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Original Article

Mesencephalic Astrocyte-Derived Neurotrophic Factor (MANF) Alleviates Sepsis-Induced Cardiomyopathy by Inhibiting Pyroptosis

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Abstract

Background: Sepsis-induced cardiomyopathy (SIC) is a common complication of sepsis accompanied by high prevalence and mortality in sepsis patients. Mesencephalic astrocyte-derived neurotrophic factor (MANF) is a neurotrophic factor, and it exerts critical functions in various diseases, including heart diseases, while its effect on SIC remains elusive. Hence, we aimed to investigate the action of MANF on SIC.

Methods: This study was under the guidance of Gongli Hospital, Shanghai, China from January 2021 to December 2021. H9c2 cells and mice were induced by LPS to establish SIC in vitro and in vivo models. qRT-PCR and Western blot were used to determine gene and protein expressions. The levels of MANF, Interleukin-1β (IL-1β), Interleukin 18 (IL-18), creatine kinase-MB (CK-MB), and cardiac troponin I (cTn I) were detected using ELISA assay. Cell pyroptosis determination was performed by flow cytometry. The DCFDA assay kit was used to determine ROS production.

Results: In SIC in vitro model, LPS induced cell pyroptosis (P<0.001) and ROS accumulation (P<0.001). Besides, MANF was decreased in LPS-induced H9c2 cells (P<0.001) and SIC patients (P<0.001). In addition, overexpression of MANF ameliorated SIC-induced injury in H9C2 cells (P<0.001). Furthermore, inhibition of NLRP3 rescued the function of MANF on SIC-induced injury in H9C2 cells (P<0.001). Moreover, enforced MANF suppressed the SIC-induced injury in vivo model (P<0.001).

Conclusion: MANF was down-regulated in SIC. Overexpressed MANF ameliorated the SIC injury by inhibiting NLRP3-mediated pyroptosis.

Keywords: Sepsis-induced cardiomyopathy; Pyroptosis

Introduction

Sepsis is a lethal disease caused by a dysregulated immune response (1). Sepsis can trigger multiple organ dysfunction (MOD) if not managed timely and properly (2, 3). Sepsis-induced cardiomyopa-

thy (SIC) is a common complication of sepsis featured by ventricular dilatation and decreased contractility and ejection fraction (4). The incidence of SIC in patients with sepsis ranges from 10 to 70% (5, 6). Besides, the mortality of patients with SIC was higher than patients without cardiomyopathy (7). Therefore, SIC is a significant threat to public health. It is essential to investigate the mechanism of SIC progression and develop new strategies for SIC therapy.

The pathological mechanism of SIC is complex. Inflammatory response, intracellular Ca^{2+} transporter dysregulation, energetic starvation, mitochondrial dysfunction, oxidative stress processes participated in the SIC progression (8, 9). Recently, cell pyroptosis, a unique form of inflammatory cell death mediated by the inflammasome, was also involved in the pathological process of SIC (10). For example, Irisin could alleviate LPS-stressed cardiac damage via suppressing inflammation and pyroptosis (10). Besides, deficiency of STING alleviated pyroptosis and LPS-induced cardiac dysfunction through activating NLRP3 (2). Thus, it may be helpful for SIC therapy via suppressing cell pyroptosis.

Mesencephalic astrocyte-derived neurotrophic factor (MANF) is a neurotrophic factor identified in recent years (11). Increasing evidence revealed that MANF exerted critical functions in various diseases (12-14). For example, MANF protected against ethanol-induced neurodegeneration through ameliorating endoplasmic reticulum stress (12). MANF suppressed liver cancer development by regulating the NF-κB/Snail signal pathway and epithelial-mesenchymal transition (13). It modulated mice's splenic macrophage differentiation (14). Besides, MANF also played essential roles in heart diseases (15, 16). MANF attenuated bacterial myocarditis by inhibiting NF-κB activation and myocardial inflammation (15). Loss of MANF increased cardiac damage during ischemia/reperfusion injury (16). Moreover, MANF was a negative regulator of LPS-mediated inflammation (17, 18).

Therefore, we conjectured that MANF might modulate SIC. However, there are few reports on the role of MANF in SIC at present. Hence, this study aimed to clarify the action of MANF on SIC progression and investigate the precise mechanism.

Methods

Human samples

The blood samples from 40 healthy volunteers and 40 sepsis-induced cardiomyopathy (SIC) patients were collected (from January 2021 to December 2021, in Gongli Hospital, Pudong New Area, Shanghai, China) to detect the MANF level.

All participants signed informed consent. Our research was authorized by the independent Ethics Committee of Gongli Hospital (No. GLYYIs2022-004) and was following the Declaration of Helsinki.

Cell culture

Mouse H9c2 cell line was bought from the Cell Bank of Shanghai Biology Institute (Shanghai, P.R. China). H9c2 cells were grown in DMEM medium with 10% FBS, 2 mM l-glutamine, and 1% penicillin/streptomycin (Thermo Fisher Scientific, Waltham, MA, USA) and maintained at a 37 °C, 5% CO_2 atmosphere. To establish the SIC model in vitro, H9c2 cells were induced by 1 μg/ml of LPS for 6 h. After LPS treatment, the H9c2 cells were harvested for further analysis.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA samples were separated using TRIzol Reagent (Invitrogen, Waltham, MA, USA) and reverse transcribed into cDNA by the cDNA synthesis kit (Thermo Fisher Scientific). In strict compliance with the supplier's protocols, DyNAmo Flash SYBR Green qPCR Kit (Thermo Fisher Scientific) was used to conduct quantitative PCR using the conditions: 95 °C for 10 minutes followed by 40 cycles of 95 °C for 15 seconds, 60 °C for 45 seconds. Relative gene expression was quantified using the $2^{-\Delta\Delta Ct}$ method. All data represent the average of three replicates. The primers sequences were: MANF

(NM_001108183.1), F: 5'-AATCGGTTGTGCTACTAC-3', R: 5'-ATCTGGCTGTCTTTCTTC-3'; GAPDH (NM_017008.4), F: 5'-GGAGTCTACTGGCGTCTTCAC-3', R: 5'-ATGAGCCCTTCCACGATGC-3'.

Western blot

Referring to our previous research (19), Western blot was uses to test the protein levels with the primary antibodies: MANF (A305-572A, Invitrogen, USA), NLRP3 (ab263899, Abcam, UK), active Caspase-1(ab138483, Abcam, UK), GSDMD-N (ab215203, Abcam, UK) and GAPDH (#5174s, CST, USA). An enhanced chemiluminescence system (Tanon, Shanghai, P.R. China) was used to detect protein expression value.

Cell transfection

Short hairpin interfering RNAs (shRNA) targeting human MANF (shMANF-1: 5'-GCGAAGTTTGTATTTCTTA-3', site: 145-163; shMANF-2, 5'- GAAGCAAGA GGCAAAGAAA-3', site: 252-270; and shMANF-3 5'-GGTTTGTAGTGTAAAGGAT-3', site: 509-527), along with a negative control siRNA (shNC, 5'-UUCUCCGAACGUGUC ACGUTT-3'), were generated (Major Industrial Co., Ltd, Shanghai, China) and constructed into lentiviral plasmids (pLKO.1).

For overexpression studies, the pLVX-puro lentiviral plasmid containing MANF (NM_001271681.2) cDNA was used along with a mock plasmid as a negative control (oeNC). Lipofectamine 3000 (Thermo Fisher Scientific, Waltham, MA, USA) was used to transfect plasmids into H9c2 cells following the manufacturer's instruction. After 48 h, cells were harvested for 48 h further analysis.

Enzyme-linked immunosorbent assay (ELISA)

After collecting cell culture media or serum, the levels of MANF, Interleukin-1β (IL-1β), Interleukin 18 (IL-18), creatine kinase-MB (CK-MB), and cardiac troponin I (cTn I) were determined using commercial ELISA kits in the manner of supplier's instruction (R&D Systems). The microplate reader (Pulangxin, China) was used to determine the OD 450 value.

Cell pyroptosis determination

To evaluate H9c2 cell pyroptosis, caspase-1 activation was quantified with a FAM-FLICA Caspase-1 Assay Kit (ImmunoChemistry Technologies) in the manner of supplier's instructions. Briefly, cells were harvested and incubated with FLICA for 1 h in the dark. Then, the cells were stained with propidium iodide (PI) for 20 min and measured using flow cytometry (BD FACSCalibur, Franklin Lakes, USA). The H9c2 cells positive for Caspase-1 and PI were identified as pyroptotic cells.

ROS determination

Cellular ROS production was detected using a DCFDA assay kit (Abcam) in the manner of the supplier's instructions. The cells were subjected to fluorospectrophotometric analysis and fluorescence microscopy in a Wallac 1420 microplate reader (PerkinElmer, Waltham, MA, USA) at an excitation wavelength of 488 nm and emission wavelength of 520 nm. The amount of intracellular ROS was proportional to DCF fluorescence intensity, which was recorded directly to indicate the relative amount of ROS. Relative changes in DCF fluorescence were expressed as fold increase over the control cells.

Establishment of sepsis-induced myocardial dysfunction (SMID) model

Male C57/B6 mice aged 8–10 weeks were acquired from Shanghai SLAC Laboratory Animal Co., Ltd. All mice were divided into Sham, SIMD-Vehicle, and SIMD-Re-MANF groups. The mice in the SIMD-Vehicle group were intraperitoneally injected with 10 mg/kg LPS to induce SMID. The mice in the Sham group were given an equal volume of saline. For mice in the SIMD-Re-MANF group, intramyocardial injection of 1×10^{11} recombinant protein MANF (Re-MANF) dissolved in 30μl PBS in five separate locations was administered before 4

weeks of 10 mg/kg LPS intraperitoneal injection. After the LPS challenge for 12 h, cardiac function was evaluated in the three groups.

Statistical analysis

Data were shown as mean ± standard deviation (SD) and analyzed using GraphPad Prism 8.4.2. Student's *t*-test and one-way ANOVA were employed to determine group differences. Survival rates were determined using the Kaplan-Meier analysis with the log-rank test. $P < 0.05$ was identified as significant difference.

Results

MANF was decreased in SIC patients and LPS-treated mouse H9c2 cells

To investigate the action of MANF in SIC, the MANF level was tested in blood samples of 40 SIC patients and healthy volunteers (Control) by ELISA assay. The results revealed that the levels of MANF also was down-regulated in SIC patients (Fig. 1A, $P<0.001$).

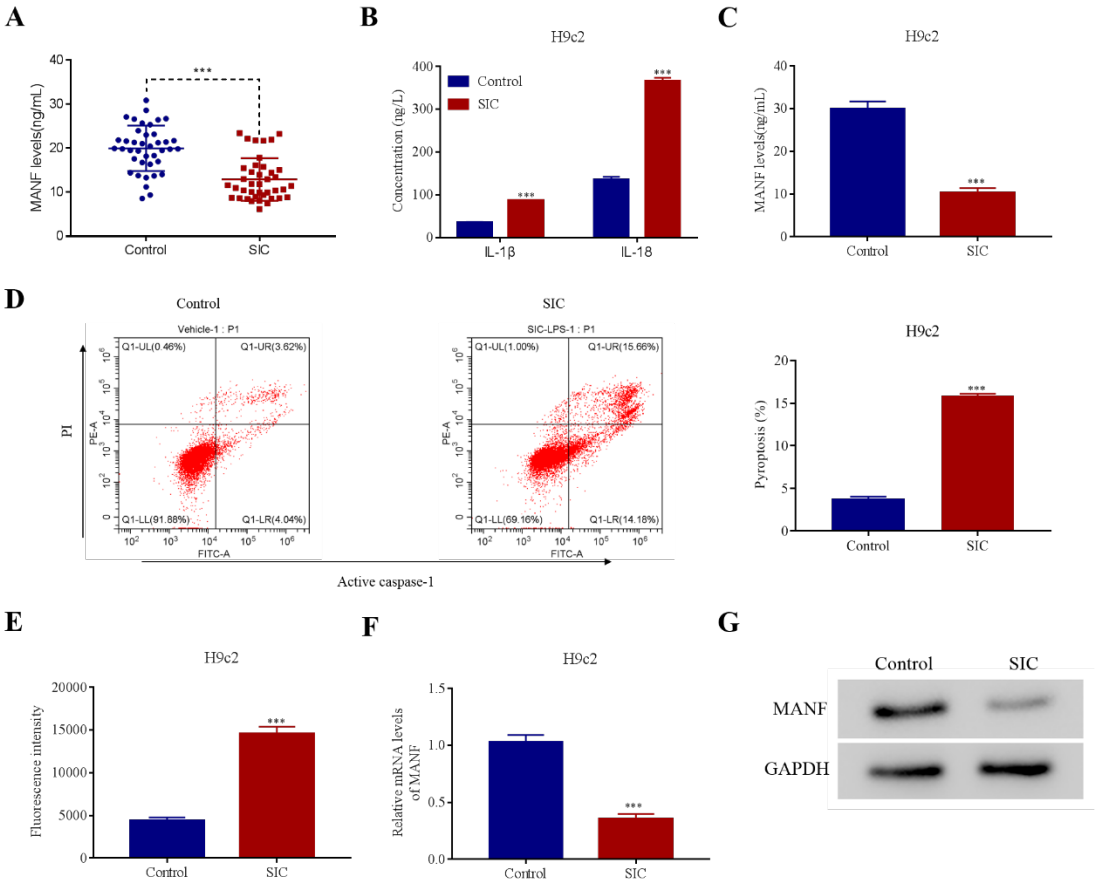

Fig. 1: MANF was decreased in SIC patients and LPS-treated mouse H9c2 cells. A. The levels of MANF in blood samples of 40 SIC patients were determined using ELISA assay. **B.** The secretions of IL-1β and IL-18 in LPS-induced H9c2 cells were detected using ELISA assay. **C.** The level of MANF in LPS-challenged H9c2 cells was determined. **D.** Cell pyroptosis of LPS-challenged H9c2 cells was measured by Flow cytometer. **E.** The accumulation of ROS was detected in LPS-induced H9c2 cells. **F.** The mRNA level of MANF in LPS-challenged H9c2 cells was analyzed by qRT-PCR. **G.** The protein level of MANF in LPS-challenged H9c2 cells was detected by Western blot. *** $P<0.001$

Furthermore, the in vitro SIC model was first constructed using H9c2 cells through the LPS challenge. It was observed that the secretions of IL-1β and IL-18 were significantly increased in the SIC cell model (Fig. 1B, $P<0.001$). The level of MANF in cell culture media was decreased in the SIC cell model (Fig. 1C, $P<0.001$). Besides, results showed that cell pyroptosis was induced in the SIC cell model (Fig. 1D, $P<0.001$). Furthermore, the accumulation of ROS in the SIC cell model was increased (Fig. 1E, $P<0.001$). Interestingly, the MANF mRNA and protein levels were significantly decreased in the SIC cell model (Fig. 1F and 1G, $P<0.001$). Therefore, MANF was decreased in SIC patients and LPS-treated mouse H9c2 cells.

Overexpression of MANF ameliorated SIC-induced injury in H9C2 cells

To validate the effect of MANF on SIC-induced injury, MANF overexpression plasmid was transfected into LPS-challenged H9c2 cells. The MANF overexpression plasmid significantly increased the MANF mRNA and protein levels (Fig. 2 A and B, $P<0.001$). Results showed that cell pyroptosis was induced in the SIC cell model but was decreased by overexpressed MANF (Fig. 2C, $P<0.001$). Besides, overexpression of MANF inhibited the LPS-induced levels of IL-1β, IL-18, cTn I, and CK-MB (Fig. 2D and 2E, $P<0.001$). Similarly, the LPS-induced ROS accumulation was suppressed by elevated MANF (Fig. 2F, $P<0.001$). Western blot results found that the protein level of MANF was decreased in LPS-treated H9c2 cells, which was elevated by MANF overexpression plasmid (Fig. 2G). Furthermore, the protein levels of NLRP3, Active caspase-1, and GSDMD-N were increased in H9c2 cells after the LPS challenge but were reversed by overexpressed MANF (Fig. 2G, $P<0.001$). Thus, overexpression of MANF ameliorated SIC-induced injury in H9C2 cells.

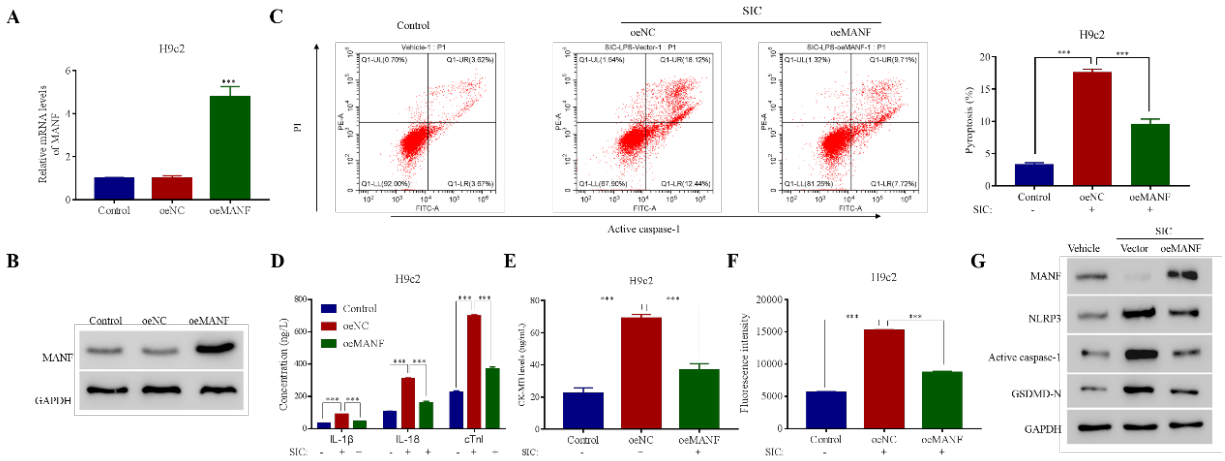

Fig. 2: Overexpression of MANF ameliorated SIC-induced injury in mouse H9C2 cells. A. The mRNA level of MANF in H9c2 cells after transfected with MANF overexpression plasmid was determined by qRT-PCR. **B.** The protein level of MANF in H9c2 cells after transfected with MANF overexpression plasmid was determined by Western blot. **C.** Cell pyroptosis in LPS-challenged H9c2 cells after transfected with MANF overexpression plasmid was measured by Flow cytometer. **D.** The levels of IL-1β, IL-18, and cTn I in LPS-challenged H9c2 cells were determined after transfected with MANF overexpression plasmid. **E.** The levels of CK-MB in LPS-challenged H9c2 cells with MANF overexpression were detected. **F.** The accumulation of ROS was detected in LPS-challenged H9c2 cells after transfected with MANF overexpression plasmid. **E.** The protein levels of MANF, NLRP3, Active caspase-1,

and GSDMD-N in LPS-challenged H9c2 cells with MANF overexpression were detected by Western blot. *** *P* <0.001

Inhibition of NLRP3 rescued the function of MANF in mouse H9C2 cells

To clarify the mechanism of MANF on SIC-induced injury, the NLRP3 inhibitor MCC950 was used to treat mouse H9C2 cells after the knockdown of MANF. The knockdown efficiency of three kinds of shMANF was confirmed by qRT-PCR and Western blot (Fig. 3 A and B, *P*<0.001). In view of the highest knockout efficiency of shMANF-1, shMANF-1 was used for subsequent experiments (Fig. 3 A and B). It was shown that knockdown of MANF increased cell pyroptosis, which was reversed by MCC950 (Fig. 3C, *P*<0.001). Besides, knockdown of MANF elevated the production of IL-1β and IL-18 in cell culture media of H9c2 (Fig. 3D, *P*<0.001). However, MCC950 suppressed the production of IL-1β and IL-18 (Fig. 3D, *P*<0.001). Furthermore, knockdown of MANF promoted the levels of Active caspase-1 and GSDMD-N in H9c2 cells but was inhibited by MCC950 (Fig. 3E, *P*<0.001). Hence, inhibition of NLRP3 rescued the function of MANF in mouse H9C2 cells.

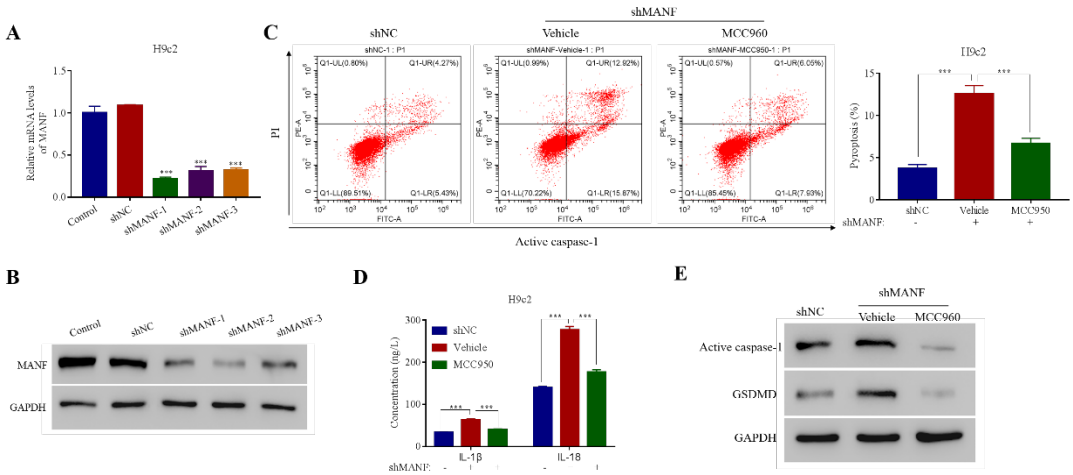

Fig. 3: Inhibition of NLRP3 rescued the function of MANF in mouse H9C2 cells. A. The mRNA level of MANF in H9c2 cells after transfected with sh-MANF was determined by qRT-PCR. **B.** The protein level of MANF in H9c2 cells after transfected with sh-MANF was determined by Western blot. **C.** Cell pyroptosis in H9c2 cells after transfected with MANF overexpression plasmid and treated with NLRP3 inhibitor MCC950 was measured by Flow cytometer. **D.** The levels of IL-1β and IL-18 in H9c2 cells after transfected with MANF overexpression plasmid and treated with NLRP3 inhibitor MCC950 were measured by ELISA assay. **E.** The protein levels of Active caspase-1 and GSDMD-N in H9c2 cells after transfected with MANF overexpression plasmid and treated with NLRP3 inhibitor MCC950 were detected by Western blot. *** *P* <0.001

The Re-MANF reduced the SIC-induced injury in vivo

To further investigate the role of MANF in SIC, the SMID mice model was established and treated by Re-MANF. Kaplan-Meier analysis results presented that the survival rate of SIMD mice was poor, which was significantly improved by overexpressed MANF (Fig. 4A). Besides, the elevated secretions of IL-1β and IL-18 in serum of SIMD mice were inhibited by overexpressed MANF (Fig. 4B, *P*<0.001). As expected, overexpressed MANF also suppressed CK-MB and cTn I levels in serum of SIMD mice (Fig. 4C and 4D, *P*<0.001). Taken together, the Re-MANF reduced the SIC-induced injury *in vivo*.

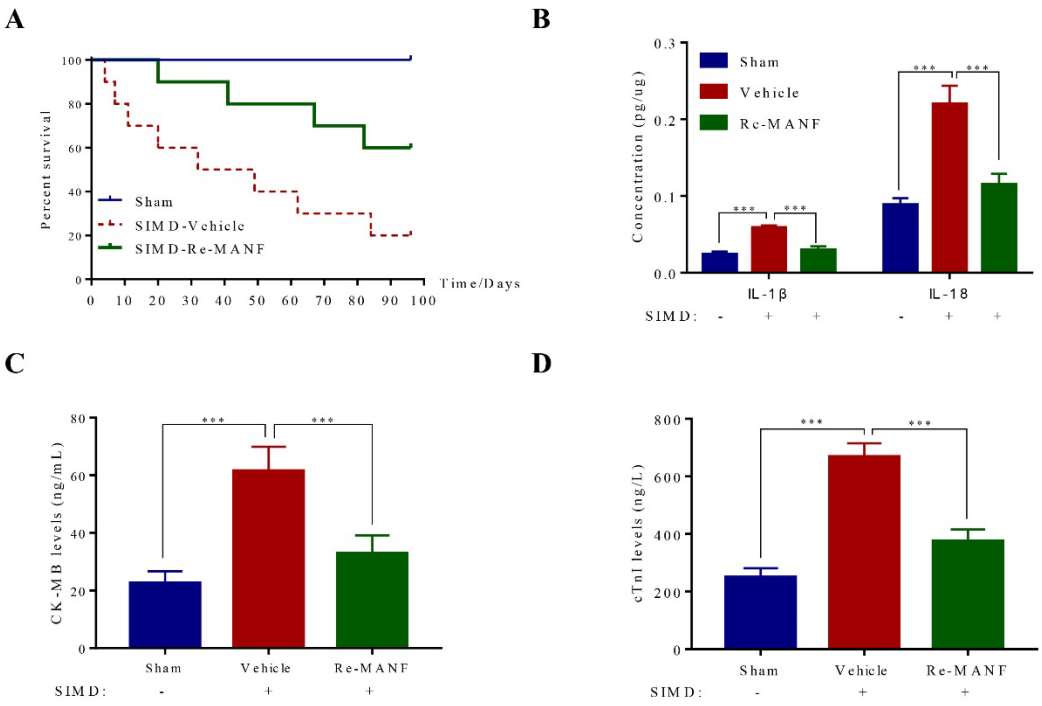

Fig. 4: The Re-MANF reduced the SIC-induced injury *in vivo*. A. The overall survival rate of the SIMD mice model with the treatment of Re-MANF was analyzed by the Kaplan-Meier method log-rank test. **B.** The levels of IL-1β and IL-18 in the SIMD mice model with the treatment of Re-MANF were assessed by ELISA assay. **C.** The levels of CK-MB in the SIMD mice model with the treatment of Re-MANF were determined. **D.** The levels of cTn I in the SIMD mice model with the treatment of Re-MANF were detected. *** P <0.001

Discussion

Sepsis is a severe disease caused by the uncontrolled immune response, and it usually induces multiple organ dysfunction such as SIC (1-3). SIC was characterized by ventricular dilatation and decreased contractility and ejection fraction, accompanied by high prevalence and mortality in sepsis patients (4-7). Therefore, it is critical to disclose the mechanism of SIC and explore new strategies for SIC therapy. MANF, a neurotrophic factor, exerted critical functions in various diseases, including heart diseases (12-16). However, the effect of MANF on SIC remained elusive. Hence, this study expounded the action of MANF on SIC.

The SIC in vitro model was constructed using H9C2 cells via the LPS challenge in the present study. Due to cell pyroptosis participating in the SIC injury development, cell pyroptosis was first determined in SIC in vitro model in this study. It was generally accepted that NLRP3 inflammasome recruited and activated caspase-1 via ASC, and activated caspase-1 could cleave GSDMD, IL-1β, and IL-18 to mature forms, thereby triggering cell pyroptosis (20, 21). Besides, ROS accumulation played a crucial role in the inflammasome activation (22, 23). Therefore, the expression of IL-1β and IL-18, the proportion of cell pyroptosis, and ROS accumulation were determined to evaluate cell pyroptosis in SIC in vitro model. We found that LPS induced cell pyroptosis and ROS accumulation in SIC in vitro model, which was consistent with the previous studies (2, 10, 24, 25). LPS enhanced cell pyroptosis in H9c2 cells (24). Bao *et al* revealed that LPS induced ROS accumulation in H9c2 cells (25). These findings suggested that SIC in vitro model was successfully established by LPS

in this study and cell pyroptosis and ROS accumulation were induced in this model.

To explore the role of MANF in SIC, MANF expression was determined in SIC in vitro model and the SIC patients. Interestingly, it was discovered that MANF was down-regulated in SIC in vitro model and the SIC patients. This is the first report that MANF is down-regulated in SIC to our knowledge. Given that cell pyroptosis was an inflammatory cell death form, and MANF was a negative regulator of LPS-mediated inflammation (17, 18, 26), we speculated that the differentially expressed MANF in SIC might regulate cell pyroptosis and SIC injury. Consistent with our conjecture, the results in this study showed that overexpression of MANF suppressed cell pyroptosis in the SIC vitro and vivo model. Up to now, this study firstly reported the regulation function of MANF on cell pyroptosis. The previous study revealed that the neurotrophic factor MANF regulated neuronal survival through directly interacting with its ER-located receptor IRE1α (27). Interestingly, hyperactivated IRE1α enhanced TXNIP mRNA stability, thereby activating NLRP3 inflammasome and promoting procaspase-1 cleavage and IL-1β secretion (28). Therefore, the regulation mechanism of MANF on cell pyroptosis in SIC may be mediated by IRE1α, which will be verified in the future study. Besides, overexpression of MANF also inhibited the levels of cTn I and CK-MB. cTn I and CK-MB levels were considered the biochemical marker of myocardial injury (29). In other words, overexpressed MANF alleviated SIC injury in vitro and *vivo* models. In addition, overexpressed MANF also improved the survival of SIC mice. The similar protective effect of MANF on heart diseases has been reported (15, 16). For example, deficiency of MANF increased LPS-caused inflammatory response in myocardium tissues and induced severe myocardial injury and lower survival rate of mice (15). These findings indicated the protection role of MANF in SIC injury. Furthermore, cardiomyocyte pyroptosis was demonstrated to promote myocardial injury (30). The treatment strategy targeting to suppress cell pyroptosis may be effective for myocardial injury prevention (31). In this study, MANF suppressed pyroptosis and myocardial injury, indicating that MANF might ameliorate the SIC injury through inhibiting pyroptosis.

To investigate the precise mechanism of MANF on SIC injury, the relationship between MANF and cell pyroptosis was further determined. NLRP3 was the crucial mediator of cell pyroptosis (20, 21). Therefore, cell pyroptosis was determined after suppressed NLRP3 and knockdown of MANF in the SIC in vitro model. The results suggested that inhibition of NLRP3 reversed the function of MANF in LPS-induced H9C2 cells. Based on this evidence, we concluded that MANF ameliorated the SIC through inhibiting NLRP3-mediated pyroptosis. Hence, the finding indicated that MANF might be a promising target for SIC therapy through suppressing cell pyroptosis.

Conclusion

MANF ameliorated the SIC by inhibiting NLRP3-mediated pyroptosis. MANF may be a promising target for SIC therapy via suppressing cell pyroptosis.

Journalism Ethical considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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Conflict of interest

The authors declare that there is no conflict of interest.

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