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Inhibition of SIRT1 by microRNA-9, the key point in process of LPS-induced severe inflammation

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ABSTRACT

Severe inflammation may lead to multiple organs dysfunction syndrome, which has a high mortality. MicroRNA is found participated in this process. In this study we developed a lipopolysaccharide-induced inflammation cell model on macrophages and a lipopolysaccharide-induced inflammation mouse model. It was found that during inflammation, microRNA-9 was increased, accompanied with the up-regulation of pro-inflammatory cytokines and anti-inflammatory cytokines. Down-regulation of microRNA-9 inhibited the up-regulation of inflammatory cytokines, promoted the up-regulation of anti-inflammatory cytokines and induced the remission of organ damage, showing a protective effect in inflammation. Bioinformatics analysis combined with luciferase reporter assay showed that SIRT1 was the target gene of microRNA-9. Transfection of microRNA-9 inhibitor could increase the level of SIRT1 and decrease the activation of NF-κB pathway in macrophages. Myeloid specific *sirt1* knockout mice were included and we found that lack of SIRT1 in mice macrophages led to aggravated inflammation, cell apoptosis and organ injury, and eliminated the protective property of microRNA-9 inhibitor. In conclusion, we demonstrated that inhibition of microRNA-9 could alleviate inflammation through the up-regulation of SIRT1 and then suppressed the activation of NF-κB pathway. This is a meaningful explore about the specific mechanism of microRNA-9 in inflammation.

1. Introduction

Inflammation is an adaptive process to the noxious stimuli [1,2]. Organ-specific receptors are activated during severe inflammation and this systemic reaction is called the acute-phase response [3]. In case the initial stimulus cleared unsuccessfully, a system-wide inflammation, even a systemic inflammatory response syndrome (SIRS) will be started, and finally lead to multiple organ dysfunction syndrome (MODS) [4]. MODS is defined as the progressive dysfunction of two or more organs and is responsible for the majority of the morbidity and mortality among patients in intensive care units [5,6]. MODS unleashes complicated systemic reactions which is initiated simultaneously by host pattern recognition and endogenous signals. Then multiple signal pathways are activated which ultimately lead to over-expression of cytokines, including TNF-α, IL-1, IL-6 and affect cellular metabolism [7–9]. MODS involves disorders of endothelial system, immune system and coagulation system [10]. It is a much more complex situation than inflammation and SIRS [11]. It is critical to control inflammation in early stage and to avoid the progression into SIRS and MODS.

NF-κB pathway regulates the expression of a large number of genes, which are crucial for the regulation of apoptosis, virus replication, tumorigenesis, inflammation and various autoimmune diseases [11–14]. Bacterial infection or LPS simulation activates NF-κB pathway which promotes the production of inflammatory cytokines [15]. Once NF-κB pathway activated, a great number of pro-inflammatory cytokines including IL-6 and IL-8 are over-expressed, which are symbols of MODS [16].

Plenty of endogenous signals including microRNAs (miRs) could activate NF-κB pathway. MiRs are non-coding RNAs which can target to mRNAs and lead to translation repression. MiRs have shown the effects to regulate inflammatory cytokines through several kinds of pathways including NF-κB [17–19]. Several miRs, such as miR-125b, miR-146a,

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miR-15a, and miR-16, could prevent NF-κB activation and prevent the translocation of the subunits to the nucleus [20,21]. It is reported that miR-9 is increased in pro-inflammatory macrophages (M1) [22–25]. Here we discuss the possible mechanism of microRNA-9 regulating severe inflammation.

2. Material and methods

2.1. Cell culture and drug administration

RAW 264.7 cells (murine macrophage cell line, ATCC, Manassas, VA) were cultured in endotoxin-free DMEM supplemented with 10% FBS (Excell Bio, China), 100IU/ml penicillin and 100 mg/ml streptomycin, and then maintained at 37 °C in a humidified incubator with 5% CO_2. LPS (Sigma-Aldrich, Saint Louis, MO, USA) was dissolved in phosphate-buffered saline (PBS). For LPS stimulation, cells were stimulated by LPS with a concentration of 1 μg/ml 6 h and 12 h after LPS stimulation, cells were harvested for PCR and western blot respectively. To inhibit SIRT1, EX527 (Selleck Chemicals, Houston, TX, USA) was used to stimulate macrophages at the concentration of 1 μM, cells were used for further experiments 24 h after disposal.

2.2. Cell transfection

The miR-9 mimic, miR-9 inhibitor as well as their negative control (GeneCopoeia, China) were transfected into RAW264.7 macrophages using Lipofectamine 2000 Transfection Reagent (Invitrogen, USA) according to the manufacturer's instructions. After 24 h, transfected cells were used for further experiments.

2.3. Quantitative real-time PCR (q-RT-PCR)

Total RNAs of cultured cells were extracted using RNA-isolation kit (Takara, Japan). 500 ng of total RNA was reverse-transcribed using Prime Script™ RT reagent Kit (Takara, Japan). The obtained cDNA was then amplified by the CFX Connect real-time system (BIO-RAD, US), using SYBR™ Premix Ex Taq™ Kit (Takara, Japan) with specific primers (Table 1). The PCR conditions were 95 °C for 30s, followed by 40 cycles of 95 °C for 30s, 60 °C for 10s, and elongation at 72 °C for 15s. The relative quantification of target gene was conducted using the $2^{-\Delta\Delta Ct}$ method, with GAPDH as an internal control. The method to quantify miR-9 was performed by stem-loop RT-PCR. MiR-9 and reverse primers were put at 65 °C for 5 min to form highly target-specific stem-loop structure. Then reverse transcriptase, RNase inhibitor, dNTPs and buffer were added for reverse transcription. Primer oligonucleotides were synthesized by Invitrogen. MicroRNA Reverse Transcription Kit and TaqMan MicroRNA assay kits (Takara, Japan) were used for qRT-PCR determination of miR-9, performed on CFX96 Real Time PCR Detection System (Bio-Rad, Hercules, CA, USA). U6 snRNA was used as the internal control to normalize sample input. The primer sequences for each gene were listed in Table 1.

Table 1
Primer sequences used for real-time-PCR analysis.

mRNA	forward primer	reverse primer
IL-1β	5′- tcctgtgtaatgaaagacggc-3′	5′-tgcttgtgaggtgctgatgta-3′
IL-6	5′-gggactgatgctggtgacaa-3′	5′-tccacgatttcccagagaaca-3′
TNF-a	5′-gaactggcagaagaggcact-3′	5′-catagaactgatgagagggagg-3′
IL-10	5′-tgctatgctgcctgctcttac-3′	5′-agaaagtcttcacctggctga-3′
CCL2	5′- gttaacgccccactcacctg -3′	5′-cccattccttcttggggtca-3′
SIRT1	5′-tattccacggtgctgaggta-3′	5′-cactttcatcttccaagggttc-3′
GAPDH	5′-gtgttcctacccccaatgtg-3′	5′-catcgaaggtggaagagtgg-3′

2.4. Western blotting

For short, 50 μg of total protein were subjected to SDS-PAGE and transferred onto PVDF membranes. Membranes were blocked with 5% non-fat milk at room temperature for 3h, incubated with primary antibodies specific to SIRT1 (1:1000, Abcam, Cambridge, UK), NF-κB p65 (1:1000, CST, USA), acetyl-NF-κB p65 (1:1000, CST, USA) and β-actin (1:1000, CST, USA) at 4 °C overnight. On the next day, membranes were incubated with HRP-conjugated secondary antibodies diluted at 1:3000 (Boster, Wuhan, China) at 37 °C for 1 h. Protein bands on the membrane were visualized with ECL Kit (Millipore, USA) using FluorChem FC system (Alpha Innotech). Results were presented as densitometric ratio between the protein of interest and the loading control (β-actin).

2.5. Animals

Healthy male C57B6/L mice, myeloid specific *sirt1* knockout mice (*sirt1*$^{-/-}$ mice) on C57B6/L background and littermate wild type mice (WT mice) weighing 20–25 g were included in this study. Mice about 6–8 weeks old were provided by Experimental Animal Center of the Fourth Military Medical University. All mice were fed *ad libitum* a standard diet and water throughout the study. They were housed separately and kept under standard conditions at room temperature (22–24 °C) under a 12:12 h light/dark cycle. All the protocols were carried out in accordance with the approved guidelines in the ethical permit that were accordance with the principles of ARRIVE approved by the Ethics Committee of Xijing Hospital, affiliated to the Fourth Military Medical University. In summary, we tried our best to lessen the numbers of animals involved and made sure that experienced researchers did the invasive manipulation. And during this process, mice were anaesthetized with isoflurane inhalation.

2.6. Mice model

Anaesthetized mice were intraperitoneally injected with LPS (Sigma-Aldrich, Saint Louis, MO, USA; 10 mg/kg) to induce inflammation. MiR-9 antagomiR or negative control was injected through cauda vein in 3 consecutive days (12.1 μmol/kg/day). LPS was injected 24 h after the last injection. Half of the mice were anaesthetized and sacrificed 24 h after LPS stimulation for detection of serum and the other half were sacrificed at 72 h to get organs tissues.

2.7. Blood and tissue acquirement and cytokines test

Mice were divided into two groups, for blood and tissue samples collection respectively. All blood was acquired 24 h after LPS injection from left ventricle of mice. Tissues from liver, kidney, cardiac and lung were obtained 72 h after LPS injection for H&E staining and TUNEL staining.

2.8. Serum examination

Blood samples were standing at room temperature for 30 min and then centrifuged for 15 min at 1000 × *g*. Serum was collected to detect levels of IL-1β, IL-6 and TNF-α using ELISA KIT (Nanjing Jiancheng, China).

2.9. Hematoxylin-eosin staining and TUNEL staining

Tissue specimens were fixed in 10% formalin, dehydrated in alcohol, embedded in paraffin, cut into 5 μm thickness sections and mounted. The sections were stained with hematoxylin and eosin (H&E) for the observation of pathological changes. Then degree of organ damage was evaluated by pathological expert according to scoring system. TUNEL staining was done according to the instruction of commercial TUNEL cell death detection kit (Roche, Switzerland). Stained slices

were observed and photographed under microscope (All-in-one FSX100, Olympus, Japan). Five high-magnification files of every slice were randomly selected. Every positive spot in DAB staining was counted as a cell and the brown spot overlapped with that was counted as apoptotic cell. The results were scored semi-quantitatively by averaging the number of apoptotic cells per field at × 400 magnification. Five fields were evaluated per tissue sample, and the results were presented as means ± SD.

2.10. Luciferase reporter assay

The 3′ untranslation region (UTR) fragments of SIRT1 containing the miR binding sites were amplified by PCR using the cDNA template obtained from RNA sample of macrophages. The wide type 3′ UTRs of SIRT1 as well as mutant 3′ UTRs with nucleotide substitutions in the putative binding sites corresponding to the seed sequence of miR-9 was cloned downstream of the firefly luciferase gene in the pGL3 vector (Promega, Madison, WI). Cells were co-transfected with miR-9 or a control miR. 48 h later, cells were washed in PBS and the luciferase activity was measured by a luminometer (Promega, Madison, WI), using dual luciferase reporter assay system.

2.11. Statistical analysis

Single blind method was used to generate data. The data were analyzed using Mann–Whitney *U* tests using the SPSS 18.0 program and presented as mean ± SD. $p < 0.05$ was considered statistically significant.

3. Results

3.1. MiR-9 is up-regulated in LPS-stimulated macrophages

To simulate inflammation environment *in vitro*, macrophages stimulated by 1 μg/ml LPS were used. As shown in Fig. 1, the mRNA levels of IL-1β, IL-6, TNF-α, CCL2 and IL-10 were significantly increased after LPS stimulation ($p < 0.01$) (Fig. 1a–b). In addition, the level of miR-9 was also increased in LPS-stimulated macrophages ($p < 0.01$) (Fig. 1c).

Macrophages were cultured for 6 h in medium alone (Control group) or in the presence of 1 μg/ml LPS (LPS group). The levels of IL-1β, IL-6, TNF-α, CCL2, IL-10 (a-b) and the level of miR-9 (c) were tested by RT-PCR. n = 4. **, $p < 0.01$.

3.2. Suppression of miR-9 decreased the inflammation induced by LPS in macrophage

To investigate the role of miR-9 *in vitro*, miR-9 inhibitor was transfected in macrophage. As shown in Fig. 2, the level of miR-9 was significantly down-regulated after miR-9 inhibitor transfection ($p < 0.01$) (Fig. 2a). The mRNA levels of pro-inflammatory cytokines TNF-α, IL-1β, IL-6, CCL2 and anti-inflammatory cytokines IL-10 were significantly increased after LPS stimulation ($p < 0.01$). After miR-9 suppression, the levels of those pro-inflammatory cytokines were significantly decreased ($p < 0.01$) (Fig. 2b), while the level of anti-inflammatory cytokine, IL-10, was significantly increased ($p < 0.01$) (Fig. 2c).

Macrophages were divided into four groups randomly: NC group (20 μM inhibitor NC), NC + LPS group (20 μM inhibitor NC + 1 μg/ml LPS), miR-9 in group (20 μM miR-9 inhibitor) and miR-9 in + LPS group (20 μM miR-9 inhibitor + 1 μg/ml LPS). The level of miR-9 was tested 6 h after LPS stimulation by RT-PCR (a). The levels of IL-1β, IL-6, TNF-α, CCL2 and IL-10 were tested 6 h after LPS stimulation by RT-PCR (b-c). n = 4. ##, $p < 0.01$ compared with NC group. **, $p < 0.01$ compared with NC + LPS group.

3.3. Inhibition of miR-9 suppressed inflammation and improved organ damage induced by LPS in mice

To verify the role of miR-9 *in vivo*, LPS-induced inflammation mice model was used. LPS was intraperitoneally injected into mice pretreated with miR-9 antagomiR or antagomiR NC to stimulate the acute organic damage induced by inflammation. Serum and tissue specimens were harvested 24 h and 72 h after injection of LPS separately. As shown in Fig. 3, inflammatory cytokines including IL-1β, IL-6 and TNF-α in blood were reduced by miR-9 inhibitor ($p < 0.01$) (Fig. 3a). Down-regulation of miR-9 alleviated organ damage, since there was obviously reduced infiltration of inflammatory cells, alleviated edema and hemorrhage, and relatively complete structures seen in specimen after H&E staining (Fig. 3b). What's more, TUNEL staining of organ tissues showed that the apoptosis of cells of mice in NC group were much more apparently than that of mice in miR-9 antagomiR group. The apoptotic ratio of heart, liver, lung and kidney in NC group were

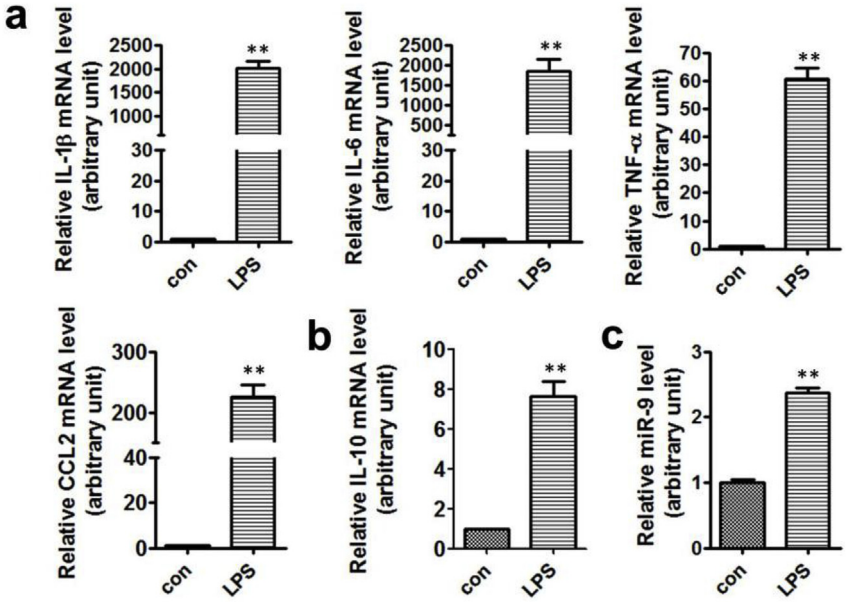

Fig. 1. MiR-9 is up-regulated in LPS-stimulated macrophages.

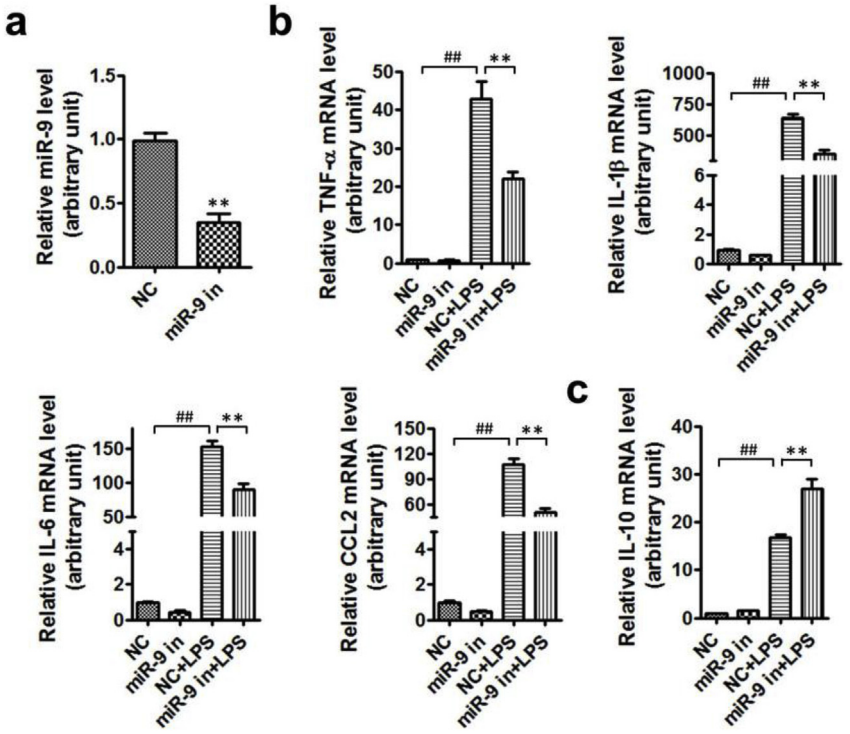

Fig. 2. Suppression of miR-9 decreased the inflammation induced by LPS in macrophages.

21.7 ± 0.5%, 19.5 ± 0.3%, 27.0 ± 0.2% and 17.1 ± 0.3% separately. The data in miR-9 antagomiR group were 16.2 ± 0.3%, 11.5 ± 0.5%, 20.0 ± 0.3% and 11.2 ± 0.3% separately (n = 6) ($p < 0.01$) (Fig. 3c).

Mice were divided into two groups randomly: NC + LPS group (12.1 μmol/kg/day antagomiR NC + 10 mg/kg LPS) and miR-9 antagomiR + LPS group (miR-9 in + LPS group) (12.1 μmol/kg/day miR-9 antagomiR + 10 mg/kg LPS). The level of IL-1β, IL-6, TNF-α in

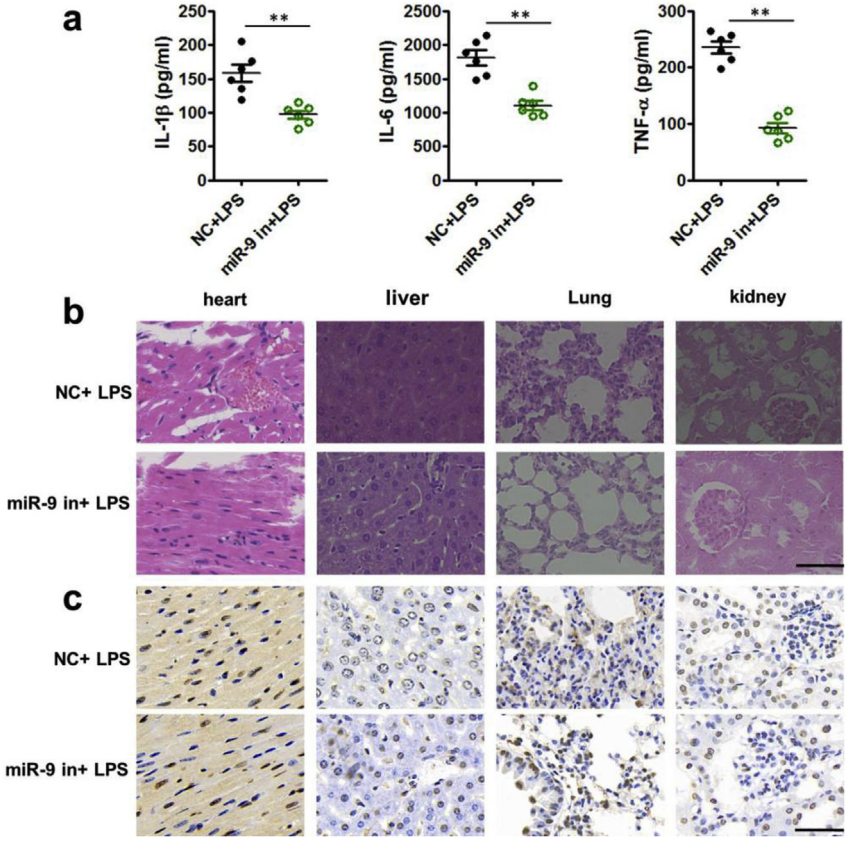

Fig. 3. Inhibition of miR-9 suppressed inflammation and improved organ damage induced by LPS in mice.

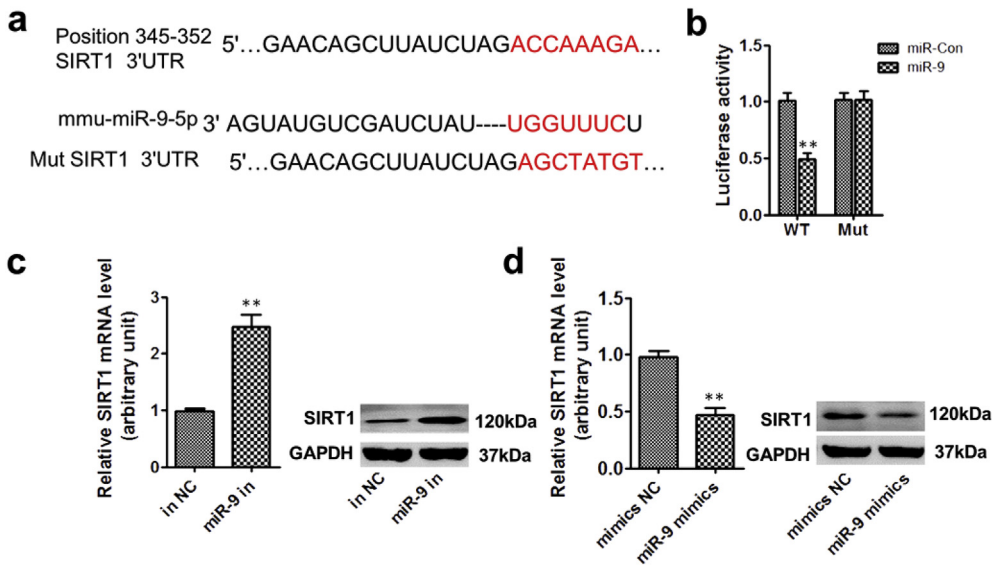

Fig. 4. SIRT1 is a target gene of miR-9.

serum were tested 24 h after LPS injection by microplate reader (a). H&E staining of tissue from heart, liver, lung and kidney were performed 72 h after LPS injection to assess the extent of damage (b). The TUNEL assay was performed 72 h after LPS injection to evaluate the apoptosis in heart, liver, lung and kidney (c). Scale bar = 50 μm n = 6. **, $p < 0.01$.

3.4. Bioinformatics analysis combined with luciferase reporter assay indicated that SIRT1 is a target of miR-9

To predict the potential targets of miR-9, databases of TargetScan, PicTar and miRanda were used. As shown in Fig. 4a, SIRT1 is a potential target of miR-9, for the 3'-UTR of SIRT1 mRNA could bind to miR-9 completely. Then luciferase reporter was used to confirm the binding of miR-9 and SIRT1. As shown in Fig. 4b, the luciferase reporter assay showed a 55% decrease in the relative luciferase activity of the wild-type SIRT1 3'-UTR vector after miR-9 mimics transfection in macrophages ($p < 0.01$). In contrast, the mutant construction completely abolished this effect, suggesting that miR-9 could directly bind to SIRT1 3'-UTR and inhibit SIRT1 expression. Furthermore, the level of SIRT1 increased after the administration of miR-9 inhibitor while decreased by miR-9 mimics ($p < 0.01$) (Fig. 4c–d), which was consistent with bioinformatics analysis and luciferase reporter assay.

Bioinformatics analysis of SIRT1 and miR-9 (a). Luciferase reporter assay showed a 55% decrease in the relative luciferase activity of the wild-type SIRT1 3'-UTR vector after miR-9 mimics transfection while the mutant construct completely abolished this effect (b). Macrophages were divided into two groups randomly: in NC group (20 μM inhibitor NC) and miR-9 in group (20 μM miR-9 inhibitor), the level of SIRT1 was detected by RT-PCR and western blotting 6 h and 12 h respectively after LPS stimulation (c). Macrophages were divided into two groups randomly: mimics NC group (20 μM mimics NC) and miR-9 mimics group (20 μM miR-9 mimics), the level of SIRT1 was detected by RT-PCR and western blotting 6 h and 12 h respectively after LPS stimulation (d). n = 4. **, $p < 0.01$.

3.5. MiR-9 affected the deacetylation of acetyl-NF-κB by regulating the expression of SIRT1

To see whether SIRT1 participates in the protective property induced by miR-9 inhibitor in LPS-induced macrophage, EX527 was used. As shown in Fig. 5, The mRNA level (Fig. 5a) as well as protein level (Fig. 5b) of SIRT1 in NC group were much higher than those in NC + LPS group ($p < 0.01$), but much lower than those in miR-9 in + LPS group ($p < 0.01$). Furthermore, western blot showed that the acetylation of NF-κB induced by LPS can be inhibited by miR-9 inhibitor ($p < 0.01$), while EX527 could partially impairment this effect ($p < 0.01$), indicated that the anti-inflammatory property of miR-9 inhibitor by deacetylation of acetyl-NF-κB is SIRT1-depended (Fig. 5c).

Macrophages were divided into four groups randomly as Fig. 2 mentioned. The levels of SIRT1 in each group were detected by RT-PCR and western blotting 6 h and 12 h respectively after LPS stimulation. (a-b). Macrophages were divided into six groups randomly: NC group (20 μM inhibitor NC), NC + LPS group (20 μM inhibitor NC + 1 μg/ml LPS), miR-9 in group (20 μM miR-9 inhibitor), miR-9 in + LPS group (20 μM miR-9 inhibitor + 1 μg/ml LPS), EX527 group (1 μM EX527) and EX527 + LPS group (1 μM EX527 + 1 μg/ml LPS). (c) The levels of acetyl-NF-κB and NF-κB in macrophages in each group were detected by western blotting. n = 4. $^{\#\#}$, $p < 0.01$ compared with NC + LPS group. **, $p < 0.01$ compared with miR-9 in + LPS group.

3.6. The myeloid specific $sirt1^{-/-}$ mice suffered from much severe inflammation and organ injury than WT mice after LPS injection

To further verify the possible mechanism *in vivo*, $sirt1^{-/-}$ mice and WT mice were used. Mice were given LPS and miR-9 antagomiR as described in mice model. Blood samples were collected to detect the levels of IL-1β, IL-6 and TNF-α. The levels of IL-1β, IL-6 and TNF-α increased significantly in $sirt1^{-/-}$ mice compared with WT mice (Fig. 6a) ($p < 0.01$). With the same dose of LPS injection, $sirt1^{-/-}$ mice suffered much more serious organ injuries compared with WT mice as is shown in H&E staining. In TUNEL staining, the ratios of apoptotic of myocardium, liver, lung and kidney were about 19.5 ± 0.2%, 18.2 ± 0.5%, 22.3 ± 0.5% and 19.0 ± 0.3% separately in WT mice. The ratios were 23.2 ± 0.3%, 21.5 ± 0.5%, 27.6 ± 0.7% and 22.4 ± 0.4% separately in $sirt1^{-/-}$ mice (Fig. 6c) ($p < 0.01$). Mice were pretreated with miR-9 antagomiR through cauda vein injection in 3 consecutive days (12.1 μmol/kg/day). LPS (10 mg/kg) was injected 24 h after the last injection. Half of the WT and $sirt1^{-/-}$ mice were anaesthetized and sacrificed 24 h after LPS stimulation for detection of IL-1β, IL-6 and TNF-α in serum by microplate reader (a). Tissues from heart, liver, lung and kidney were acquired from the other half anaesthetized and sacrificed mice at 72 h after LPS stimulation. H&E staining was used to assess organ damage (b). TUNEL staining of organ tissues was used to assess the extent of cell apoptosis (c). Scale bar = 50 μm n = 6. **, $p < 0.01$.

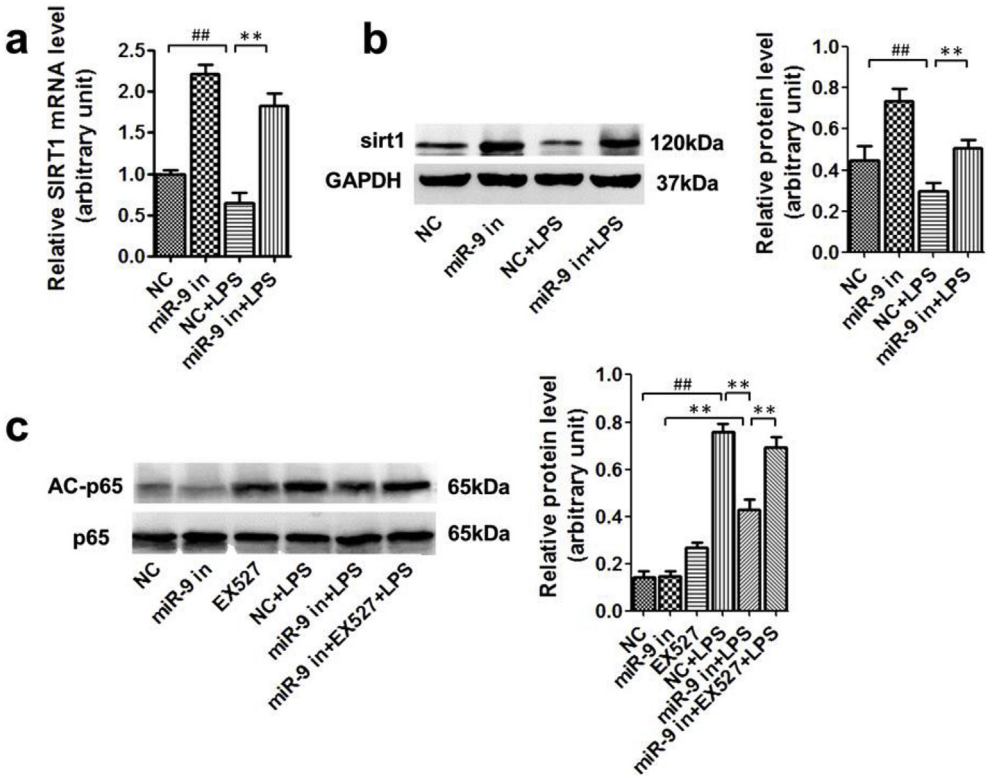

Fig. 5. MiR-9 affected the deacetylation of acetyl-NF-κB by regulating the expression of SIRT1.

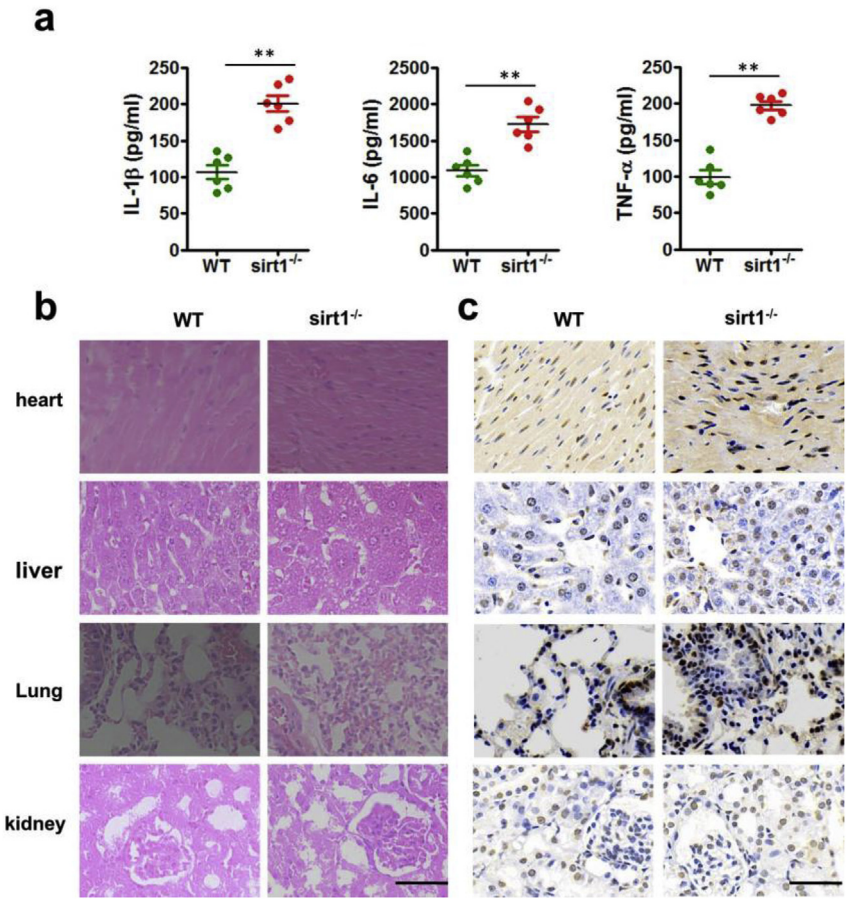

Fig. 6. The myeloid specific *sirt1*$^{-/-}$ mice suffered from much severe inflammation and organ injury than WT mice after LPS injection.

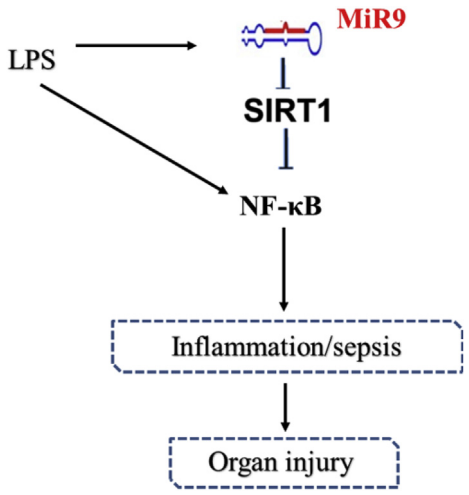

Fig. 7. MiR-9-SIRT1 axis participated in LPS-induced organ injury.

4. Discussion

Severe inflammation and MODS greatly threat patients' life. The increasing prevalence of drug resistant compels us to find new and effective approaches for the diagnosis and therapy of severe inflammation [26]. MicroRNAs such as miR-9 have been reported to be involved in inflammation and to play an important role in preventing inflammation developing to MODS, while the specific mechanism is still unclear [27–30]. In this study, we explored the potential effects of miR-9 on multiple organs injury in inflammation and the mechanism involved. Macrophages participate in the regulation of inflammatory responses and protect organ function damage [31,32]. We constructed an inflammation macrophages model and found the expression of pro-inflammation cytokines as well as anti-inflammatory cytokine increased significantly in LPS induced macrophages. MiR-9 also increased in this process, consistent with results drawn from previous study [27]. So we suspected that miR-9 might play a role in inflammation of LPS stimulated macrophages. To testify that, miR-9 inhibitor was used and the results showed that the suppression of miR-9 could inhibit inflammation and alleviate tissue damage induced by LPS.

MiRs have emerged as key regulators of the posttranscriptional control of gene expression by recognizing the specific sites in the 3'UTR of target mRNAs. Both tumor suppressor and oncogenic roles of miR-9 have been deeply reported in tumors, by binding to corresponding genes, such as E-cadherin, SOCS5, HES1, CXCR4, TLN1, NF-κB1 and so on [33–37]. Apart from tumor, miR-9 also plays an irreplaceable role in many kinds of inflammation. During viral infection, miR-9 was reported to act as a intermediary in NF-κB activation induced by nucleocapsid protein of human coronavirus OC43 [38]. In human atherosclerosis inflammation cell models, miR-9 inhibits NLRP3 inflammasome activation through JAK1/STAT signaling pathway [24]. In knee osteoarthritis, down-regulated miR-9 facilitate proliferation and anti-apoptosis of chondrocytes by directly binding to NF-κB1 [39]. During inflammatory response following cerebral ischemia, miR-9 is down-regulated and up-regulated miR-9 relief inflammatory injury by inhibit the expression and nuclear translocation of NF-κB p65 [40], While others demonstrate that MCPIP1 is the direct target of miR-9 in microglial and the LPS-induced miR-9 promote microglial activation by MCPIP1-NF-κB pathway [25]. In addition, the impacts of miR-9 on macrophage polarization were also noticeable [41–43]. MiR-9, the only miRNA (out of the 365 tested) consistently induced by LPS in a MyD88- and NF-κB-dependent manner in both monocytes and polymorphonuclear neutrophils [27], has been shown to promote M1 polarization by target peroxisome proliferator-activated receptor δ (PPARδ) [23]. Furthermore, miR-9 mediated down-regulation of Dusp6 and enhanced ERK-mediated signal transduction was reported to involved in classical activation of CCL2 pre-treated macrophages [44].

As declared above, the mechanisms of miR-9 involvement in multiple pathological processes have been studied in depth over the past years. However, understanding of connection between miR-9 and inflammation is far from enough. Numerous studies have implicated the involvement of NF-κB signaling in sepsis and the direct interaction of miR-9 and the 3'UTR of NF-κB1 is well illustrated, whether there is a intermediary between them attracts our interest. So TargetScan, PicTar and miRanda were used to explore the key gene in the downstream of miR-9 and found that SIRT1, a NAD^+ dependent deacetylase enzyme, which was broadly reported to be a crucial gene in inflammation [45,46], was the potential target of miR-9. We found that down-regulation of miR-9 could increase the level of SIRT1, and vice verse. Furthermore, luciferase reporter assay verified that SIRT1 was the target of miR-9. All in all, we have enough evidence to accept that SIRT1 was the downstream gene of miR-9.

Acetylation and deacetylation are important mechanism to regulate the activity of NF-κB, and the imbalance of acetylation and deacetylation plays an important role in the occurrence and development of many diseases [47–49]. It is well demonstrated that activation of SIRT1 leads to deacetylation of Re1A/p65, thereby leading to transcriptional repression of various inflammation-related genes [50,51], consistent with our results in LPS induced macrophages. To further verify the miR-9-SIRT1-NF-κB pathway *in vivo*, myeloid specific *sirt1* knockout mice were used. It showed that the protective effects of miR-9 inhibitor were abolished because of the lack of SIRT1. Levels of pro-inflammatory cytokines increased and the pathological injury aggravated in *sirt1*$^{-/-}$ mice than those of WT mice.

In conclusion, we demonstrated that miR-9 plays an important role in severe inflammation and MODS. Inhibition of microRNA-9 could promote the deacetylation of Re1A/p65 and eventually alleviate inflammation and organ injuries by up-regulating the expression of SIRT1 (Fig. 7). This is a potential target for the prevention and treatment of inflammation.

LPS leads to the up-regulation of miR-9 and causes uncontrolled inflammation and MODS, ended with multiple organ failures and death. Inhibition of miR-9, which targets to SIRT1, could promote the deacetylation of NF-κB and improve the organ injuries caused by inflammation.

Competing interests' statement

None.

Author contribution

Wen Yin and Xiaozhi Bai conceived and designed the experiments; Mengyuan Cao, Lincheng Li and Mingchuan Liu performed the experiments; Junjie Li, Wanfu Zhang and Julei Zhang analyzed the data; Wanfu Zhang wrote the paper, Wen Yin and Xiaozhi Bai revised the paper.

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