

IL-39 increases ROS production and promotes the phosphorylation of p38 MAPK in the apoptotic cardiomyocytes

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Abstract

Introduction. The cytokine interleukin (IL)-39 is a novel member of the IL-12 family. Our previous study found that the serum level of IL-39 significantly increased in patients with acute myocardial infarction. However, the role of IL-39 in cardiomyocyte apoptosis remains unclear.

Material and methods. In this study, the cultured mouse HL-1 cardiomyocytes were incubated with PBS, 0–100 ng/mL IL-39, 200 μ M H₂O₂ or 20 μ M Trolox.

Results. IL-39 promoted the production of intracellular reactive oxygen species (ROS) in a concentration-dependent manner in HL-1 cardiomyocytes. IL-39 and H₂O₂ both significantly promoted the production of intracellular ROS, increased the level of intracellular CCL2, stimulated the apoptotic progress of cardiomyocytes, increased the mRNA and protein expression levels of Bax, caspase-3, and p-p38 MAPK, and decreased the mRNA and protein expression levels of Bcl-2. ROS production, CCL2 level, cardiomyocyte apoptosis, and expression of Bax, caspase-3, and p-p38 MAPK were significantly amplified by the administration of IL-39 combined with H₂O₂, and these processes were significantly alleviated by an antioxidant Trolox.

Conclusion. This study was novel in revealing that IL-39 promoted apoptosis by stimulating the phosphorylation of p38 MAPK in mouse HL-1 cardiomyocytes. (*Folia Histochemica et Cytobiologica* 2021, Vol. 59, No. 3, 195–202)

Key words: apoptosis; HL-1 cardiomyocyte; interleukin-39; p38 MAPK; ROS

Introduction

Acute myocardial infarction is the most common cause of mortality and morbidity worldwide [1]. Rep-

erfusion therapy is the most effective procedure to save ischemic cardiomyocytes and limit infarct size [2]. However, the restoration of blood flow may aggravate the injury to the original ischemic myocardium, which is defined as myocardial ischemia–reperfusion injury (MIRI) and lead to myocardial stunning, hemodynamic abnormalities, ventricular arrhythmia, and development of heart failure [3]. The sophisticated pathophysiological process of MIRI needs to be clarified and methods need to be developed to improve the prognosis of these patients [4].

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The MIRI process involves excess reactive oxygen species (ROS) production, neutrophil aggregation, intracellular calcium overload, and mitochondrial dysfunction [5, 6]. The activation of immune system, including innate immunity and adaptive immunity, was found to be closely associated with MIRI [7, 8]. The members of the interleukin-12 (IL-12) family are critical cytokines mediating the inflammatory process and playing a critical role in immune responses [9]. As a novel member of the IL-12 family, IL-39 was recently reported to enhance inflammatory response through activating signal transducer and activator of transcription in lupus-like mice [10]. However, the relationship between IL-39 and MIRI has not been investigated yet.

The fate of cardiomyocytes in infarcted myocardium is either cellular necrosis or apoptosis. Cardiomyocyte apoptosis is the most common in ischemic regions close to the infarcted myocardium [11]. Our previous study found that the serum level of IL-39 significantly increased in patients with acute ST-segment elevation myocardial infarction (STEMI) and was related to left ventricular systolic dysfunction [12]. IL-17 and IL-23 contribute to cardiomyocyte apoptosis and MIRI [13, 14]; however, the role of IL-39 is worth investigating. This study examined the relationship between IL-39 and cardiomyocyte apoptosis in an *in vitro* model to better understand the role of IL-39 in cardiovascular diseases.

Materials and methods

Cell culture. The mouse HL-1 cardiomyocyte line obtained from Enzyme Research Biotech INC (China) was cultured in DMEM (Gibco, USA) with 10% FBS (Gibco), penicillin (100 unit/mL), and streptomycin (100 μ g/mL) in the humidified incubator in the atmosphere of 5% CO₂ at 37°C. The cells between passages 3 and 5 were employed for experiments. The cells seeded at a density of 5×10^4 cells/well were cultured for 24 h and subsequently treated with phosphate-buffered saline (PBS, vehicle), 0–100 ng/mL IL-39 (R&D, USA), 200 μ M H₂O₂ (Sigma–Aldrich, Germany), 60 ng/mL IL-39 combined with 200 μ M H₂O₂, and 20 μ M Trolox (Solarbio, China) for 24 h.

Intracellular ROS detection. A dichlorodihydrofluorescein diacetate (DCFH-DA) ROS assay kit (Beyotime Biotechnology, China) was employed to investigate intracellular ROS production in HL-1 cardiomyocytes following the manufacturer's protocol. In brief, the medium was discarded 24 h after the cells were cultured and treated using the method described previously. Then, 1.5 mL of DCFH-DA (10 μ M) solution was added, and the cells were subsequently incubated for 30 min at 37°C in the presence of 5% CO₂. The intracellular fluorescence intensities were detected at 488 and 525 nm under a fluorescence microscope (Olympus, Japan).

Enzyme-linked immunosorbent assay. HL-1 cardiomyocytes were cultured and treated with PBS, IL-39, H₂O₂, and/or Trolox. The supernatants of cultured cells were harvested and centrifuged at 2000 rpm for 20 min at 4°C 24 h after the treatment. The level of chemokine C-C motif ligand 2 (CCL2) was measured using an enzyme-linked immunosorbent assay (ELISA) kit (J&L Biotech, China) following the manufacturer's protocol.

Apoptosis assay. The apoptosis of HL-1 cardiomyocytes was analyzed by flow cytometry using a Dead Cell Apoptosis Kit with Annexin V Alexa Fluor 488 and PI (Thermo Fisher, USA). Briefly, the cells were collected and resuspended in 500 μ L of binding buffer, and 1 μ L of Annexin V and 5 μ L of PI were successively added to stain the cells 24 h after the treatment. The samples were determined using flow cytometry (BD Biosciences, USA). The percentage of apoptotic cells were calculated which represented Annexin V positive/ PI positive cells.

Quantitative real-time PCR. Total RNAs were isolated after the cells were treated for 24 h using an RNeasy Mini Kit (Qiagen, Germany) and reversed to cDNA using a One-Step PrimeScript miRNA cDNA Synthesis Kit (TaKaRa, Japan) to quantify target genes in HL-1 cardiomyocytes. Quantitative PCR of Bcl-2, Bax, caspase-3, and phosphorylated-p38 mitogen-activated protein kinase (p-p38 MAPK) were performed using an ABI 7500 Fast Real-Time PCR System (Applied Biosystems, USA) with an SYBR Premix Ex Taq kit (TaKaRa). U6 was used as an internal control. All primers were synthesized by Sangon (China). The relative expression of target genes was calculated using the following equation: Relative expression level = $2^{-(\Delta C_t \text{ sample} - \Delta C_t \text{ control})}$.

Western blot analysis. For Western blot analysis, 20 μ g of total protein extracted from the HL-1 cardiomyocytes after the cells were treated for 24 h was resolved on 10% SDS-polyacrylamide gels and electrotransferred onto nitrocellulose membranes. The membranes were blocked with 5% nonfat milk in TBS containing 0.3% Tween-20 and then incubated overnight with polyclonal rabbit anti-mouse antibodies against Bcl-2 (1:1000 dilution, Abcam, USA), Bax (1:500 dilution, Abcam), caspase-3 (1:1000 dilution, Cell Signaling, USA), and p-p38 MAPK (1:1000 dilution, Cell Signaling). The polyclonal rabbit anti-mouse GAPDH antibody (1:1000 dilution, Abcam) served as control. The goat anti-rabbit horseradish peroxidase-conjugated secondary antibody was subsequently added. ECL was administered to detect protein signals using Quant RT ECL cold CCD imaging system (General Electric, USA).

Statistical analysis. The statistical analyses were carried out using SPSS 16.0 (SPSS Inc, USA). Data were presented as mean \pm standard deviation. The mean value in the vehicle group was defined as 100% for relative gene expression.

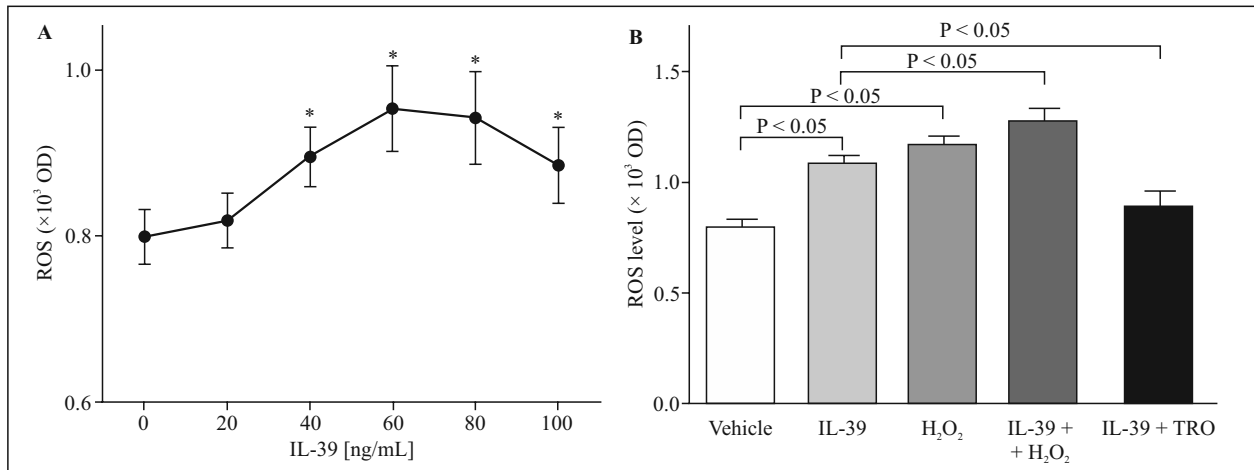


Figure 1. IL-39 promoted ROS production in HL-1 cardiomyocytes. **A.** IL-39 promoted ROS production in a concentration-dependent manner ($n = 6$); $P < 0.05$ vs. 0 ng/mL IL-39. **B.** IL-39 and H₂O₂ increased the ROS level in HL-1 cardiomyocytes ($n = 6$). ROS levels were determined as described in Methods section.

Overall data were compared using analysis of variance and *post-hoc* least significant difference statistics. A P value of less than 0.05 indicated a statistically significant difference.

Results

IL-39 promoted ROS production in HL-1 cardiomyocytes

Different concentrations of IL-39 were added to cultures of HL-1 cardiomyocytes to identify the relationship between ROS production and IL-39 levels. As shown in Figure 1A, IL-39 promoted ROS production in a concentration-dependent manner; the highest level of ROS production was induced by IL-39 at a concentration of 60 ng/mL (vs. 0 ng/mL IL-39, $P < 0.05$). Both 60 ng/mL IL-39 and 200 μ M H₂O₂ promoted ROS production in HL-1 cardiomyocytes (vs. vehicle, $P < 0.05$). The elevation of ROS production induced by 60 ng/mL IL-39 alone was amplified by the stimulation of IL-39 60 ng/mL combined with 200 μ M H₂O₂ (IL-39 + H₂O₂ vs. IL-39, $P < 0.05$), but was decreased by the antioxidant 20 μ M Trolox (IL-39 + TRO vs. IL-39, $P < 0.05$) (Fig. 1B).

IL-39 increased the level of CCL2 in HL-1 cardiomyocytes

The chemokine CCL2 levels in HL-1 cardiomyocytes were investigated using ELISA. Both IL-39 and oxidative stress significantly increased the level of CCL2 (vs. vehicle, $P < 0.05$). The elevation of CCL2 level in HL-1 cardiomyocytes induced by 60 ng/mL IL-39 alone was amplified by the addition of 200 μ M H₂O₂ (IL-39 + H₂O₂ vs. IL-39, $P < 0.05$), however, it was significantly decreased by 20 μ M Trolox (IL-39 + TRO vs. IL-39, $P < 0.05$) (Fig. 2).

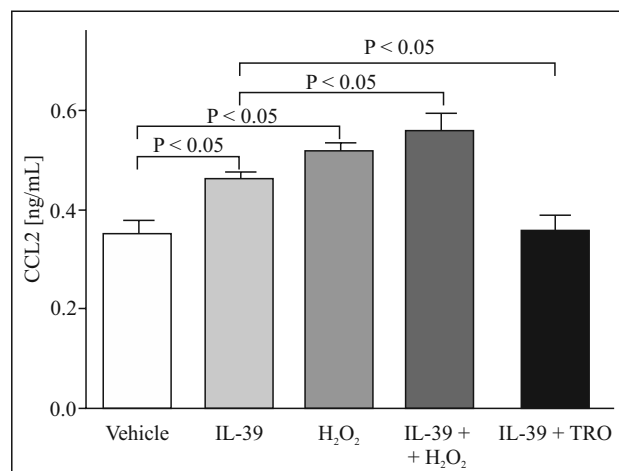


Figure 2. IL-39 increased the level of CCL2 chemokine in HL-1 cardiomyocytes incubated with IL-39 (60 ng/mL), 200 μ M H₂O₂ or IL-39 at 60 ng/mL and 20 μ M Trolox ($n = 6$). CCL2 concentration was determined by ELISA as described in Methods section.

IL-39 stimulates the apoptosis of HL-1 cardiomyocytes

Flow cytometry was used to evaluate the apoptosis of HL-1 cardiomyocytes, the real-time PCR and Western blot analyses were used to examine the mRNA and protein levels of Bcl-2, Bax and caspase-3. Both 60 ng/mL IL-39 and 200 μ M H₂O₂ induced significant apoptosis in HL-1 cells (vs. vehicle, $P < 0.05$). IL-39 demonstrated a more potent ability to promote HL-1 cardiomyocyte apoptosis (IL-39 vs. vehicle, $P < 0.01$). The apoptosis of HL-1 cardiomyocytes induced by 60 ng/mL IL-39 alone was amplified by 200 μ M H₂O₂ (IL-39 + H₂O₂ vs. IL-39, $P < 0.05$), and was

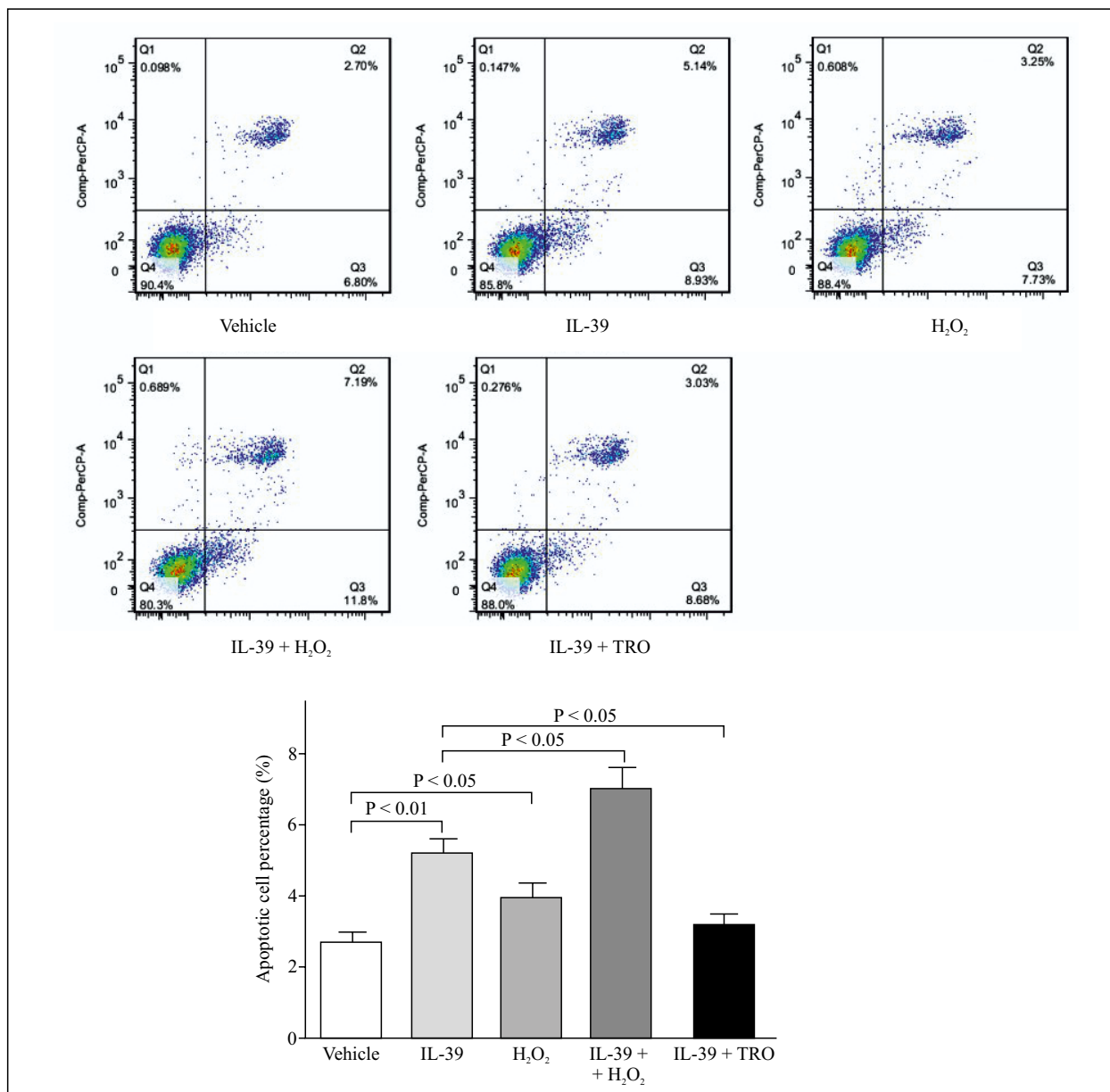


Figure 3. IL-39 promoted the apoptosis of HL-1 cardiomyocytes ($n = 6$). The concentrations of IL-39, H₂O₂, and Trolox were as in the description of Figure 2. Apoptosis was determined by flow cytometry as described in Methods section.

significantly alleviated by 20 μ M Trolox (IL-39 + TRO vs. IL-39, $P < 0.05$) (Fig. 3). The transcription level of Bcl-2 was significantly decreased by 60 ng/mL IL-39 and 200 μ M H₂O₂ (vs. vehicle, $P < 0.05$). The decreased transcription level of Bcl-2 in HL-1 cardiomyocytes induced by 60 ng/mL IL-39 alone was amplified by 200 μ M H₂O₂ (IL-39 + H₂O₂ vs. IL-39, $P < 0.01$), and was significantly alleviated by 20 μ M Trolox (IL-39 + TRO vs. IL-39, $P < 0.05$) (Fig. 4A and 4B). The transcription levels of Bax and caspase-3 were significantly elevated by 60 ng/mL IL-39 and 200 μ M H₂O₂ (vs. vehicle, $P < 0.05$). The increased transcription levels of Bax and caspase-3

in HL-1 cardiomyocytes induced by 60 ng/mL IL-39 alone were amplified by 200 μ M H₂O₂ (IL-39 + H₂O₂ vs. IL-39, $P < 0.05$), and were significantly alleviated by 20 μ M Trolox (IL-39 + TRO vs. IL-39, $P < 0.05$) (Fig. 4A, 4C, 4D).

IL-39 stimulates the phosphorylation of p38 MAPK in the apoptotic cardiomyocytes

To identify the potential molecular mechanism of IL-39 regulating the apoptosis of HL-1 cardiomyocytes, the mRNA and protein levels of phosphorylated p38 MAPK were examined using real-time PCR and Western blot analysis, respectively. As shown in

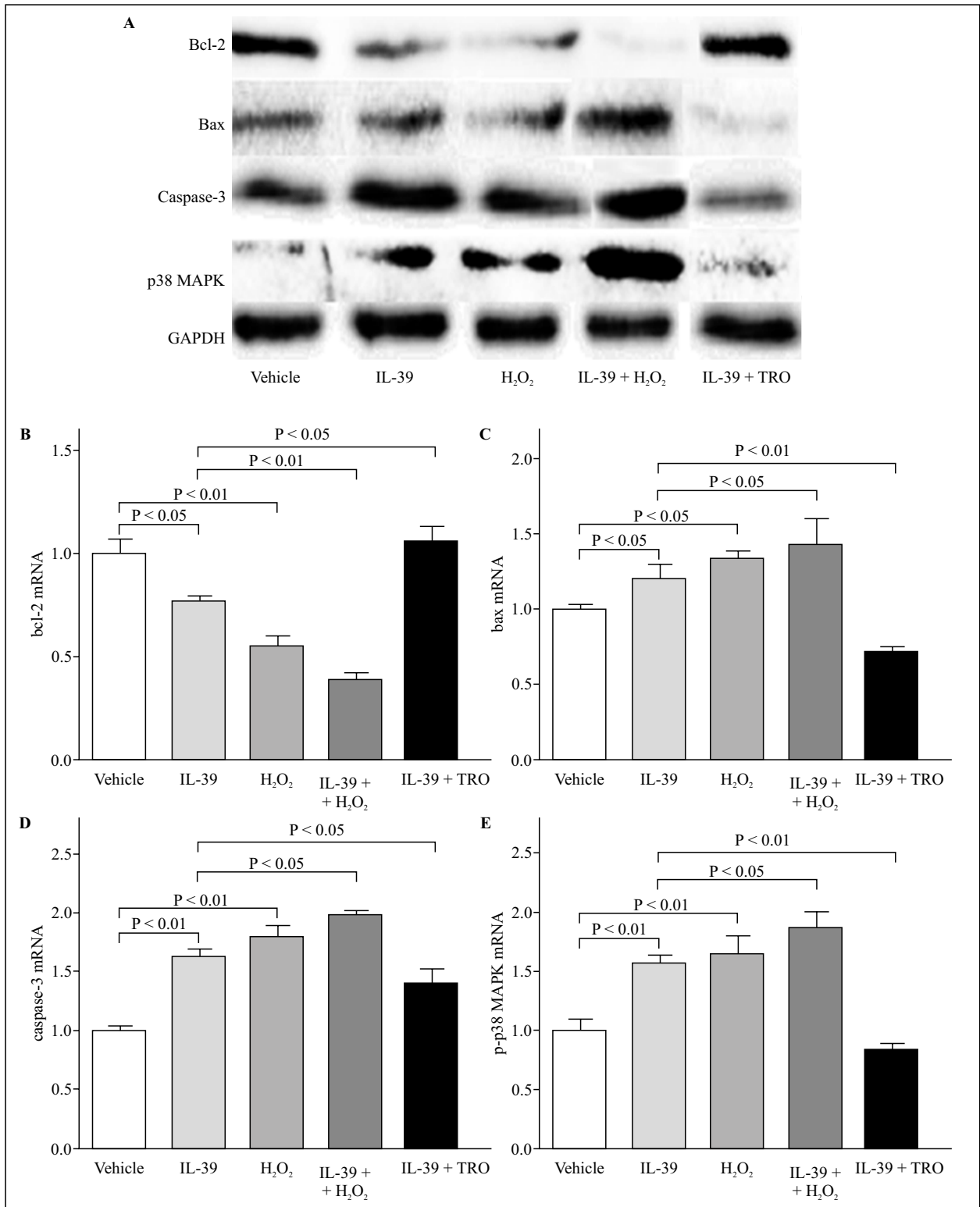


Figure 4. IL-39 promoted the expression of *p*-p38 MAPK in HL-1 cardiomyocytes. **A.** Protein levels in HL-1 cardiomyocytes were identified using Western blot analysis ($n = 3$). **B.** IL-39 significantly decreased the transcription level of Bcl-2 in HL-1 cardiomyocytes ($n = 6$). **C.** IL-39 significantly increased the transcription level of Bax in HL-1 cardiomyocytes ($n = 6$). **D.** IL-39 significantly increased the transcription level of caspase-3 in HL-1 cardiomyocytes ($n = 6$). **E.** IL-39 significantly increased the phosphorylation level of p38 MAPK in HL-1 cardiomyocytes ($n = 6$). The concentrations of IL-30, H₂O₂, and Trolox were as in the description of Figure 2.

Figure 4A and 4E, the transcription level of *p*-p38 MAPK was significantly elevated by 60 ng/mL IL-39 and 200 μ M H₂O₂ (vs. vehicle, $P < 0.05$). The increased transcription level of *p*-p38 MAPK in HL-1 cardiomyocytes induced by 60 ng/mL IL-39 alone was amplified by 200 μ M H₂O₂ (IL-39 + H₂O₂ vs. IL-39, $P < 0.05$), and was significantly alleviated by 20 μ M Trolox (IL-39 + TRO vs. IL-39, $P < 0.05$).

Discussion

In the previous study [12], we found that the serum level of IL-39 in patients with STEMI significantly increased, indicating that IL-39 might be involved in the process of myocardial injury and remodeling. The present study showed that IL-39 promoted ROS production, CCL2 secretion, and cardiomyocyte apoptosis; the underlying mechanism was related to the increased expression of *p*-p38 MAPK.

The MIRI is mediated by several factors including excessive ROS production. The increased accumulation of ROS in cardiomyocytes results in oxidative stress, mitochondrial dysfunction, and subsequent cell death [5]. Antioxidant therapies hold great potential in attenuating cardiac injury induced by ischemia-reperfusion [15]. The data revealed that IL-39 promoted ROS production in a concentration-dependent manner, highlighting its association with MIRI.

Inflammation response is the major pathological feature in cardiac repair after acute myocardial infarction [16]. Chemokines play a critical role in the acute phase of myocardial infarction. CCL2 plays crucial roles in recruiting inflammatory cells and contributes to cardiac remodeling after myocardial infarction [17–19]. The level of CCL2 was also elevated in cardiomyocytes incubated with IL-37 and patients with acute coronary syndrome; hence, CCL2 can be treated as a biomarker and a potential target for therapy [20–22]. The results showed that IL-39 significantly increased the CCL2 level in HL-1 cardiomyocytes. Interestingly, CCL2 protected mouse neonatal cardiac myocytes from hypoxia-induced apoptosis [23]. However, the role of CCL2 in cardiomyocyte apoptosis was not investigated in the present study. Hence, how IL-39 increased the CCL2 level and their association in cardiomyocyte apoptosis deserve further investigation.

Cellular apoptosis is initiated by the activation of cell-surface receptors (the extrinsic pathway) or by the alteration of mitochondria permeability (the intrinsic pathway) [24]. Accumulating evidence indicated the critical role of the Bcl-2 family in determining the cell death process through the extrinsic pathway. The Bcl-2 family comprises two classes of

regulatory proteins: pro-apoptotic members (Bak and Bax) and anti-apoptotic members (Bcl-2 and Bcl-xL) [25, 26]. At the intersection of the intrinsic pathway and the extrinsic pathway, caspase-3 is ultimately stimulated by pro-apoptotic signals and the apoptosis process is completed in the nucleus [27]. IL-39 significantly stimulated the apoptosis of HL-1 cardiomyocytes *in vitro* in this study; the ability to induce apoptosis was more potent than that of H₂O₂. The evidence that decreased the expression of Bcl-2 and increased the expression of Bax and caspase-3 supported the opinion that IL-39 promoted cardiomyocyte apoptosis through the extrinsic pathway.

MAPK families play important roles in a wide variety of cellular programs, including cell growth, proliferation, differentiation, and apoptosis [28, 29]. Characterized as a member of MAPK families, p38 MAPK is activated to balance cell survival and death in response to both extracellular and intracellular stresses [30]. Also, p38 MAPK regulated the phosphorylation of Bcl-2 in the early induction of apoptosis under cellular stress [31]. The cardiomyocyte apoptosis induced by hypoxia/reoxygenation was mediated by the ROS-activated MAPK pathway and inhibited by the p38 MAPK inhibitor [32]. The inhibition of p38 MAPK reduced cardiac injury and improved cardiac function after acute myocardial infarction, indicating its critical role in cardiac remodeling [33–35]. In mice with lupus-like phenotype, IL-39 promoted inflammatory response through the STAT1/STAT3 signaling pathway [10]. The present study found that IL-39-induced cardiomyocyte apoptosis involved the elevated expression of *p*-p38 MAPK. However, the upstream and downstream proteins of p38 MAPK were not explored in the present study. Whether p38 MAPK signaling plays a crucial role in IL-39-induced cardiomyocyte apoptosis is unclear. The role and function of IL-39 in physiological and pathological statuses still remain controversial [36] and require further investigation.

In conclusion, this study was novel in demonstrating that IL-39 promoted ROS production and stimulated the phosphorylation of p38 MAPK. Further studies should be accomplished to better understand the role of IL-39 and the molecular mechanism in the pathophysiological process of cardiovascular diseases and its prospects as a therapeutic target.

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Authors' contributions

WX and YL conceived the study. JL, JR, CN, and RL performed the experiments. FL and BH analyzed the data. WX drafted the manuscript. WX, HC, and YL revised the manuscript. All authors read and approved the final manuscript.

Availability of data and materials

All data generated or analyzed in this study are included in this manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Conflicts of interest

The authors declare no conflicts of interest.

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