Original Article

Innate Immunity

Innate Immunity
2021, Vol. 27(5) 377–387

DOI: 10.1177/17534259211034221
journals.sagepub.com/home/ini
SAGE

Long non-coding RNA ZFAS1 alleviates sepsis-induced myocardial injury via target miR-34b-5p/SIRT1

Dan-Dan Chen[1], Hong-Wu Wang[1] and Xing-Jun Cai[2]

Abstract

Long non-coding RNA ZFAS1 is down-regulated in sepsis. However, whether ZFAS1 participates in sepsis-induced cardiomyopathy (SIC) remains largely unknown. LPS injection to rats was used to establish an *in vivo* sepsis model, while LPS stimulation with H9C2 cell was used to mimic an *in vitro* sepsis-induced myocardial injury model. Western blots and quantitative RT-PCR were performed to evaluate protein and mRNA levels, respectively. ELISA was conducted to determine cytokine levels in supernatant. Flow cytometry was used to test apoptosis. Dual-luciferase assay was performed to validate binding between ZFAS1 and miR-34b-5p, miR-34b-5p and SIRT1. Our data revealed that ZFAS1 and SIRT1 were down-regulated, while miR-34b-5p was up-regulated in LPS-induced H9C2 cells. Inhibition of miR-34b-5p or overexpression of ZFAS1 alleviated inflammatory response and cell apoptosis in LPS-stimulated H9C2 cells. A mechanism study revealed that ZFAS1 sponged miR-34b-5p and thus elevated expression of SIRT1, which was prohibited by miR-34b-5p. ZFAS1 alleviated inflammatory response and cell apoptosis in LPS-stimulated H9C2 cells via the miR-34b-5p/SIRT1 axis, providing novel potential therapeutic targets for SIC.

Keywords

lncRNA ZFAS1, miR-34b-5p, SIC, sepsis, SIRT1

Date received: 14 January 2021; revised: 1 July 2021; accepted: 5 July 2021

Introduction

With life-threatening organ dysfunction, sepsis is defined as a comprehensive systemic disease.[1] Sepsis always induces multiple complications in its clinical appearance, including cardiomyopathy. It has been demonstrated that sepsis-induced cardiomyopathy (SIC) contributes significantly to patient mortality.[2] Several mechanisms have been unveiled in sepsis-induced myocardial injury, including extravagant inflammatory response and cardiomyocytes apoptosis. Thus, investigating the regulation of these two processes might provide novel therapeutic interest for SIC.[3]

Long non-coding RNA (lncRNA) comprises a set of long RNAs (>200 nt) that have no ability to encode protein.[4] Increasing evidence has revealed that dys-regulated circulating lncRNAs could be biomarkers for sepsis.[4] In addition, the regulatory function of lncRNAs has also been studied in the progress of sepsis, including SIC. It was reported that by regulating miR-24 and thus elevating XIAP, lncRNA CYTOR alleviated septic myocardial injury.[5] A recent study also found that lncRNA KCNQ1OT1 has a critical role in attenuating myocardial injury in sepsis.[6] Moreover, lncRNA ZFAS1 is a recently identified lncRNA with high expression in several cancer types.[7] A clinical observational study reported low lncRNA ZFAS1 in sepsis patients. Expression of lncRNA ZFAS1 was negatively correlated with cytokine levels and disease severity and might have good diagnostic value in sepsis patients.[8] However, whether

[1]Department of Critical Care Medicine, Haikou Hospital, Xiangya Medical College, Central South University, P.R. China
[2]Department of Pulmonary and Critical Care Medicine, Hainan General Hospital, P.R. China

Corresponding author:
Xing-Jun Cai, Department of Pulmonary and Critical Care Medicine, Hainan General Hospital, No.19, Xiuhua Road, Haikou 570311, Hainan Province, P.R. China.
Email: hkcxj1974@163.com

ZFAS1 has a regulatory role in sepsis, especially SIC, requires further investigation.

MicroRNA (miRNA) is another type of non-coding RNA with a length of 21–25 nt and exhibiting post-transcription via mRNA degradation.[9] Similar to lncRNA, many miRNAs have also been demonstrated to be a vital regulator or indicator in sepsis.[10,11] Specifically, a broadly conserved miRNA, miR-34b-5p, was reported to promote LPS-aggravated acute lung injury via regulating progranulin.[12] Another study reconfirmed this role of miR-34b-5p by proving that lncRNA TUG1 alleviates septic acute lung injury via competitively binding with miR-34b-5p.[13] Based on these previous studies, it is possible that inhibition of miR-34b-5p also benefits sepsis-induced myocardial injury.

In the present study, we found that ZFAS1 was down-regulated, while miR-34b-5p was up-regulated in LPS-induced myocardial injury. Overexpression of ZFAS1 alleviated the inflammatory response and apoptosis in LPS-treated cardiomyocytes. The mechanic study revealed that ZFAS1 exerted these protective effects via sponging miR-34b-5p. Thus, up-regulated miR-34b-5p suppressed SIRT1. Our work might provide novel insight to reveal the mechanism and treatment of SIC.

Methods

Cell culture and LPS induction

Rat cardiomyocyte H9C2 was purchased from Cell Bank of Chinese Academy of Sciences (Shanghai, P. R. China) and was cultured in DMEM (Invitrogen, Carlsbad, CA) with 10% FBS (Gibco, Grand Island, NY), 100 IU/ml penicillin and 100 μg/ml streptomycin (Invitrogen). Cells were maintained in a humidified incubator with 5% CO_2 at 37°C. Upon achieving 80–90% confluence, cells were used for the indicated assays. To mimic *in vitro* sepsis-induced myocardial injury, 10 μg/ml LPS (Sigma–Aldrich, St Louis, MO) was added to the cultured medium for the indicated period.

Animal model

Male Sprague Dawley rats (weighing 200–230 g) were purchased from Hunan SJA Laboratory Animal (Changsha, Hunan, P.R. China) and were raised in a specific pathogen-free (SPF) environment on a 12 h light/12 h dark cycle at 25°C and with access to standard food and water *ad libitum*. To generate a rat SIC model, rats received a sublethal dose of LPS (5 mg/kg) intraperitoneally. Rats in the control group received an equal volume of saline. Twelve h after LPS injection, rats were sacrificed by sodium pentobarbital (200 mg/kg). The heart tissue was collected from each group. This animal study was reviewed and approved by the Ethical Committee of Central South University.

Plasmid construction and transfection

siRNA for ZFAS1, overexpression plasmid of ZFAS1 (pcDNA3.1-ZFAS1) and SIRT1(pcDNA3.1-SIRT1), miR-34b-5p inhibitor, miR-34b-5p mimics and respective negative controls (siNC, pcDNA3.1, NC inhibitor, NC mimics) were synthesised and purchased from RiboBio (Guangzhou, P.R. China). Lipofectamine 3000 was purchased for H9C2 cell transfection (Invitrogen, Shanghai, P.R. China) according to the manufacturer's instructions. Forty-eight h after transfection, cells were applied to subsequent experiments.

Quantitative RT-PCR

Total RNA from the rats' heart tissues or H9C2 cells was extracted by Trizol reagent (Invitrogen). A commercial reverse transcription system (Takara, Dalian, P.R. China) was used to synthesise cDNA from purified total RNA. qRT-PCR was conducted using a SYBR premix EX Taq II kit (Takara) on a thermocycler (ABI-7500). The sequences of primers used are listed in Table 1. U6 was used as an internal control for miR-34b-5p. GAPDH was used as an internal control for other indicated genes. The relative expression of a target gene was determined by the $2^{-\Delta\Delta Ct}$ method.

Table 1. Sequences of primers (5′-3′).

Primer	Forward	Reverse
ZFAS1	GCTATTGTCCTGCCCGTTAG	TCGTCAGGAGATCGAAGGTT
miR-34b-5p	GGGTAGGCAGTGTCATTAGC	AACAACCAACACAACCCAAC
SIRT1	TGCCGGAAACAATACCTCCA	AGACACCCCAGCTCCAGTTA
TNF-α	GGGGCCACCACGCTCTTCTGTC	TGGGCTACGGGCTTGTCACTCG
IL-1β	CCAGGATGAGGACCCAAGCA	TCCCGACCATTGCTGTTTCC
IL-6	TAGCCGCCCCACACAGACAG	GGCTGGCATTTGTGGTTGGG
U6	CTCGCTTCGGCAGCACA	AACGCTTCAGAATTTGCCT
GAPDH	GAGTCAACGGATTTGGTCGTT	TTGATTTTGGAGGGATCTCG

Flow cytometry for cell apoptosis

H9C2 cells were seeded on 6 cm plates at a density of 2×10^6. After transfection and/or LPS stimulation for the indicated time, the apoptosis rate was determined with an annexin V/propidium iodide apoptotic kit (Beyotime, Jiangsu, P.R. China) according to the manufacturer's instructions.[14] Briefly, H9C2 cells were collected and rinsed twice with cold PBS. The washed cells were re-suspended in binding buffer. Then, FITC-Annexin V and propidium iodide were added to the buffer for 10 min in dark incubation at 25°C. After staining, the cell mixture was loaded in a flow cytometry system. The apoptosis rate was calculated as a sum of late apoptosis (upper-right quadrant) and early apoptosis (down-right quadrant).

ELISA of inflammatory cytokines

H9C2 cells was subjected to ELISA after transfection and/or LPS stimulation. Cytokines were detected in the supernatant of the H9C2 culture system. Briefly, the supernatant of cultured cells was collected using ELISA kits (Boster Biological Technology, Wuhan, P.R. China) according to the manual.

Western blotting

H9C2 cells or heart tissues were lysed by RIPA buffer on ice, and the concentration of total protein was determined with a BCA kit (Beyotime). Equalised 20 μg protein samples were then loaded in SDS-PAGE gel (10%) and subjected to electrophoresis, and then the gels were transferred onto PVDF membrane. Blocking was performed by incubation membrane with 5% non-fat milk-PBS at 25°C for 1 h. After blocking, the membranes were incubated with primary Ab at 4°C overnight. Information of primary Abs were shown as follows: SIRT1 (#8469, 1:1000; Cell Signaling Technology, Danvers, MA), cleaved caspase-3 (#9664, 1:1000; Cell Signaling Technology), Bcl-2 (#8469, 1:1000;Cell Signaling Technology), Bax (#ab196495, 1:1000; Abcam, Cambridge, MA) and β-actin (#4970, 1:1000; Cell Signaling Technology). The next day, the membrane was probed by HRP-conjugated secondary Ab (1:10,000' Beyotime) at 25°C. After the ECL substrate was added, bands were photographed using an imaging system (ChemiScope 6000; Clinx, Shanghai, P.R. China). ImageJ Software was used to perform the quantification, and statistical analysis was done based on at least three bands from the independent sample. β-Actin was used as an internal control.

Dual-luciferase reporter assay

Binding sites between ZFAS1 and miR-34b-5p, miR-34b-5p and SIRT1 were predicted by Starbase v2.0. 3′ UTR containing predictive sequences of wild type (wt) ZFAS1 or wt SIRT1 or mutated (mut) ZFAS1 or mut SIRT1 was inserted into a pGL3 vector (Promega, Madison, WI). Mutated binding sites were generated by using a QuikChange Mutagenesis kit (Stratagene, La Jolla, CA). All constructs were sequenced to verify the sequence. H9C2 was co-transfected with modified pGL3 vector, and NC mimics or NC inhibitor, miR-34b-5p inhibitor, miR-34b-5p mimics. After 48 h, the activity of luciferase was determined. Renilla luciferase activity was used for normalisation.

Statistical analysis

Data are shown as the mean ± SD. Before statistical analysis, each group of data was tested for normality and homogeneity of variance, and the experimental data in this study were all in line with homogeneity of variance and normal distribution. Data between the two groups were analysed by Student's *t*-test. One-way ANOVA followed by Tukey's *post hoc* test was used for multiple comparisons. $P < 0.05$ was considered as statistical significance. All statistical analysis was conducted using GraphPad Prism v8.0 (GraphPad Software, La Jolla, CA).

Results

Dys-regulated expression of ZFAS1, miR-34b-5p and SIRT1 in LPS-induced myocardial injury

First, we aimed to confirm the dys-regulated of ZFAS1, miR-34b-5p and SIRT1 in hearts taken from LPS-treated rats and LPS-treated H9C2 cells. The results showed that ZFAS1 in sepsis group was significantly down-regulated compared to that in the control group, whereas miR-34b-5p was elevated in the sepsis group (Figure 1a). SIRT1 is a well-known protective protein in sepsis.[3,15] We also confirmed that LPS attenuated SIRT1 in heart tissue compared to the control group (Figure 1b). The expression profile was also validated in H9C2 cells. LPS stimulation led to an attenuation of ZFAS1 and SIRT1 and an elevation of miR-34b-5p compared to the control group (Figure 1c and d). These results confirmed the dys-regulation of ZFAS1, miR-34b-5p and SIRT1 in LPS-induced myocardial injury.

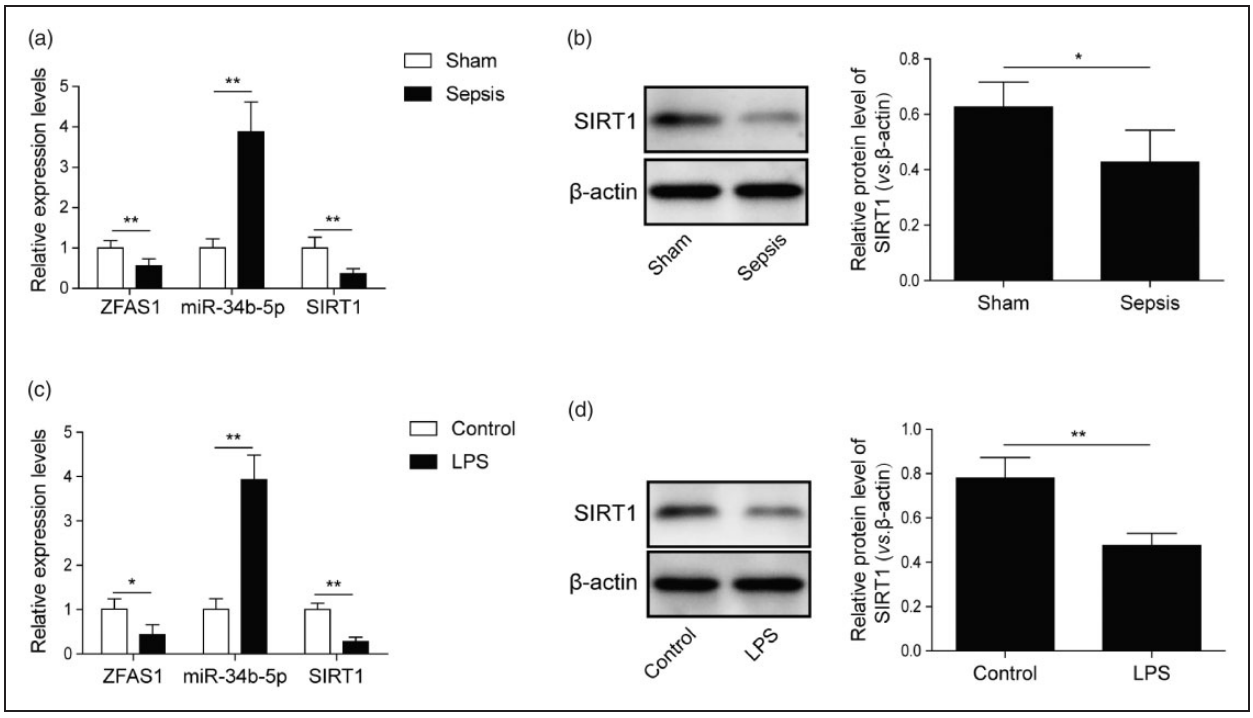

Figure 1. Dys-regulated expression of ZFAS1, miR-34b-5p and SIRT1 in LPS-induced myocardial injury. (a and b) Rats in the LPS group ($n = 5$) received a sublethal dose of LPS (5 mg/kg) intraperitoneally; rats in the control group ($n=3$) received and equal volume of saline. Twelve h after injection, the heart tissue from each group was collected. (a) Quantitative RT-PCR was used to detect the expression of ZFAS1, miR-34b-5p and SIRT1. (b) Western blot was used to detect the protein expression of SIRT1. (c) and (d) LPS (10 μg/ml) was added to the cultured medium of H9C2 for 12 h. (c) Quantitative RT-PCR was conducted to evaluate the expression of ZFAS1, miR-34b-5p and SIRT1. (d) Western blot was used to detect protein expression of SIRT1. Data are shown as the mean ± SD from three independent experiments. *$P < 0.05$; **$P < 0.01$.

Knockdown of miR-34b-5p alleviates inflammatory response and cell apoptosis in LPS-stimulated H9C2 cells

To investigate whether miR-34b-5p participates in LPS-induced myocardial injury, we used a miR-34b-5p inhibitor to knock down miR-34b-5p in H9C2 cells. The efficiency of transfection was evaluated by quantitative RT-PCR. The level of miR-34b-5p was inhibited in the miR-34b-5p inhibitor group (Figure 2a). Forty-eight h after transfection, H9C2 cells were stimulated with LPS for 12 h. As shown in Figure 2b and c, quantitative RT-PCR and ELISA showed that LPS substantially elevated the levels of TNF-α, IL-1β and IL-6 compared to the control group, while miR-34b-5p knockdown inhibited LPS-induced releasing of those cytokines. An apoptosis assay showed that LPS stimulation resulted in an elevated apoptosis rate, which was significantly attenuated by miR-34b-5p inhibition (Figure 2d). These results were further supported by Western blots. LPS stimulation resulted in an increased expression of pro-apoptotic marker Bax and cleaved caspase 3 while down-regulating anti-apoptotic marker, Bcl-2. Silencing of miR-34b-5p largely compromised this expression pattern induced by LPS (Figure 2e). Therefore, in this part, we validated that target miR-34b-5p might benefit LPS-induced myocardial injury.

miR-34b-5p directly targets SIRT1

To investigate the mechanism by which miR-34b-5p promoted inflammatory response and cell apoptosis in LPS-induced myocardial injury, we predicted the target genes of miR-34b-5p through Starbase v2.0. The results showed that miR-34b-5p could bind to the 3′-UTR of SIRT1 (Figure 3a). Furthermore, the results of the dual-luciferase reporter assay revealed that the relative luciferase activity of reporter containing the wt SIRT1 sequence was restrained in a miR-34b-5p mimics group compared to NC mimics group, whereas the relative luciferase activity of reporter containing the mutated SIRT1 sequence was not influenced by miR-34b-5p mimics (Figure 3b). To determine further whether miR-34b-5p attenuated expression of SIRT1, miR-34b-5p inhibitor or miR-34b-5p mimics were transfected into H9C2 cells to depress or overexpress the expression of miR-34b-5p (Figure 3c). Results of quantitative RT-PCR

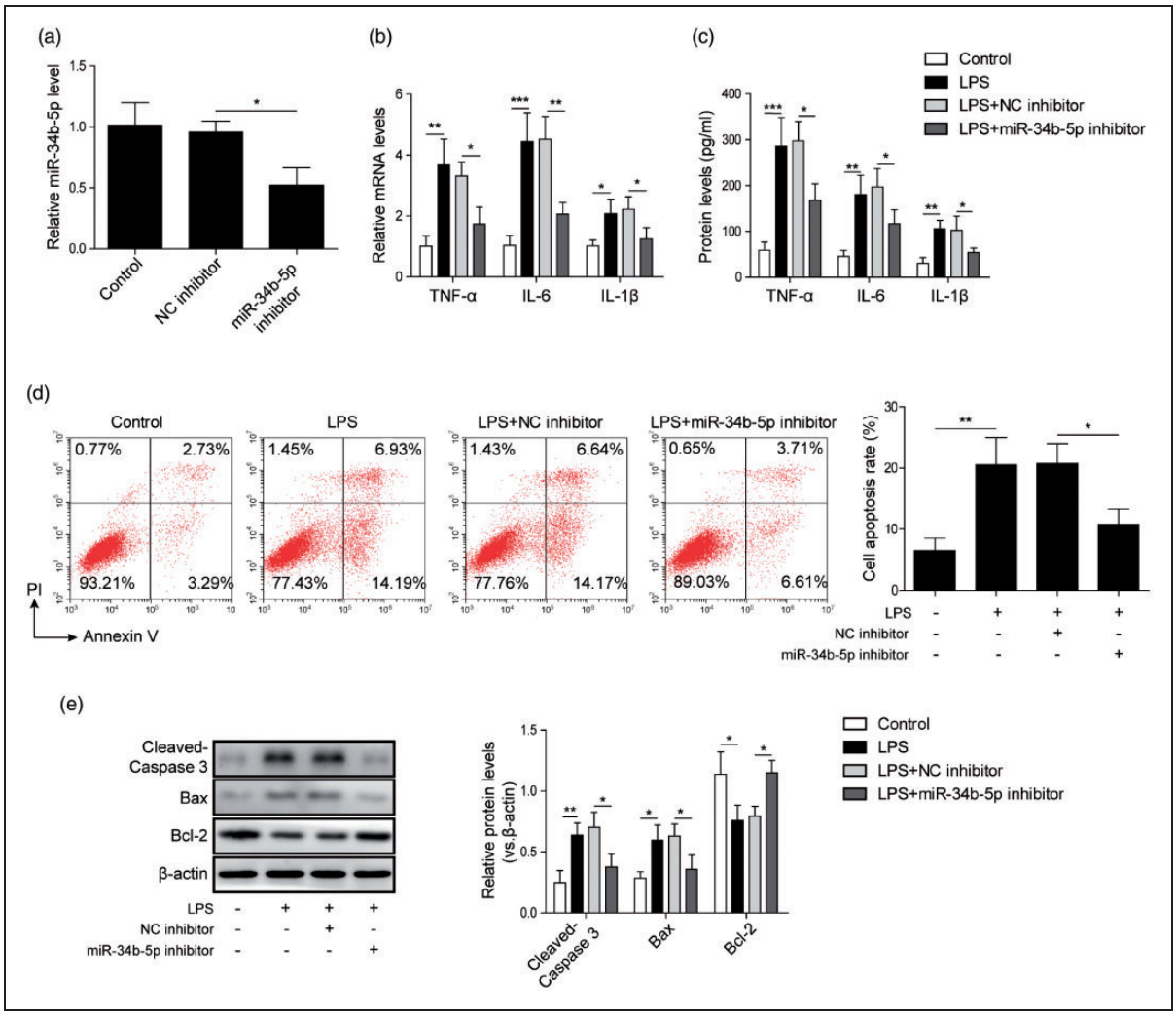

Figure 2. Knockdown of miR-34b-5p alleviated inflammatory response and cell apoptosis in LPS-stimulated H9C2 cells. H9C2 cells were transfected with NC inhibitor or miR-34b-5p inhibitor. Forty-eight h after transfection, cells were then stimulated with 10 μg/ml LPS for 12 h. (a) Quantitative RT-PCR was used to detect the expression of miR-34b-5p. (b) Quantitative RT-PCR was used to detect the expression of TNF-α, IL-1β and IL-6. (c) ELISA was used to evaluate the expression of TNF-α, IL-1β and IL-6 in supernatant. (d) Annexin V/propidium iodide (PI) staining with flow cytometry was performed to detect apoptosis. (e) Western blot was used to detect the protein expression of Bcl-2, cleaved caspase 3 and Bax. Data are shown as the mean ± SD from three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

(Figure 3d) and Western blots (Figure 3e) revealed that inhibition of miR-34b-5p increased mRNA and protein level of SIRT1, whereas overexpression of miR-34b-5p had the opposite effect on SIRT1. Therefore, our results implied that miR-34b-5p bound to and negatively regulated SIRT1 in H9C2 cells.

miR-34b-5p aggravates inflammatory response and cell apoptosis via targeting SIRT1 in LPS-stimulated H9C2 cells

To investigate further whether inhibition of SIRT1 was critical for miR-34b-5p aggravated apoptosis and inflammatory upon LPS stimulation, we simultaneously overexpressed miR-34b-5p and SIRT1 in H9C2 cells before LPS stimulation. As shown in Figure 4a, miR-34b-5p mimics treatment elevated the expression of miR-34b-5p and inhibited the expression of SIRT1. Overexpression of SIRT1 in pcDNA3.1-SIRT1 group restored miR-34b-5p restrained expression. In line with results depicted in Figure 2, quantitative RT-PCR and ELISA showed that LPS dramatically elevated the pro-inflammatory cytokine level, which was further aggravated by miR-34b-5p overexpression. However, the enhancement of miR-34b-5p was prohibited by co-overexpression of SIRT1 (Figure 4b and c). Consistently, overexpression of miR-34b-5p further raised the apoptosis rate induced by

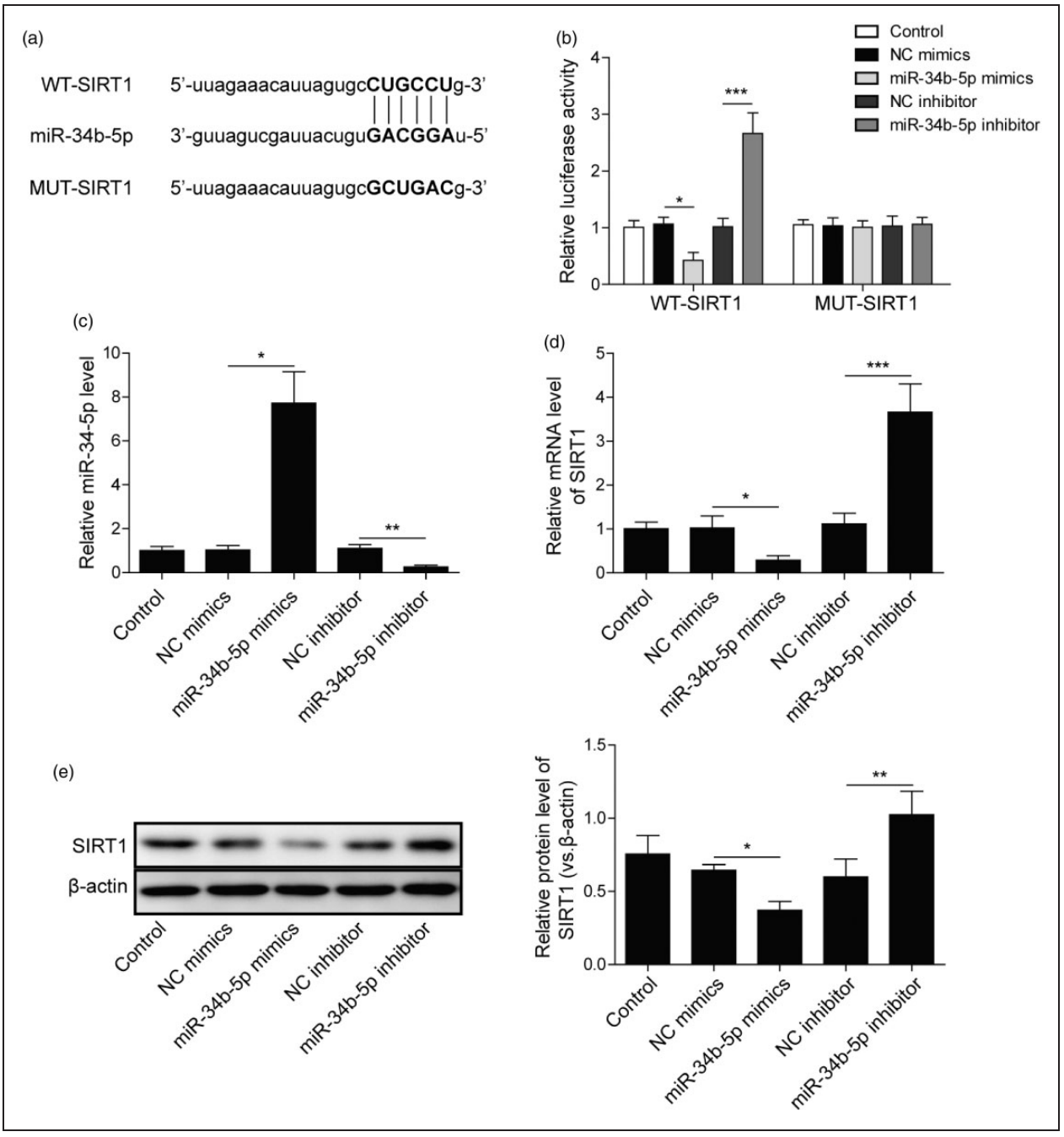

Figure 3. MiR-34b-5p directly targeted SIRT1. H9C2 cells were transfected with miR-34b-5p inhibitor, miR-34b-5p mimics or NC inhibitor, NC mimics. (a) Illustration for predicted binding site between miR-34b-5p and SIRT1. (b) Dual-luciferase activity assay was used to validate the binding. (c) Quantitative RT-PCR was used to detect the expression of miR-34b-5p. (d) Quantitative RT-PCR was used to detect the expression of SIRT1. (e) Western blot was performed to examine the protein expression of SIRT1. Data are shown as the mean ± SD from three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

LPS stimulation, whereas co-overexpression of SIRT1 compromised the enhancement of miR-34b-5p (Figure 4d and e). Protein patterns of Bax, cleaved caspase-3 and Bcl-2 were in line with the results of flow cytometry, with Bax and cleaved caspase-3 attenuated and Bcl-2 stimulated by co-overexpression of SIRT1 (Figure 4f). Therefore, this rescue experiment validated that miR-34b-5p aggravated inflammatory response and cell apoptosis in LPS-stimulated H9C2 cells at least partly dependent on inhibition of SIRT1.

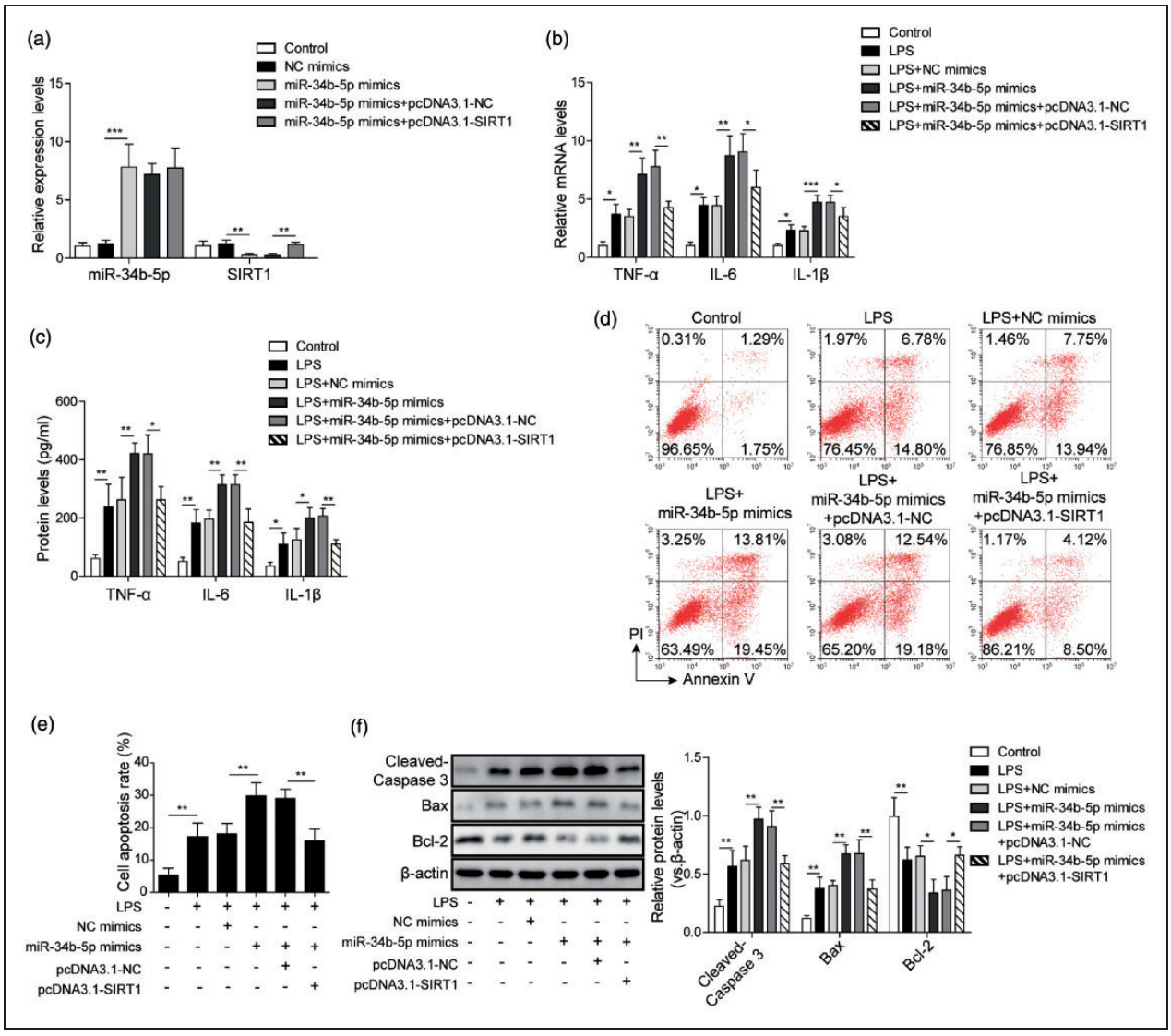

Figure 4. MiR-34b-5p aggravated inflammatory response and cell apoptosis in LPS-stimulated H9C2 cells at least partly dependent on the inhibition of SIRT1. H9C2 cells were solely transfected with miR-34b-5p mimics or NC mimics or simultaneously transfected miR-34b-5p mimics/NC mimics and pcDNA3.1-SIRT1 or pcDNA3.1. Forty-eight h after transfection, cells were stimulated with 10 μg/ml LPS for 12 h. (a) Quantitative RT-PCR was used to detect the expression of miR-34b-5p and SIRT1. (b) Quantitative RT-PCR was used to detect the expression of TNF-α, IL-1β and IL-6. (c) ELISA was used to evaluate the expression of TNF-α, IL-1β and IL-6 in supernatant. (d) and (e) Annexin V/PI staining with flow cytometry was performed to detect apoptosis. (f) Western blot was used to detect protein expression of Bcl-2, cleaved caspase 3 and Bax. Data are shown as the mean ± SD from three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

ZFAS1 acts as a sponge for miR-34b-5p

We used Starbase v2.0 for prediction and found a potential binding site of miR-34b-5p in the sequence of ZFAS1 (Figure 5a). Dual-luciferase reporters containing ZFAS1 binding sequence and miR-34b-5p mimics were co-transfected into H9C2 cells. The relative luciferase activity of reporter containing the wt ZFAS1 sequence was prohibited in the miR-34b-5p mimics group compared to the NC mimics group, while the relative luciferase activity of reporter containing the mutated ZFAS1 sequence was not affected by miR-34b-5p mimics (Figure 5b). To investigate whether ZFAS1 had a regulatory effect on the expression of miR-34b-5p and SIRT1, specific siRNA or overexpressing plasmid were used to knock down or overexpress ZFAS1 in H9C2, and the efficiency was validated by quantitative RT-PCR (Figure 5c). Expression of miR-34b-5p was decreased by overexpressed ZFAS1 and promoted by ZFAS1 knockdown (Figure 5d). In addition, as expected, SIRT1 was positively correlated with ZFAS1, as overexpressed

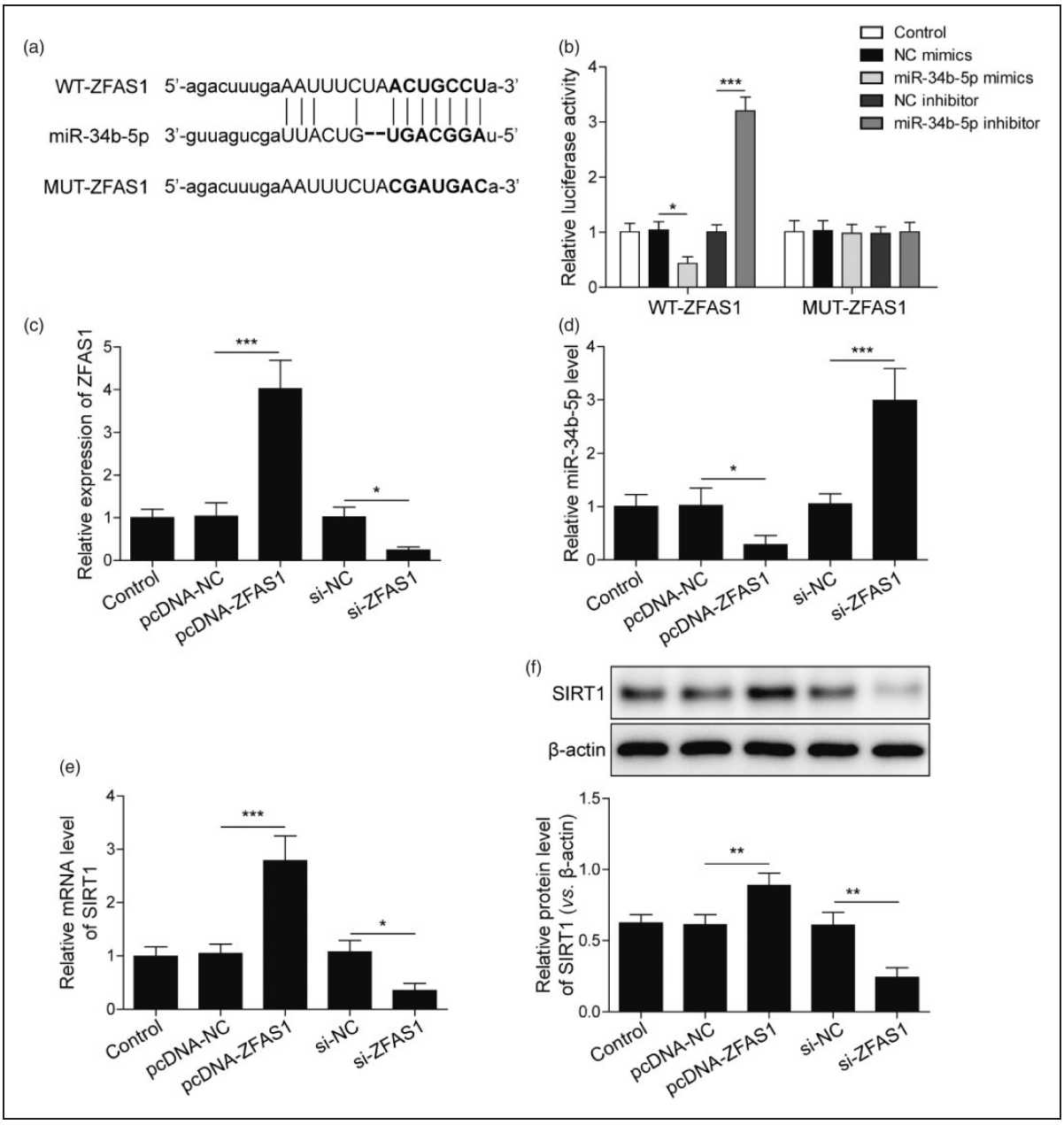

Figure 5. ZFAS1 sponged miR-34b-5p. (a) and (b) H9C2 cells were transfected with NC mimics or miR-34b-5p mimics. (a) Illustration for predicted binding site between miR-34b-5p and ZFAS1. (b) Dual-luciferase activity assay was used to validate the binding. (c–e) H9C2 cells were transfected with siRNA of ZFAS1 or overexpressed plasmid of ZFAS1(pcDNA3.1-ZFAS1) or respective control. (c) Quantitative RT-PCR was performed to examine the expression of ZFAS1. (d) Quantitative RT-PCR was conducted to determine the expression of miR-34b-5p. (e) Quantitative RT-PCR was used to detect the expression of SIRT1. (f) Western blot was used to detect the expression of SIRT1. Data are shown as the mean ± SD from three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

ZFAS1 elevated and knockdown of ZFAS1 attenuated mRNA and protein expression of SIRT1 (Figure 5e and f). These findings implied that lncRNA ZFAS1 negatively regulated miR-34b-5p expression in H9C2 cells via direct sponging.

ZFAS1 alleviated inflammatory response and cell apoptosis in LPS-stimulated H9C2 cells at least partly depends on the miR-34b-5p/SIRT1 axis

To investigate whether ZFAS1 had the capacity to alleviate apoptosis and inflammation upon LPS

stimulation and reveal the underlying mechanism, we solely overexpressed ZFAS1 or simultaneously overexpressed miR-34b-5p and ZFAS1 in H9C2 cells before LPS stimulation. As shown in Figure 6a, the overexpressed plasmid of ZFAS1 elevated the expression of ZFAS1 and prohibited the expression of miR-34b-5p, while miR-34b-5p mimics restored the expression of miR-34b-5p. Quantitative RT-PCR and ELISA showed that ZFAS1 overexpression largely attenuated the LPS-induced cytokine level. However, the anti-inflammatory effect of ZFAS1 was reversed by co-overexpression of miR-34b-5p (Figure 6b and c). In terms of apoptosis, overexpression of ZFAS1 inhibited the apoptosis rate induced by LPS stimulation, whereas co-overexpression of miR-34b-5p compromised the anti-apoptotic effects of ZFAS1 (Figure 6d and e). The results of Western blots showed that LPS-induced Bax and cleaved caspase-3 was attenuated by

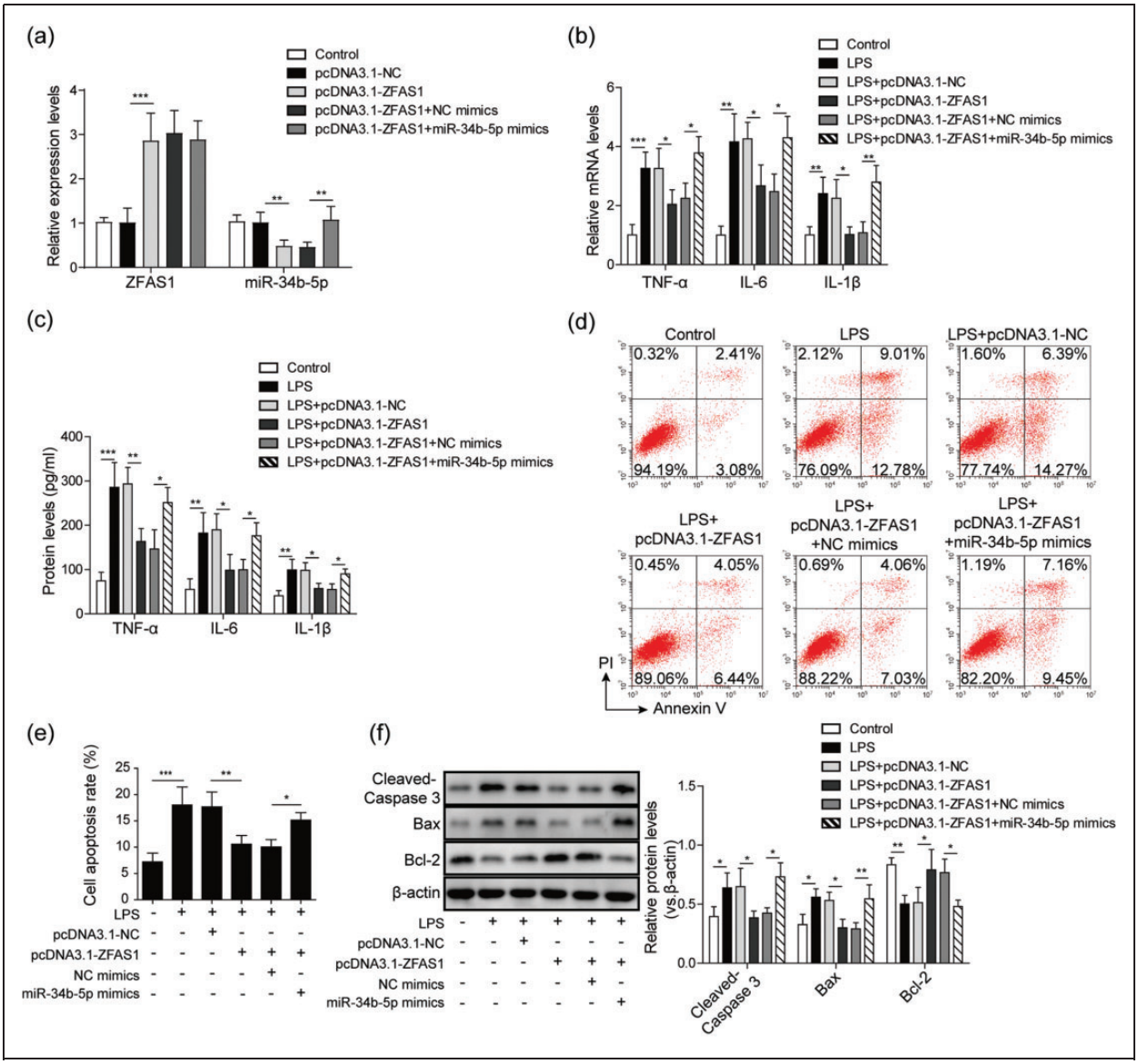

Figure 6. ZFAS1 alleviated inflammatory response and cell apoptosis in LPS-stimulated H9C2 cells at least partly dependent on the miR-34b-5p/SIRT1 axis. H9C2 cells were solely transfected with pcDNA3.1-ZFAS1 or pcDNA3.1 or simultaneously transfected miR-34b-5p mimics/NC mimics and pcDNA3.1-ZFAS1 or pcDNA3.1. Forty-eight h after transfection, cells were stimulated with 10 μg/ml LPS for 12 h. (a) Quantitative RT-PCR was used to detect the expression of miR-34b-5p and ZFAS1. (b) Quantitative RT-PCR was used to detect expression of TNF-α, IL-1β and IL-6. (c) ELISA was used to examine the expression of TNF-α, IL-1β and IL-6 in supernatant. (d and e) Annexin V/PI staining with flow cytometry was performed to detect apoptosis. (f) Western blot was applied to detect protein expression of Bcl-2, cleaved caspase 3 and Bax. Data are shown as the mean ± SD from three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

ZFAS1 overexpression, and LPS-inhibited Bcl-2 was restored by overexpression of ZFAS1 (Figure 6f). However, co-overexpression of miR-34b-5p reversed the effect of ZFAS1 on these protein expressions. Therefore, this rescue experiment validated that ZFAS1 alleviated the inflammatory response and cell apoptosis in LPS-stimulated H9C2 cells via the miR-34b-5p/SIRT1 axis.

Discussion

To the best of our knowledge, our results are the first to describe a protective role of ZFAS1 in LPS-induced myocardial injury. Overexpression of ZFAS1 alleviated inflammation and apoptosis induced by LPS via the miR-34b-5p/SIRT1 axis. Additionally, inhibition of miR-34b-5p mimics the effect of overexpression of ZFAS1.

It has previously been reported that circulating ZFAS1 is an indicator of sepsis pathology.[8] Our study revealed a protective function of ZFAS1 in LPS-induced myocardial injury by repressing inflammation and apoptosis, which are two critical progress of SIC.[3] Zhang et al. previously revealed that ZFAS1 improved neuronal injury by alleviating inflammation and apoptosis via sponging miR-582 and up-regulated NOS3 (eNOS).[16] eNOS is a well-known protective factor in sepsis-induced extravagant inflammatory response.[17] Thus, ZFAS1 might also exert a protective role in SIC via regulation of eNOS. However, different from a single report about ZFAS1 in inflammation, the function of ZFAS1 in apoptosis was controversial. A number of studies have unveiled the pro-apoptotic role of ZFAS1 in acute myocardial infarction and myocardial ischaemia/reperfusion injury.[18–20] Contrary to these reports, the present study reports that ZFAS1 has an anti-apoptotic role in myocardial infarction, which is consistent with the conclusion of Jiao et al., who demonstrated that silencing ZFAS1 abrogated ischaemia-induced apoptosis in cardiomyocytes via mitochondria apoptosis.[21] Therefore, the expression of ZFAS1 is inconsistent under different pathology. These reports remind us to draw any conclusions more cautiously.

ZFAS1 has been reported to up-regulate downstream gene expression by acting like a sponge for miRNA to attenuate the binding between miRNA and mRNA.[7] In the present study, we revealed for the first time that ZFAS1 served as a sponge for miR-34b-5p. A previous study indicated that miR-34b-5p aggravated LPS-induced acute lung injury.[12,13] Consistent with the previous literature, our study found a detrimental role of miR-34b-5p in SIC. Inhibition of miR-34b-5p alleviated the stimulation of LPS-induced myocardial injury by inhibiting the inflammatory response and apoptosis. On the contrary, it was also reported that p53 could inhibit non-canonical NF-κB signalling by up-regulating miR-34b-5p, which indicated that miR-34b-5p might have an anti-inflammatory function. Further investigation should be focus on these different conclusions.

SIRT1 is a protein deacetylase which participates in variety of cellular progress.[22] It had been widely demonstrated to be beneficial to sepsis-induced injury.[23,24] Specifically, it has also been reported that SIRT1 had a protective effect in SIC,[3] which might contribute to the negative regulative effect on NF-κB signalling, which is a major pathway in LPS-induced inflammatory response.[25] Meanwhile, SIRT1 has also been reported to be an anti-apoptotic regulator in many diseases by regulating expression of multiple apoptotic proteins.[26,27] For instance, p53 was deacetylated by SIRT1 and thus lead to apoptosis suppression.[28] Consistently, our work illustrated that SIRT1 participated in miR-34b-5p-mediated inflammation and apoptosis in SIC.

In the present study, we reveal a novel protective role of ZFAS1 in LPS-induced myocardial injury, which was achieved by regulating the miR-34b-5p/SIRT1 axis. Our findings might provide insight to show a novel mechanism in SIC. However, *in vivo* studies about the functional validation of ZFAS1 should be done before our current work can be translated to SIC pathology.

Acknowledgements

We would like to give our sincere gratitude to the reviewers for their constructive comments.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship and/or publication of this article: This work was supported by The Key Research and Development Plan Project of Hainan Province (ZDYF2018150).

ORCID iD

Xing-Jun Cai https://orcid.org/0000-0001-8244-2416

Supplemental material

Supplemental material for this article is available online.

References

1. Shankar-Hari M, Phillips GS, Levy ML, et al. Developing a new definition and assessing new clinical criteria for septic shock: for the Third International

Consensus Definitions for Sepsis and Septic Shock (Sepsis-3). *JAMA* 2016; 315: 775–787.
2. Hochstadt A, Meroz Y and Landesberg G. Myocardial dysfunction in severe sepsis and septic shock: more questions than answers? *J Cardiothorac Vasc Anesth* 2011; 25: 526–535.
3. Jiang W, Li W, Hu X, et al. CTRP1 prevents sepsis-induced cardiomyopathy via Sirt1-dependent pathways. *Free Radic Biol Med* 2020; 152: 810–820.
4. Zhang P, Yi L, Qu S, et al. The biomarker TCONS_00016233 drives septic AKI by targeting the miR-22-3p/AIFM1 signaling axis. *Mol Ther Nucleic Acids* 2020; 19: 1027–1042.
5. Chen T, Zhu C and Ye C. LncRNA CYTOR attenuates sepsis-induced myocardial injury via regulating miR-24/XIAP. *Cell Biochem Funct* 2020; 38: 976–985.
6. Sun F, Yuan W, Wu H, et al. LncRNA KCNQ1OT1 attenuates sepsis-induced myocardial injury via regulating miR-192-5p/XIAP axis. *Exp Biol Med* 2020; 245: 620–630.
7. Peng J, Liu F, Zheng H, et al. Long noncoding RNA ZFAS1 promotes tumorigenesis and metastasis in nasopharyngeal carcinoma by sponging miR-892b to upregulate LPAR1 expression. *J Cell Mol Med* 2020; 24: 1437–1450.
8. Xu Y and Shao B. Circulating long noncoding RNA ZNFX1 antisense RNA negatively correlates with disease risk, severity, inflammatory markers, and predicts poor prognosis in sepsis patients. *Medicine* 2019; 98: e14558.
9. Pan Y, Wang J, Xue Y, et al. GSKJ4 protects mice against early sepsis via reducing proinflammatory factors and up-regulating MiR-146a. *Front Immunol* 2018; 9: 2272.
10. Xue Z, Xi Q, Liu H, et al. miR-21 promotes NLRP3 inflammasome activation to mediate pyroptosis and endotoxic shock. *Cell Death Dis* 2019; 10: 461.
11. Jiang K, Yang J, Guo S, et al. Peripheral circulating exosome-mediated delivery of miR-155 as a novel mechanism for acute lung inflammation. *Mol Ther* 2019; 27: 1758–1771.
12. Xie W, Lu Q, Wang K, et al. miR-34b-5p inhibition attenuates lung inflammation and apoptosis in an LPS-induced acute lung injury mouse model by targeting progranulin. *J Cell Physiol* 2018; 233: 6615–6631.
13. Qiu N, Xu X and He Y. LncRNA TUG1 alleviates sepsis-induced acute lung injury by targeting miR-34b-5p/GAB1. *BMC Pulm Med* 2020; 20: 49.
14. Liao K, Li J and Wang Z. Dihydroartemisinin inhibits cell proliferation via AKT/GSK3beta/cyclinD1 pathway and induces apoptosis in A549 lung cancer cells. *Int J Clin Exp Pathol* 2014; 7: 8684–8691.
15. Wang QL, Yang L, Peng Y, et al. Ginsenoside Rg1 regulates SIRT1 to ameliorate sepsis-induced lung inflammation and injury via inhibiting endoplasmic reticulum stress and inflammation. *Mediators Inflamm* 2019; 2019: 6453296.
16. Zhang Y and Zhang Y. lncRNA ZFAS1 improves neuronal injury and inhibits inflammation, oxidative stress, and apoptosis by sponging miR-582 and upregulating NOS3 expression in cerebral ischemia/reperfusion injury. *Inflammation* 2020; 43: 1337–1350.
17. Zeng N, Xu J, Yao W, et al. Brain-derived neurotrophic factor attenuates septic myocardial dysfunction via eNOS/NO pathway in rats. *Oxid Med Cell Longev* 2017; 2017: 1721434.
18. Li Z, Qin X, Bian W, et al. Exosomal lncRNA ZFAS1 regulates esophageal squamous cell carcinoma cell proliferation, invasion, migration and apoptosis via microRNA-124/STAT3 axis. *J Exp Clin Cancer Res* 2019; 38: 477.
19. Tong H, Zhuang X, Cai J, et al. Long noncoding RNA ZFAS1 promotes progression of papillary thyroid carcinoma by sponging miR-590-3p and upregulating HMGA2 expression. *Onco Targets Ther* 2019; 12: 7501–7512.
20. Su L, Kong H, Wu F, et al. Long non-coding RNA zinc finger antisense 1 functions as an oncogene in acute promyelocytic leukemia cells. *Oncol Lett* 2019; 18: 6331–6338.
21. Jiao L, Li M, Shao Y, et al. lncRNA-ZFAS1 induces mitochondria-mediated apoptosis by causing cytosolic Ca(2+) overload in myocardial infarction mice model. *Cell Death Dis* 2019; 10: 942.
22. Opal SM, Ellis JL, Suri V, et al. Pharmacological Sirt1 activation improves mortality and markedly alters transcriptional profiles that accompany experimental sepsis. *Shock* 2016; 45: 411–418.
23. Liu Y, Guan H, Zhang JL, et al. Acute down-regulation of miR-199a attenuates sepsis-induced acute lung injury by targeting SIRT1. *Am J Physiol Cell Physiol* 2018; 314: C449–C455.
24. Li L, Liu M, Cao M, et al. Research progress on SIRT1 and sepsis. *Histol Histopathol* 2019; 34: 1205–1215.
25. Yeung F, Hoberg JE, Ramsey CS, et al. Modulation of NF-kappaB-dependent transcription and cell survival by the SIRT1 deacetylase. *EMBO J* 2004; 23: 2369–2380.
26. Zhu H, Yang Y, Wang Y, et al. MicroRNA-195 promotes palmitate-induced apoptosis in cardiomyocytes by down-regulating Sirt1. *Cardiovasc Res* 2011; 92: 75–84.
27. Zhang M, Zhang Q, Hu Y, et al. miR-181a increases FoxO1 acetylation and promotes granulosa cell apoptosis via SIRT1 down-regulation. *Cell Death Dis* 2017; 8: e3088.
28. Zhang YY and Zhou LM. Omentin-1, a new adipokine, promotes apoptosis through regulating Sirt1-dependent p53 deacetylation in hepatocellular carcinoma cells. *Eur J Pharmacol* 2013; 698: 137–144.