of normal saline once per day for seven days. Then, 10 d after the operation, no mouse deaths were observed, indicating that the establishment of models was successful. Subsequently, the mice were anesthetized with 2% sodium pentobarbital via intraperitoneal injection and fixed in the prone position. The skin overlying the abdomen was incised to collect liver tissues. One part of the tissue was stored in liquid nitrogen. The other part was fixed in 10% neutral buffered formalin for 24 h, dehydrated with graded ethanol, and embedded in paraffin.

Hematoxylin-eosin staining

Paraffin-embedded specimens were sliced into 5 μ m serial sections, followed by extension at 45°C and drying for 1 h at 60°C. After dewaxing by xylene I and xylene II for 5 min respectively, liver samples were dehydrated with graded ethanol at 100%, 95%, 80%, and 70% (each for 2 min), rinsed with distilled water for 2 $\times$ 5 min, and hydrated. Then, the samples were stained using hematoxylin-eosin (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) for 3 min, followed by rinsing under running water for 5 min. Subsequently, the samples were dehydrated with 70% ethanol for 5 min and 80% ethanol for 5 min, cleaned with xylene I and xylene II for 5 min, and then sealed with neutral gum with cover glass. The inflammatory cell infiltration and damage to liver cells in the transplanted liver in the sham operation, immune tolerance, and acute rejection groups were observed under a light microscope (XP-330, Shanghai, Bingyu Optical Instrument Co., Ltd., Shanghai, China).

Immunohistochemistry

Paraffin-embedded sections were immersed in 3% H_2O_2 , dewaxed with xylene I and xylene II for 10 min, respectively, and dehydrated with graded ethanol at 100%, 95%, 80%, and 70% (each for 2 min). Next, the sections were rinsed with distilled water for 2 $\times$ 5 min on a shaking table. After immersion in 3% H_2O_2 for 10 min, the sections were rinsed with distilled water, followed by antigen repair at high pressure for 50 s. Next, the sections were allowed to cool to room temperature and rinsed with PBS, blocked with 5% bovine serum albumin (BSA), incubated at 37°C for 30 min, and then incubated with rabbit anti-mouse CD3⁺ primary antibody (ab16669, dilution ratio of 1:400, Abcam, London, UK) at 4°C overnight. Subsequently, the sections were incubated with goat anti-rabbit IgG-HRP secondary antibody (SE134, Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) at 37°C for 30 min, reacted with streptavidin-biotin (SAB) working fluid, stained with diaminobenzidine (DAB), and counterstained by hematoxylin for 5 min, followed by rinsing under running water for 10 min, dehydration, transparency, sealing, and microscopy. PBS was used to replace the primary antibody as the NC. The criteria for immunohistochemistry staining results [20] were as follows: the percentage of cells in positive expression was more than 10%, and cells with brownish yellow membranes, cytoplasm and vascular endothelium were regarded as CD3⁺-positive cells. A total of 5 fields under a high-power lens were randomly selected to detect the position of positive expression and the positive expression of CD3⁺. Pathology images and a character report management system (HPIAS 1000) were adopted to detect the mean OD value of positive cells. The experiment was repeated three times.

Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling (TUNEL) assay

Paraffin-embedded sections were dewaxed with xylene I and xylene II for 10 min, respectively, followed by immersion in ethanol at 100%, 95%, 80%, and 70% (each for 2 min) and rinsing with PBS for 3 $\times$ 5 min. Next, the sections were incubated with proteinase K at 37°C for 30 min, rinsed with PBS for 3 $\times$ 5 min, fixed in paraformaldehyde for 2 h, and rinsed with PBS for 3 $\times$ 5 min. Subsequently, the sections were blocked with methanol containing 3% H_2O_2 for 10 min, rinsed with PBS for 3 $\times$ 5 min, and then immersed in 20% sucrose phosphate buffer at 4°C overnight. The following day, the specimens were sliced into 20 μ m serial sections using a cutting machine at -22°C. A total of 10 sections from each mouse were stained using TUNEL assay, and the TUNEL kit was purchased from Boehringer Mannheim Company (Germany). Cells with dark particles, observed under a light microscope (Leica DM4 P, Shanghai Meijing Electronic Co., Ltd., Shanghai, China), were regarded as apoptotic cells. In addition, 10 fields under high power lens sections in each group were randomly selected to determine the apoptotic nuclei, total number of cells, and apoptotic index (AI = apoptotic cells/total cells). The experiment was conducted three times.

Immunofluorescence assay

The levels of α -smooth muscle actin (α -SMA), as a marker of HSCs in the activation phase, and desmin, as a marker of HSCs in the resting phase, were detected using immunofluorescent double staining to detect the activation of HSCs in mouse models. The frozen liver tissues were embedded with an embedding agent, sliced into 5 μ m sections with a freezing microtome, and dried overnight. Next, the sections were fixed in cold acetone for 10 min and rinsed with PBS for 3 $\times$ 5 min. After being permeated with 0.2% Triton X-100, the sections were rinsed with PBS for 3 $\times$ 5 min, fixed with goat serum for 30 min at room temperature, followed by rinsing with PBS for 3 $\times$ 5 min, and then incubated with FITC-labeled rabbit anti-mouse antibodies of

α -SMA (dilution ratio of 1:500, ab5831, Abcam, London, UK) and desmin (dilution ratio of 1:500, ab15200, Abcam, London, UK) at 4°C overnight. After rinsing with PBS for 3 × 5 min, the sections were washed with distilled water to remove residual PBS, mounted in glycerol and observed under a fluorescence microscope.

Establishment of mouse models of CCl₄-induced liver fibrosis

A total of 6 C57BL/6 mice were randomly assigned into the control₁ group and the CCl₄ group with three mice in each group. The mice were acclimated to the surrounding conditions of 22°C in temperature, 55% humidity, and ample food and water in a 12-h light/dark cycle. CCl₄ (20 ml) was mixed with olive oil (30 ml) and stirred with a magnetic stirrer for 8-12 h. Next, a mixture of CCl₄/olive oil with 40% volume fraction was prepared. Mice were administered intraperitoneal injections of prepared 2 ml/kg CCl₄/olive oil (namely, 0.8 ml/kg CCl₄) two times per week to establish mouse models of liver fibrosis. Mice in the control group₁ were injected with equal amounts of olive oil. There were no deaths during model establishment. Six weeks after injection, normal mice were observed to be responsive with normal eating and drinking, as well as normal weights. The mice in the CCl₄ group were found to be overweight, unresponsive, and presented with rough fur. Failed mouse models were removed, while all of the mouse models were established successfully. After 6 weeks, the mice were administered intraperitoneal injections of 2% sodium pentobarbital for deep anesthesia. Then, whole blood in eyeballs (0.4-0.6 ml) from each mouse was extracted and preserved in serum tubes. Next, the skin overlying the abdomen was incised immediately. Liver tissues (100 mg) from the left lobe were collected, placed in frozen tubes, and preserved in liquid nitrogen for further use. The remnant liver from the left lobe was collected and fixed in 10% neutral formalin buffer for 12-24 h. Next, the samples were embedded in paraffin and sliced into sections for further experimentation.

Masson staining of liver lobule tissues in the control₁ and CCl₄ groups

Paraffin-embedded sections were immersed in 3% H₂O₂, dewaxed with xylene I and xylene II (each for 10 min), and dehydrated in graded ethanol at 100%, 95%, 80%, and 70% (each for 2 min). Next, the sections were rinsed with distilled water for 2 × 5 min on a shaking table and stained with hematoxylin-eosin (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) for 15 min. After being rinsed with 1% acetic acid for 10 min, the sample sections were reacted with phospho molybdate and aniline blue (each for 10 min) and rinsed with 1% acetic acid, followed by dehydration with ethanol and clearing with xylene. Next, the samples were mounted in neutral gum, and liver fibrosis in liver lobules in the control₁ and CCl₄ groups was observed under a light microscope (XP-330, Shanghai, Bingyu Optical Instrument Co., Ltd., Shanghai, China).

Mouse groupings

A total of 3 C57BL/6 mice (donor) and 6 C3H mice (receptor) were randomly selected to conduct further grouping. The C3H mice were randomly classified into the control₂ group and the UO126 group, with three mice in each group. In addition, C3H mice were used to establish mouse models of immune tolerance. At days 2, 4, and 6 after the operation, the C3H mice were injected with a MAPK signaling pathway inhibitor, UO126. After 10 d, the C3H mice were administered a 10% intraperitoneal injection of 2% sodium pentobarbital. Blood from the eyeballs of the mice was extracted once the mice were appropriately anesthetized. The whole blood (0.4-0.6 ml) of each mouse was preserved and placed in serum tubes. Next, the skin overlying the abdomen of mice was incised immediately. The liver tissues (each group 100 mg) were extracted, placed in frozen tubes, and preserved for further use in liquid nitrogen. The remnant tissues were collected and fixed in 10% neutral formalin buffer for 12-24 h. Next, the samples were embedded in paraffin and sliced into sections for the following experiments.

Statistical analysis

Statistical analyses were performed using SPSS software, version 21.0 (IBM Corp Armonk, NY, USA). Measurement data are expressed as the mean ± standard deviation (SD). Comparisons between two groups were performed using the *t*-test, and comparisons among multiple groups were analyzed by one-way analysis of variance (ANOVA). A level of *p* < 0.05 was considered to be statistically significant.

Results

Galectin-1 expression is decreased in the Galectin-1-sh groups and increased in the Galectin-1 OE group

First, the results of western blot analysis (Fig. 1A-B) revealed that, compared with the NC group, the expression of galectin-1 was found to be decreased in the other groups ($p < 0.05$). Among the Galectin-1-sh1, Galectin-1-sh2, and Galectin-1-sh3 groups, the expression of galectin-1 in the Galectin-1-sh3 group was the lowest, indicating that the Galectin-1-sh3 group exhibited the best interference effect. Therefore, cells in the Galectin-1-sh3 group were selected for the following experiments.

Additionally, the results of western blot analysis are shown in Fig. 1C-D. It was observed that the protein expression of galectin-1 was much higher in the Galectin-1 OE group, compared to the NC group ($p < 0.05$).

Changes of T-cell proliferation rate, percentage of CD4⁺ and CD8⁺ cells and expression levels of IL-2, IL-10 and TGF- β

Next, CFSE, flow cytometry and ELISA were employed to determine the T-cell proliferation rate, percentage of CD4⁺ and CD8⁺ cells and expression levels of IL-2, IL-10 and TGF- β , respectively. The outcomes of CFSE and flow cytometry are shown in Fig. 2A. The results revealed that the proliferation rate of T-cells in the Galectin-1 OE U0126 group was the lowest among the seven groups. Compared with the control group, the proliferation rate of T-cells was found to be increased in the Galectin-1 shRNA group, whereas reduced rates were observed in the Galectin-1 OE and Galectin-1 OE U0126 groups ($p < 0.05$). There were no significant differences in the proliferation rate of T-cells among the control, Galectin-1 OE SB431542, Galectin-1 OE Sulforaphane, and Galectin-1 OE Y27632 groups (all $p > 0.05$).

The results of flow cytometry shown in Fig. 2B indicated that, since the 3rd day, compared with the control group, the ratio of CD4⁺ to CD8⁺ T-cells was found to be obviously elevated in the Galectin-1 OE group but notably decreased in the Galectin-1 shRNA, Galectin-1 OE SB431542, Galectin-1 OE Sulforaphane, Galectin-1 OE Y27632, and Galectin-1 OE U0126 groups (all $p < 0.05$). Compared to the Galectin-1 OE group, the ratio of CD4⁺ to CD8⁺ T-cells was significantly reduced in the Galectin-1 OE SB431542, Galectin-1 OE Sulforaphane, Galectin-1 OE Y27632, and Galectin-1 OE U0126 groups (all $p < 0.05$). The results suggested that overexpression of galectin-1 enhanced the percentage of CD4⁺ T-cells but decreased the percentage of CD8⁺ T-cells, exerting an immunosuppressive effect. In addition, the immunosuppressive effect could be enhanced by the addition of U0126, an inhibitor of the MAPK/ERK signaling pathway.

Fig. 1. Galectin-1 expression is decreased in the Galectin-1-sh groups and increased in the Galectin-1 OE group. Panels A and B, western blot analysis showed that the Galectin-1-sh3 group exhibited lower Galectin-1 protein levels, compared to the other two groups; Panels C and D, western blot analysis suggested that the Galectin-1 OE group had higher Galectin-1 protein levels than the NC group; NC, negative control; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; *, $p < 0.05$, vs. the NC group; #, $p < 0.05$, vs. the Galectin-1-sh1 and Galectin-1-sh2 groups. The t-test was used for the comparison between the two groups, while one-way analysis of variance (ANOVA) was used for the comparison among groups. The experiment was performed in triplicate, and the result was three times the mean $\pm$ standard deviation.

Fig. 2. Changes in T-cell proliferation rate, percentages of CD4⁺ and CD8⁺ cells and expression levels of IL-2, IL-10 and TGF- β . Panel A, CFSE together with flow cytometry outcomes showed that T-cell proliferation rate was higher in the Galectin-1 shRNA group; Panel B, results of flow cytometry showed that Galectin-1 overexpression enhanced the percentage of CD4⁺ T-cells but decreased the percentage of CD8⁺ T-cells, which exerted immunosuppressive effects, while the immunosuppressive effect could be enhanced by addition of UO126, an inhibitor of the MAPK/ERK signaling pathway; Panel C, the findings of ELISA showed that Galectin-1 overexpression inhibited IL-2 levels but promoted the levels of IL-10 and TGF- β , which suppressed inflammatory responses. However, the immunosuppressive effect can be enhanced by the addition of UO126, an inhibitor of the MAPK/ERK signaling pathway; *, $p < 0.05$, vs. the NC group; #, $p < 0.05$, vs. the Galectin-1 OE group. One-way analysis of variance (ANOVA) was used for the comparison among groups. The experiment was performed in triplicate, and the result was three times the mean $\pm$ standard deviation. NC, negative control; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; shRNA, short hairpin RNA; CFSE, carboxyfluorescein diacetate succinimidyl ester; IL-2, interleukin-2; IL-10, interleukin-10; TGF- β , transforming growth factor beta.

ELISA results (Fig. 2C) showed that, since the 3rd day, compared with the control group, the IL-2 level was found to be remarkably elevated, and the levels of IL-10 and TGF- β were notably decreased in the Galectin-1 shRNA group, while the opposite trends were observed in the Galectin-1 OE group (all $p < 0.05$). Since the 5th day, compared with the control group, the Galectin-1 OE UO126 group showed decreased IL-2 levels and increased levels of IL-10 and TGF- β (all $p < 0.05$). Compared with the Galectin-1 OE group, IL-2 levels were found to be significantly elevated, and the levels of IL-10 and TGF- β were decreased in the Galectin-1 OE SB431542, Galectin-1 OE Sulforaphane, and Galectin-1 OE Y27632 groups, while the opposite trends were observed in the Galectin-1 OE UO126 group (all $p < 0.05$). The results suggested that overexpression of galectin-1 inhibited IL-2 levels but increased the levels of IL-10 and TGF- β , which suppressed inflammatory responses. In addition, the immunosuppressive effect could be enhanced by the addition of UO126, an inhibitor of the MAPK/ERK signaling pathway.

Histopathological changes, positive expression rate of CD3⁺, HSCs apoptosis, galectin-1 protein expression and the activation of HSCs in the sham operation and immune tolerance, acute rejection groups

Next, HE staining, immunohistochemistry, TUNEL assay, western blot analysis and immunofluorescence assay were performed to conduct histopathological examination, evaluation of CD3⁺ positive rate, HSC apoptosis, galectin-1 protein expression and activation of HSCs, respectively. The results of HE staining (Fig. 3A) indicated that the sham operation group showed no evident inflammatory cell infiltration or cell necrosis. The immune tolerance group presented with local proliferation with inflammatory cell infiltration, cell necrosis, and congestion. Further, the acute rejection group showed obvious inflammatory cell infiltration and congestion, in addition to more cell necrosis.

The results of immunohistochemistry (Fig. 3B) showed that, 10 d after model establishment, the positive expression rate of CD3⁺ was much higher in the immune

Fig. 3. Observation of histopathological changes, positive expression rate of CD3⁺, HSC apoptosis, Galectin-1 protein expression and the activation of HSCs in the sham operation and immune tolerance, acute rejection groups. Panel A, the immune tolerance group presented local proliferation with inflammatory cell infiltration, cell necrosis, and congestion evaluated by HE staining ($\times 200$); scale = 50 μm ; Panel B, the positive expression rate of CD3⁺ was higher in the acute rejection group than in the immune tolerance group by immunohistochemistry ($\times 200$); scale = 50 μm ; Panel C, the hepatocyte apoptotic index in the immune tolerance group was decreased compared to that in the acute rejection group but still relatively serious by TUNEL staining ($\times 100$); scale = 100 μm ; Panel D, the protein expression of Galectin-1 increased in the immune tolerance group but showed a more significant increase in the acute rejection group by western blot analysis; Panel E, HSCs were massively activated in the acute rejection group by immunofluorescence assay ($\times 200$); scale = 50 μm ; *, $p < 0.05$, vs. the sham operation group; #, $p < 0.05$, vs. the immune tolerance group. One-way analysis of variance (ANOVA) was used for the comparison among groups. The experiment was carried out in triplicate, and the result was three times the mean $\pm$ standard deviation. HE, hematoxylin-eosin; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DAPI, 4', 6-diamidino-2-phenylindole; α -SMA, α -smooth muscle actin; HSC, hepatic stellate cell.

tolerance and acute rejection groups, compared to the sham operation group (all $p < 0.05$). In the acute rejection group, the positive expression rate of CD3⁺ was found to be higher than in the immune tolerance group ($p < 0.05$).

The TUNEL assay results (Fig. 3C) showed that the apoptotic index was found to be evidently elevated in the immune tolerance and acute rejection groups, compared to the sham group (all $p < 0.05$). The apoptotic index showed an evident increase in the acute rejection group, compared to the immune tolerance group (all $p < 0.05$). The results indicated that allotransplantation of the liver led to hepatocyte apoptosis, and the hepatocyte apoptotic index in the immune tolerance group was decreased compared to the acute rejection group but still relatively serious.

The results of western blot analysis (Fig. 3D) indicated that, compared with the sham operation group, the protein expression of galectin-1 was found to be increased in the immune tolerance group, but it showed a more significant increase in the acute rejection group (all $p < 0.05$).

The results of immunofluorescence assay (Fig. 3E) indicated that, compared with the sham operation group, desmin levels in the immune tolerance group significantly decreased with elevated levels of α -SMA, while the acute rejection group exhibited the opposite results. Compared with the immune tolerance group, the level of desmin in the acute rejection group was found to be notably increased with reduced levels of α -SMA. The results revealed that HSCs were in a stationary state at the normal stage. When the HSCs underwent injuries, the HSCs were activated, and the phenotype of the HSCs changed from the stationary type to the activated type. In addition, HSCs in the immune tolerance group were found to be significantly activated.

Histopathological changes of liver lobule tissues, changes of liver fibrosis, levels of ALT, AST, TBIL, and Hyp, and protein expression of galectin-1 in the control₁ and CCl₄ groups

Subsequently, HE staining, Masson staining, ELISA, western blot analysis and immunofluorescence assay were employed to measure histopathological changes of liver lobule tissues, changes in liver fibrosis, levels of ALT, AST, TBIL, and Hyp, protein expression of galectin-1 and activation of HSCs. HE staining results (Fig. 4A) indicated that the control₁ group showed no obvious inflammatory cell infiltration or cell necrosis, in addition to clear liver lobule structures. The CCl₄ group presented local proliferation with inflammatory cell infiltration, cell necrosis, and congestion in some parts.

Fig. 4. Observation of histopathological changes of liver lobule tissues, changes of liver fibrosis, levels of ALT, AST, TBIL, and Hyp, and protein expression of Galectin-1 in the control₁ and CCl₄ groups. Panel A, the CCl₄ group presented local proliferation with inflammatory cell infiltration, cell necrosis, and congestion in some parts observed by HE staining ($\times 200$), scale = 50 μ m; Panel B, liver fibrosis evidently increased in the CCl₄ group detected by Masson staining ($\times 200$), scale = 50 μ m; Panel C, CCl₄ increased levels of ALT, AST, TBIL and Hyp, and liver damage was serious in the CCl₄ group by ELISA, n = 3; Panel D, protein expression of Galectin-1 was increased in CCl₄ group by western blot analysis; Panel E, the level of desmin in the CCl₄ group significantly decreased with elevated levels of α -SMA, evaluated by immunofluorescence assay ($\times 200$), scale = 50 μ m. *, p < 0.05, vs. the control₁ group; HE, hematoxylin-eosin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; TBIL, total bilirubin; Hyp, hydroxyproline; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DAPI, 4', 6-diamidino-2-phenylindole; α -SMA, α -smooth muscle actin; HSC, hepatic stellate cell.

Masson staining results (Fig. 4B) indicated that the liver tissues were normal, and liver fibrosis was decreased in the control₁ group, while liver fibrosis was found to be evidently increased in the CCl₄ group.

The ELISA results (Fig. 4C) showed that levels of ALT, AST, TBIL, and Hyp were found to be notably elevated in the CCl₄ group compared to the control₁ group (all $p < 0.05$), indicating that there was serious liver damage in the CCl₄ group.

The results of western blot analysis (Fig. 4D) showed that protein expression of galectin-1 was found to be notably elevated in the CCl₄ group compared to the control₁ group (all $p < 0.05$).

The results of immunofluorescence assay (Fig. 4E) indicated that, compared with the control₁ group, the level of desmin in the CCl₄ group was found to be significantly decreased, in addition to having elevated α -SMA. At the normal stage, the HSCs were observed to be in the stationary state. When HSCs underwent injury, the HSCs were activated, and subsequently, the phenotype of HSCs changed from the stationary type to the activated type.

Immune tolerance of liver transplantation, histopathological changes of liver lobule tissues, expression levels of IL-2, IL-10 and TGF- β , percentage of CD4+ and CD8+ cells, and protein expression of galectin-1

In the following experiments, immunofluorescence staining, HE staining, ELISA, flow cytometry and western blot analysis were employed to measure the immune tolerance of liver transplantation, histopathological changes of liver lobule tissues, expression levels of IL-2, IL-10 and TGF- β , percentage of CD4+ and CD8+ cells, and protein expression of galectin-1, respectively. The results of immunofluorescence assay (Fig. 5A) indicated that, compared with the control₂ group, the expression level of desmin was found to be significantly elevated in the UO126 group, and the expression level of α -SMA decreased evidently. The control₂

Fig. 5. Observation of immune tolerance of liver transplantation, histopathological changes of liver lobule tissues, expression levels of IL-2, IL-10 and TGF- β , percentage of CD4+ and CD8+ cells, and protein expression of Galectin-1. Panel A, results of immunofluorescence assay showed that the MAPK/ERK inhibitor UO126 exerted protective effects on liver damage ($\times 200$), scale = 50 μ m; Panel B, UO126 reduced inflammatory responses and liver lesions by inhibiting the MAPK/ERK signaling pathway observed after HE staining ($\times 200$), scale = 50 μ m; Panel C, MAPK/ERK inhibitor UO126 inhibits inflammatory responses by ELISA; Panel D, the MAPK/ERK inhibitor UO126 increased the percentages

of CD4+ and CD3+ T-cells and reduced the percentage of CD8+ T-cells to promote immunosuppressive effect by flow cytometry; Panel E, UO126 increased the protein expression of Galectin-1 suggested by western blot analysis; *, $p < 0.05$, vs. the control₂ group; IL-2, interleukin-2; IL-10, interleukin-10; TGF- β , transforming growth factor beta; DAPI, 4', 6-diamidino-2-phenylindole; α -SMA, α -smooth muscle actin; HE, hematoxylin-eosin; ELISA, enzyme-linked immunosorbent assay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

group was regarded as the mouse model of immune tolerance, and the most of the HSCs were found to be activated. After treatment with the MAPK/ERK inhibitor UO126, HSCs changed to the stationary state, indicating that the MAPK/ERK inhibitor UO126 exerted protective effects against liver damage.

The HE staining results (Fig. 5B) indicated that the control₂ group exhibited unclear liver lobule structures, irregular cell arrangement, expanded sinus hepaticus to some extent, apparent inflammatory cell infiltration, and cell necrosis. The UO126 group showed clear liver lobule structures, regular cell arrangement, expanded sinus hepaticus to some extent, no obvious inflammatory cell infiltration and cell necrosis. The aforementioned findings indicated that UO126 could reduce inflammatory responses and liver lesions by inhibiting the MAPK/ERK signaling pathway.

The ELISA results (Fig. 5C) showed that, compared with the control₂ group, the UO126 group exhibited decreased IL-2 levels and elevated levels of IL-10 and TGF- β (all $p < 0.05$), indicating that the MAPK/ERK inhibitor UO126 inhibited inflammatory responses.

Flow cytometric detection (Fig. 5D) revealed that, compared with the control₂ group, the percentages of CD4⁺ and CD3⁺ T-cells were found to be obviously increased, whereas the percentage of CD8⁺ T-cells evidently decreased in the UO126 group (all $p < 0.05$). The results revealed that the MAPK/ERK inhibitor UO126 increased the percentages of CD4⁺ and CD3⁺ T-cells and reduced the percentage of CD8⁺ T-cells to promote immunosuppressive effects, namely, inflammatory response inhibition.

The results of western blot analysis (Fig. 5E) showed that, compared with the control₂ group, the protein expression of galectin-1 was found to be elevated in the UO126 group ($p < 0.05$). The results suggested that UO126 increased the protein expression of galectin-1.

Discussion

The induction of specific immune tolerance has been reported to be a feasible approach to treating organ rejection [21]. Galalpha1-3Gal epitopes were expressed in pig tissues and the binding of anti-Galalpha1-3Gal led to endothelial cell activation and complement-mediated hyperacute graft rejection [22]. Recently, galectin-1 was demonstrated to significantly attenuate allogeneic immune responses in murine LT models [5]. In addition, galectin-1 has been noted to play a role in the mediation of immune tolerance in autoimmune and inflammatory settings [23]. Due to the lack of knowledge and research regarding galectin-1 and immune tolerance, the current study was performed aiming to investigate the effects of galectin-1 on immune tolerance in LT via the mediation of HSCs.

First, the results of the current study indicated that galectin-1 overexpression can enhance immune tolerance by inhibiting the proliferation of T-cells, decreasing IL-2 levels and increasing levels of IL-10 and TGF- β in LT. Interestingly, IL-2, IL-10 and TGF- β are cytokines regarded as key mediators in the induction and effector phases of all immune and inflammatory responses [24]. Previously, it was proved that IL-2 supports the proliferation of T-cells, suggesting that IL-2 levels are positively related to T-cell proliferation [25]. TGF- β and IL-10 are known functional mediators of immunosuppression, and they exert great effects on controlling excessive immune responses [26]. In addition, it was reported that IL-10 and TGF- β treatment inhibited the development of inflammatory responses [27]. Furthermore, a previous study strongly supported the hypothesis that galectin-1 participates in multiple biological processes in different tissues and cells, such as cell proliferation, apoptosis, and immunosuppression [28]. As an evolutionarily conserved glycan-binding protein, galectin-1 contributes to the creation of an immunosuppressed microenvironment at sites of tumor growth [29]. Moreover, galectin-1 was demonstrated to suppress cell viability and proliferation of nonmalignant T-cells in patients with leukemic cutaneous T-cell lymphoma [30]. There has been growing evidence showing that galectin-1 exerts anti-inflammatory effects in concanavalin A-induced hepatitis, and upregulation of galectin-1 in activated HSCs can modulate immune cell functioning [31, 32]. The aforementioned evidence and findings

suggested that galectin-1 inhibits T lymphocyte proliferation by regulating the levels of IL-2, IL-10 and TGF- β to promote immune tolerance in LT. The Th1/Th2 balance exerts its function in transplantation immunology, and IL-2 and IL-10, two representative cytokines, play essential roles in regulating this balance [33]. In addition, highly upregulated galectin-1 in HSCs has been explored quite intensively in recent years, and the results have indicated that galectin-1 is generated by activated HSCs [34]. Furthermore, accumulating evidence has suggested that HSCs play an important role in liver transplant immune tolerance by inducing T lymphocyte apoptosis and regulating the expression of TGF- β and IL-10 [3].

TGF- β , a polypeptide member of the transforming growth factor beta superfamily of cytokines, plays a pleiotropic role in acquired immunity and participates in T-cell regulation and regulatory responses. Interestingly, it has been reported that various immune cells and non-immune cells (peripheral lymphoid cells) can secrete TGF- β or activate the TGF- β /Smad pathway to inhibit T-cell proliferation [35]. The current study found that overexpression of galectin-1 could induce TGF- β 1 secretion and inhibit the proliferation of T-cells, while silencing of galectin-1 inhibited the secretion of TGF- β 1 and promoted the proliferation of T-cells, but the inhibition of proliferation could be reversed using a TGF- β /Smad inhibitor. Simultaneously, it was also found that the addition of U0126, the MAPK/ERK pathway inhibitor in the U0126 group, significantly upregulated galectin-1 and increased TGF- β 1 secretion, and it enhanced the inhibition of proliferation of T-cells. In addition, a previous study revealed that the PI3K/AKT pathway inhibited the activation of NF- κ B by activating AKT, mTOR and C/EBP β to promote immunosuppression [36]. Thus, it can be suggested that the addition of the PI3K/AKT pathway can also rescue the inhibition of proliferation induced by overexpression of galectin-1. Additionally, the Rho/ROCK pathway, closely related to the TGF- β pathway, has been demonstrated to activate the TGF- β /Smad pathway and induce epithelial-mesenchymal transition (EMT) processes [37, 38]. Thereby, it can be concluded that the addition of Rho/ROCK pathway inhibitor also had the same rescue effect as the addition of TGF- β /Smad inhibitor.

The overexpression of galectin-1 plays an immunosuppressive role and has been known to result in an increase in the percentage of CD4⁺/CD8. As previously mentioned, TGF- β /Smad and PI3K/AKT pathways can mediate immunosuppression, which is trivial for maintaining homeostasis. Once the aforementioned pathways are abnormally blocked, it leads to an autoimmune reaction *in vivo*. In agreement with the aforementioned, the current study revealed that, compared with the Galectin-1 OE group, the ratio of CD4⁺/CD8⁺ in the Galectin-1 OE + pathway inhibitor group was found to be decreased, and the addition of these pathway inhibitors reversed the overexpression of galectin-1 and increased the CD4⁺/CD8⁺ ratio. Conversely, the MAPK/ERK pathway was found to promote the inflammatory response, and inhibition of the MAPK/ERK pathway resulted in an increased CD4⁺/CD8⁺ ratio. Furthermore, in follow-up experiments, repeated experiments in each group on the ratio of CD4⁺/CD8⁺ revealed that, on the 7th day, the CD4⁺/CD8⁺ ratio in the Galectin-1 OE + U0126 group was significantly upregulated compared with the Galectin-1 OE group, and addition of U0126 could enhance the immunosuppression ability of galectin-1 overexpression.

In addition, the results of the current study revealed that high activation of HSCs was correlated with the enhancement of immune tolerance. HSCs, as unique nonparenchymal cells, play a potent immunoregulatory role in cotransplantation, with allogeneic islets effectively protecting the islet allografts from rejection, suggesting that HSCs possess immune regulatory effects [7]. Previously, it was indicated that allogeneic CD4⁺ T-cells, including Tregs, are closely related to HSCs [10]. Tregs play a significant role in inducing peripheral tolerance to self and foreign antigens [39]. As previously reported, the xenograft survival was longer in CD4⁺ T-cell-depleted mice, in contrast to in CD4⁺ T-cell mice [40]. In the current study, the results revealed increased expression of α -SMA in CCl₄-induced hepatic fibrosis; however, the expression of desmin was found to be decreased. It is known that intracellular desmin and α -SMA have emerged as markers of quiescent and activated HSCs, respectively [41]. Additionally, α -SMA overexpression has been reported to be correlated with increased liver fibrosis [42].

CCl₄-induced hepatic fibrosis has been reported to be associated with apoptosis and proinflammatory cytokine genes, including TGF-β [43]. Thus, the special effects of TGF-β on hepatic fibrosis and immune tolerance might be an important research direction in achieving better prognoses in liver transplant recipients. In addition, liver function markers in serum comprised of Hyp ALT, AST, TBIL, and Hyp were used in our study in a bid to explore the effects of galectin-1 on immune tolerance and hepatic fibrosis in LT [44]. A previous study demonstrated that serum levels of ALT, AST, TBIL, and Hyp were found to be significantly elevated in liver tissues in mouse models of liver fibrosis [45]. The current study also revealed that the activation might be a contributor to immune tolerance enhancement. In addition, various studies have indicated that induced HSCs resulted in increased extracellular matrix production and collagen degradation during the progression of liver fibrosis, and they are considered to be the major producers of fibrotic nonmatrix in liver injury [46-48].

In conclusion, the current study demonstrated that overexpression of galectin-1 inhibited the proliferation of T-cells by means of HSC activation, which reduced the inflammatory response by exerting immunosuppressive effects and furthermore enhanced immune tolerance and alleviated hepatic fibrosis in LT. The findings of the current study might provide a new therapeutic target for immunological rejection after LT. However, further studies are warranted to explore the underlying mechanism in the future, based on larger sample sizes.

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Disclosure Statement

The authors declare that they have no competing interests.

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