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VEGF-C attenuates ischemia reperfusion injury of liver graft in rats

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ABSTRACT

Background: Vascular endothelial growth factor receptor-3 (VEGFR-3) / vascular endothelial growth factor -c (VEGF-C) signaling is reported to negatively regulate TLR4-triggered inflammation of macrophages. This study aims to clarify whether the VEGFR-3/VEGF-C signaling can suppress Kupffer cells (KCs) activation and attenuate hepatic ischemia-reperfusion injury (IRI) after liver transplantation.

Methods: A rat model of liver transplantation was performed. Donor livers were perfused with VEGF-C injection via portal vein during cold preservation, and controls were perfused with UW solution. Serum levels of alanine transaminase (ALT), total bilirubin (TBIL) and inflammatory cytokines, as well as histology were analyzed after 24 h. KCs were isolated from grafts, RT-PCR and immunofluorescence were used to evaluate polarization-specific marker genes, western bolt was employed to assess the expression of suppressor of cytokine signaling 1(SOCS1) and phosphorylated glycogen synthase kinase 3β (p-GSK3β), and EMSA was utilized to quantify the NF-κB transcriptional activity.

Results: Compared with controls, VEGF-C perfusion reduced ALT and TBIL levels and alleviated liver damage. Furthermore, VEGF-C perfusion suppressed serum proinflammatory cytokines secretion and increased IL-10.In addition, the VEGFR-3 mRNA of KCs was increased after reperfusion. VEGF-C perfusion suppressed NF-κB activity and up-regulated the expression of SOCS1 and p-GSK3β in KCs, and shifted the M1/M2 balance toward an anti-inflammatory profile.

Conclusion: Exogenous VEGF-C protects liver graft from IRI by regulating the inflammatory response and modifying polarization of KCs.

1. Introduction

Liver transplantation is an effective method for treating liver malignancy and end-stage liver disease, but ischemia-reperfusion injury (IRI) is still a problem unsolved for liver transplantation. Liver injury caused by ischemia-reperfusion not only leads to up to 10% failure of early graft function, but also increases the risk of organ rejection and liver dysfunction [1]. The mechanism of liver IRI during transplantation is complex. The current studies suggest that the inflammatory cascade is a key factor in IRI development [2], and the activation of Kupffer cells (KCs) in the early stage of transplantation is an important cause of downstream inflammatory cascade [3]. Toll-like receptor 4 (TLR4), a pathogen recognition receptor, coordinates innate and adaptive immune responses and plays a central role in transplantation immunity [4]. Our previous research finds that inhibition of TLR4 - nuclear factor κB (NF-κB) signaling of KCs can reduce the release of inflammatory mediators such as tumor necrosis factor-alpha (TNF-α) and interluekin-6 (IL-6), and attenuate IRI of liver grafts [5,6]. However, further research is still needed to investigate how to regulate KCs activation during liver transplantation.

KCs, the resident macrophages of the liver, can exhibit distinctly different phenotype and function in different microenvironments [7]. Recent finding suggests that the vascular endothelial growth factor receptor-3 (VEGFR-3) and its ligand vascular endothelial growth factor-C (VEGF-C) are up-regulated in macrophages under the lipopolysaccharide (LPS)-induced condition. VEGFR-3 ligation by VEGF-C not only restrains TLR4-NF-κB pathway, but also increases anti-inflammatory cytokine production in vitro [8].

2. Original hypothesis

Portal vein (PV) occlusion during liver transplantation results in

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intestinal wall congestion and increased bacterial translocation, as well as provides LPS from gram-negative bacteria [9]. We hypothesize that VEGFR-3 / VEGF-C signaling may play a key role in regulating KCs function during liver transplantation. Therefore, using the rat model of orthotopic liver transplantation (OLT), the purpose of the present study is to investigate the role of exogenous VEGF-C on hepatic IRI as well as the underlying mechanism.

3. Materials and methods

3.1. Animals and experimental protocol

Male Lewis rats (weighing 200–250 g) were purchased from Animal Experimental Center of Chongqing Medical University. The experimental procedure was approved by the Animal Care and Use Committee of Chongqing Medical University. The rats were randomly divided into VEGF-C group, the control group and the sham-operated group. The donor livers of VEGF-C group ($n = 6$) were continuously perfused with VEGF-C injection (R&D Systems, Inc., Minneapolis, MN, USA, 5 ng/kg/min) via PV cannulation using a syringe pump for 1 h at the beginning of cold preservation. The donor livers of the control group were treated with the same volume of University of Wisconsin (UW) solution by the same way ($n = 6$). The sham-operated group performed only abdominal open surgery (n = 6). All the experimental animals survived 24 h postoperative. The rats were euthanized by carbon dioxide 24 h after reperfusion. Blood samples were collected through PV and liver tissues were collected for histology analysis and KCs isolation.

3.2. Animal model

Orthotopic liver transplantation was performed according to Kamada's two cuff technique [10]. The liver grafts were preserved in 0–4 °C UW solution for 18 h [3,11]. The details of surgical procedures are described in our previous report [12]. Recipients were allowed to take only water 4 h post surgery.

3.3. Liver function and histology

Plasma alanine transaminase (ALT) and total bilirubin (TBIL) levels were measured by an autoanalyzer (Beckman CX7, USA). Paraffin-embedded liver tissue samples were prepared, sectioned at 5 μm, and stained with hematoxylin-eosin (HE). According to Suzuki's criteria, the sections were observed by microscopy (400 × magnification) and graded from 0 to 4 [13]. The grading criteria are as follows [13]: grade 0, none congestion, none vacuolization, none necrosis; grade 1, minimal congestion, minimal vacuolization, single-cell necrosis; grade 2, mild congestion, mild vacuolization, −30% necrosis; grade 3, moderate congestion, moderate vacuolization, −60% necrosis; grade 4, severe congestion, severe vacuolization, > 60% necrosis.

3.4. Inflammatory cytokines levels

Enzyme-linked immunosorbent assay (ELISA) kits (4A Biotech, China) were used for analyzing serum concentrations of TNF-α, IL-10 and IL-6.

3.5. Isolation of KCs

KCs were isolated according to previous study [14]. Briefly, the liver was perfused in situ with phosphate-buffered saline at a rate of 10 ml/min for 5 mins. The liver tissue was then minced and incubated in a water bath containing 30 ml of 1 mg/mL collagenase type IV (Sigma, USA) at 37 °C for 30 mins. Then, after removing undigested tissue fragments, KCs were obtained by discontinuous density gradient centrifugation. Isolated KCs were purified by removing non-adherent cells after incubation at 37 °C in 5% CO_2 for 2 h, and collected at a density of 1×10^6 cells/well.

3.6. Immunofluorescence

In brief, isolated KCs were fixed in 4% paraformaldehyde for 30 mins. After that, the cells were treated with 0.4% Triton X-100 for 45 mins and then treated with 3% H_2O_2 for 30 mins. After blocked with goat serum for 30 mins, the KCs were incubated with arginase 1 (Arg1) or nitric oxide synthase 2 (Nos2) antibody (rabbit, 1:100, santa cruz biotechnology) at 4 °C for 60 mins, and then with fluorescein isothiocyanate (DyLight488)–labeled goat anti-rabbit IgG (1:100) in darkness for 30 mins. The KCs were examined at HPF (800 × magnification) by laser scanning confocal microscope (LSCM, TCS NT 160195, Leica, Germany) and the fluorescence intensity was analyzed by image pro plus 6.0.

3.7. Real-time reverse transcription-polymerase chain reaction

The total RNA of KCs was extracted according to the kit (Biyotime biotech, China) and reverse transcribed into cDNA. The levels of VEGFR-3 and polarization-specific marker genes were detected by quantitative real-time PCR. The following primers were used: VEGFR-3 (forward: 5′-TGAAAGACGGCACACGAATG-3′, reverse: 5′-CCTCGCTTTAGGGTCTCCAG-3′), TNF-α (forward: 5′- CATACCAGGAGAAAGTCAGC -3′, reverse: 5′- CTAAGTACTTGGGCAGGTTG-3′), IL-6 (forward: 5′-GTTCTCTGGGAAATCGTGGA-3′, reverse: 5′- TGTACTCCAGGTAGCTA -3′), IL-10 (forward: 5′-TGGGTAGACAGCAGTGCCAC-3′, reverse: 5′-GCCCACAAGATGGACAGGG-3′). Mrc1 (forward: 5′- AGTCTGCCTTAA CCTGGCAC -3′, reverse: 5′- AGGCACATCACTTTCCGAGG -3′), Ct values were normalized to GAPDH (forward: 5′ -TCAACGGGGGACATAAA AGT-3′, reverse: 5′-TGCATTGTTTTACCAGTGTCAA-3′). The relative expression was calculated using the ΔΔCt-method.

3.8. Electrophoretic mobility shift assay

The electrophoretic mobility shift assay (EMSA) commercial kit (Thermo Fisher Scientific, USA.) assay was used for analyzing NF-κB transcriptional activity. Nuclear protein of KCs was extracted by commercial assay kit (Bio-Rad, USA). The NF-κB oligonucleotide probe (5'-AGTTGAGGGGACTTTCCCAGGC-3′, 3'-TCAACTCCCCTGAAAGGGT CCG-5′) labeled with biotin terminal was purchased from (Biyotime biotech, China). For super-shift assays, nuclear extracts were incubated with anti-P65 antibody (Santa Cruz Biotechnology, USA) at room temperature for 20 mins and then incubated with the NF-κB probe. The samples were electrophoresed on a 4% polyacrylamide gel. All steps followed the manufacturer's instructions.

3.9. Western blot analysis

Protein was extracted from KCs (1×10^6) by RIPA lysis buffer (Beyotime, China) and quantified by the BCA protein quantitative kit (Beyotime, China). The protein samples were electrophoresed in 8% sodium dodecyl sulfate polyacrylamide gel and transferred to polyvinylidene fluoride membranes. After blocked with skim milk powder at 4 °C for 1 h, the membranes were incubated with anti- phosphorylation of glycogen synthase kinase 3β at serine 9 (p-GSK3β (Ser9)) or anti- suppressor of cytokine signaling 1(SOCS1) (rabbit, 1:100, Cell Signaling Technology, USA) at 4 °C overnight. Then the membranes were reacted with horseradish peroxidase -conjugated secondary antibody at 37 °C for 1 h and stained with chemiluminescence detection buffer.

3.10. Data analysis

The data are presented as mean ± SD. ANOVA was used to compare multiple groups and Student's *t*-test was used for two groups. *P*

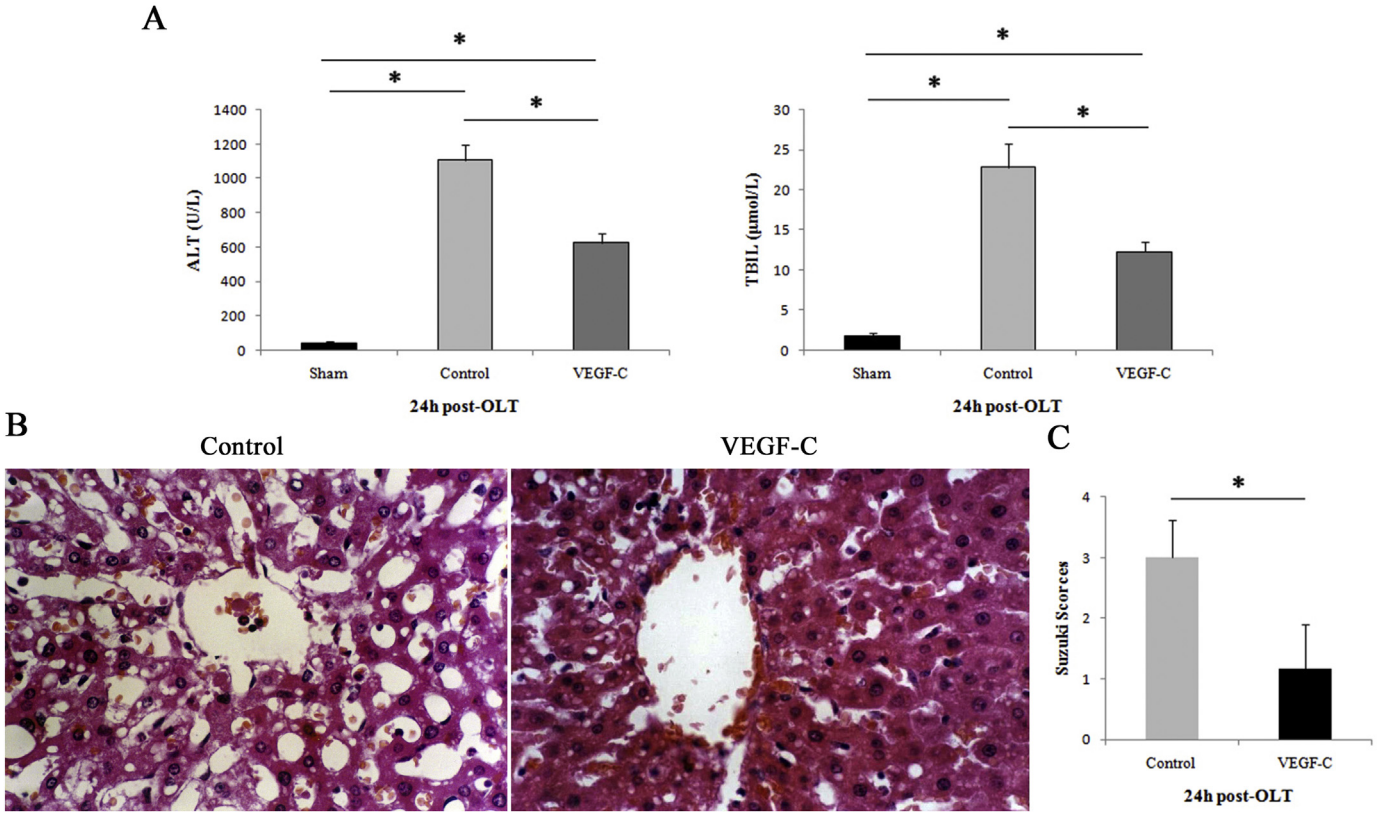

Fig. 1. Liver function and histopathological analysis at 24 h after liver transplantation. A, serum ALT and TBIL levels. (*$P < .05$, $n = 6$). B, HE staining (×400). The control group exhibited mild hepatic sinusoid congestion and vacuolization and the VEGF-C group revealed minimal hepatic sinusoid congestion and vacuolization and single-cell necrosis. C, Qualification of Suzuki's histological score. (*$p < .05$, n = 6).

values < .05 were considered statistically significant.

4. Results

4.1. VEGF-C attenuates IRI of liver grafts

Firstly, we observed the role of exogenous VEGF-C on transplanted liver IRI. Compared with the sham-operated group, serum ALT and TBIL levels increased obviously 24 h after surgery in the control group and VEGF-C group (Fig. 1A). Compared with the control group, VEGF-C treatment significantly decreased postoperative ALT and TBIL levels (Fig. 1A). Through histologic analysis, the control group exhibited mild hepatic sinusoid congestion and vacuolization and the VEGF-C group revealed minimal hepatic sinusoid congestion and vacuolization and single-cell necrosis (Fig. 1B). Compared with the control group, the suzuki score was lower in VEGF-C group (Fig. 1C).

4.2. VEGF-C modulates KCs polarization of grafts

Previous study suggests that VEGF-C not only restrains TLR4-NF-κB pathway, but also increases anti-inflammatory cytokine production in vitro [8]. Therefore, we isolated KCs and examined the polarization-specific marker genes in vivo. Compared with the sham-operated group, we found that the mRNA levels of VEGFR-3 in KCs were increased in the control group and VEGF-C group (Fig. 2A). However, there was no significant deference between the control group and VEGF-C group (Fig. 2A). The expression of M2-specific marker genes, including IL-10 and Mrc1, were significantly increased in VEGF-C group while the M1-specific marker genes, including IL-6 and TNF-α, were obviously decreased (Fig. 2B). In addition, immunofluorescence showed that the expression of Arg 1 (marker of M2) was higher in VEGF-C group and that of Nos2 (marker of M1) was lower (Fig. 2C).

4.3. VEGF-C suppresses NF-κB activity and up-regulates SOCS1 and p-GSK3β

Reportedly, up-regulation of VEGFR-3-VEGF-C signaling suppressed TLR4-NF-κB pathway by enhancing SOCS1 [8]. Compared with the control group, the NF-κB transcriptional activity of KCs in VEGF-C group was found obviously inhibited (Fig. 3A) and the expression of SOCS1 was increased (Fig. 3B). The phosphorylated GSK3β at serine 9 (p-GSK3β (Ser9)) can reduce GSK3β activity, and inhibition of GSK3β is reported to augmenting IL-10 production [15]. We found that the expression of p-GSK3β (Ser9) was higher in VEGF-C group than that in the control group (Fig. 3B).

4.4. VEGF-C reduces proinflammatory cytokines and increases IL-10

Finally, we analyzed the inflammatory cytokines in serum. The results showed that, compared with the control group, VEGF-C perfusion reduced the production of proinflammatory cytokines, including IL-6 and TNF-α, but increased IL-10 level (Fig. 4).

5. Discussion

VEGFR-3/VEGF-C signaling plays a critical role in lymphangiogenesis and angiogenesis [16,17]. Recent research finds that VEGFR-3/VEGF-C signaling negatively regulates TLR4-triggered inflammation of macrophages in vitro [8]. We found that VEGF-C could orchestrate KCs activation post liver transplant. It is also the first report that VEGF-C plays a key role in improving hepatic IRI after liver transplantation. Perfusion of exogenous VEGF-C through PV significantly reduced post-transplant ALT and TBIL levels, and protected pathological morphology 24 h after surgery.

As the largest number of macrophages in the body, KCs are the main

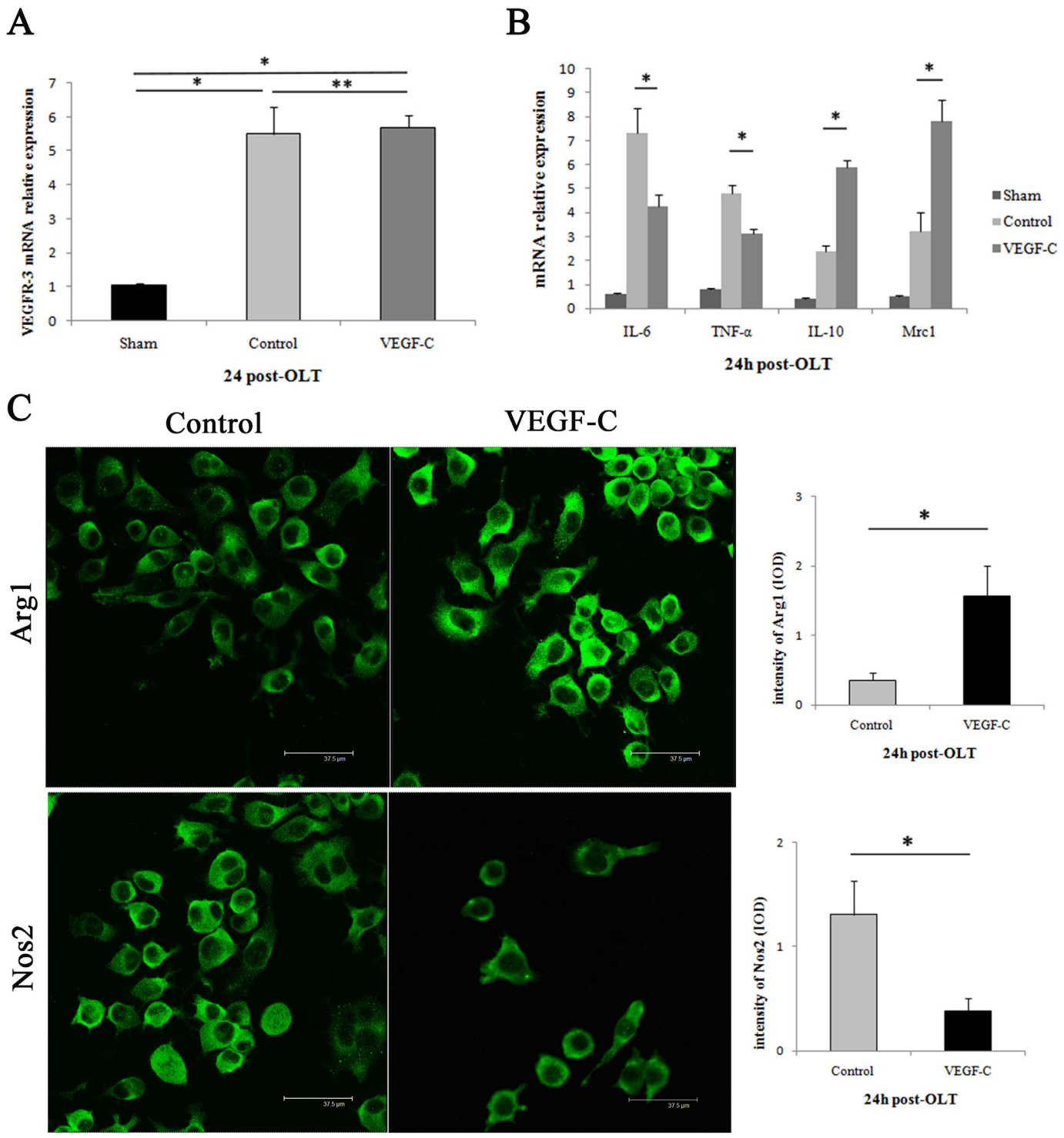

Fig. 2. VEGF-C modulates KCs polarization in vivo after liver transplantation. A, VEGFR-3 mRNA levels of KCs. (*P < .05, **P > .05, n = 6). B, Real-time PCR for the mRNA of polarization-specific marker genes in KCs (* P < .05, n = 6). C, Immunofluorescence (×800). The protein expression was semi-quantified as the fluorescence intensity per HPF (* P < .05, n = 6).

source of pro-inflammatory cytokines in the liver [18,19]. KCs activation triggered by ischemia promotes the production of pro-inflammatory cytokines and reactive oxygen species, and plays an important role in initiation of hepatic IRI [3]. Previous studies find that, when KCs are activated by the TLR4-NF-κB pathway, the production of pro-inflammatory cytokines (such as TNF-α and IL-6) are increased and the IRI of the liver graft is aggravated [5,6]. However, KCs can also release anti-inflammatory cytokine IL-10 and attenuate hepatic IRI [20,21]. This difference is related to different phenotype of KCs which named classically (M1) and alter-natively (M2) activated macrophages [7]. The M1 macrophages induced by lipopolysaccharide (LPS) and interferon-γ (IFN γ) elicit rapid proinflammatory responses. In contrast, the M2 macrophages induced by interleukin 4 (IL-4) and interleukin 13 (IL-13) show anti-inflammatory activities. In the present study, we found that the levels of VEGFR-3 mRNA in KCs were up-regulated post-transplant. In addition, exogenous VEGF-C up-regulated the expression

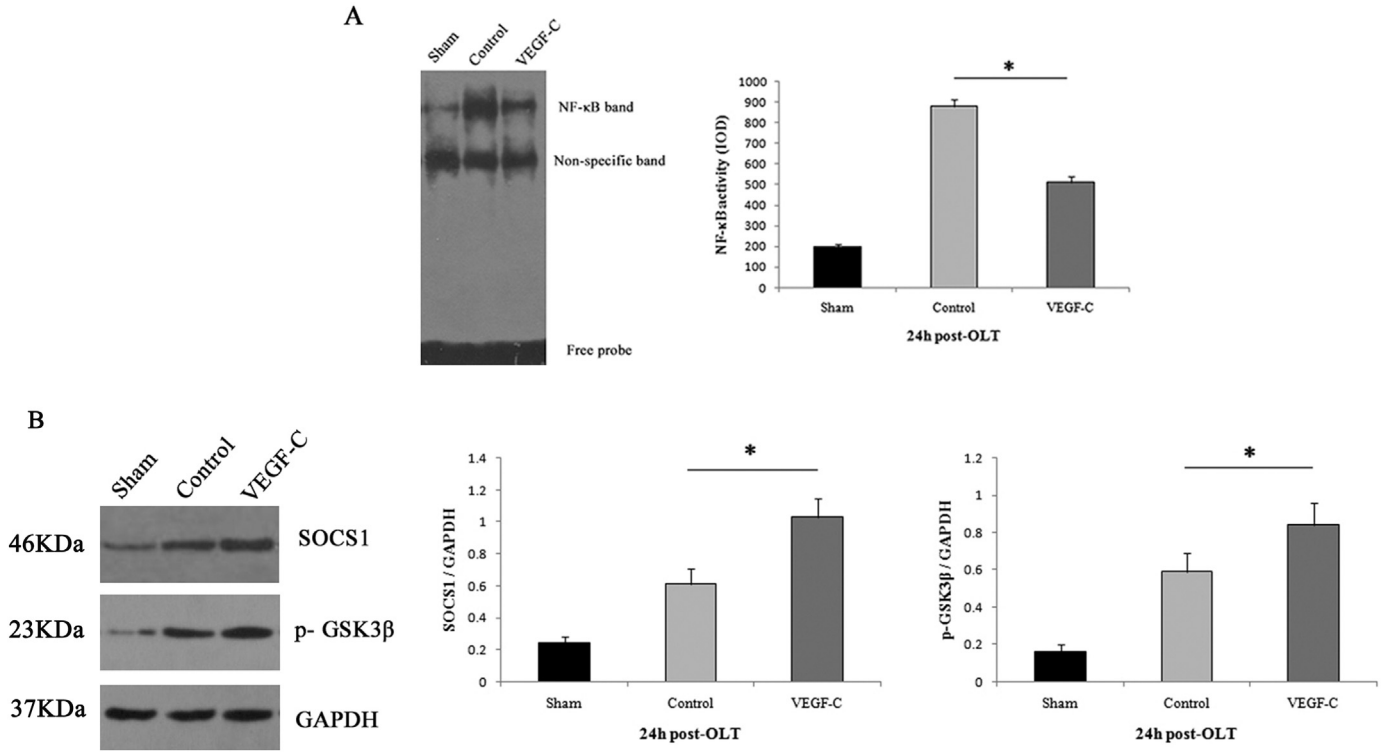

Fig. 3. Effect of VEGF-C on NF-κB activity and p-GSK3β/SOCS1 expression. A, EMSA for NF-κB transcriptional activity of KCs (* P < .05, n = 3). B, Western blot for the expression of SOCS1 and p-GSK3β (Ser9) in KCs (* P < .05, n = 3).

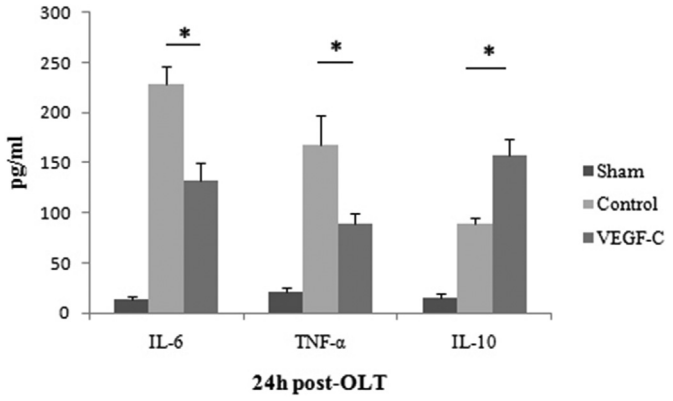

Fig. 4. VEGF-C reduces pro-inflammatory cytokines and increases IL-10. ELISA for inflammatory cytokines in serum. (* P < .05, n = 6.)

of M2-specific marker genes in KCs and reduced the M1-specific marker genes. These results suggest that VEGFR-3/VEGF-C signaling plays a key role in shifting KCs from M1 to M2 during liver transplantation. Furthermore, VEGF-C treatment reduced serum proinflammatory cytokines and increased IL-10 concentration. The effect of VEGF-C on other types of cells during liver transplantation, such as hepatocyte, needs further research.

The SOCS1 is reported to negative regulate TLR4- NF-κB pathway in macrophages [22]. Inhibition of GSK3β by p-GSK3β (serine 9) is documented to increase the production of IL-10 and ameliorate hepatic IRI [23]. In the present study, we found that VEGF-C up-regulated the expression of SOCS1 and p-GSK3β, and suppressed the NF-κB activity. Recent findings suggest that up-regulation of SOCS1 and p-GSK3β is related to activation of the phosphatidylinositol-3 kinase (PI3K)-Akt1 signaling [8,23]. The exact mechanism needs further research.

6. Conclusions

In conclusion, we demonstrated that exogenous VEGF-C protects liver graft from IRI by regulating the inflammatory response and modifying polarization of KCs from M1 to M2. The mechanism is related to up-regulated expression of SOCS1 and p-GSK3β, and suppressed NF-κB activity. This research indicates that VEGFR-3/VEGF-C signaling may be a novel therapeutic target for preventing hepatic IRI.

Conflicts of interest

The authors declare no conflicts of interest.

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