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RESEARCH ARTICLE

# IL-11 Attenuates Liver Ischemia/Reperfusion Injury (IRI) through STAT3 Signaling Pathway in Mice

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## Abstract

### Background

The protective role of IL-11, an IL-6 family cytokine, has been implicated in ischemia/reperfusion injury (IRI) in the heart and kidney, but its role has not been elucidated in liver IRI. This study was designed to evaluate the effects of IL-11 and its mechanism of action on liver IRI in a mouse model.

### Methods

A partial (70%) warm liver IRI was induced by interrupting the artery/portal vein blood supply to the left/middle liver lobes. IL-11 mRNA expression of ischemic liver after reperfusion was analyzed. Signal transducer and activator of transcription 3 (STAT3) was analyzed following IL-11 treatment in vivo and in vitro. Next, IL-11 was injected intraperitoneally (ip) 1 hour before ischemia. Liver injury was assessed based on serum alanine aminotransferase levels and histopathology. Apoptosis and inflammation were also determined in the ischemic liver. To analyze the role of STAT3 in IL-11 treatment, STAT3 siRNA or non-specific (NS) siRNA was used in vitro and in vivo.

### Results

IL-11 mRNA expression was significantly increased after reperfusion in the ischemic liver. STAT3, as a target of IL-11, was activated in hepatocytes after IL-11 treatment in vivo and in vitro. Next, effects of IL-11/STAT3 signaling pathway were assessed in liver IRI, which showed IL-11 treatment significantly attenuated liver IRI, as evidenced by reduced hepatocellular function and hepatocellular necrosis/apoptosis. In addition, IL-11 treatment significantly inhibited the gene expressions of pro-inflammatory cytokines (TNF-α and IL-10) and chemokines (IP-10 and MCP-1). To determine the role of STAT3 in the hepatoprotective

effects of IL-11, STAT3 siRNA or NS siRNA was used prior to IL-11 treatment. The results showed STAT3 knockdown abrogated the protective effects of IL-11 in vitro and in vivo.

## Conclusions

This work provides first-time evidence for the protective effect of IL-11 treatment on hepatocyte in liver IRI, through the activation of the STAT3 pathway.

## Introduction

Ischemia-reperfusion injury (IRI) is a key contributing factor in liver dysfunction and failure after hepatic trauma, resection, liver transplantation, and circulatory shock [1–4]. An effective method for preventing or minimizing liver IRI is urgently needed in liver surgery. The factors/pathways have been involved in the hepatic IRI process include anaerobic metabolism, mitochondria damage, oxidative stress, endoplasmic reticulum stress, intracellular calcium overload, Kupffer cell (KC) activation, neutrophil infiltrations, and production of cytokines and chemokines [1–3]. The adverse factors mentioned above finally lead to cell death/apoptosis, which indicates that cell death/apoptosis is a significant and perhaps principal contributor to liver IRI [5–7]. Thus, understanding the sequence of events central to the cell death/apoptosis mechanism may potentially lead to treatments for liver IRI.

IL-11 is a hematopoietic IL-6 family cytokine with multifunctional effects. Indeed, IL-11 has thrombopoietic activity, and recombinant human IL-11 has been used for thrombocytopenia in clinical settings [8]. Different from other IL-6 family cytokines, IL-11 holds anti-inflammatory function against chronic inflammatory diseases, lipopolysaccharide-induced sepsis, etc [9–11]. Kimura's group reported that IL-11 played a cardioprotective role, and conferred resistance to heart IRI in a mouse model by enabling significant anti-necrotic/apoptotic effects [12]. In addition, IL-11 pretreatment also reduces IR-induced cell death/apoptosis by up-regulating Bcl-2 [13]. More importantly, IL-11 shares some similar effects to other IL-6 family members. It has been reported that IL-11 binding with gp130 receptor induces activation of STAT3, which is involved in many physiological and pathological processes [14]. Inhibitors of STAT3 phosphorylation or dominant-negative STAT3 mutants facilitate the expression of pro-apoptosis factors, suggesting that STAT3 plays a critical role in regulating cell proliferation and anti-apoptosis [15]. Furthermore, STAT3 knockout mice exhibit complete embryonic lethality [16]. Conditional ablation of STAT3 in myocardial cells leads to higher susceptibility to drug-induced heart failure [17]. To the best of our knowledge, there has been no report on IL-11 preconditioning before liver IRI. In the present study, we tested the hypothesis that exogenous IL-11 attenuates liver IRI by STAT3-mediated anti-necrotic/apoptotic effects.

## Materials and Methods

### Animals

Male C57BL/6 mice were purchased from the Laboratory Animal Resources Center of Nanjing Medical University (NMU). The animals were fed a laboratory diet with water and food and kept under constant environmental conditions with 12h light–dark cycles. Procedures were carried out in accordance with the Guidelines for Laboratory Animal Care. The animal protocol had been approved by the Institutional Animal Care & Use Committee (IACUC) of Nanjing Medical University (Protocol Number NMU08-092).

## Surgical procedure and IL-11 treatment

The present study used a well-established mouse model of partial (70%) warm hepatic IRI [18]. Anesthesia was induced by 10% chloral hydrate (0.3g/kg, intraperitoneally). Mice were injected with heparin (100U/kg), and an atraumatic clip was used to interrupt the artery/portal vein blood supply to the left/middle liver lobes. After 90 minutes of ischemia, the clip was removed, and the mice were sacrificed (cervical dislocation) at required times after reperfusion. Some mice received a single injection of recombinant human IL-IL-11 (500μg/kg, ip) (PeproTech, Rocky Hill, NJ) or medium (PBS) 1 hour prior to ischemia. PBS injection was used as a control. Sham-operated controls underwent the same procedure but without vascular occlusion. To access effects of STAT3 on IL-11 treatment, STAT3 siRNA or NS siRNA (2mg/kg) was given intravenously 4 hours prior to ischemia [19]. Reports have previously documented the efficacy of this siRNA approach in the liver, with>40% of intravenously infused siRNA accumulating in the ischemic mouse livers [20].

## Serum biochemical examination

Blood samples collected 6h after reperfusion was centrifuged to obtain serum. The serum level of alanine aminotransferase (sALT) or supernatant level of lactate dehydrogenase (LDH) was measured to assess the extent of hepatocyte damage using an automated chemical analyzer (Olympus Automated Chemistry Analyzer AU5400, Tokyo, Japan).

## Histopathologic study

Liver specimens were fixed with 10% neutral formaldehyde and then embedded in paraffin. The specimens were sectioned at 4μm and stained with hematoxylin and eosin. The sections were used in histopathologic analysis by light microscopy. Sections were scored from 0 to 4 for sinusoidal congestion, vacuolization of hepatocyte cytoplasm, and parenchymal, as described by Suzuki et al [21].

## Caspase-3 activity assay

Caspase-3 activity was assayed in liver tissues 6h after reperfusion. Frozen samples of ischemic tissues were homogenized with a Polytron homogenizer and centrifuged at 16,000g for 20 minutes. Activity was measured with an assay kit (Calbiochem) according to the manufacturer's instructions.

## Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining

Paraffin sections (4μm in thickness) were deparaffinized in toluene and then dehydrated in a graded series of ethanol solutions. Sections were stained by TUNEL using a commercially available kit (in situ cell death detection kit, Roche-Boehringer Mannheim, Germany).

## Western blot analysis

Proteins were extracted from liver tissues subjected to ischemia or from cell lysates, and their concentrations were determined by the Bradford assay (Bio-Rad, CA). About 30 μg protein was resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Sunshine Biotechnology, China). These membranes were blocked in skim milk powder (5% wt/vol) with phosphate buffered saline containing 0.1% Tween 20 (PBS-T) at 4°C overnight. Membranes were then incubated with primary antibodies for cleaved Caspase-3, P-STAT3, Bcl-2, Bax, β-actin (Cell Signaling Technology, Danvers, MA), and

STAT3 (Santa Cruz Biotechnology, Santa Cruz, CA). Following three washes with PBS-T, the membranes were incubated for 1h at room temperature with peroxidase-conjugated secondary antibody (Cell Signaling Technology, Danvers, MA). The final results were obtained by exposure to autoradiographic film (Kodak XAR film), and then visualized via a chemiluminescent detection system (ECL Substrate Western blot detection system, Pierce, IL).

## Quantitative real-time PCR

Quantitative real-time PCR was performed using the DNA Engine with a Chromo 4 Detector (MJ Research, Waltham, MA). In a final reaction volume of 25μl, the following were added: 1×SuperMix (Platinum SYBR Green qPCR Kit; Invitrogen), cDNA, and 2.5μM of each primer. The amplification conditions were as follows: 50°C (2 min), 95°C (5 min), followed by 50 cycles at 95°C (15 sec) and 60°C (30 sec). The expression of the target genes (IL11, TNF-α, IL-6, IP-10 and MCP-1) (Invitrogen, Shanghai, China) was calculated based on the ratio of the gene of interest to the housekeeping gene HPRT. Primer sets (sense sequence and antisense sequence, respectively) for the following genes were: HPRT forward, 5'- TCA ACG GGG GAC ATA AAA GT-3', reverse, 5'- TGC ATT GTT TTA CCA GTG TCA A'; IL-11 forward: 5'- CTG CCC ACC TTG GCC ATG AG-3'; IL-11 reverse: 5'- CCA GGC GAG ACA TCA AGA AAG A-3'; TNF-α forward, 5'- GCC TCT TCT CAT TCC TGC TTG T-3', reverse, 5'- TTG AGA TCC ATG CCG TTG-3'; IL-6 forward, 5'- GCT ACC AAA CTG GAT ATA ATC AGG A-3', reverse, 5'- CCA GGT AGC TAT GGT ACT CCA GAA-3'; IP-10 forward. 5'-GCT GCC GTC ATT TTC TGC-3', reverse, 5'-TCT CAC TGG CCC GTC ATC-3'; MCP-1 forward, GGT GAT AAC CGC CCT AGC-3', reverse, 5'-TGT GTC GGC TGG ATA GGC-3'.

## Cell culture and treatment

Mouse hepatocytes were isolated using a two-step in situ collagenase perfusion procedure [18]. Livers from the C57BL/6 mice were perfused in situ through the portal vein with ethylene glycol tetraacetic acid (EGTA) buffer (0.5mM EGTA, 137mM NaCl, 4.7mM KCl, 1.2mM KH2PO4, 0.65mM MgSO4, and 10.07mM HEPES at pH 7.4) at a flow rate of 5ml/min for 10 min, followed by collagenase buffer (67mM NaCl, 6.7mM KCl, 4.76mM CaCl2, 0.035% collagenase type II, and 10.07mM HEPES at pH 7.6) at a flow rate of 5ml/min for 15 min. After centrifugation, the hepatocytes were collected and seeded in DMEM containing 10% FBS, 100units/ml penicillin, and 100μg/ml streptomycin. Cells were preincubated with IL-11 (1μg/ml for 1h), then $H_2O_2$ (200μm for 24h) to induce cell death.

## Knockdown of STAT3 expression using STAT3 siRNA transfection

Hepatocytes were grown and transiently transfected with STAT3 siRNA or NS siRNA using Transfection Reagent LipofectamineTM RNAiMAX (Invitrogen, CA, USA) according to the manufacturer's instructions. In brief, cells were seeded at 1 x $10^6$ per well in 1.5ml of OPTI-medium (Invitrogen, CA, USA) in a 6-well plate. After 20h, the cells were transfected with 20nmol/ml STAT3 siRNA or NS siRNA. About 6h after transfection, the medium was changed to a regular medium, and the cells were treated as described above after 24h.

## Statistical analysis

The data are presented as the mean ± SEM from at least three independent experiments. One-way analysis of variance test [—] was used in comparisons of three groups. Student's t-test [Π] was used for comparison of two groups. All P values were two-sided, and $P<0.05$ was considered to be statistically significant.

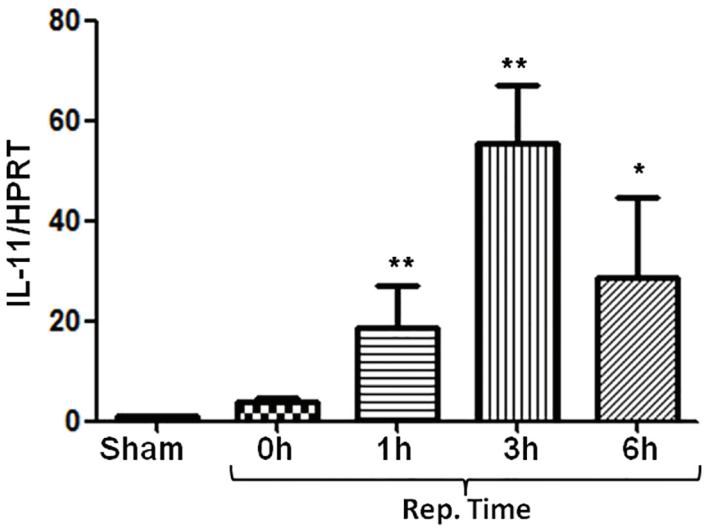


**Fig 1. IL-11 expression was increased after IR.** Mice were subjected to 90min of partial liver ischemia, followed by 0h, 1h, 3h and 6h reperfusion. Kinetics of IL-11 gene expression was analyzed in ischemic liver by RT-PCR. Expression of IL-11 was normalized with that of HPRT. Data are expressed as mean±SD (n = 6/group). *P<0.05, **P<0.001 vs sham group.

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## Results

### IL-11 is elevated in IR-stressed liver

To determine the effects of IL-11 on liver IRI, we first analyzed the gene expression of IL-11 in ischemic livers after various reperfusion time points. As shown in Fig 1, the expression of IL-11 was increased at 0h post reperfusion and reached its peak 3h post reperfusion. The data indicate that IL-11 was present in the liver 3h after reperfusion.

### IL-11 activates STAT3 in liver and hepatocytes

IL-11, an IL-6 family cytokine, was supposed to activate STAT3. Here we determined whether administration of IL-11 stimulates STAT3 in liver by western blotting with antibody (Fig 2A). Phosphorylation of STAT3 was rapidly induced and recovered nearly to baseline after 2 hours. To further ascertain that phosphorylation of STAT3 occurred in hepatocytes, we analyzed the effects of IL-11 on STAT3 activity of hepatocytes in vitro. Fig 2B shows phosphorylation of STAT3 was also significantly elevated after IL-11 treatment, indicating that IL-11 administration may activate STAT3 within parenchymal cells in the liver.

### IL-11 attenuates liver IRI

Next, we analyzed effects of IL-11 administration in liver IRI. Mouse livers were subjected to 90 min of warm ischemia 6h after reperfusion. sALT levels in each group were analyzed (Fig 3A). sALT levels were markedly increased in the IR group compared with that of the sham group (33.33±5.49 and 10610.00±1393.00, respectively; $P<0.01$). By contrast, when mice were pretreated with IL-11, sALT levels (3832.00±834.90, $P<0.01$) were significantly decreased compared with those in the IR control. Liver serum enzyme data were in line with liver pathological analysis (Fig 3B and 3C). The histological parameters in the sham (0.25±0.25), IR (3.20±0.37), and IL-11 administration (1.8±0.37) groups were observed according to Suzuki et al [26]. These data indicate that IL-11 treatment significantly attenuates IR-induced liver injury.

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