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Original Article

FOXO1 Alleviates Liver Ischemia-reperfusion Injury by Regulating the Th17/Treg Ratio through the AKT/Stat3/FOXO1 Pathway

Hao-Zhen Ren[1,2,3#], Sen-Zhe Xia[1,2#], Xue-Qian Qin[1,3], An-Yin Hu[1] and Jing-Lin Wang[1,2,3*]

[1]*Department of Hepatobiliary Surgery, Affiliated Drum Tower Hospital of Nanjing University Medical School, Nanjing, Jiangsu, China;* [2]*Department of Hepatobiliary Surgery, Nanjing Drum Tower Hospital Clinical College of Jiangsu University, Nanjing, Jiangsu, China;* [3]*Department of Hepatobiliary Surgery, Nanjing Drum Tower Hospital Clinical College of Traditional Chinese and Western Medicine, Nanjing University of Chinese Medicine, Nanjing, Jiangsu, China*

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Abstract

Background and Aims: Hepatic ischemic reperfusion injury (IRI) occurring during surgery seriously affects patient prognosis. The specific mechanism of IRI has not been fully elucidated. The study aim was to explore the changes of inflammatory environment, and the relationship of the Th17/Treg cell ratio and FOXO1 expression in hepatic IRI. ***Methods:*** Liver samples at different ischemic times were collected from patients and mice. The expression of inflammatory markers and FOXO1 in the liver was detected by western blotting and qPCR. Phenotypic changes of liver lymphocytes were analyzed by flow cytometry. The AKT/Stat3/FOXO1 pathway was verified by targeting AKT with GSK2141795. The role of FOXO1 in liver inflammation and changes in lymphocyte phenotype was confirmed by upregulating FOXO1 with resveratrol. ***Results:*** Prolonged ischemic time aggravates liver injury in both humans and mouse models of hepatic IRI. IR-stress caused Th17/Treg imbalance and FOXO1 down-regulation by activating the AKT/Stat3/FOXO1 signaling pathway. Upregulation of FOXO1 reversed the Th17/Treg cytokine imbalance and altered the inflammation environment in the liver. ***Conclusions:*** Liver IRI induced Th17/Treg imbalance. Upregulation of FOXO1 reversed the imbalance and alleviated liver inflammation.

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Keywords: Liver ischemia-reperfusion injury; Inflammatory factors; Th17; Treg; AKT/Stat3/FOXO1 pathway.

Abbreviations: AKT, protein kinase B; ALT, alanine transaminase; AST, aspartate aminotransferase; DAMP, damage associated molecular pattern; FOXO1, forkhead box transcription factor 1; Foxp3, transcription factor forkhead box P3; GVHD, graft-versus-host disease; IL10, interleukin-10; IL17A, interleukin-17A; IR, ischemic-reperfusion; IRI, ischemic-reperfusion injury; PBS, phosphate-buffered saline; Stat3, signal transducer and activator of transcription 3; TGF-β, transforming growth factor beta; Th17, T helper type 17; Treg, regulatory T cells.

[#]Contributed equally to this work.

[*]**Correspondence to:** Jing-Lin Wang, Department of Hepatobiliary Surgery, Affiliated Drum Tower Hospital of Nanjing University Medical School, No. 321, Zhongshan Road, Gulou District, Nanjing, Jiangsu 210008, China. ORCID: https://orcid.org/0000-0002-4349-750X. Tel/Fax: +86-25-83106666, E-mail: cw20120817@163.com

Introduction

Ischemia-reperfusion injury (IRI) during liver surgery causes liver dysfunction and liver failure after transplantation.[1] Multiple types of immune cells are involved in hepatic IRI. Ischemic stress can damage sinusoidal endothelial cells and hepatocytes, and the release of damage-associated molecular patterns. Neutrophils, natural killer cells, dendritic cells, macrophages, and various lymphocytes are involved in liver inflammatory injury that leads to liver immune microenvironment disorders.[2,3] However, the target and molecular mechanisms of liver IRI are complex and still not fully understood, and there are no effective treatments for liver IRI.

As the main mediator of IRI, CD4+T cells promote the activation of Kupffer cells, amplify their effects, and trigger an immune cascade reaction.[4] The actions initiate of IRI inflammation and ultimately lead to liver-cell dysfunction.[5] Many studies have shown that T helper type 17 (Th17) cells, a subtype of CD4+ T lymphocytes, participate in liver IRI.[6] Interleukin-17A (IL17A), a cytokine secreted by Th17 cells, is significantly increased in hepatic IRI, and stimulates macrophages to secrete pro-inflammatory factors that lead to increased inflammation in the liver.[7] Regulatory T (Treg) cells, known as inhibitory lymphocyte relieve liver IRI by releasing TGF-β.[8] The balance between Th17 and Tregs is a focus of IRI research, and we have previously shown that endoplasmic reticulum stress in Kupffer cells leads to IL-6-mediated transformation of natural Tregs into Th17 cells, thus aggravating liver inflammation and damage.[9] Others have found that the Th17/Treg balance can be regulated by forkhead box transcription factor 1 (FOXO1), miR-21, Rab4b, intestinal flora, and other factors.[10–13] FOXO1 is a transcription factor that regulates apoptosis, autophagy, the cell cycle, oxidative stress, and immune responses,[14] but the role of FOXO1 in liver IRI is not clear. FOXO1 has been shown to regulate the differentiation and function of Th17 and Tregs. Knocking out FOXO1 was reported to increase the differentiation of Th17 cells, indicating that FOXO1 has a negative regulatory effect on Th17 cells,[12] but whether FOXO1 regulates Th17/Treg balance and inflammatory injury in liver IRI is not clear. The aim of this study was to determine the relationship between FOXO1 and the immune microenvironment. Our results showed that FOXO1 regulated the Th17/Treg to protect the liver from IRI.

Methods

Clinical liver IRI study

Human ischemic liver tissues and peripheral blood samples were obtained from patients with partial hepatectomy at the Drum Tower Hospital Affiliated with Medical College of Nanjing University. Eighteen samples of liver lesions that were confirmed benign by clinical diagnosis and pathology, were also selected. All patients signed the informed consent before operation and specimen acquisition. All clinical samples were cryopreserved in liquid nitrogen.

Mouse liver IRI model

Male C57BL/6 mice from 6 to 8 weeks of age were purchased from the Experimental Animal Center of Drum Tower Hospital, Nanjing University of Medical School. The mouse liver ischemia-reperfusion injury model was established by blocking the hepatic artery/portal vein for 90 minutes and a sham group was established without blocking the blood vessels. Mice in the treatment groups were continuously injected with a FOXO1 agonist resveratrol (501-36-0; SelleckChem, Houston, TX, USA, 20 mg/kg intraperitoneally) or AKT inhibitor GSK2141795 (1047634-65-0; SelleckChem, Houston, TX, USA, 100 mg/kg, orally) every day for 2 weeks. Groups of eight mice each were anesthetized and sacrificed at 1, 3, 6, 12 and 24 h after reperfusion. Liver and serum samples were collected for analysis. National Institutes of Health Laboratory Animal Care Guidelines were used.

Alanine transaminase (ALT) and aspartate aminotransferase (AST) measurement

Serum ALT and AST levels in clinical patients and mice were measured with an automatic biochemistry analyzer (Fuji, Tokyo, Japan)

Western blot analysis

Total protein was extracted from liver tissue, and a bicinchoninic acid assay (Sigma-Aldrich, St Louis, MO, USA) was used to determine protein concentration. Equal amounts of protein were loaded on gels for separation by sodium dodecyl-sulfate polyacrylamide gel electrophoresis, transferred to. membranes, incubated with primary antibodies (Supplementary Table 1) and horseradish peroxidase-conjugated secondary antibodies, developed with an electrochemiluminescence system, and exposed on X-ray film (Kodak, Japan). The signal intensity was quantified using Image J software (National Institutes of Health, Bethesda, MD, USA).

Real-time quantitative polymerase chain reaction (qPCR) assay

TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA from frozen liver tissue. The RNA was reverse transcribed into cDNA using Superscript II Reverse Transcriptase Kits (Invitrogen, Carlsbad, CA, USA). qPCR was performed with Power SYBR Green PCR Master Mix (Takara, Tokyo, Japan). Normalized gene expression was calculated using the $2^{-\Delta\Delta CT}$ method with β-actin as the housekeeping gene. The primer sequences are shown in Supplementary Table 2.

Isolation of liver lymphocytes

Mice were sacrificed after anesthesia and the liver was perfused *in situ* with 1 × Hanks balanced salt solution (HBSS) for 5 m before removal. The liver was placed on a 70 µm cell filter and gently pressed to obtain a cell suspension. The cells were pelleted by centrifugation at 300×*g* (5810R; Eppendorf, Hamburg, Germany) 4°C for 10 m. The supernatant was discarded, the pellet was resuspended in 5 mL 40% Percoll (P4937, Sigma-Aldrich, St Louis, MO, USA), 5 mL 70% Percoll was slowly added. The suspension was centrifuged at 1,300×*g* for 30 m with a slow deceleration. The middle interface was transferred to a 15 mL centrifuge tube with 1×HBSS and centrifuged at 1,300×*g* for 10 m. After discarding the supernatant, the pellet was resuspended in 2 mL RPMI 1640 and transferred to a six-well plate. After incubation for 15 m at 37°C, the cells that were not adherent or weakly adherent were transferred to a 15 mL centrifuge tube in phosphate buffered saline and centrifuged at 1,500×*g* for 10 m. The supernatant was discarded and the pellet, which contained liver lymphocytes except for macrophages, was resuspended in 1 mL staining buffer and assayed by fluorescence-activated cell sorting (FACS) flow cytometry. FlowJo software (Media Cybernetics, TreeStar, Ashland, OR, USA) was used for analysis.

Isolation of peripheral blood lymphocytes

Mouse blood samples were diluted with 1× phosphate-buffered saline (PBS) at a 1:1 ratio. 2 mL Ficoll (BS159; Biosharp, Hefei, China) separation solution was added to a 15 mL centrifuge tube, the diluted blood was slowly added to the upper layer of Ficoll and centrifuged at 2,000 rpm for 20 m with slow deceleration. After removing the peripheral blood mononuclear cell (PBMC) layer, 1 mL 1× PBS was used to resuspend and gently wash the pellet, followed by centrifugation at 1,200 rpm for 5 m. After discarding the supernatant, the peripheral blood lymphocytes were stained and analyzed by flow cytometry.

Flow cytometry

Treg surface markers CD4, CD25, and the intracellular marker Foxp3; Th17 surface markers CD4, CD3, and intracellular marker IL17A were analyzed by flow cytometry. The antibodies against mouse lymphocyte surface markers were: fluoresceine isothiocyanate-conjugated anti-mouse CD4 (100405; Biolegend, San Diego, CA, USA), allophycocyanin-conjugated anti-mouse CD3 (100235; Biolegend, San Diego, CA, USA), allophycocyanin-conjugated anti-mouse CD25 (101909; Biolegend, San Diego, CA, USA), PE anti-mouse Foxp3 (563101; Becton-Dickinson, San Jose, USA), phycoerythrin-conjugated anti-mouse IL-17A (559502; Becton-Dickinson, San Jose, USA). Th17 cells were stimulated with 1 µL cell stimulation cocktail (00-4970-03; Invitrogen, Carlsbad, CA, USA) in 100 µL RPMI 1640 and incubated at 5% CO_2 and 37°C for 4–6 h before staining. Cells were incubated with surface marker antibody for 30 m, centrifuged at 500×*g* for 5 m, resuspended, and fixed for 30 m with Cytofix/Cytoperm solution (554715; Becton-Dickinson, San Jose, USA). The fixed cells were washed and permeabilized in 250 µL 1×perm/wash buffer (554723; Becton-Dickinson, San Jose, USA), centrifuged at 500×*g* for 5 m, resuspended in 50 µL 1×Perm/Wash buffer, and incubated 30 m with intracellu-

Table 1. The relationship between liver injury and ischemia time in clinical samples

n=18	No occlusion	0 m < occlusion < 60 m	Occlusion ≥ 60 m	*p*-value
Occlusion Time, m	0	30±13.98	71.4±16.58	<0.05$^{\#}$
Sex	All women	All women	2 men 4 women	–
Age, years	47±11	51.8±13.97	52±11.55	–
hepatitis virus	No	No	No	–
ALT before operation	12.43±6.47	21.28±7.6	23.68±15.44	–
AST before operation	16.17±2.12	18.65±1.46	18.4±4.46	–
ALT after operation	96.2±9.68	194.08±27.01	327.68±107.26	<0.01$^{\#\#}$
AST after operation	83.17±17.32	182.23±45.77	265.14±118.91	<0.05$^{\#}$
p-value	<0.01**	<0.01**	<0.01**	

Data are means ± SEM, *p <0.05, **p <0.01, $^{\#}$p <0.05, $^{\#\#}$p <0.01, Student *t*-test. ALT, alanine transaminase; AST, aspartate aminotransferase.

lar antibodies After incubation, the cells were washed with PBS, centrifuged at 500×*g* for 5 m, resuspended in 100 µL stain buffer, and analyzed by flow cytometry.

Histopathology

Liver tissue was fixed with 4% paraformaldehyde, embedded in paraffin, and the blocks were sectioned at 5×10^3 µm, three slices per liver. The sections were stained with hematoxylin and eosin for pathological evaluation. Image-Pro Plus 5.0 (Media Cybernetics, Bethesda, MD, USA) was used to analyze the images.

Immunofluorescence staining

We used immunofluorescence staining to analyze the changes of FOXO1 and p-FOXO1 in the liver after ischemia-reperfusion. Patient and mouse liver samples were stained with anti-FOXO1 antibody (1:100; Ab52857; Abcam, Cambridge, UK) and anti-p-FOXO1 antibody (1:100; Ab131339/Ab259337; Abcam, Cambridge, UK). Liver tissue was fixed in 4% paraformaldehyde in PBS (Sigma-Aldrich, St Louis, MO, USA), washed, and stored in 30% sucrose in PBS for 24 h. The samples were embedded into Optimum Cutting Temperature gel (Tissue-Tek, Torrance, CA, USA), frozen (CM30505; Leica, Wetzlar, Germany), and cut into 10 µm sections. The slides were incubated with goat serum (Gibco, Grand Island, NY, USA) blocking buffer for 1 h at room temperature and then incubated with primary antibody at 4°C overnight. After washing, the slides were incubated with secondary antibody at room temperature for 1 h. After washing with PBS for 3 times, the slides were incubated with diamidino-phenylindole for 10 m, dried, and mounted. Image-Pro Plus 5.0 was used to analyze FOXO1 and p-FOXO1 signals.

Statistical analysis

All experiments in this study were repeated at least three times. GraphPad Prism software (version 6.0; GraphPad Software Inc., La Jolla, CA, USA) was used to perform the statistical analysis, and the results were reported as means ± SD. Student's *t*-test was used to determine significant differences. Significant correlations were determined with Spearman's coefficient and *p*-value. Flow cytometry results were analyzed using FlowJo version10 (Media Cybernetics, TreeStar, Ashland, OR, USA).

Results

Prolonged ischemia aggravates liver injury in human hepatic IRI

To explore the relationship between liver damage and ischemic time, we grouped clinical samples into no occlusion, 0 m < occlusion < 60 m and occlusion ≥ 60 m groups by the time of portal vein occlusion. Preoperative ALT and AST levels (Table 1) were not significantly different between groups before surgery (p >0.05), but were significantly increased after surgery (p<0.05), and the trend was correlated with the ischemic time. We also measured the levels of apoptosis-related proteins Caspase 3 and cleaved-Caspase 3 in the liver, and found that the ratio was significantly increased after IRI, indicating that cell apoptosis had been increased (Fig. 1A). In addition, the mRNA (Fig. 1B) and protein (Fig. 1C) expression of inflammatory factors such as tumor necrosis factor alpha (TNF-α) increased after IR-stress and the increase was correlated with the length of blocking time. The findings indicate that liver damage became more severe with the cumulative increase of blocking time.

Correlation between reperfusion time and liver injury in mouse hepatic IRI

To determine the correlation between liver damage and reperfusion time in mice, we performed liver ischemia 90 m and reperfusion for 1, 3, 6, 12, and 24 h. Compared with the sham group, the serum levels of ALT and AST (Fig. 2A) increased continuously with the extension of reperfusion time, and reached a peak at 6 h after reperfusion, which was consistent with the hematoxylin and eosin staining results (Fig. 2B). At 6 h after reperfusion, obvious congestion occurred in the liver, inflammatory-cell infiltration increased, and hepatocytes included vacuole-like phenotypes. These results show that the liver damage was the most severe at 6 h of reperfusion following 1 h of ischemia. Those times were selected for follow-up procedures.

We also found that after liver IRI, the ratio of Caspase-3 and cleaved-Caspase-3 increased (Fig. 2C), indicating that apoptosis increased. The expression of inflammatory factors also increased significantly (Fig. 2D), and the expression of TNF-α protein showed an upward trend (Fig. 2E), which was consistent with the findings in clinical samples. The results showed that the IR stress induced liver inflammation and apoptosis in mice, and that the injury peaked at 6 h after reperfusion.

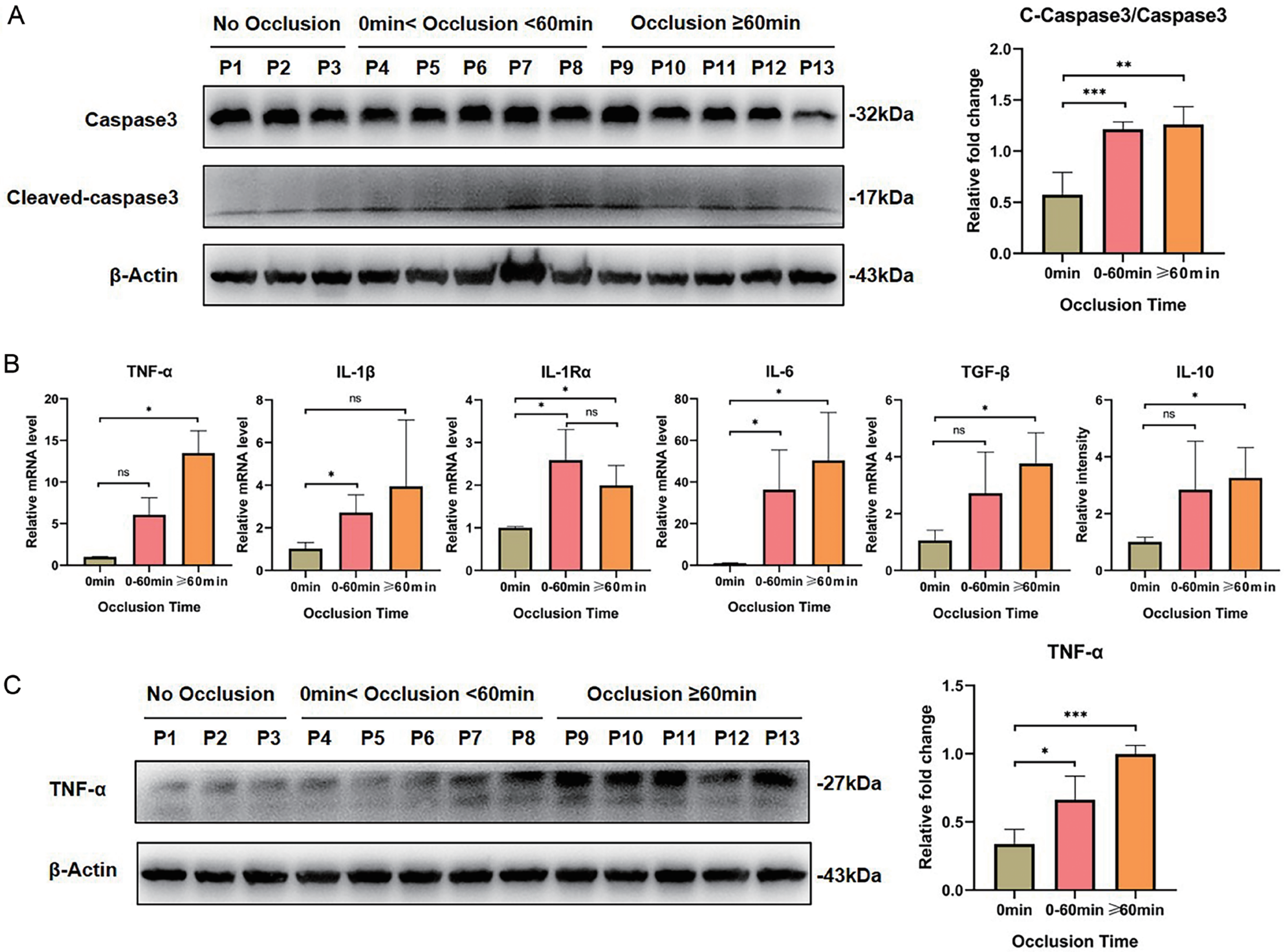

Fig. 1. Expression of apoptosis and inflammatory factors increased in clinical samples after liver IR. (A) Western blots of Caspase 3 and cleaved-Caspase 3. (B) mRNA levels of TNF-α, IL6, IL1β, TGF-β, IL1Ra, IL10. (C) Western blots analysis of TNF-α. Data are means ± SEM, $*p < 0.05$, Student *t*-test. TNF-α, tumor necrosis factor alpha; IL6, interleukin 6; IL1β, interleukin 1 beta; TGF-β, transforming growth factor beta; IL1Ra, interleukin 1 receptor alpha; IL10, interleukin 10; IR, ischemic-reperfusion.

IR-stress induced Th17/Treg cell imbalance in liver tissue

As T cells have a crucial role in liver inflammation and the imbalance of Th17/Treg mediates liver inflammation in chronic liver graft versus host disease,[15] we measured the changes of Th17/Treg ratio and their cytokines in liver tissue after hepatic IRI. In both clinical and mouse liver samples, the levels of Th17 cytokine IL17A and its receptor IL17RA (Fig. 3A) were significantly upregulated after IR stress, which was confirmed by western blotting (Fig. 3B). On the other hand, the expression of the Treg cytokine Foxp3 was downregulated (Fig. 3A).

To further clarify the change of Th17/Treg ratio after hepatic IR-stress, we isolated peripheral blood and intrahepatic lymphocytes from mice for flow cytometry. We found that after IRI, the proportion of CD4+ IL17A+ Th17 cells in peripheral blood and liver increased, and the proportion of CD4+ CD25+ Foxp3+ Treg cells decreased (Fig. 3C). In addition, in human peripheral blood lymphocytes and mouse liver lymphocytes, the expression of Th17 indicators showed an upward trend and the expression of Treg-related cytokine Foxp3 decreased (Fig. 3D). The findings indicate that the Th17/Treg cell ratio and their cytokines in liver became imbalanced after IRI.

Expression of FOXO1 decreased after hepatic IRI

Previous studies have reported that FOXO1 was involved in the regulation of Th17 and Treg cell differentiation and function,[16] and participated in liver IRI.[17] We measured FOXO1 levels in clinical and mouse liver samples to explore the changes expression associated with the stress related to liver IRI. We found that after liver IRI, FOXO1 expression decreased in clinical liver samples, and the reduction was positively correlated with the blocking time. In mice, the decreasing trend of FOXO1 expression was related to the degree of liver damage. FOXO1 expression reached a minimum after 6 h of reperfusion, which was consistent with the changes of liver damage (Fig. 4A, B). The change of FOXO1 expression was further confirmed by immunohistochemical staining (Fig. 4C). Similarly, it was also found in mice liver lymphocytes that the expression of FOXO1 decreased significantly after experiencing ischemic stress (Fig. 4D). The results suggested that the expression of FOXO1 in liver de-

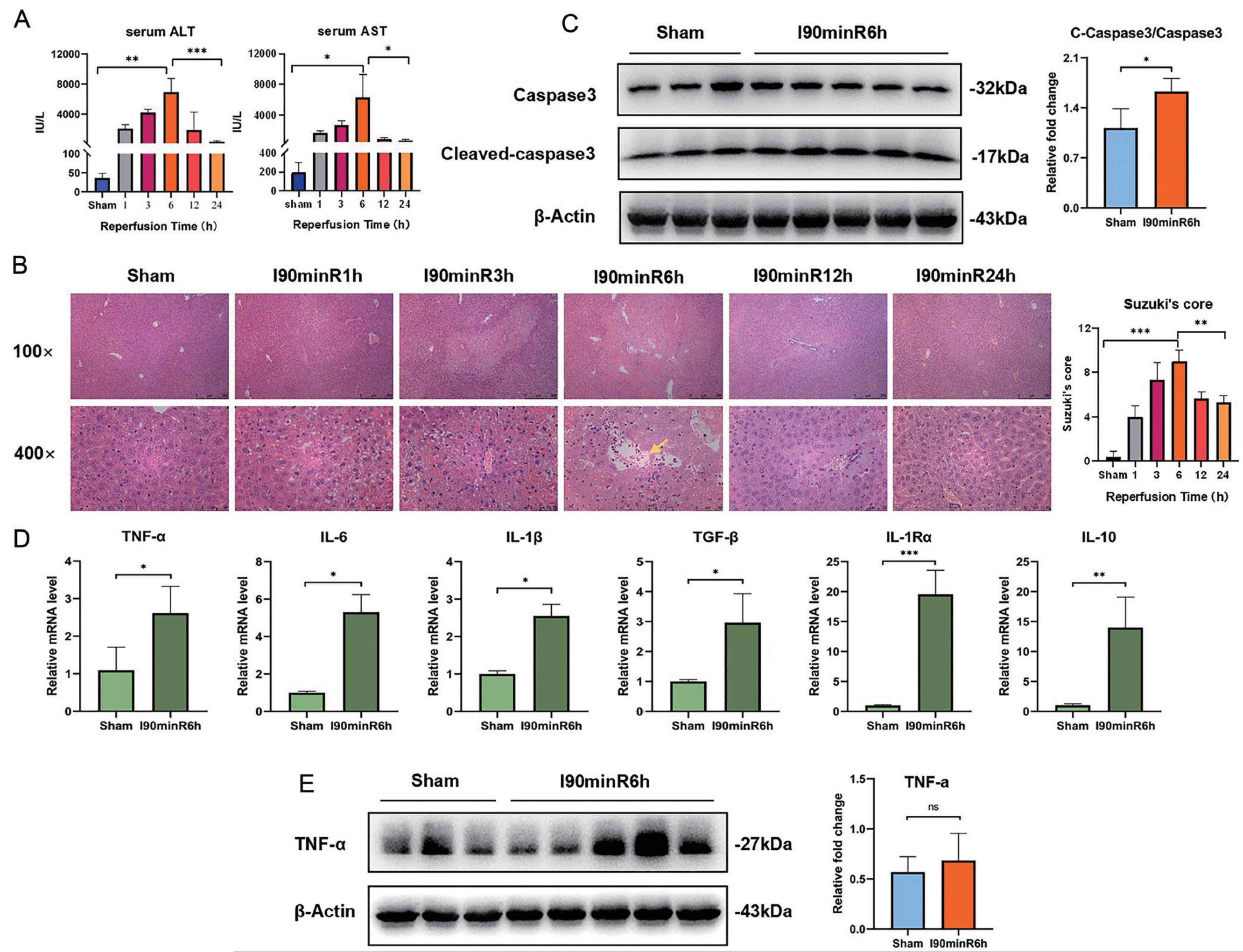

Fig. 2. Expression of apoptosis and inflammatory factors increased in mice liver after IR-stress. (A) Serum ALT and AST levels after reperfusion for 1, 3, 6, 12, and 24h. ALT and AST levels peaked at 6 h. (B) Representative liver tissue (hematoxylin and eosin, 100×, 400×; *n*=8/group). (C) Western blots of TNF-α expression. (D) mRNA levels of TNF-α, IL6, IL1β, TGF-β, IL1Ra, IL10. (E) Western blots of TNF-α. Data are means ± SEM, **p* <0.05, Student's *t*-test. ALT, alanine transaminase; AST, aspartate aminotransferase.

creased after IRI and was correlated with ischemic time.

The AKT/Stat3/FOXO1 signaling pathway was activated after liver IRI

It has been shown that phosphorylation of FOXO1 through the Akt pathway reduce its transcriptional activity,[18] and that Stat3 pathway has an important role in regulating FOXO1. To explore whether AKT pathway reduced FOXO1 transcriptional activity during liver IRI, we examined the AKT/FOXO1 pathway in clinical samples and animal models. We found that expression of p-FOXO1 increased significantly after liver IRI (Fig. 5A), and the trend was dependent on the blocking time (Fig. 5B). The activation of the Stat3 pathway was reduced in clinical and mouse liver samples after IRI (Fig. 5C), and the Akt inhibitor GSK2141795 (100 mg/kg) inhibited AKT activity after liver IRI. The expression of FOXO1 was up-regulated and the expression of p-FOXO1 was downregulated (Fig. 5D). The findings indicate that FOXO1 participated in the liver IRI process through the AKT/Stat3/FOXO1 pathway.

Up-regulation of FOXO1 reversed Th17/Treg imbalance to alleviate liver IRI

Previous studies have found that FOXO1 regulated the function of Th17/Treg cells. To explore the mechanism of FOXO1 in liver IRI, and determine whether it affected liver IRI and inflammatory infiltration by regulating Th17/Treg cytokines, we injected resveratrol (20 mg/kg), a FOXO1 agonist,[19] into mice intraperitoneally for 2 weeks before the operation (Fig. 6A). After resveratrol treatment, the reduction of FOXO1 in lymphocytes caused by liver IRI was partially reversed, the Th17/Treg imbalance was reversed, the expression of IL17A and its receptor IL17RA was reduced, and the expression of Treg cytokine Foxp3 increased (Fig. 6B). Changes in the proportions of Treg and Th17 cells are shown in (Fig. 6C). After up-regulation of FOXO1 expression, liver injury was significantly reduced, vacuolar degeneration of hepatocytes was alleviated (Fig. 6D, E), and the expression of inflammatory factors was inhibited (Fig. 6F). The findings indicated that FOXO1 up-regulation alleviated the inflammatory damage induced by liver IRI by regulating the ratio and function of Th17 and Treg cells.

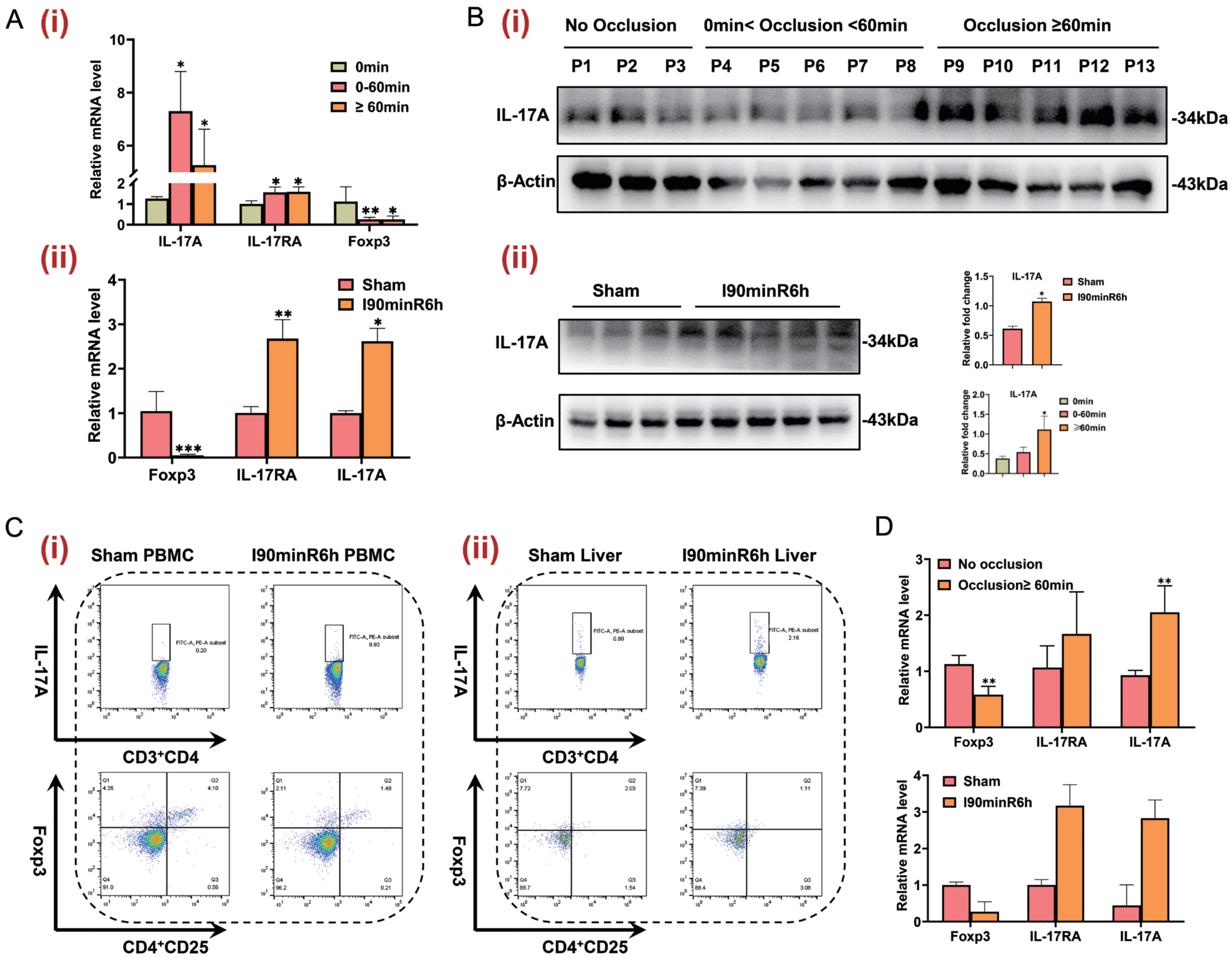

Fig. 3. Imbalance of Th17 and Treg cells proportion in liver induced by IR-stress. (A) mRNA levels of IL17A, IL17Ra, and Foxp3 in human (i) and mouse (ii) liver after IR-stress. (B) Western blots of IL17A in human (i) and mouse (ii) liver. (C) Flow cytometry of lymphocyte phenotype transition. The proportion of CD4+ IL17A+ Th17 cells in peripheral blood (i) and liver (ii) increased; that of CD4+ CD25+ Foxp3+ Treg cells decreased. (D) mRNA levels of IL17A, IL17Ra, and Foxp3 in human peripheral blood and mouse liver lymphocytes. Data are means ± SEM, **p* <0.05, Student's *t*-test. IL17A, interleukin-17A; IL17Ra, interleukin 17 receptor alpha; Foxp3, transcription factor forkhead box P3; Th17, T helper type 17; Treg, regulatory T.

Discussion

Hepatic IRI is a common pathophysiological event during liver surgery, and results in disturbed liver metabolism and immune inflammation that directly affect disease prognosis and the survival. Ischemia and reperfusion time affect the severity of liver IRI. In this study, evaluation of clinical samples with no occlusion, 0 m < occlusion < 60 m, and ≥ 60 m occlusion intraoperative occlusion time, found that the expression of markers of liver injury were closely related to the time of occlusion. The correlation was verified in a mouse IRI model. Liver damage was the most severe at 6 h after reperfusion, which was chosen to establish the mouse model. Increased inflammation after liver IRI has been confirmed in previous studies.[20] The levels of TNF-α, IL1β, IL1Rα, and IL6 inflammatory factors were significantly increased after liver IRI. In other studies, the Treg cytokines TGF-β and IL10 were down-regulated after liver IRI,[21] but they were significantly increased after liver IRI in this study. A possible reason for the discrepancy is that those cytokines have both anti- and pro-inflammatory effects, and are not secreted only by Treg cells. In this study, it is not clear whether they served as pro-inflammatory factors or anti-inflammatory factors. Further study of the activity of TGF-β and IL10 in IRI is needed.

Recent studies have shown that Th17/Treg cell imbalance is involved in liver IRI.[22] Treg cells are thought to have an anti-inflammatory role during immune response, and Th17 is usually described as a pro-inflammatory cell. Our study found an imbalance of Th17/Treg cells after hepatic IRI, in which the proportion of Th17 cells and its cytokines IL17A and IL17RA increased, and the proportion of Tregs and its cytokine Foxp3 decreased. The expression of IL17A, IL17RA, and Foxp3 in peripheral blood lymphocytes from clinical samples and mouse liver tissue showed the same trend. The increase of Th17 and its cytokine IL17A may promote the inflammatory immune response of liver, thereby mediating liver IRI. To further study the mechanism of Th17/Treg imbalance, we determined the expression of FOXO1, a highly conserved transcription factor that regulates the differentiation and function of Th17/Treg cells,[16] but its role in organ IRI is not clear. We found that the

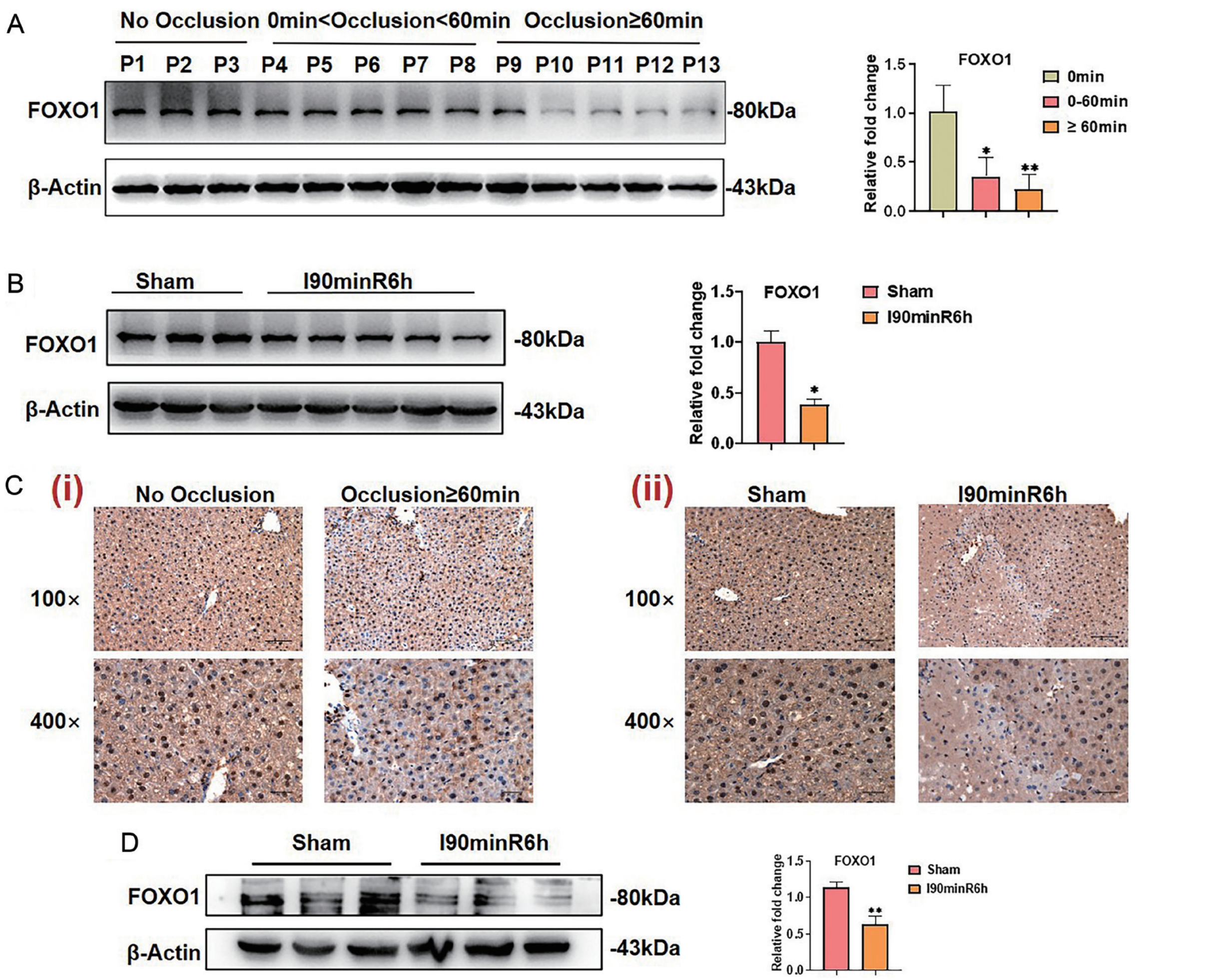

Fig. 4. FOXO1 expression of decreased after hepatic IRI. (A, B) Western blots of FOXO1 in human and mouse liver. (C) Immunohistochemical staining of FOXO1 (200×, 400×) in human (i) and mouse (ii) liver. (D) Western blots of FOXO1 in mouse liver lymphocytes. Data are means ± SEM, *p <0.05, Student's *t*-test. FOXO1, forkhead box transcription factor 1.

AKT/FOXO1 pathway was activated in hepatic IRI, the nuclear translocation of FOXO1 decreased, and the cytoplasmic p-FOXO1 increased. The result is consistent with other studies that found that FOXO1 expression increased and p-FOXO1 decreased after liver IRI. Activation of the AKT/FOXO1 pathway reduced FOXO1 and increased p-FOXO1 to reduce liver IRI.[23] Further study is needed to reveal the role of FOXO1 in the liver. Based on the findings, we speculate that the reduced expression of FOXO1 in the liver after IRI led to an imbalance of Th17/Treg ratio that caused the increase of IL-17A and IL-17RA, and decrease of Foxp3. The changes ultimately resulted in an increased inflammatory response and liver damage (Supplementary Fig. 1). The study results showed that upregulation of FOXO1 by resveratrol reversed the Th17/Treg imbalance, significantly reduced the expression IL-17A and its receptor IL-17RA, and increased the expression of the Treg cytokine Foxp3. FOXO1 upregulation reduced the infiltration of inflammatory factors and relieved liver IRI.

In conclusion, this study showed that the activation of AKT/FOXO1 pathway after hepatic IRI reduced FOXO1 activity and caused Th17/Treg imbalance. Upregulation of FOXO1 reversed the changes and reduce liver IRI. These results suggest that targeting FOXO1 might be a new direction for the prevention and treatment of liver IRI in the clinic, and warrant continuing research.

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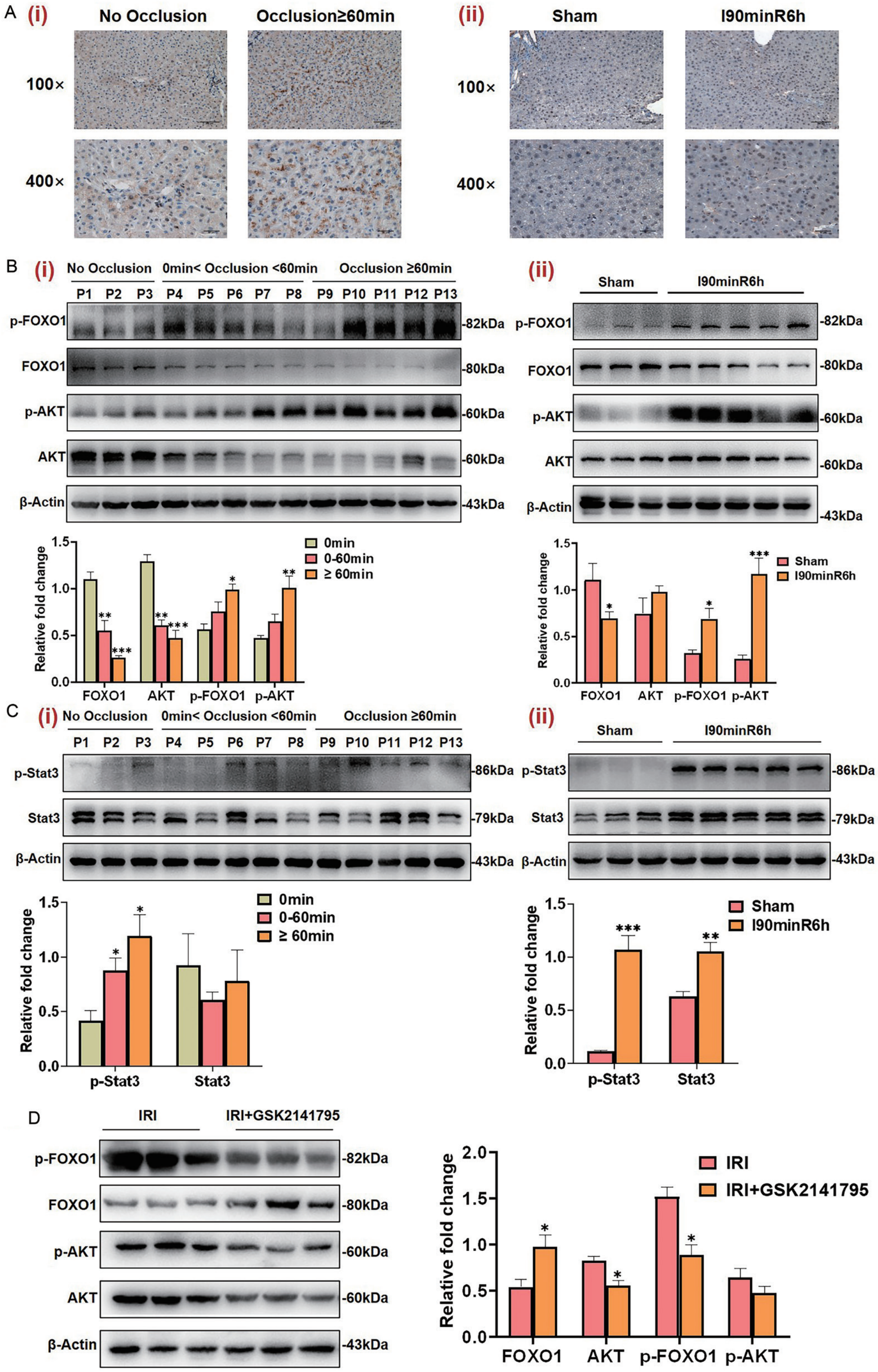

Fig. 5. AKT/p-FOXO1 signaling pathway is activated after liver IRI. (A) Immunohistochemical staining of p-FOXO1 (200×, 400×) in human (i) and mouse (ii) liver. (B) Western blots of FOXO1, p-FOXO1, AKT, and p-AKT in human (i) and m (ii) liver after IR-stress. (C) Western blots of Stat3, and p-Stat3 in human (i) and mouse (ii) liver. (D) Western blots of FOXO1, p-FOXO1, AKT, and p-AKT after GSK2141795 treatment. Data are means ± SEM, *p <0.05, Student's t-test. AKT, protein kinase B; Stat3, signal transducer and activator of transcription 3; IRI, ischemic-reperfusion injury.

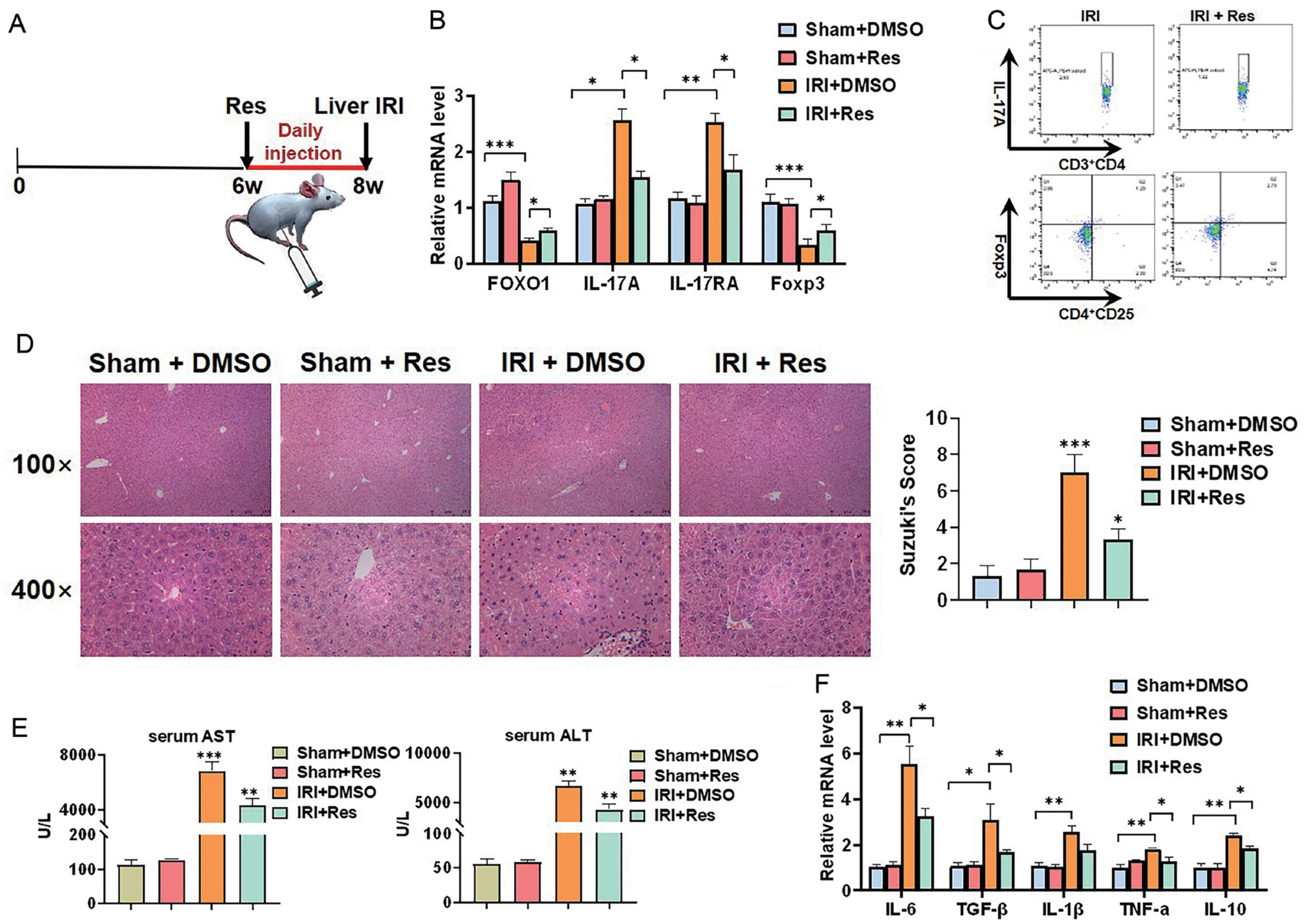

Fig. 6. Up-regulation of FOXO1 reverses Th17/Treg cytokine imbalance to alleviate liver IRI. (A) Effect of up-regulated FOXO1 in IRI model mice. Male mice were injected with Res (20 mg/kg) at 6 weeks of age, and the IRI model was established 2 weeks after resveratrol injection. (B) mRNA levels of FOXO1, Foxp3, IL-17A, and IL-17Ra. (C) Lymphocyte phenotype transition assayed by flow cytometry after resveratrol treatment. (D) Representative liver tissue (hematoxylin and eosin, 100×, 400×; *n*=8/group) after resveratrol injection (E) Serum ALT and AST levels after FOXO1 up-regulation. (F) mRNA levels of TNF-α, IL1β, IL1Ra, IL6, TGF-β, and IL10. Data are means ± SEM, **p* <0.05, Student's *t*-test. Res, resveratrol; DMSO, Dimethyl sulfoxide.

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Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Conceived and designed the study (RHZ, XSZ, QXQ, HAY, WJL), collected and assembled the data (RHZ, XSZ, QXQ, HAY, WJL), performed the data analysis and interpretation (RHZ, XSZ, QXQ, HAY, WJL), wrote the manuscript (RHZ, XSZ, QXQ), and provided financial support and gave final approval of the manuscript (RHZ, WJL). All authors read and approved the manuscript.

Ethical statement

All animal experiments were performed with the approval of Ethics Committee for Animal Experimentation of the Affiliated Drum Tower Hospital of Nanjing University Medical School (approval no. 2020AE01042). The humane endpoint was judged by the assessment of activity (e.g., hunched back, rest, poor grooming, and wrinkles) or weight loss (> 20% of total body weight) in mice. All surgery was performed under isoflurane anesthesia (induction concentration of 3–4%, and maintenance concentration of 1–1.5%). Mice were euthanized by cervical dislocation, and death was confirmed by observing movement, including breathing movement in the chest. All efforts were made to minimize animal suffering. The study was approved by The Affiliated Drum Tower Hospital of Nanjing University Medical School (Ethical approval No.2019-157-001).

Data sharing statement

The datasets generated during and/or analyzed during the study are available from the corresponding author on reasonable request.

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