Am J Transl Res 2021;13(3):1155-1169
www.ajtr.org /ISSN:1943-8141/AJTR0126567

Original Article

Irg1-itaconate axis protects against acute kidney injury via activation of Nrf2

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Received November 18, 2020; Accepted December 29, 2020; Epub March 15, 2021; Published March 30, 2021

Abstract: Acute kidney injury (AKI) is a common clinical implication with increased tissue damage, uncontrolled immune responses, and risk of mortality, in which ischemia-reperfusion injury (IRI) is one of the leading causes. As critical role for metabolic remodeling in inflammation, Irg1-itaconate axis has received much attention for its immunomodulation in the control of the inflammation. However, its role in the AKI and IRI remains unknown. Here, we found that Irg1 expression was negatively correlated with the expression of inflammatory cytokines during ischemia-reperfusion injury. And Irg1 deficiency promotes renal inflammation and ischemia-reperfusion injury *in vivo*. Itaconate treatment promoted the survival of WT mice from lethal ischemia and protected against renal IRI and systemic inflammation. Mechanistically, dimethyl itaconate protected renal cells from oxidative stress and prevented macrophage activation by enhancing the translocation of Nrf2 into the nuclei. Our study highlighted the importance of the Irg1-itaconate axis in the protecting against ischemia-reperfusion injury and acute kidney injury, providing potential therapeutic targets to control AKI.

Keywords: Acute kidney injury, ischemia-reperfusion injury, Irg1, itaconate, Nrf2, inflammation

Introduction

Acute kidney injury (AKI) is a globally common and severe clinical implications, leading to increased clinical costs, development of chronic kidney disease, and risk of mortality [1, 2]. AKI was directly characterized by tubular injury and vascular damage, as well as un-controlled inflammatory responses. However, current therapies for AKI are still unsatisfactory in the clinic. Ischemia-reperfusion injury (IRI) is a common cause of AKI [3, 4]. More and more evidences indicate that IRI is an acute inflammatory disease, which involves in multiple immune cell infiltration and cytokine secretion, and cell apoptosis in the kidneys [5]. Suppression of inflammation can inhibit acute injury and facilitate recovery after renal IRI [6].

Growing studies have shown that profound metabolic remodeling in host immune and non-immune cells after exposure to endogenous or exogenous stimuli is critical for the process of inflammation [7]. Among them, the tricarboxylic acid (TCA) cycle exerts essential roles during the trigger and final resolution of the inflammatory responses and tissue damages [8, 9]. As a product of decarboxylation of cis-aconitate mediated by immune-responsive gene 1 (Irg1) in the TCA cycle, itaconate has received much attention for its immunomodulation in the control of the inflammation and induction of immunological tolerance [10, 11]. With the stimulation of macrophages by lipopolysaccharide, type I interferons promoted the expression of Irg1 and itaconate production, which in turn limited the interferon response, indicating a crucial anti-inflammatory role of Irg1 and itaconate via activation of transcription factor Nrf2 [12]. In a mouse model of fungal keratitis, dimethyl itaconate was proved to limit fungal keratitis by the activation of Nrf2/HO-1 pathway [13]. Mechanistically, itaconate can directly alkylate cysteine residues in KEAP1, promoting Nrf2 nuclear transportation in macrophages during endotoxemia [12]. These novel findings provide

sufficient evidences for Irg1 and itaconate in the control of inflammatory responses, which suggests their role in the tissue damage and treatment of inflammatory diseases. However, whether Irg1-Itaconate Axis involved in the AKI and IRI remains unknown.

In this study, we found that Irg1 expression was negatively correlated with the expression of inflammatory cytokines during ischemia-reperfusion injury. And Irg1 deficiency promotes renal inflammation and ischemia-reperfusion injury *in vivo*. Itaconate treatment promoted the survival of WT mice from lethal ischemia and protected against renal IRI and systemic inflammation. Mechanistically, dimethyl itaconate protected renal cells from oxidative stress and prevented macrophage activation by enhancing the translocation of Nrf2 into the nuclei. These data demonstrate the importance of the Irg1-itaconate axis in the protecting against ischemia-reperfusion injury and acute kidney injury.

Methods

Mice and IRI models

C57BL/6J (B6) mice (male 8 to 10-week-old) were purchased from Cavens lab (Changzhou, China). Irg1 deficient mice were gifted by Dr. Shulei Yin from Navy Medical University. Renal IRI was performed by transiently clamping the bilateral renal pedicles for 20 minutes (unlethal model) or for 40 minutes (lethal model) as previous reported [14]. Mice were housed in specific pathogen-free conditions in Navy Medical University. Before operation, mice were anesthetized by gas anesthesia (2% isoflurane). After operation, mice received gentamicin sulfate (10 mg/kg) for 7 days to prevent postoperative infections. All animal experiments were approved by the Scientific Investigation Board of Navy Medical University (Shanghai, China).

Bone marrow and kidney transplantation

Bone marrow cells harvested from wild-type or $Irg1^{-/-}$ mice were used for bone marrow transplantation. Recipient mice were lethally irradiated with a single dose of 11 Gy. Then 2×10^6 donor BMs were administered to each recipient by tail vein injection within 24 hours. IRI modeling was performed 8 weeks after BM transplantation.

For kidney transplantation, surgery was performed as described [15]. In brief, 8-week-old mice were anesthetized with isoflurane and the left donor kidney was attached to a cuff of the aorta and the renal vein with a small vena cava cuff, and the ureter was removed. Donor kidneys saved in 4°C Ringer-lactate solution. After left nephrectomy of the recipients, the vascular cuffs were anastomosed to the recipient abdominal aorta and vena cava. The ureter was directly anastomosed to the bladder. Normally, the grafts would undergo 25~30 min warm ischemia and about 1-hour cold ischemia.

Cell culture and hypoxia-reoxygenation (H/R) treatment

Mouse RAW264.7 macrophage cells were grown in RPMI-1640 medium (BasalMedia, L220KJ) supplemented with 10% fetal bovine serum (Gibco, 10099-141). Murine renal cells were obtained from suckling mice. Briefly, kidneys were obtained from one-day-old suckling mice and digested by 100 μg/ml Liberase TL (Roche, 05401020001) for 20~30 min. Subsequently, the cells were purified by differential velocity adherent and cultured in Dulbecco's modified Eagle's medium/F-12 medium (Gibco, 319-075-CL) supplemented with 10% fetal bovine serum (Gibco, 10099-141), penicillin (100 U/mL), and streptomycin (100 μg/mL), and maintained at 37°C in 5% CO_2 in a humidified incubator. For hypoxia treatment, cells were incubated for 2 hrs in a hypoxia chamber (Coy Laboratory Products, Ann Arbor, MI, USA) to maintain oxygen concentration at 1% (94% N_2 and 5% CO_2). After the hypoxia treatment, the cells were reoxygenated by returning them to normoxic conditions for 6 hrs. The control cells were incubated under a normoxic condition. For siRNA transfection, RAW264.7 or renal cells were transfected with double-stranded siRNA (**Table 2**) or negative control (NC) using RFect transfection reagent (Biodai, 11015).

Measurement of intracellular ROS and renal hydroxyproline content

Intracellular ROS generation was measured using a dichlorofluorescein diacetate (DCF-DA)-based flow cytometric assay. Renal cells were incubated for 20 minutes with a DCF-DA (5 mm; Thermo Fisher Scientific), followed by stimulation with phorbol 12-myristate 13-acetate (50

Table 1. Primers for RT-qPCR

Genes	Forward primers (5'-3')	Reverse primers (5'-3')
Irg1	TGCTGCTGCGTCCAAGTTT	GGGGCTTAGTCTGAGTGGC
Nlrp3	TCTTGGAGTAAGTCGAGAAGTGT	GTTGAAACTGAGCGAAAAAGGC
Hmox1	AAGCCGAGAATGCTGAGTTCA	GCCGTGTAGATATGGTACAAGGA
Gapdh	GGCTGTATTCCCCTCCATCG	CCAGTTGGTAACAATGCCATGT

Table 2. siRNA sequence

Gene	siRNA Name	Target Sequence
Irf1	siIrf1-1#	GGCTAGAGATGCAGATTAATTCC
	siIrf1-2*	GACCTTATGAAGCTCTTTGAACA
	siIrf1-3	GGCTGTGATACAAAAAAAGCTAG
Cebpβ	siCebpb-1*,#	CCCTGAGTAATCACTTAAAGATG
	siCebpb-2	GGGGTTGTTGATGTTTTTGGTTT
	siCebpb-3	GAGAAAAGAGGCGTATGTATATT

*Sequences used in function experiments of RAW264.7 cells.
#Sequences used in function experiments of murine renal cells.

ng/ml; Sigma-Aldrich) for 2 hours and measurement of the intracellular dichlorofluorescein of cells by flow cytometry. The hydroxyproline content in the kidney was measured by the Hydroxyproline Colorimetric Assay Kit (BioVision Inc.) and expressed as hydroxyproline (micrograms) content per kidney weight (milligrams).

H&E

After treatment, kidney tissues were fixed with 4% paraformaldehyde overnight, and then embedded in paraffin wax. The Skiving Machine Slicer (Leica) diced 4-μm sections, which were stained with hematoxylin and eosin (H&E) according to the instruction. For ischemia-reperfusion injury evaluation, light microscope (Olympus) was used at ×20 magnification.

ELISA

Expression levels of TNF-α and IL-1β in serum and culture supernatant were measured using an ELISA kit (R&D Systems) according to the manufacturers' protocols. Serum creatinine and urea nitrogen levels were measured using a QuantiChrom BUN or Creatinine assay kit (BioAssay Systems).

Cell transfection

To knock down Nrf2 genes, the shRNA lentiviral transduction particles were transfected into cells with the FuGene 6 reagent (Promega). A lentiviral transduction particle containing the pLKO.1 empty vector was used as the control. The transfected cells were recovered in an antibiotic-free DMEM medium for 6 h, followed by selection using DMEM with 1 μg/mL puromycin medium. Then, the individual puromycin-resistant cells were collected and examined the knockdown efficiency of the target genes.

Bone marrow reconstitution

Bone marrow from long bones of $Irg^{-/-}$ mice and WT littermates was flushed under sterile conditions with phosphate buffered solution (PBS). Bone marrow cells were filtered, collected, and checked for viability using trypan blue. Recipient mice were given 11 Gy total-body irradiation (at 7-8 weeks of age) and all mice received bone marrow cells from either $Irg^{-/-}$ mice or WT littermates within 4 hours. The mice were used after 8 weeks of bone marrow transplantation.

RNA quantification

Total RNA from kidney tissues and cells was extracted with TRIzol reagent according to the manufacturer's instructions (Thermo). Reverse transcription was performed with the FSQ-201 ReverTra Ace qPCR RT Kit (Toyobo). Real-time PCR analysis was performed with diluted cDNA and SYBR Green Realtime PCR Master Mix (Toyobo) using a LightCycler1.5 PCR system (Roche). Primer sequences were followed in **Table 1**.

Western blot

Renal tissue or cells were lysed by M-PER Protein Extraction Reagent (Pierce) with a protease inhibitor cocktail (Sigma), and protein concentrations in the extracts were measured with BCA Protein Assay Kit (Pierce). Equal amounts of extracts were separated by SDS-PAGE and then transferred onto nitrocellulose membranes for immunoblot analysis. Lysates were stained with anti-Irg1, anti-Caspase3, anti-Nrf2, anti-a-SMA, anti-pP38, anti-P38, anti-pP65, anti-P65, anti-Erk, anti-pErk, anti-JNK, anti-pJNK, anti-β-actin, anti-Irf1 and anti-Cebpβ antibodies (Cell Signaling Technology) at a 1:1000 dilution.

Statistical analyses

The data was analyzed using Statistical Package for the Social Sciences (SPSS) software for Windows. For most study, unpaired Student's t-test was used for comparisons of the mean between two groups, and Kaplan-Meier test was for survival rate. Statistical significance was two-tailed and set at *P* value <0.05.

Results

Irg1 expression was negatively correlated with the expression of inflammatory cytokines during ischemia-reperfusion injury

To determine the expression pattern of Irg1 in IRI, we detected the Irg1 expression in PBMC and kidney tissues during the process of ischemia-reperfusion. Both mRNA and protein level of Irg1 was up-regulated in PBMC and kidney tissues at 24-36 h after ischemia-reperfusion (**Figure 1A** and **1B**). We also detected the inflammatory cytokine TNF-α and IL-1β in serum of mice. The peak of cytokines appeared at 6-12 h after ischemia-reperfusion, and was degraded at 24-36 h (**Figure 1C**).

Next, the underlying mechanism for inducible expression of Irg1 in renal cell and PBMC was investigated. We first analyzed Irg1 expression in RAW264.7 and murine renal cells under various inflammatory conditions induced either by kidney lysis (200 ng protein/mL), H_2O_2 (100 μM) or mTNF-α (50 ng/mL) exposures. After stimulation, the expression levels of Irg1 were highly induced following the cell lysis or TNFα treatment in RAW264.7, while the highest Irg1 levels obtained after H_2O_2 exposure in renal cells (**Figure 2A**).

To identify the potential transcriptional factors of Irg1, we analyzed transcriptional regulatory atlas of murine Irg1 in SPP database (https://www.signalingpathways.org/) [16], top 5 TFs were showed in **Figure 2B**. Aravind Tallam *et al* also identified Irf1, Cebpβ, Cebpδ, PRDM1 and STAT1 as top 5 scoring transcriptional factors in mouse through computer-aided bioinformatics algorithms [17]. Based on the above results, we considered Irf1, Cebpβ and p65 as the candidate TFs regulating Irg1. The siRNAs and small molecule inhibitors is widely used in "loss-of-function" study. Thus we designed siRNA for knocking-down Irf1 and Cebpβ and use PDTC (pyrrolidine dithiocarbamate) for blocking p65. The interference efficiency of the siRNA was detected first to confirm the feasibility (**Figure 2C**). Results indicated knocking-down of both Irf1 and Cebpβ resulted in decrease of the Irg1 mRNA levels when compared to the NC in H/R-treated RAW264.7 cells (**Figure 2D**). Correspondingly, Irg1 expression was decreased in Cebpβ silenced renal cells as well as p65 blocking renal cells (**Figure 2D**). The above results suggested that Irg1 is ROS-induced molecule via p65/Cebpβ pathway and it may be related to the regression of inflammation.

Irg1 deficiency promotes renal inflammation and injury

Next, we used Irg1 deficient mice to further determine the role of Irg1 in IRI. The survival of mice after IRI in Irg1 deficient mice was significantly decreased (**Figure 3A**). Consistently, the results of H&E showed that the kidney injury was aggravated after irg1 deficiency (**Figure 3B**). Also, the clearance rate of creatinine and urea nitrogen decreased and increased significantly in serum (**Figure 3C**), suggesting the role of Irg1 in protecting against IRI. Then we detected the serum cytokines, which showed that the cytokine TNF-α and IL-1β were not degraded at 24-36 h after Irg1 deficiency (**Figure 3D**). Collectively, Irg1 deficiency promotes renal inflammation and ischemia-reperfusion injury *in vivo*.

Both Irg1 in renal and bone marrow-derived cells protects against ischemia-reperfusion injury

Then, we wanted to know whether Irg1 in bone marrow cells or renal cells were responsible for the protective effect. Bone marrow reconstitution showed that both $Irg1^{-/-}$ mice receiving bone marrow of WT littermates or WT littermates receiving bone marrow of $Irg1^{-/-}$ mice exhibited lower survival time (**Figure 4A**). H&E showed that the kidney injury was aggravated in those two groups (**Figure 4B**). Consistently, serum creatinine and urea nitrogen increased significantly (**Figure 4C**), and serum cytokine TNF-α and IL-1β were not degraded at 24-36 h in those two groups (**Figure 4D**). These data suggest that Irg1 present on host renal cells, and also on BMDCs, is critical for renal ischemia-reperfusion injury and systemic inflammation.

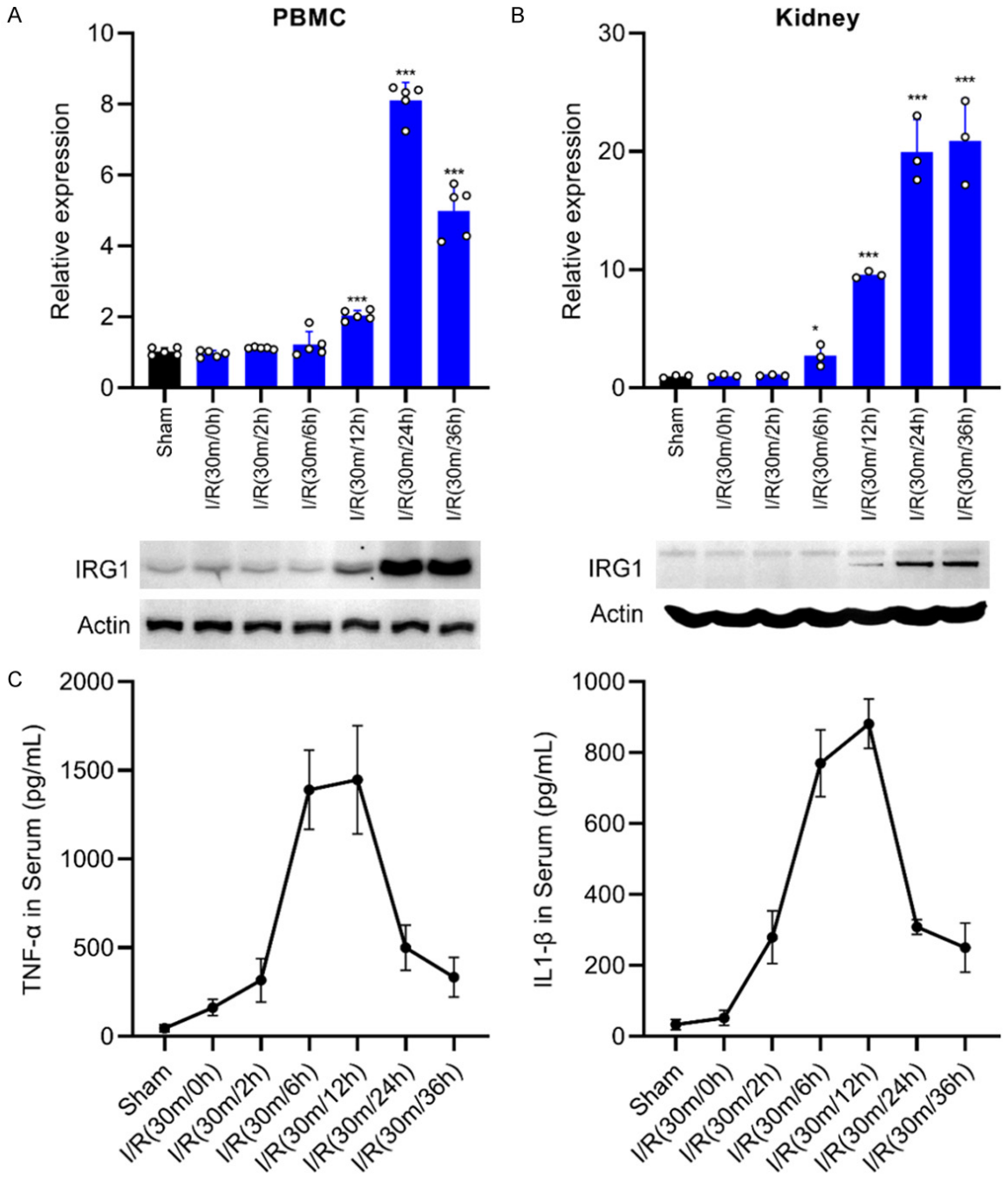
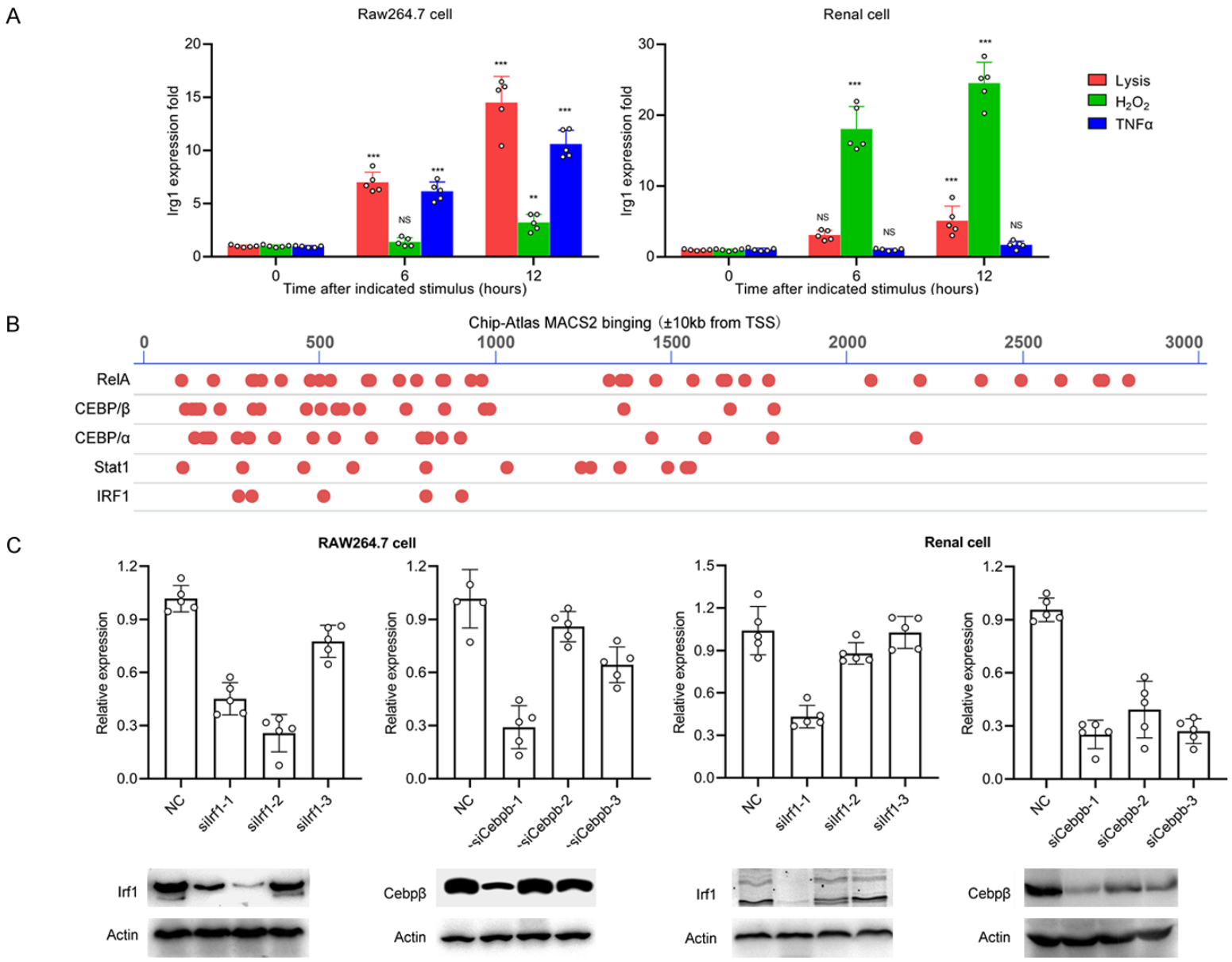


Figure 1. IRG1 expression was negatively correlated with the expression of inflammatory cytokines during IRI. (A, B) Quantitative PCR (upper) and immunoblot analysis (below) of IRG1 expression in the PBMC (A) and kidney (B) after ischemia for 30 min and then reperfusion for 0-36 h. The data were normalized to the sham group as shown in the first column. β-actin was assayed as a control. (C) Quantification of TNF-α and IL-1β in serum after ischemia for 30 min and then reperfusion for 0-36 h. Data are mean ± s.d. of one representative experiment. Similar results were seen in five independent experiments. Unpaired Student's t-tests, *P<0.05, ***P<0.001.

Irg1 protects against renal ischemia-reperfusion injury and systemic inflammation through itaconate

Previous studies have shown that Irg1 is an enzyme that converts cis-aconitate to the metabolite itaconate, which plays important roles in inflammation and tissue damage [18]. We therefore speculated that the effect of Irg1 in IRI may be mediated by downstream product itaconate. As dimethyl itaconate (DI) has been shown to cross the plasma membrane and


A
Raw264.7 cell
Renal cell
Irg1 expression fold
Time after indicated stimulus (hours)
Lysis
H2O2
TNFα
B
Chip-Atlas MACS2 binging (±10kb from TSS)
RelA
CEBP/β
CEBP/α
Stat1
IRF1
C
RAW264.7 cell
Renal cell
Relative expression
NC
siIrf1-1
siIrf1-2
siIrf1-3
siCebpb-1
siCebpb-2
siCebpb-3
Irf1
Cebpβ
Actin

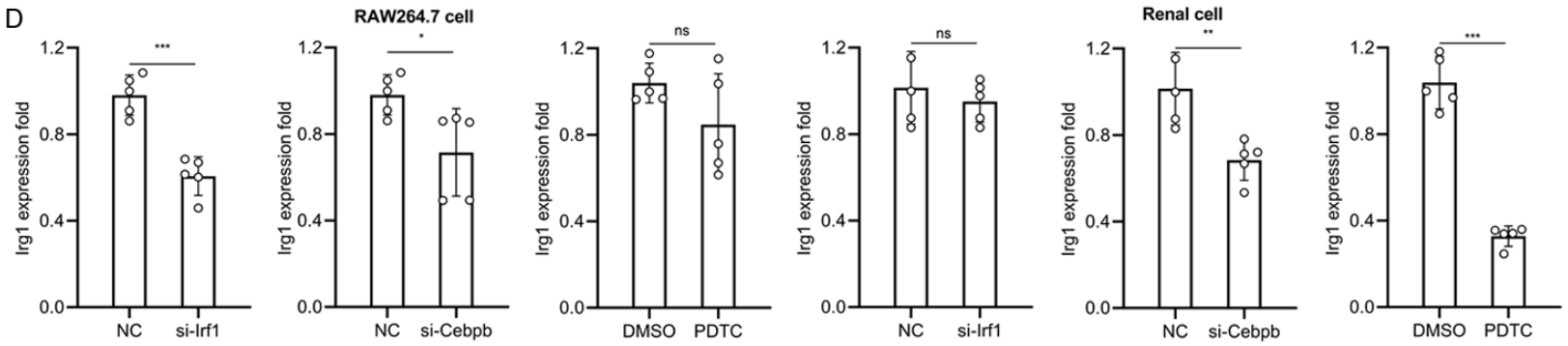


Figure 2. Irg1 is a ROS-induced molecule via p65/Cebpβ pathway. A. RAW264.7 and murine renal cells were stimulated by various inflammatory conditions including kidney lysis (200 ng protein/mL), H_2O_2 (100 μM) or mTNF-α (50 ng/mL). After stimulation, quantification of the expression levels of Irg1 were measured. B. Mining of TFs (top 5) binding to Irg1-promter in the SPP knowledgebase. C. Raw264.7 cells and renal cells were transfected with siRNA for Irf1 or Cebpβ, or negative control RNA (NC). Relative expression level of indicated genes was measured by qPCR and Western-blot. D. Raw264.7 cells and renal cells were transfected with siRNA for Irf1 or Cebpβ, or negative control RNA (NC). For p65 signal blocking, cells were pretreated with 200 μM ammonium pyrrolidinedithiocarbamate (PDTC) for 1 hour. H/R treatment was performed 48 hrs after transfection. Irg1 expression was measured by qPCR. Similar results were seen in three independent experiments. Unpaired Student's t-tests, ns, not significant, *P<0.05, **P<0.01, ***P<0.001.

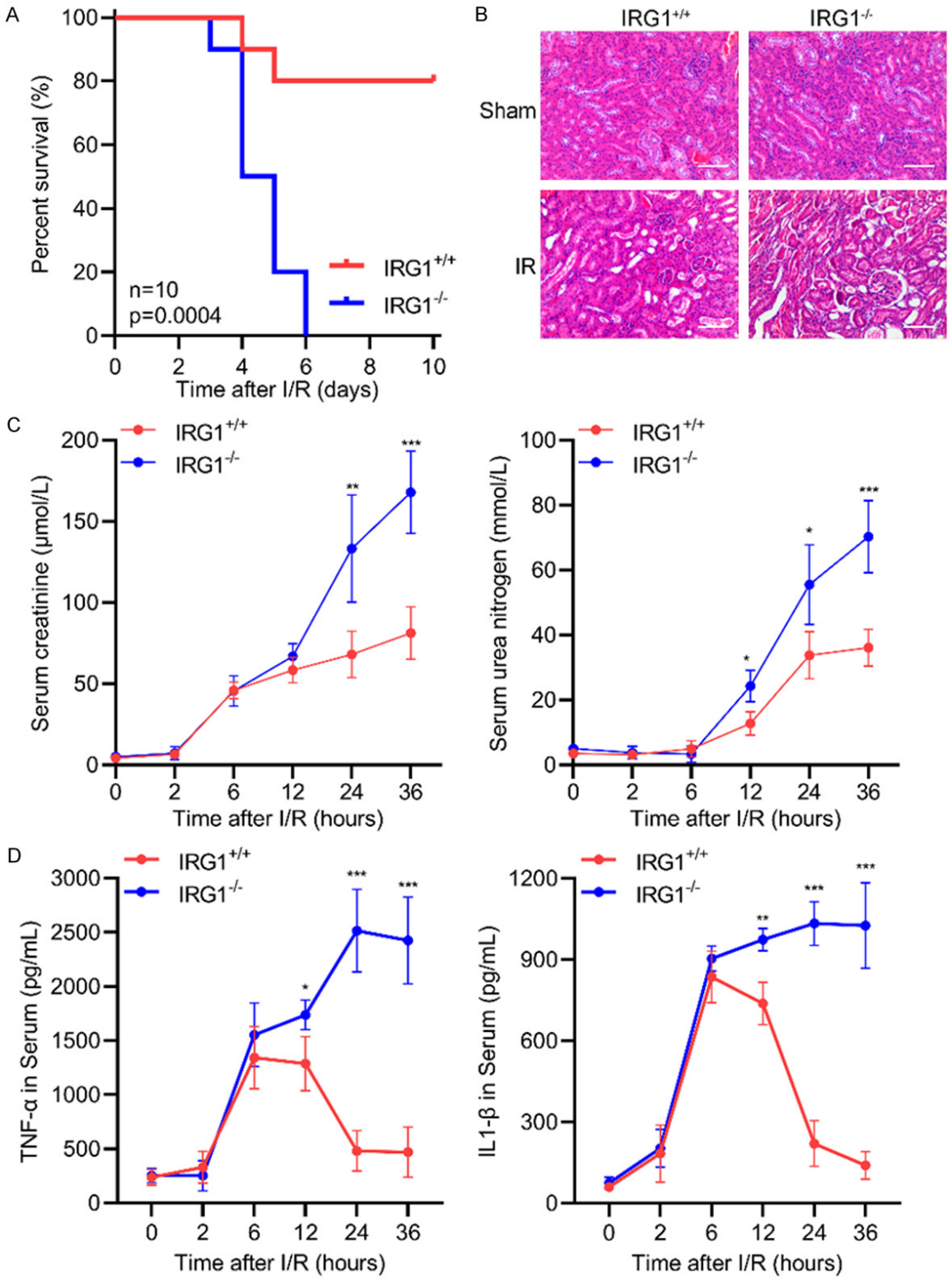


Figure 3. IRG1 deficiency promotes renal inflammation and injury. (A, B) Survival of Irg1$^{-/-}$ mice or WT littermates (n=10 each) after ischemia-reperfusion (P=0.0004; Kaplan-Meier test). (B) H&E-stained kidney sections of Irg1$^{-/-}$ mice or WT littermates (n=10 each) after ischemia-reperfusion. Scale bars, 50 um. (C) Quantification of creatinine and urea nitrogen in serum after ischemia for 30 min and then reperfusion for 0-36 h. (D) Quantification of TNF-α and IL-1β in serum after ischemia for 30 min and then reperfusion for 0-36 h. Data are mean ± s.d. of one representative experiment. Similar results (C and D) were seen in ten independent experiments. Unpaired Student's t-tests unless noted, *P<0.05, **P<0.01, ***P<0.001.

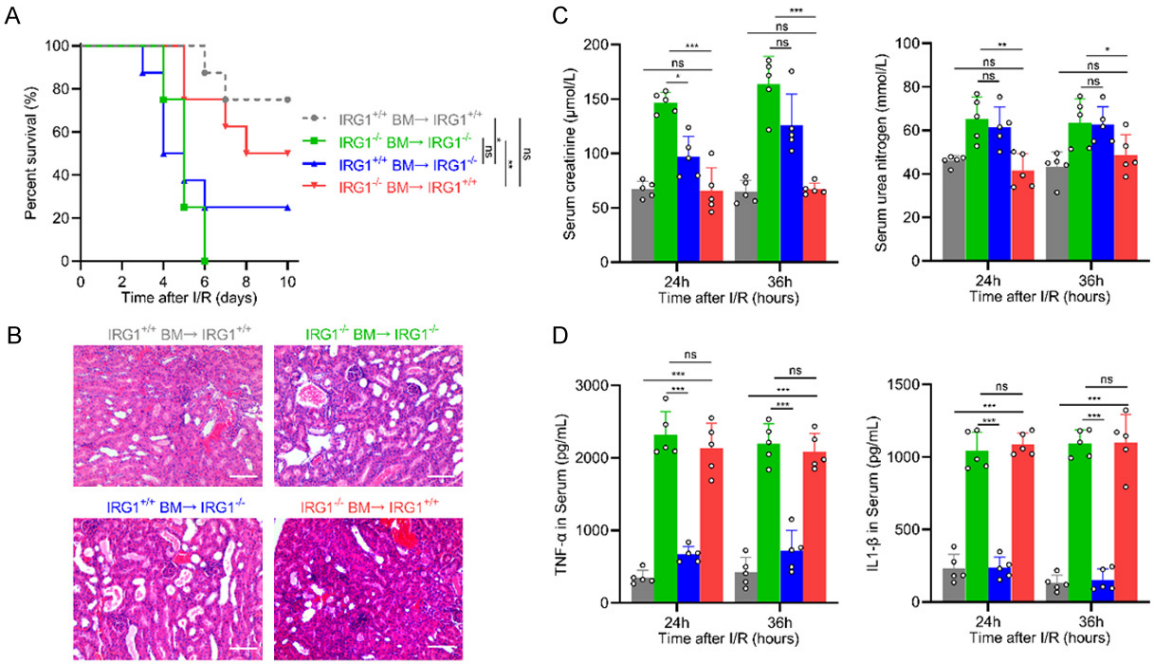


Figure 4. Both IRG1 in renal and bone marrow-derived cells protects against ischemia-reperfusion Injury. (A, B) Survival of four different groups of bone marrow transplantation after ischemia-reperfusion (Kaplan-Meier test). (C) Quantification of creatinine and urea nitrogen in serum of four different groups of bone marrow transplantation after ischemia for 30 min and then reperfusion for 24 h or 36 h. (B) H&E-stained kidney sections of four different groups of bone marrow transplantation after ischemia-reperfusion. Scale bars, 50 um. (D) Quantification of TNF-α and IL-1β in serum of four different groups of bone marrow transplantation after ischemia for 30 min and then reperfusion for 24 h or 36 h. Data are mean ± s.d. of one representative experiment. Similar results in (C and D) were seen in five independent experiments. Unpaired Student's t-tests unless noted, NS, not significant, *P<0.05, **P<0.01, ***P<0.001.

increase intracellular concentrations of itaconate [11], we examined whether DI protects IRI. Results indicated DI exposure significantly reduced promoted the survival of Irg1$^{+/+}$ mice from lethal ischemia (40 min) (**Figure 5A**). Moreover, the mortality of Irg1 deficient mice was significantly reduced by DI treatment (**Figure 5B**). This suggested that the itaconate is likely a significant factor contributing to the reduced IRI-caused death in the Irg1 mice. Considering both immunocytes and renal cells were engaged in IRI, we firstly examined the survival rate in irradiated IRG$^{+/+}$ mice reconstituted with a Irg1$^{-/-}$ immune system via bone marrow transplantation upon renal IR challenge. Result showed DI treatment rescued the death of chimeric mice (**Figure 5C**) by decreasing the levels of pro-inflammatory cytokines (**Figure 5D**). Next, IRG$^{+/+}$ recipients syngeneic transplanted with Irg1$^{-/-}$ kidneys to exam whether Irg1-itaconate axis in renal cells affects IRI. It was found that DI treatment significantly reduced the renal injury with lower serum creatinine level and pathological changes (**Figure 5E** and **5F**). In sum, Irg1 protects against renal IRI and systemic inflammation through Irg1-itaconate axis.

Dimethyl itaconate protects renal cells from oxidative stress injury by activating Nrf2

Previous study reported that Irg1/itaconate pathway activated Nrf2-mediated anti-oxidative response to limit the immune responses [12]. Firstly, we detected the level of reactive oxygen species (ROS) by DCFH-DA probe after IRI. The results showed that dimethyl itaconate could significantly inhibit the production of ROS in renal cells (**Figure 6A**). Then, the LDH level representing cell death in the culture supernatant after IRI was detected. The cell death was significantly reduced by dimethyl itaconate treatment (**Figure 6B**). Consistently, caspase-3 activation detected by WB showed that dimethyl itaconate treatment could significantly reduce the activation of Caspase-3 (**Figure 6C**).

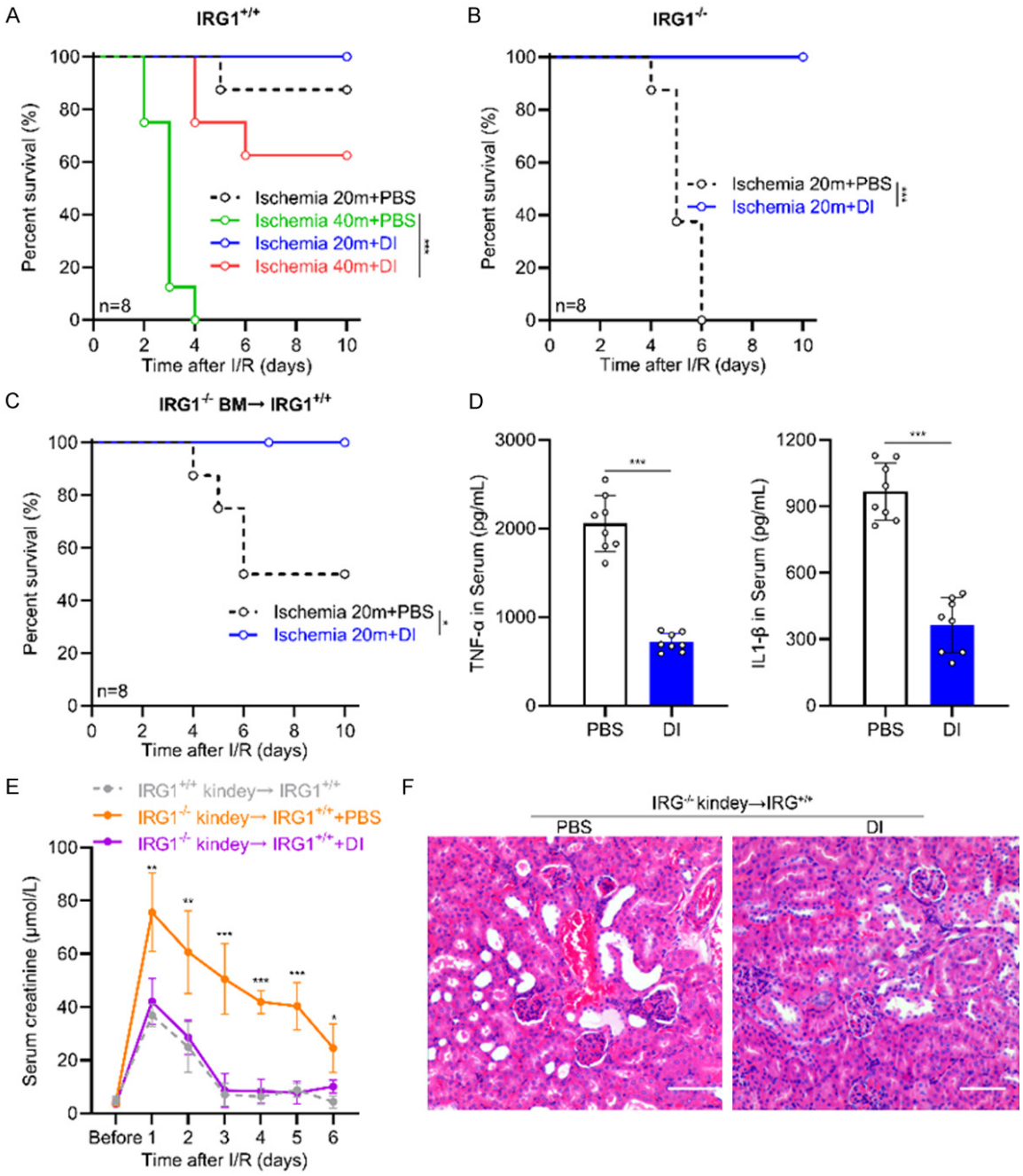


Figure 5. IRG1 protects against renal ischemia-reperfusion injury and systemic inflammation through dimethyl itaconate. (A) Survival curves for IRG1$^{+/+}$ mice after bilateral renal ischemia-reperfusion upon 50 mg/kg DI or PBS treatment. (B) Survival curves for IRG1$^{-/-}$ mice after renal ischemia (20 min)-reperfusion upon indicated treatment. (C) Bone marrow (BM) from IRG1$^{-/-}$ mice transplanted into IRG1$^{+/+}$ mice. After recovery, mice were underwent bilateral renal ischemia (20 min)-reperfusion. (D) Serum was obtained from Mice in (C) 24 hrs post operation via orbital sinus. Then TNF-α and IL-1β were measured by ELISA. (E) Left kidneys from IRG1$^{+/+}$ or IRG1$^{-/-}$ mice were subjected to 25~30 min of warm ischemia and followed by 1 hrs of cold ischemia at 4 °C Lactated Ringer's solution, then transplanted into IRG1$^{+/+}$ mice. 4 hrs post operation, opposite side kidneys of recipients were removed. Serum creatinine was measured as indicated. (F) Mice were treated as in (E), representative H&E-stained grafts were showed. Data are mean ± s.d. of one representative experiment. Similar results were seen in eight independent experiments. *P<0.05, **P<0.01, ***P<0.001.

Next, the activation of Nrf2 and its downstream genes were detected. The results showed that dimethyl itaconate could significantly enhance the translocation of Nrf2 into the nuclei, which

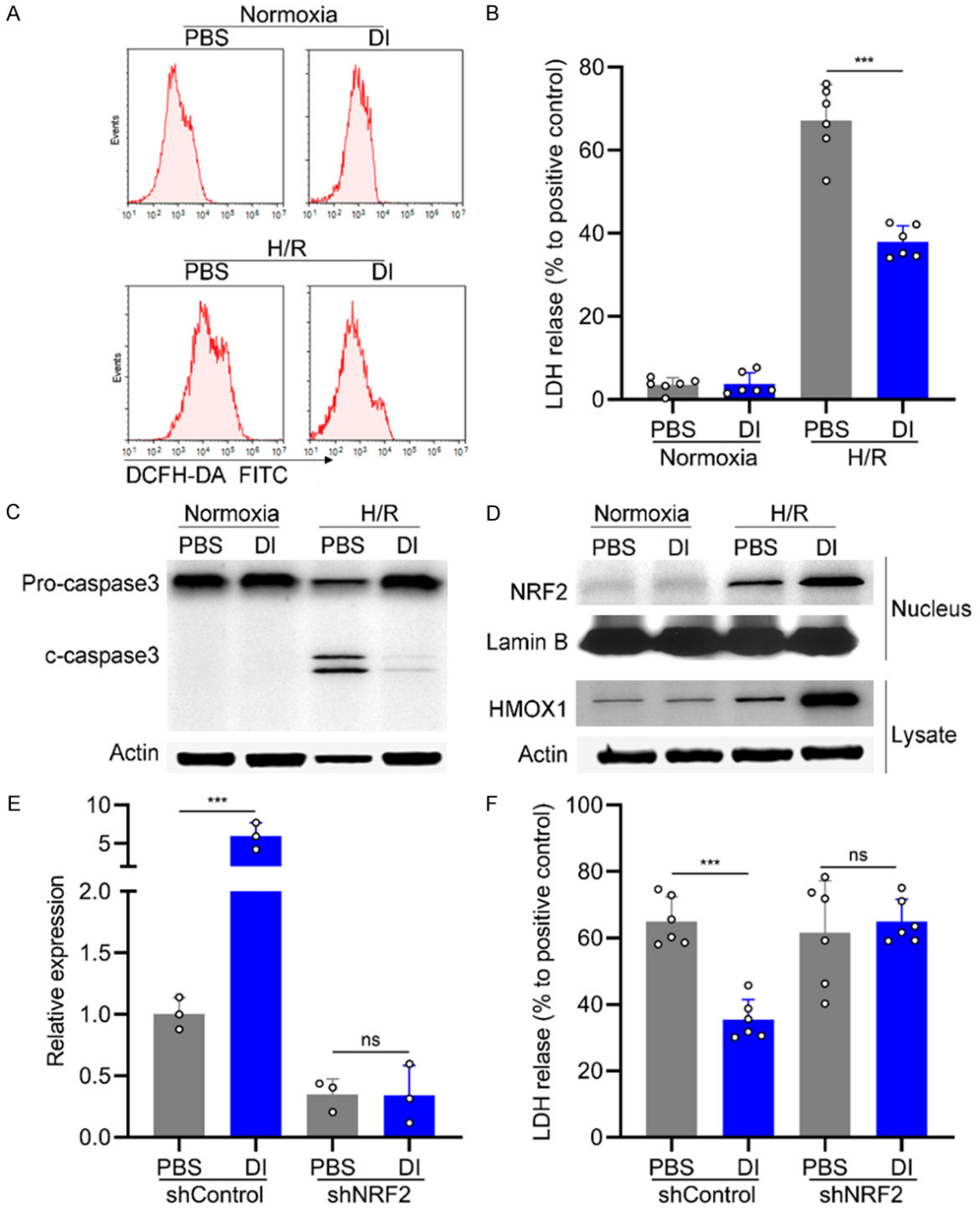


Figure 6. Dimethyl itaconate protects renal cells from oxidative stress injury by activating NRF2. (A) The intracellular ROS in suckling mice renal cells was monitored using a fluorogenic DCFH-DA probe. (B) The LDH release after H/R was determined and presented as % cytotoxicity compared with maximum LDH release. (C) Apoptotic cells were detected by cleaved Caspase3 after H/R. (D) Translocation of Nrf2 from cytosol to nucleus and its down streaming molecular HMOX1 were determined by Western-blot. (E) After H/R treatment, qPCR measuring HMOX1 mRNA level changes upon shRNA-mediated NRF2 knockdown. (F) After H/R treatment, The LDH release changes upon shRNA-mediated NRF2 knockdown were determined and presented as % cytotoxicity compared with maximum LDH release. Data are mean ± s.d. of one representative experiment. Similar results were seen in three (C) or six (B, F) independent experiments. Unpaired Student's t-tests unless noted, NS, not significant, *P<0.05, **P<0.01, ***P<0.001.

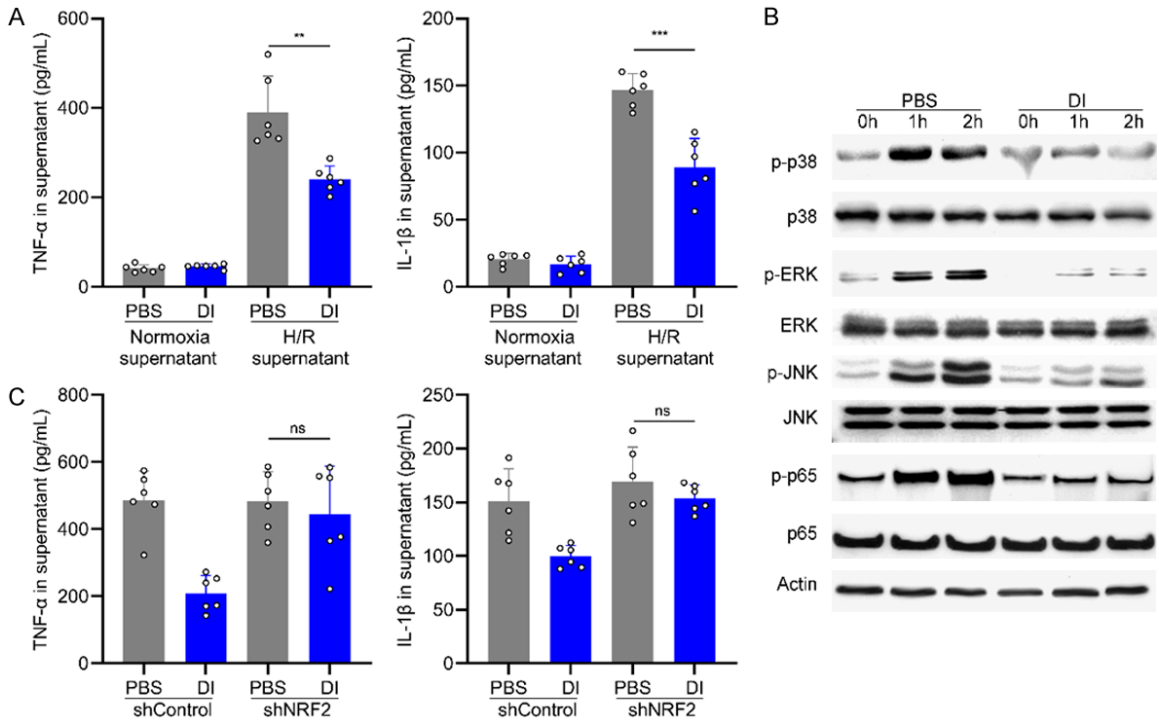


Figure 7. Dimethyl itaconate inhibits inflammation through NRF2 pathway. (A) Quantification of TNF-α and IL-1β of macrophages pretreated with dimethyl itaconate or PBS and then culture supernatant of neonatal rat renal cells treated with H/R or normoxia. (B) Immunoblot analysis of p-P38, P38, p-P65, P65, Erk, p-Erk, JNK, and p-JNK expression in the macrophages pretreated with dimethyl itaconate or PBS. β-actin was assayed as a control. (C) Quantification of TNF-α and IL-1β in the culture supernatant of macrophages pretreated with dimethyl itaconate or PBS with or without shNRF2. Data are mean ± s.d. of one representative experiment. Similar results (A and C) were seen in six independent experiments. Unpaired Student's t-tests, NS, not significant, **P<0.01, ***P<0.001.

induced the expression of downstream gene HMOX1 (**Figure 6D**). After intervention on Nrf2 expression by shRNA, HMOX1 expression was reversed and LDH in supernatant was increased (**Figure 6E** and **6F**). Thus, dimethyl itaconate protects neonatal rat kidney cells from oxidative stress injury by activating Nrf2.

Dimethyl itaconate inhibits inflammation through Nrf2 pathway

Since the Irg1 in bone marrow cells were also responsible for the protective effect, we wanted to know how bone marrow cells were involved in the process. Macrophages are critical important in the sensing of cell death to trigger the immune responses [19]. Thus, we used the supernatant of neonatal rat renal cells treated with H/R to stimulate the activation of macrophages. Pretreatment with dimethyl itaconate can reduce the secretion of inflammatory cytokine TNF-α and IL-1β in macrophages (**Figure 7A**). Mechanistically, dimethyl itaconate can reduce the activation of MAPK and NF-κB pathway in macrophages treated with the supernatant of neonatal rat renal cells after H/R (**Figure 7B**). After intervention on Nrf2 expression by shRNA, the secretion of TNF-α and IL-1β was reversed (**Figure 7C**). These results indicated that dimethyl itaconate inhibited macrophage activation through Nrf2 pathway.

Discussion

Irg1-itaconate axis has now been identified to play an anti-inflammatory role for induction of immune tolerance and control of the innate immunity, especially in activated macrophages [20]. Acute kidney injury, including ischemia-reperfusion injury, is characterized by inflammation-associated tubular injury and vascular damage [21, 22], suggesting the possible role of Irg1-itaconate axis in AKI. Here, we reported that Irg1-itaconate axis protected against acute kidney injury via activation of Nrf2.

Irg1 was well-identified to link metabolism with inflammation by catalyzing endogenous itaconate production [23]. Irg1, with cis-aconitate

decarboxylating activity, is highly upregulated in macrophages during inflammation [22]. Macrophage Irg1 gene silencing may lead to decreased itaconate levels and reduced antimicrobial activity during microbial infections [24]. Moreover, itaconate has been shown to inhibit the enzyme activity of succinate dehydrogenase, which is important in promoting inflammation through oxidizing succinate [18]. Furthermore, itaconate was crucial for the activation of Nrf2, an anti-inflammatory transcription factor, in lipopolysaccharide-stimulated macrophages. Itaconate can directly modify proteins via alkylation of cysteine residues, promoting the Nrf2 activation and downstream gene expression to play anti-inflammatory and anti-oxidant roles [12]. In accordance, we showed that dimethyl itaconate inhibited macrophage activation through Nrf2 pathway in AKI, providing more clues for Irg1-itaconate axis in multiple diseases.

Despite the important biological functions of Irg1-itaconate axis, the insight mechanisms that regulate Irg1 expression have not yet been fully identified. As reported, Irg1 may be regulated by TLRs cascades [25], Myd88-dependent [26] or independent pathway [27] and HO-1 [28]. One possible explanation is that Irg1 would respond to different stimuli and regulate different cellular processes by a complex transcriptional machinery. Our data revealed inducible Irg1 is regulated by p65/Cebpβ in renal cells upon ROS stimulation, and it would be upregulated by Irg1/Cebpβ in macrophages upon DAMP or pro-inflammatory cytokines exposure.

We also identified that both Irg1 in renal and bone marrow-derived cells protected against ischemia-reperfusion injury, suggesting the role of Irg1-itaconate axis in non-immune cells. Recent studies have also reported the Irg1 expression in non-immune cells. mRNA and protein expression of Irg1 was significantly increased in myometrium with human term labor, compared to no labor samples [29]. In an ischemia-reperfusion model, hepatocytes upregulated Irg1 in response to ischemia-reperfusion, which elicited anti-oxidative response in hepatocytes to protect against liver injury [30]. Irg1 expression was also induced in alveolar epithelial cells after respiratory syncytial virus (RSV) infection. And we found that itaconate protected renal cells from oxidative stress injury by activating Nrf2 [31]. Therefore, the Irg1 expressed in non-immune cells is needed to be investigated intensively.

Nrf2 is a master transcriptional regulator of genes involved in the resolution of inflammation and oxidative stress [32, 33]. Induced by oxidative stress, Nrf2 promotes the expression of antioxidants, as well as anti-inflammatory profile. Itaconate can promote the Nrf2 activation and downstream gene expression to elicit anti-inflammatory and anti-oxidant roles [12]. As oxidative stress is also key driver of various kidney diseases, Nrf2 was reported to protect against kidney disease via negative regulation of reactive oxygen species (ROS) [34]. Decreased Nrf2 levels are found in many kidney diseases with high levels of ROS [35]. And many studies have reported that Nrf2 played the anti-inflammatory role by regulating the recruitment of inflammatory cells and expression of inflammatory gene through the antioxidant response element (ARE) [36]. Nrf2 deletion amplified the pathogenic inflammation pathways and led to autoimmune nephritis [37]. Therefore, strategies using Nrf2 inducer to reduce tissue ROS levels to prevent kidney diseases have been developed [37]. However, Nrf2 is highly activated, which is related to high tumor burden and poor prognosis in several subsets of renal cancer. HMOX1, which is a downstream gene of Nrf2, can remove toxic heme, produce biliverdin, iron ions and carbon monoxide. HMOX1 and related products give beneficial effects via the protection against oxidative injury, regulation of apoptosis, and modulation of inflammation [38]. In this study, we found that itaconate protected renal cells from oxidative stress and inhibited inflammation by activating Nrf2/HMOX1 pathway. More studies are needed to uncover the role of Nrf2 pathway in kidney diseases.

In summary, Irg1-itaconate axis protected renal cells from oxidative stress and also prevented macrophage activation by enhancing the activation of Nrf2 pathway. Our study highlighted the importance of the Irg1-itaconate axis in the protecting against ischemia-reperfusion injury and acute kidney injury, providing potential therapeutic targets to control AKI.

Acknowledgements

This study was supported by A Special Supportive Program for Organ transplantation by COTDF (2019JYJH11).

Disclosure of conflict of interest

None.

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