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Isorhynchophylline exerts anti-inflammatory and anti-oxidative activities in LPS-stimulated murine alveolar macrophages

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ABSTRACT

Aims: Excessive inflammatory response and oxidative stress are considered as important pathogenic factors in the development of acute lung injury. Isorhynchophylline (IRN), a tetracyclic oxindole alkaloid isolated from *Uncaria rhynchophylla*, possesses anti-inflammatory and anti-oxidant activities. Our study aimed to investigate the effects and potential mechanisms of IRN on lipopolysaccharide (LPS)-stimulated murine alveolar macrophage cell lines MH-S and NR8383.

Main methods: CCK-8 assay was used to evaluate the cytotoxicity of IRN and LPS. Inflammatory response was assessed by detecting the mRNA expressions and release of tumor necrosis factor α (TNF-α), interleukin (IL)-1β, IL-6, and plasminogen activator inhibitor-1 (PAI-1) using qRT-PCR and ELISA. The expressions of inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2 were examined by qRT-PCR and western blot. Oxidative stress was evaluated by detecting malondialdehyde (MDA) level and the activities of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and catalase (CAT). The changes of the toll like receptor (TLR4)/nuclear factor-kappa B (NF-κB)/nod-like receptor protein 3 (NLRP3) inflammasome pathway was detected by western blot.

Key findings: Treatment with LPS or IRN for 24 h showed no cytotoxicity on MH-S and NR8383 cells. IRN pretreatment inhibited LPS-induced production of inflammatory cytokines, expressions of iNOS and COX-2, and oxidative stress in murine alveolar macrophages. Additionally, IRN inhibited LPS-induced activation of TLR4/NF-κB/NLRP3 inflammasome pathway in MH-S cells. Mechanistically, inhibition of TLR4/NF-κB/NLRP3 inflammasome pathway by si-TLR4 suppressed LPS-induced inflammation and oxidative stress in murine alveolar macrophages.

Significance: IRN exerted anti-inflammatory and anti-oxidant effects on LPS-stimulated murine alveolar macrophages via inhibition of the TLR4/NF-κB/NLRP3 inflammasome pathway.

1. Introduction

Acute lung injury (ALI) and its advanced stage, acute respiratory distress syndrome (ARDS), caused by many factors such as aspiration pneumonia, sepsis, and trauma, has been recognized as a severe clinical inflammatory syndrome of the respiratory system, which leads to the development of hypoxemia, pulmonary edema, as well as respiratory failure [1]. The pathogenesis of ALI is somewhat complicated and associated with various molecular mechanisms that involve excessive inflammatory response and oxidative damage in pulmonary cells, including pulmonary alveolar type II epithelial cells, pulmonary vascular endothelial cells, and alveolar macrophages (AMs) [2,3]. AMs are predominant immune cells in the bronchoalveolar lavage fluid (BALF) and play a pivotal role in lung inflammation and ALI pathogenesis [4]. Therefore, inhibiting inflammatory response and oxidative stress may be effective therapeutic approaches for the prevention and treatment of ALI.

Isorhynchophylline (IRN, the chemical structure is shown in Fig. 1A) is a tetracyclic oxindole alkaloid isolated from the Chinese herbal medicine *Uncaria rhynchophylla*, which has already been routinely used in traditional Chinese medicine to treat various cardiovascular and central nervous system disorders [5,6]. Moreover, a growing body of evidence has demonstrated that IRN possesses diverse biological activities, including anti-oxidant, anti-inflammatory, anti-coagulation, anti-proliferation and neuroprotection activities [7–9]. For example, IRN was reported to inhibit pulmonary arterial smooth muscle

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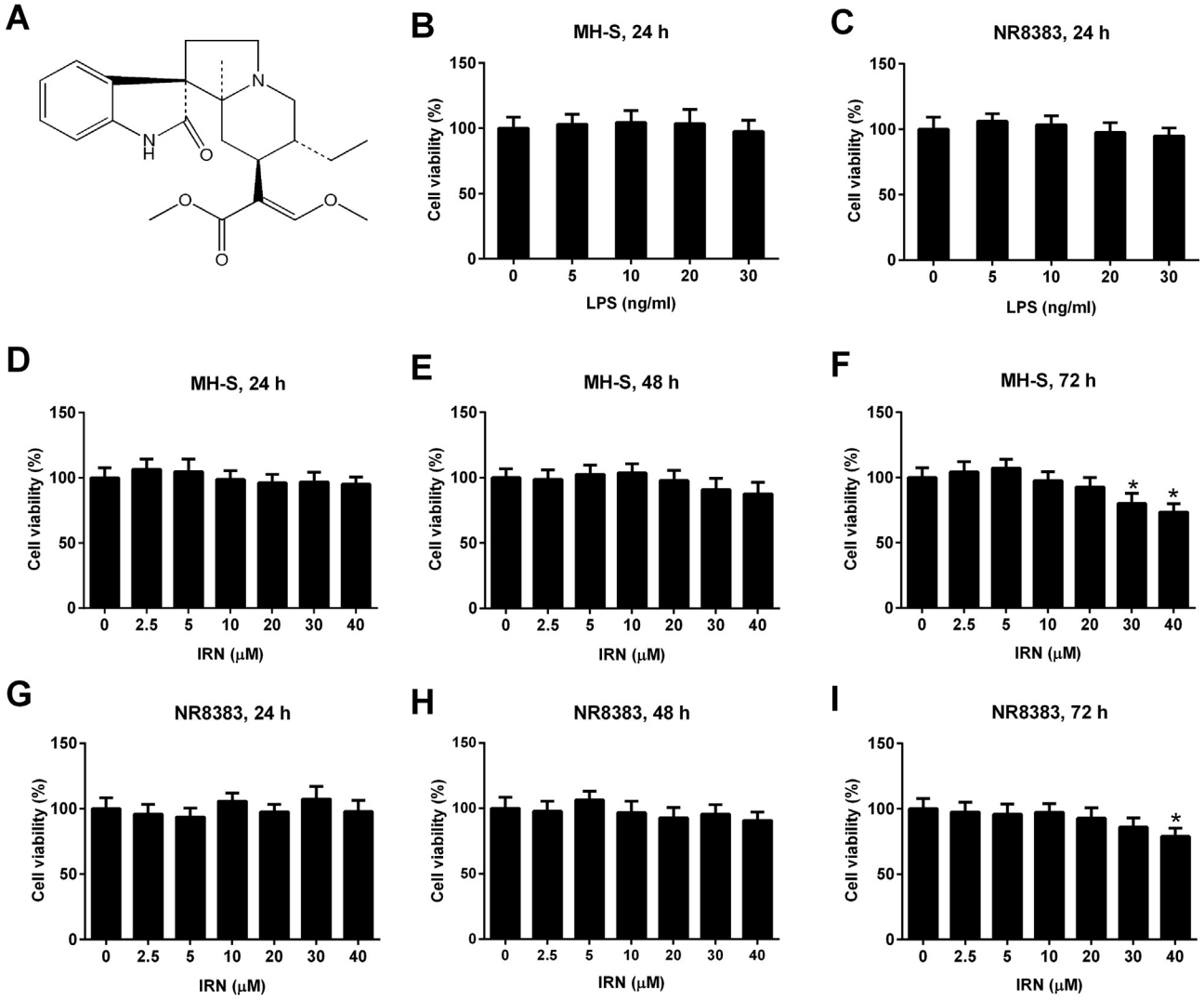

Fig. 1. Effects of IRN or LPS on the viability of murine alveolar macrophages. (A) The chemical structure of IRN. (B and C) CCK-8 assay was performed to detect the viability of MH-S and NR8383 cells after treatment with different doses of LPS (0, 5, 10, 20, or 30 ng/mL) for 24 h. (D–F) CCK-8 assay was conducted to evaluate the viability of MH-S cells after exposure to a series of IRN (0, 2.5, 5, 10, 20, 30, or 40 μM) for 24, 48, and 72 h. (G–I) CCK-8 assay was conducted to evaluate the viability of NR8383 cells after exposure to IRN (0, 2.5, 5, 10, 20, 30, or 40 μM) for 24, 48, and 72 h. Data are expressed as the mean ± SD of three independent experiments performed in triplicate. $^{*}P < 0.05$.

cells proliferation and attenuate pulmonary vascular remodeling in monocrotaline (MCT)-induced pulmonary arterial hypertension (PAH) [10]. IRN was previously demonstrated to confer neuroprotective effects on ischemia- and glutamate-induced neuronal damage in the rat hippocampus and suppress 5-hydroxytryptamine (5-HT) receptor function in the mouse brain [11–13]. IRN was also reported to attenuate LPS-induced production of inflammatory cytokines in mouse microglial cells [7]. However, whether IRN could exert potential anti-inflammatory and anti-oxidative activities on ALI remains largely unknown.

Lipopolysaccharide (LPS), the major outer member component of Gram-negative bacteria, has been identified as a key risk factor in the pathogenesis of ALI and thus has been widely used to induce pharmacological research models for drug development against ALI [14]. Stimulation of AM cells by LPS triggers the over-release of inflammatory cytokines, which enlarge the inflammatory response and further induce ALI [15]. Therefore, our study aimed to investigate the anti-inflammatory and anti-oxidative effects of IRN on LPS-stimulated mouse AMs and the related molecular mechanisms.

2. Materials and methods

2.1. Cell culture and treatment

The mouse alveolar macrophage cell line MH-S and the rat alveolar macrophage cell line NR8383 were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and were routinely passed in RPMI-1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, Gibco), 100 U/mL penicillin and 100 μg/mL streptomycin under a humidified atmosphere of 5% CO_2 at 37 °C. When grown to 70–80% confluence, MH-S and NR8383 cells were treated with appointed concentrations of IRN (purity > 98%, Aktin Chemicals, Inc., Chengdu, China) for 24, 48, and 72 h, or pretreated with 20 μM IRN for 20 min, followed by stimulation with 10 ng/mL LPS (*Escherichia coli* 055:B5, Sigma-Aldrich, St Louis, MO, USA) for 24 h. SiRNA targeting toll like receptor (TLR4) (si-TLR4) and its negative control (si-Con) were purchased from GenePharma Co., Ltd. (Shanghai, China) and transient transfection with si-TLR4 or si-Con into MH-S cells was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

2.2. Cell viability assay

Cell Counting Kit-8 (CCK-8) assay was performed to evaluate the effects of LPS or IRN on the viability of MH-S cells. MH-S and NR8383 cells were seeded into 96-well plates and then treated with different doses of LPS (0, 5, 10, 20, or 30 ng/mL) for 24 h or IRN (0, 2.5, 5, 10, 20, 30, or 40 μM) for 24, 48, and 72 h. Then, 10 μL of CCK-8 solution (Dojindo, Kumamoto, Japan) was added into each well, followed by incubation at 37 °C for 2 h. The absorbance at 450 nm was measured by the ELx800 Absorbance Microplate Reader (Bio-rad, Hercules, CA, USA).

2.3. Oxidative stress measurement

After different treatments, MH-S and NR8383 cells were harvested and homogenized, and the supernatants were collected by centrifugation. Then, the level of malondialdehyde (MDA) and the activities of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and catalase (CAT) were determined by corresponding commercial kits according to the manufacturer's instructions. MDA level was determined according to the thiobarbituric acid method using a MDA Assay Kit (Jiancheng Bioengineering Institute, Nanjing, China). The absorbance was measured at 532 nm using an absorbance microplate reader (Bio-rad). SOD activity was determined according to the water soluble tetrazolium salts assay method using a Superoxide Dismutase Assay Kit (Jiancheng Bioengineering Institute). The absorbance was measured at 450 nm using an absorbance microplate reader (Bio-rad). GSH-Px activity was determined by determination of reduced glutathione using a GSH-Px Assay Kit (Jiancheng Bioengineering Institute). The absorbance was measured at 412 nm using an absorbance microplate reader (Bio-rad). CAT activity was determined using a CAT Assay Kit (Jiancheng Bioengineering Institute). The action of CAT decomposing H_2O_2 could be stopped with ammonium molybdate, and the absorbance values of the resulting compound with straw yellow were determined at 405 nm using an absorbance microplate reader (Bio-rad).

2.4. Enzyme-linked immunosorbent assay (ELISA)

The concentrations of tumor necrosis factor α (TNF-α), interleukin (IL)-1β, and IL-6 in the supernatants of treated MH-S and NR8383 cells were measured by respective specific ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

2.5. Quantitative real-time PCR (qRT-PCR)

Total RNAs were extracted from treated MH-S and NR8383 cells using TRIzol reagent (Invitrogen). Complementary DNA (cDNA) synthesis was carried out from approximately 2 μg of total RNA using RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Waltham, MA, USA). The mRNA expressions of TNF-α, IL-1β, IL-6, inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2 were performed using SYBR Green Mastermix kit (Takara, Tokyo, Japan) on the CFX96 Touch™ Real-Time PCR Detection System (Bio-rad). The mRNA expression level was calculated using $2^{-\Delta\Delta Ct}$ method and normalized to GAPDH. The primer sequences were displayed as follows: mouse TNF-α, forward 5′-CTT CTC ATT CCT GCT TGT G-3′, reverse 5′-ACT TGG TGG TTT GCT ACG-3′; rat TNF-α, forward 5′-AAG CCC GTA GCC CAC GTC GTA-3′, reverse 5′-GCC CGC AAT CCA GGC CAC TAC-3′; mouse and rat IL-6, forward 5′-CTG CAA GAG ACT TCC ATC CAG-3′, reverse 5′-AGT GGT ATA GAC AGG TCT GTT GG-3′; mouse and rat IL-1β forward 5′-AAC CTG CTG GTG TGT GAC GTT C-3′, reverse 5′-CAG CAC GAG GCT TTT TTG TTG T-3′; mouse PAI-1, forward 5′-TCC TCA TCC TGC CTA AGT TCT C-3′, reverse 5′-GTG CCG CDC TCG TTT ACC TC-3′; rat PAI-1, forward 5′-TGA GAT CAG TAC TGC GGA CG-3′, reverse 5′-GAG GGG CAC ATC TTT TTC AA-3′; mouse iNOS forward 5′-CTC AGC CCA ACA ATA CAA G-3′, reverse 5′-CTA CAG TTC CGA GCG TCA-3′, mouse COX-2, forward 5′-TTC AAA TGA GAT TGT GGG AAA AT-3′, reverse 5′-AGA TCA TCT CTG CCT GAG TAT CTT-3′; mouse GAPDH, forward 5′-AAC GTG TCA GTC GTG GAC CTG-3′, reverse 5′-AGT GGG TGT CGC TGT FGA AGT-3′; rat GAPDH, forward 5′-GCA CCG TCA AGC TGA GAA C-3′, reverse 5′-TGG TGA AGA CGC CAG TGG A-3′.

2.6. Western blot analysis

The MH-S cells after different treatments were harvested and lysed using RIPA lysis buffer (Beyotime, Shanghai, China) containing 1% protease inhibitor (Beyotime). The cellular supernatant was collected by centrifugation at 12,000*g* for 15 min and the protein concentrations were measured with a BCA protein assay kit (Bio-rad). The protein samples (20 μg/lane) were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA). The membranes were blocked with 5% non-fat milk proteins for 2 h at room temperature and then probed with primary antibodies against iNOS (Abcam, Cambridge, MA, USA), COX-2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), TLR4 (Abcam), myeloid differentiation factor 88 (MyD88) (Santa Cruz Biotechnology), phosphorylated nuclear factor-kappa B (NF-κB) p65 (NF-κB p-p65) (Cell Signaling Technology Inc., Beverly, MA, USA), NF-κB p65 (Cell Signaling Technology Inc.), nod-like receptor protein 3 (NLRP3) (Abcam), and β-actin (Abcam) overnight at 4 °C. Subsequently, the membranes were incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) for 1 h at 37 °C. Finally, protein bands were visualized using an enhanced chemiluminescence (ECL) system (Thermo Fisher Scientific) and analyzed using Quantity Onesoftware (Bio-rad).

2.7. Statistical analysis

All experimental results are expressed as the mean ± standard deviation (SD). Statistical analysis was performed using the GraphPad Prism version 5.0 (GraphPad Software, CA, USA) with Student's *t*-test or one-way analysis of variance (ANOVA). Differences were considered significant when P values were < 0.05.

3. Results

3.1. Effect of IRN or LPS on the viability of murine alveolar macrophages

Firstly, we evaluated the cytotoxicity of LPS and IRN on MH-S and NR8383 cells using CCK-8 assay. As shown in Fig. 1B and C, treatment with LPS for 24 h showed nontoxic effect on MH-S and NR8383 cells. Fig. 1D and E showed that exposure to various concentrations of IRN for 24 and 48 h exhibited no significant effect on the viability of MH-S cells. After 72 h of exposure, IRN at 2.5, 5, 10, and 20 μM did not affect the viability of MH-S cells, but cell viability was decreased in the presence of 30 and 40 μM of IRN (Fig. 1F). The viability of NR8383 cells was unchanged after treatment with appointed concentrations of IRN for 24 and 48 h (Fig. 1G and H). After 72 h of treatment, the viability of NR8383 cells was reduced when the concentration reached 40 μM (Fig. 1I). The cell viability was unchanged after exposure to 20 μM concentration of IRN for 24, 48, and 72 h, ensuring that the anti-inflammatory and anti-oxidative activities were not affected by the cell viability. Therefore, 20 μM concentration of IRN was selected for the following experiments.

3.2. IRN exerts anti-inflammatory activities in LPS-stimulated murine alveolar macrophages

PAI-1 is considered as a pro-inflammatory risk marker and its role in the pathogenesis of lung inflammation is evidenced by its upregulation in ALI [16,17]. To investigate the anti-inflammatory effects of IRN, the

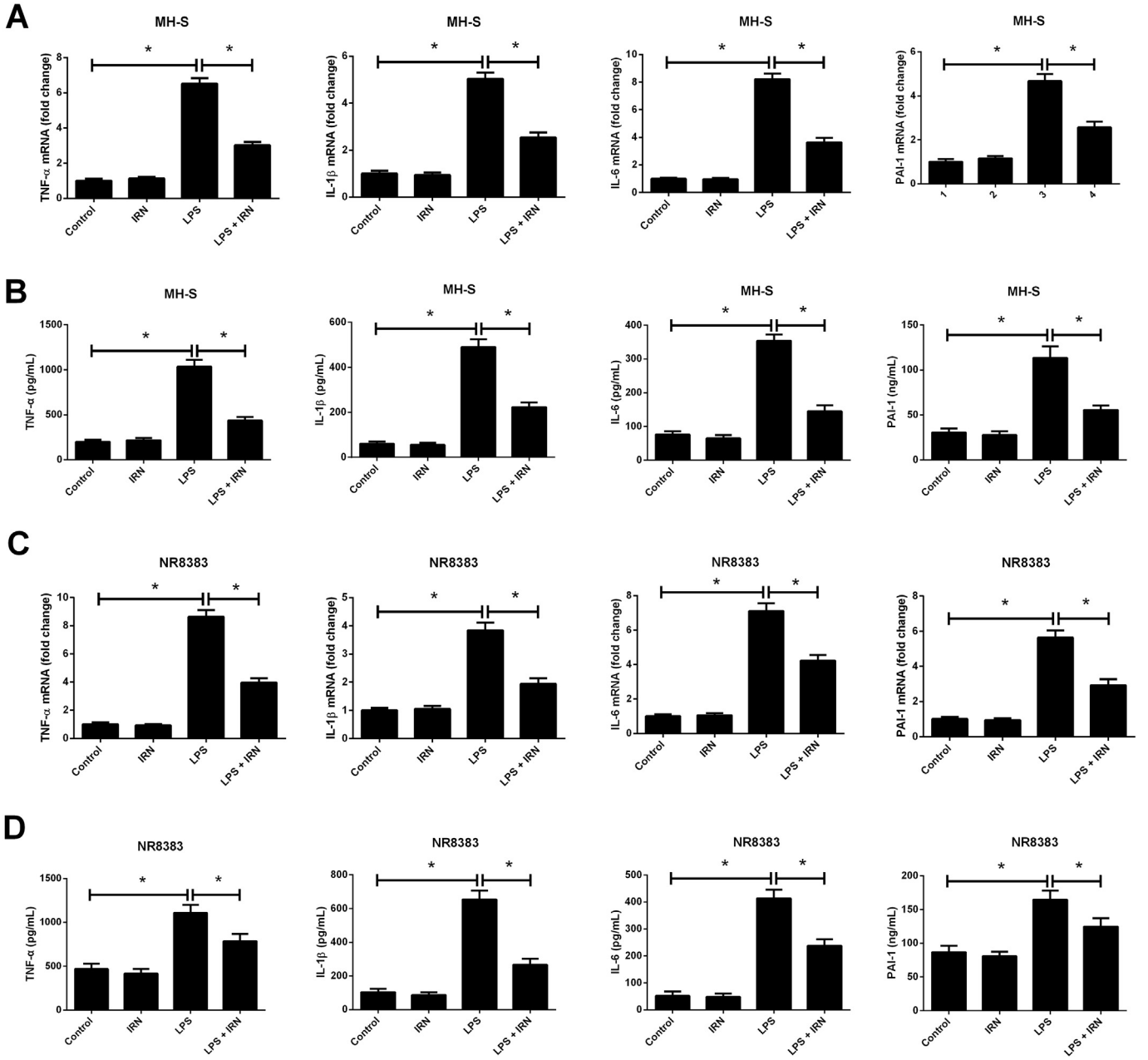

Fig. 2. Effects of IRN on LPS-induced production of pro-inflammatory cytokines and PAI-1 in murine alveolar macrophages. MH-S and NR8383 cells were treated with 20 μM IRN for 24 h, or pretreated with 20 μM IRN for 20 min, followed by stimulation with 10 ng/mL LPS for 24 h. (A) qRT-PCR analysis of mRNA expressions of TNF-α, IL-1β, IL-6, and API-1 in treated MH-S cells. (B) The concentrations of TNF-α, IL-1β, IL-6 and API-1 in the supernatants of treated MH-S cells were measured by ELISA assay. (C) qRT-PCR analysis of mRNA expressions of TNF-α, IL-1β, IL-6, and API-1 in treated NR8383 cells. (D) The concentrations of TNF-α, IL-1β, IL-6 and API-1 in the supernatants of treated NR8383 cells were measured by ELISA assay. Data are expressed as the mean ± SD of three independent experiments performed in triplicate. $^*P < 0.05$.

levels of pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) and PAI-1 in LPS-stimulated MH-S cells were determined by qRT-PCR and ELISA. The results manifested that IRN treatment alone did not affect the mRNA expressions and secretion of TNF-α, IL-1β, IL-6, and PAI-1 in MH-S cells and LPS exposure remarkably increased the mRNA expressions and secretion of TNF-α, IL-1β, IL-6, and PAI-1 in MH-S cells. However, pretreatment with IRN reduced LPS-induced increase of TNF-α, IL-1β, IL-6, and PAI-1 levels in MH-S cells (Fig. 2A and B). To confirm the anti-inflammatory effects of IRN, qRT-PCR and ELISA were repeated using another murine alveolar macrophage cell line NR8383. We found that IRN treatment alone did not affect the mRNA expressions and secretion of TNF-α, IL-1β, IL-6, and PAI-1 in NR8383 cells. Pretreatment with IRN attenuated LPS-induced increase of TNF-α, IL-1β, IL-6, and PAI-1 levels in NR8383 cells (Fig. 2C and D).

3.3. IRN inhibited LPS-induced expressions of iNOS and COX-2 in MH-S cells

iNOS and COX-2 are important inflammatory mediators that induce inflammation in ALI and are often used to assess the severity of inflammation [18]. We further explored the effects of IRN on the expressions of iNOS and COX-2 in MH-S cells by qRT-PCR and western blot. The results showed that LPS treatment enhanced the expressions of iNOS (Fig. 3A and C) and COX-2 (Fig. 3B and D) in MH-S cells at mRNA and protein levels in MH-S cells. However, pretreatment with IRN attenuated LPS-induced expressions of iNOS and COX-2 in MH-S

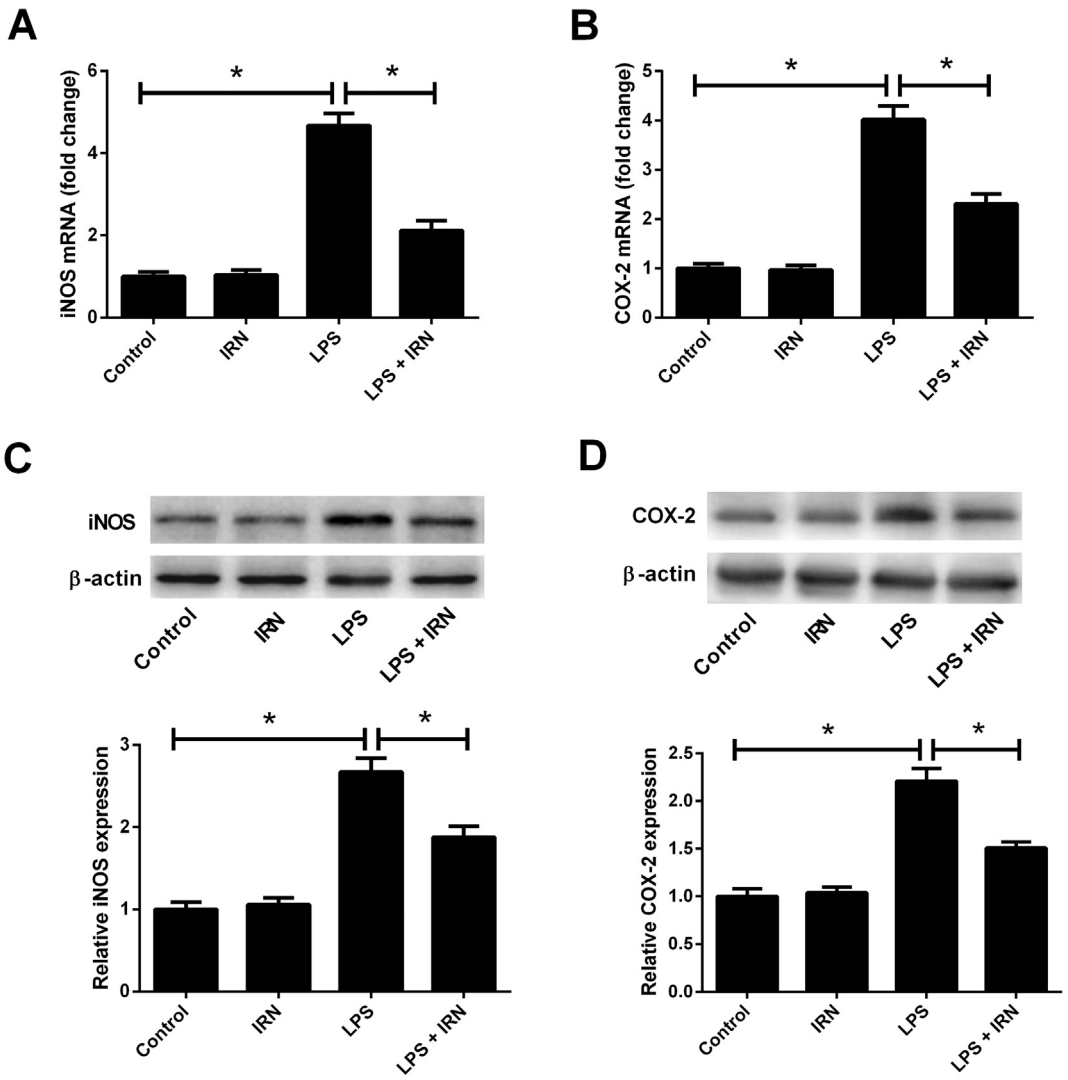

Fig. 3. Effects of IRN on LPS-induced expressions of iNOS and COX-2 in MH-S cells. MH-S cells were treated with 20 μM IRN for 24 h, or pretreated with 20 μM IRN for 20 min, followed by stimulation with 10 ng/mL LPS for 24 h. The mRNA and protein expression levels of iNOS (A and C) and COX-2 (B and D) in the treated MH-S cells were determined by qRT-PCR and western blot. Data are expressed as the mean ± SD of three independent experiments. *$P < 0.05$.

cells.

3.4. IRN suppressed LPS-induced oxidative stress in murine alveolar macrophages

To investigate the anti-oxidant effect of IRN in LPS-stimulated MH-S cells, the level of oxidative stress marker MDA and anti-oxidant enzymes including SOD, GSH-Px, and CAT were measured. It was shown that LPS stimulation increased MDA level (Fig. 4A) and reduced the activities of SOD (Fig. 4B), GSH-Px (Fig. 4C), and CAT (Fig. 4D) in MH-S cells, which were reversed by IRN pretreatment. These results suggested that IRN showed anti-oxidant activity on LPS-induced oxidative stress in MH-S cells. To confirm the anti-oxidant activity of IRN, MDA level and enzyme activities of SOD, GSH-Px, and CAT were also determined in NR8383 cells. IRN treatment alone did not affect the MDA levels and enzyme activities of SOD, GSH-Px, and CAT in NR8383 cells. Pretreatment with IRN reversed LPS-induced increase in MDA level (Fig. 4E) and reduction in activities of SOD (Fig. 4F), GSH-Px (Fig. 4G), and CAT (Fig. 4H) in NR8383 cells.

3.5. IRN inhibited LPS-induced activation of TLR4/NF-κB/NLRP3 inflammasome pathway in MH-S cells

It is well-known that the TLR4/MyD88, NF-κB and NLRP3 inflammasome pathways are involved in LPS-induced inflammatory response and thus is implicated in the pathogenesis of ALI [19,20]. To explore the related mechanism of the IRN effects on LPS-induced MH-S cells, the effects of IRN on TLR4/MyD88, NF-κB and NLRP3 inflammasome pathways were evaluated by western blot. As compared with control group, LPS-stimulated MH-S cells showed an elevation in the protein levels of TLR4, MyD88, NF-κB p-p65 and NLRP3 and no significant difference of these protein expressions was observed in IRN-treated MH-S cells. Notably, cotreatment with IRN and LPS inhibited the effects of LPS on these protein levels in MH-S cells (Fig. 5), suggesting that IRN inhibited LPS-induced activation of TLR4/NF-κB/NLRP3 inflammasome pathway in MH-S cells.

3.6. Inhibition of the TLR4/NF-κB/NLRP3 inflammasome pathway suppressed LPS-induced inflammation and oxidative stress in MH-S cells

To further determine the effects of TLR4/NF-κB/NLRP3 inflammasome pathway on the inflammatory responses and oxidative stress in

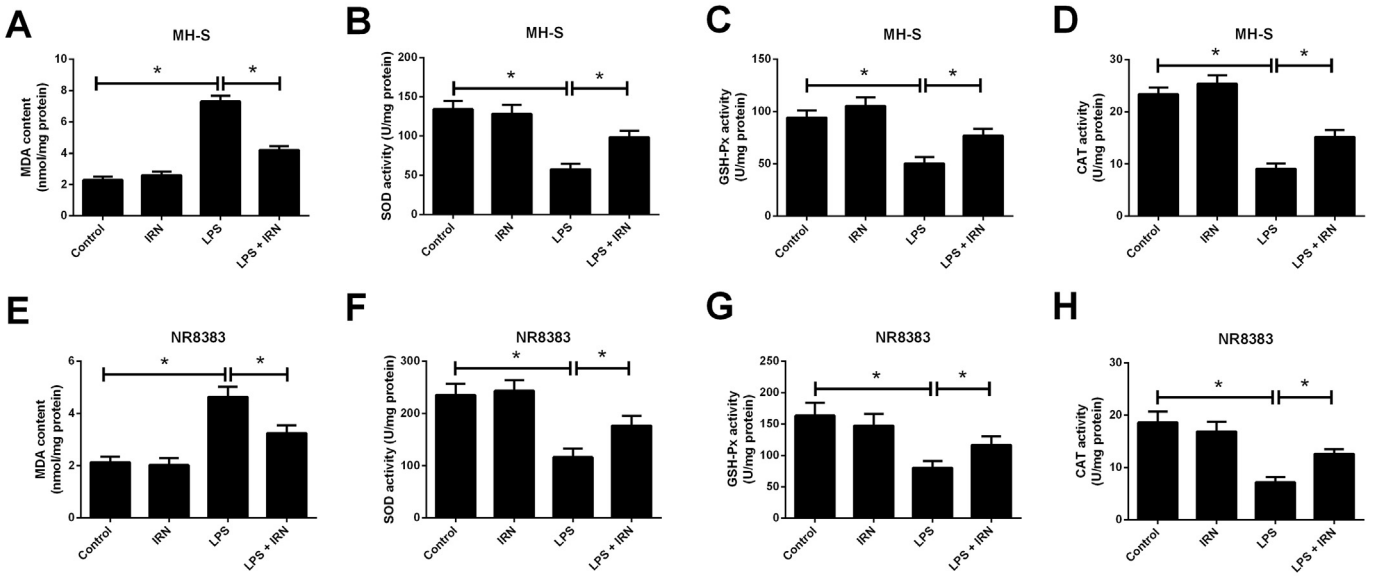

Fig. 4. Effects of IRN on LPS-induced oxidative stress in murine alveolar macrophages. MH-S and NR8383 cells were exposed to 20 μM IRN for 24 h, or pretreated with 20 μM IRN for 20 min and then stimulated with 10 ng/mL LPS for 24 h. (A–D) MDA level and the activities of SOD, GSH-Px, and CAT in MH-S cells were determined using corresponding commercial kits. (E–H) MDA level and the activities of SOD, GSH-Px, and CAT in NR8383 cells were determined using corresponding commercial kits. Data are expressed as the mean ± SD of three independent experiments performed in triplicate. $^*P < 0.05$.

LPS-stimulated MH-S cells, MH-S cells were transfected with si-TLR4 or si-Con to inhibit the TLR4/NF-κB/NLRP3 inflammasome pathway. TLR4 knockdown inhibited the protein levels of TLR4, MyD88, NF-κB p-p65 and NLRP3 in MH-S cells (Fig. 6A). Subsequently, ELISA assay showed that TLR4 silencing antagonized LPS-induced release of TNF-α, IL-1β, and IL-6 in MH-S cells (Fig. 6B). We also discovered that depletion of TLR4 greatly undermined the increase of iNOS and COX-2 expressions induced by LPS in MH-S cells (Fig. 6C). In addition, LPS-induced increase of MDA level and decrease of SOD, GSH-Px, and CAT activities in MH-S cells were abolished by downregulation of TLR4 (Fig. 6D). Collectively, these results demonstrated that inhibition of the TLR4/NF-κB/NLRP3 inflammasome pathway suppressed LPS-induced inflammation and oxidative stress in MH-S cells.

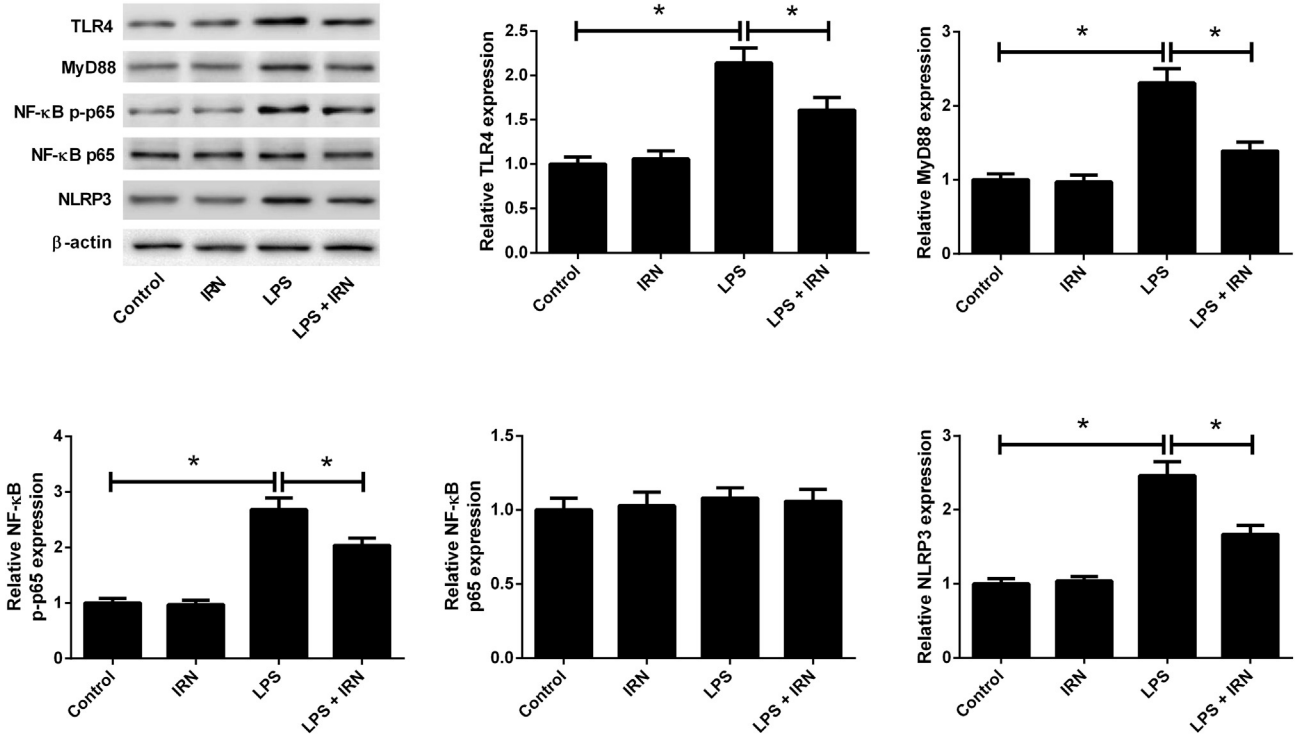

Fig. 5. Effects of IRN on the TLR4/NF-κB/NLRP3 inflammasome pathway in LPS-stimulated MH-S cells. Western blot analysis was performed to detect the protein levels of TLR4, MyD88, NF-κB p-p65, NF-κB p65 and NLRP3 in MH-S cells after treatment with 20 μM IRN for 24 h, or pretreatment with 20 μM IRN for 20 min and then stimulation with 10 ng/mL LPS for 24 h. Data are expressed as the mean ± SD of three independent experiments. $^*P < 0.05$.

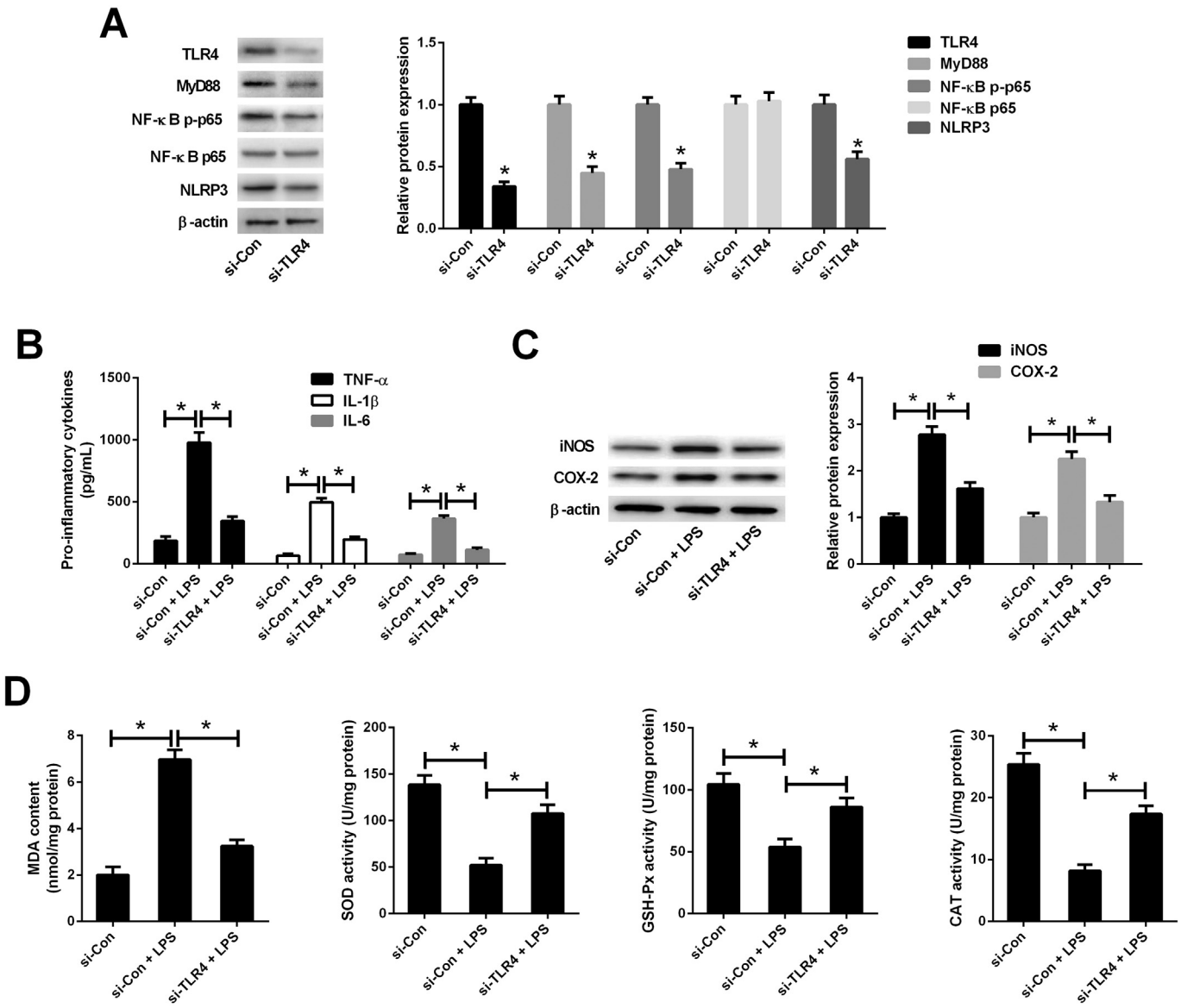

Fig. 6. Inhibition of the TLR4/NF-κB/NLRP3 inflammasome pathway suppressed LPS-induced inflammation and oxidative stress in MH-S cells. (A) Western blot was carried out to detect the protein levels of TLR4, MyD88, NF-κB p-p65, NF-κB p65 and NLRP3 in si-TLR4- or si-Con-transfected MH-S cells. Data are expressed as the mean ± SD of three independent experiments. *$P < 0.05$. (B) ELISA was used to measure the concentrations of TNF-α, IL-1β, and IL-6 in MH-S cells after transfection with si-TLR4 or si-Con for another 24 h, and then stimulation with 10 ng/mL LPS for 24 h. Data are expressed as the mean ± SD of three independent experiments performed in triplicate. *$P < 0.05$. (C) The protein levels of iNOS and COX-2 were examined by western blot in MH-S cells introduced with si-TLR4 or si-Con for 24 h and then stimulated with 10 ng/mL LPS for another 24 h. Data are expressed as the mean ± SD of three independent experiments. *$P < 0.05$. (D) MDA level and the activities of SOD, GSH-Px, and CAT were measured using corresponding commercial kits in MH-S cells introduced with si-TLR4 or si-Con and then treated with 10 ng/mL LPS for another 24 h. Data are expressed as the mean ± SD of three independent experiments performed in triplicate. *$P < 0.05$.

4. Discussion

ALI has been reported as a severe life-threatening disease that contributes to more critical illness and poses a serious threat to human health worldwide [1]. Regardless of recent improvements in understanding for the pathophysiology and therapy of ALI, the clinical therapeutic effects remain poor with high rates of morbidity and mortality [21]. Since pharmacological approaches for the treatment of ALI/ARDS are lacking, it is urgently needed to search for novel and effective therapeutic strategies for ALI. IRN, an active alkaloid component of *Uncaria rhynchophylla*, has been shown to possess anti-oxidant and anti-inflammatory properties. However, there is no direct evidence concerning the effects of IRN on ALI and the underlying molecular mechanisms. In the current study, we, for the first time, demonstrated that IRN attenuated LPS-induced inflammatory response and oxidative stress in AM cells via inhibition of TLR4/NF-κB/NLRP3 inflammasome pathway.

A number of evidence has shown that excessive inflammatory response and oxidative stress are closely linked and considered as important pathogenic factors in the development of ALI [22,23]. Oxidative stress, caused by an imbalance between oxidant and anti-oxidant systems, accelerates inflammation by triggering recruitment and activation of inflammatory cells, while in turn cause induction of oxidative stress [24]. Increased levels of inflammatory mediators such as TNF-α, IL-6, IL-1β, iNOS and COX-2 and disturbance of oxidative balance have been observed in LPS-induced ALI [25]. Thus, increasing researches have focused on the elevated oxidative stress and inflammatory response during ALI progression [26]. Data showed that suppressing inflammatory response and oxidative stress could ameliorate LPS-induced ALI [27]. In the present study, we demonstrated that IRN showed no

cytotoxicity on MH-S cells. In addition, we found that IRN inhibited the production of inflammatory mediators including TNF-α, IL-6, IL-1β, iNOS and COX-2 in LPS-stimulated MH-S cells. Treatment with IRN also attenuated LPS-induced increase of MDA level and decrease of SOD, GSH-Px, and CAT in MH-S cells. On the basis of these results, we confirmed the anti-inflammatory and anti-oxidant effects of IRN on LPS-induced ALI, suggesting that IRN may be a promising therapeutic agent for the treatment of ALI. Similarly, previous studies found that IRN ameliorated D-galactose-induced mouse memory deficits through enhancing the anti-oxidant status and anti-inflammatory effect in brain tissues via activation of NF-κB signaling pathway [28]. IRN protected rat pheochromocytoma PC12 cells against β-amyloid-induced neurotoxicity via inhibiting oxidative stress and repressing apoptosis [29], and the protective effect might be involved in the phosphatidylinositol 3-kinase (PI3K)/Akt/glycogen synthase kinase 3β (GSK-3β) pathway [30]. Yuan et al. reported that IRN suppressed inflammatory response in LPS-stimulated murine microglial N9 cells, and this effect was mediated by the NF-κB and extracellular signal-regulated kinase (ERK) and p38 MAPK pathways [7]. A previous study suggested that IRN inhibited the proliferation of pulmonary arterial smooth muscle cells and attenuated monocrotaline-induced pulmonary vascular remodeling, and these beneficial effects were partially regulated by the inhibition of PDGF-Rβ phosphorylation and its downstream Akt/GSK-3β, ERK1/2 and signal transducers and activators of transcription 3 (STAT3) pathways [10].

TLR signaling plays crucial roles in innate immunity system which is responsible for inflammation process in ALI [31,32]. Among TLRs, TLR4 is a dominant signaling receptor for LPS and associated with the pathogenesis of LPS-induced ALI [33]. MyD88 is a key and universal downstream adapter in response to interaction of TLR4 with LPS [34]. Upon stimulation by LPS, TLR4 could activate the NF-κB through MyD88 pathway, which induces the release of inflammatory cytokines to modulate the inflammatory reaction [35]. Previously, it was reported that inhibition of the TLR4/MyD88/NF-κB pathway attenuated inflammation, oxidative stress and mitochondrial dysfunction in LPS-induced ALI mice [36]. NLRP3 inflammasome, the most fully characterized inflammasome, is a major multimeric protein complex of the innate immune system including NLRP3, caspase-1, and apoptosis-associated speck-like protein adaptor (ASC) [37]. NLRP3 inflammasome has been reported to regulate the maturation and production of inflammatory cytokines and be involved in the pathogenesis of ALI induced by LPS [38]. A growing body of evidence has shown that the NLRP3 inflammasome also participates in TLR4/NF-κB signaling pathway-mediated inflammatory response [39,40]. In the present study, we verified that LPS challenge increased the expressions of TLR4, MyD88, NF-κB p-p65 and NLRP3 in MH-S cells, suggesting that LPS induced activation of the TLR4/NF-κB/NLRP3 inflammasome pathway. However, IRN pretreatment inhibited LPS-induced activation of the TLR4/NF-κB/NLRP3 inflammasome pathway in MH-S cells. Furthermore, our study manifested that inhibition of TLR4/NF-κB/NLRP3 inflammasome pathway by si-TLR4 suppressed LPS-induced release of inflammatory mediators and oxidative stress in MH-S cells. According to the above results, we could conclude that IRN attenuate LPS-induced inflammation and oxidative stress in AM cells via inhibition of the TLR4/NF-κB/NLRP3 inflammasome pathway. However, the current study did not elaborate how IRN affected the TLR4/NF-κB/NLRP3 inflammasome pathway. Further studies are needed to clarify the underlying mechanisms.

5. Conclusion

In summary, we demonstrated for the first time that IRN exerted anti-inflammatory and anti-oxidant effects on LPS-stimulated MH-S cells, which was involved in inhibition of the TLR4/NF-κB/NLRP3 inflammasome pathway, providing novel insight into the effects and molecular mechanism of IRN in ALI. Thus, IRN may represent a promising therapeutic strategy for treating ALI. Further ALI animal models are required to evaluate the effects of IRN in vivo.

Conflict of interest

The authors declare no conflict of interest.

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