ORIGINAL ARTICLE

LncRNA MIAT Promotes Inflammation and Oxidative Stress in Sepsis-Induced Cardiac Injury by Targeting miR-330-5p/TRAF6/NF-κB Axis

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Abstract

Sepsis is a whole-body inflammation and main cause of death in intensive care units worldwide. We aimed to investigate the roles of lncRNA MIAT and miR-330-5p in modulating inflammatory responses and oxidative stress in lipopolysachariden (LPS)-induced septic cardiomyopathy. Serum and heart tissue were collected from in vivo septic mice model, ELISA and qRT-PCR were used to measure the expression of pro-inflammation cytokines, MIAT and miR-330-5p, respectively. The knockdown of MIAT and overexpression of miR-330-5p were conducted to assess their effects on regulating inflammation response and intracellular oxidative stress in LPS-stimulated HL-1 cells. The reactive oxygen (ROS) level, mitochondrial membrane potential (MMP), GSH/GSSH ratio, and lipid peroxidation assessment (MDA) were used to evaluate the intracellular oxidative stress. Dual-luciferase reporter assay was performed to identify the association between MIAT and miR-330-5p, TRAF6 and miR-330-5p, respectively. In septic mice, the expression of MIAT and pro-inflammation cytokines was elevated while the expression of miR-330-5p decreased. Knockdown of MIAT or overexpression of miR-330-5p restrained inflammation and oxidative stress induced by LPS in vitro; MIAT directly targeted miR-330-5p to regulate NF-κB signaling, and miR-330-5p targeted against TRAF6 to suppress the activation of NF-κB signaling. We determined that lncRNA MIAT directly binds to miR-330-5p to activate TRAF6/NF-κB signaling axis and further promotes inflammation response as well as oxidative stress in LPS-induced septic cardiomyopathy. This finding suggests the potential therapeutic role of lncRNA MIAT and miR-330-5p in LPS-induced myocardial injury.

Keywords MIAT · miR-330-5p · TRAF6 · NF-κB · LPS-induced sepsis · Cardiomyopathy

Background

Sepsis is a systematic inflammation disease triggered by pathogens after infection, severe trauma, major surgeries and usually accompanied with multiple organ failure (Wu 2016). It is one of the leading causes of death in intensive care units globally (Zou 2014). Evidence demonstrated that myocardial dysfunction is a fatal complication that develops in patients with septic shock or sever sepsis (Tsolaki 2017). Sepsis-induced cardiomyopathy could be observed in over 50% of septic patients, which suggests a worse prognosis (Rudiger and Singer 2007). The increased reactive oxygen (ROS) and nitrogen species (RNS) production from cardiac mitochondria are highly associated with myocardial injury, leading to the formation of oxidative stress which is a major contributor to septic cardiomyopathy (Tsolaki 2017; Takasu 2013). Several animal models have shown oxidative stress and excessive expression of inflammatory cytokines such as IL-1β, IL-6 and TNF in LPS-induced septic cardiomyopathy (Okazaki 2014; Lorigados et al. 2010; Sandt 2013). As such, it is of great significance to investigate the effective approaches to alleviate oxidative stress and modulating inflammatory cytokine release during sepsis-induced cardiomyopathy.

Long non-coding RNAs (lncRNAs) refer to non-coding RNAs with more than 200 nucleotides in length (Qureshi and Mehler 2012). The roles of lncRNAs are implicated at epigenetic, transcriptional and post-transcriptional level. Mounting studies have shown its functions in multiple biological events such as cancer biology, age-associated cardiovascular aging and inflammatory responses (Yang 2013; Pearson and Jones 2016; Greco et al. 2015). The association of lncRNA myocardial infarction associated transcript (MIAT) and inflammation of metabolic syndrome (MetS) or sepsis has been identified earlier by RNA-sequencing, revealing MIAT was significantly upregulated in sepsis-related myocardial dysfunction (Meydan et al. 2018). Evidence further revealed the implication of lncRNA MIAT in myocardial diseases such as myocardial infarction in the rat model. It was demonstrated that MIAT can competitively bind to miR-93 and upregulate TLR4 via inactivating PI3K/Akt/mTOR pathway, which eventually resulted in cardiac hypertrophy (Li 2018). Inspired by these findings, we speculated that MIAT is the potential regulator during septic cardiomyopathy. However, the underlying molecular mechanisms responsible for MIAT's regulatory functions on septic cardiomyopathy remains unclear, which worth further investigation.

MiRNAs are small non-coding RNAs with ~22 nucleotide in length, which play key roles in gene expression through specifically binding to 3′-untranslate region (Oliveira et al. 2017) of their target mRNAs. Researches revealed the abnormal expression of miR-330-5p was observed in various cancers, including epithelial ovarian cancer (EOC), hepatocellular carcinoma and lung cancer (Cui 2018; Kong 2017; Shao 2018; Wang 2018; Xiao 2018). Liu et al. demonstrated that miR-330-5p was associated with oxidative stress and inflammation response in human macrophages in vitro (Liu et al. 2018). Additionally, study has reported that MIAT can directly bind to miR-330-5p in EOC disease model (Shao 2018). However, the role of miR-330-5p in sepsis has not yet been reported and thereby requiring further explorations.

Tumor necrosis factor receptor-associated factor 6 (TRAF6), is a signal transducer in the activation of nuclear factor-κB (NF-κB) signaling pathway to mediate the incidence of inflammation response. Reports suggested TRAF6 play negative roles in sepsis-induced myocardial dysfunction (An 2018; Abdullah et al. 2017). Based on previous bioinformatic analysis, miR-330-5p can specifically target TRAF6. Therefore, we speculated miR-330-5p might regulate TRAF6 expression in septic cardiomyopathy.

In the present study, we explored the association of lncRNA MIAT and miR-330-5p and their effects on sepsis-induced intracellular oxidative stress and inflammatory responses during the septic cardiomyopathy. Taken together, our findings suggested that MIAT promotes inflammation response and oxidative stress in LPS-induced septic cardiomyopathy through activating TRAF6/NF-κB signaling axis by miR-330-5p.

Methods

Cell Culture and Treatment

The murine HL-1 atrial myocyte was purchased from American Type Culture Collection (ATCC) and maintained in Claycomb medium (51800C, Signal-Aldrich) supplemented with 10% fetal bovine serum (Gibco, USA), 0.1 mM noradrenaline, 2 mM L-glutamine and 1% penicillin/streptomycin in a humidified atmosphere (95% air and 5% CO_2) at 37 °C. The cells were subsequently diluted to 1×10^6 cells/ml and seeded to six well plates followed by culturing for 48 h to 70% confluence and then incubated with 1 µg/ml Lipopolysaccharides (LPS) or saline solution for 12 h before using.

Animal Experiments

Male BALB/c mice (25–30 g) were purchased from Beijing Laboratory Animal Research Center (Beijing, China) and housed with free access to food and water for acclimation under controlled environmental conditions. Mice at the age of 8–10 weeks were randomly assigned to three groups. After anesthetization, one group received intraperitoneal injection of LPS (15 mg/kg, Sigma-Aldrich, Shanghai, China) and named as LPS-induced sepsis group ($n=6$), another group was administrated with equivalent amount of saline solution and named as control group ($n=6$), the third group was blank control group ($n=6$) without any administrations. Mice were sacrificed by euthanasia 12 h after the injection. The heart tissue was collected for the following qRT-PCR analysis. The blood samples were collected from these mice and serum was isolated for the subsequent inflammatory cytokine analysis. All animal experiments were approved by the Institutional Animal Care and Use Committee of the Shanghai Sixth People`s Hospital East.

Cell Transfection

The siRNA against MIAT (si-MIAT), siRNA negative control (si-NC) miR-330-5p mimic and mimic negative control (mimic NC) were purchased from Shanghai GenePharm Co., Ltd. (GenePharm, Shanghai, China). HL-1 atrial myocyte was seeded to 24-well plates and transfected with plasmids or oligonucleotides using Lipofectamine 3000 (Invitrogen, CA, USA) according to manufacturer's instruction.

Quantitative Real-Time PCR (qRT-PCR)

Total RNA from HL-1 atrial myocyte and mice heart tissue were harvested using Trizol Reagent (Invitrogen, CA, USA) according to manufacturer's protocol. Then, 1 μg of total RNA was reversed into first-strand complementary DNA (cDNA) using Superscript II First-Strand cDNA synthesis kit (Invitrogen, CA, USA). PCR analysis was performed using SYBR Green qPCR MasterMix (Applied Biosystems, CA, USA) for lncRNA and mRNA and using the TaqMan MicroRNA Assay kit for miRNA according to the manufacturer's instructions (Applied Biosystems, CA, USA). U6 and GAPDH were loaded as the internal control. Relative mRNA expression was determined using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Primers used were as follows:

MIAT,
F: 5′- GGGCTTAGGGGAGTCCAAAC-3′
R: 5′- CTCACTACCAACCCCAACCC-3′

MiR-330-5p,
F: 5′- CTGATCTCTGGGCCTGTGTC-3'
R: 5′-GTGCAGGGTCCGAGGT-3'

U6,
F: 5′-CTCGCTTCGGCAGCACA-3'
R: 5′-AACGCTTCACGAATTTGCGT-3'

TNF-α,
F: 5′-CCGATGGGTTGTACCTTGTC-3′
R: 5′-TGGAAGACTCCTCCCAGGTA-3′

IL-6,
F: 5′-TGCAAGAGACTTCCATCCAG-3′
R: 5′-TCCACGATTTCCCAGAGAAC-3′

IL-1β,
F: 5′-GCCACCTTTTGACAGTGATGAG-3'
R: 5′-AAGGTCCACGGGAAAGACAC-3'

GAPDH,
F: 5′-CCAGGTGGTCTCCTCTGA-3′
R: 5′-GCTGTAGCCAAATCGTTGT-3′

Western Blot Analysis

Cells were lysed using RIPA buffer and whole proteins were extracted by centrifuging at 1200 rpm for 5 min at 4 °C. The protein content was then quantified using a BCA protein assay kit (Beyotime, China). Equal amounts of proteins were separated using SDS-PAGE gel and then electro-blotted on to PVDF membranes, which were blocked with TBS containing 5% non-fat milk for 1 h at room temperature. Blots were then incubated overnight at 4 °C with the primary antibodies against: TRAF6 (sc-8409 HRP, at 1:1000, Santa Cruz, TX, USA), p65 (sc-8008 HRP, at 1:1000, Santa Cruz, TX, USA) and p-p65 (sc-136548 HRP, at 1:1000, Santa Cruz, TX, USA) and loaded with β-actin as an internal control (sc-4778 HRP, at 1:1000, Santa Cruz, TX, USA). The membranes were subsequently washed by TBS-T (0.1% Tween) buffer and incubated with HRP-conjugated secondary antibody for 1 h. The blotting was visualized using ECL Select Western Blotting Detection Reagent (GE Healthcare, UK) and analyzed by Image J software.

ELISA

The sera secretion of TNF-α, IL-6, IL-1β was measured using mouse TNF-α, IL-6, IL-1β ELISA kit (PT512, PI326, PI301, Beyotime, China) as per manufacturer's instructions. The results were expressed as pg/ml.

Mitochondrial ROS Measurement

Mitochondrial ROS (mtROS) production in murine HL-1 cells was assessed by mitochondrial superoxide indicator MitoSOX (Molecular Probes, Thermofisher, USA). The cells in each experimental group were stained with 5 mM MitoSOX in serum free culture medium for 30 min in the darkness at 37 ℃. The cells were then washed with PBS and photographed using a fluorescent inverted microscope (Nikon, Japan).

Mitochondrial Membrane Potential (MMP, Δψm) Measurement

The mitochondrial membrane potential (MMP, Δψm) was determined using the cationic dye JC-1 (Invitrogen, USA) as introduced previously (Mo 2019). Briefly, HL-1 cells in each experimental group were incubated for 30 min at 37 °C in 1 ml

of culture medium supplementary with 2 μM JC-1, followed by washing with PBS. The mean green fluorescence (FL-1 channel) and orange-red fluorescence (FL-2 channel) were measured using flow cytometry (BD FACS, BD Biosciences, USA). The mean red to green fluorescence ratio was employed to express Δψm. The non-stained control cells were used to determine baseline fluorescence.

GSH/GSSH Ratio

The GSH/GSSG ration was determined according to previous methods (Gyuraszova 2018). To measure the GSH, HL-1 cells were washed with ice-cold PBS and resuspended with O-phthalaldehyde solution (1 mg/ml) and phosphate buffer solution (100 mmol/l, 2.5 mM EDTA-Na$_2$) and then homogenized. The suspension was incubated 15 min at room temperature followed by measurement on specific fluorescence at $\lambda_{ex} = 350$ nm, $\lambda_{em} = 460$ nm. GSSG was determined by resuspending HL-1 cells in N-ethylmaleimide (5 g/ml) and incubation for 40 min at room temperature. The suspension was transferred to new microtiter plate supplementary with O-phthalaldehyde (1 mg/ml) and NaOH (0.1 mol/l), which followed by 15 min's incubation at room temperature. The specific fluorescence was measured at $\lambda_{ex} = 350$ nm, $\lambda_{ex} = 460$ nm. GSH values were divided with GSSG values to assess their ratio.

Lipid Peroxidation Assessment (MDA)

HL-1 cells were washed with ice-cold PBS and resuspended by phosphate buffer and homogenized. The suspension was centrifuged for 15 min at 5000 rpm. The supernatant was then collected and used to test MDA level using the specific assay kit (NJJC Bio, Nanjing, China).

Dual-Luciferase Analysis

Online prediction tool Starbase (https://starbase.sysu.edu.cn/) validated that miR-330-5p is a predictive target of lncRNA MIAT; the potential binding of miR-330-5p and TRAF6 was predicted by TargetScan (https://www.targetscan.org/). The full length wild type or mutant sequence of mouse MIAT or TRAF6 containing the predicted miR-330-5p binding sites was inserted into 3′UTR of firefly luciferase gene in pGL3-basic vector (Promega) and named as MIAT-WT/TRAF6-WT and MIAT-MUT/TRAF6-MUT, respectively. A Renilla luciferase reporter vector was used as internal control. Cells were co-transfected in miR-330-5p mimic or mimic NC and pGL3 vectors containing WT/MUT sequence for 48 h. The binding of miR-330-5p and wild type sequence causes the inhibition of transcription of firefly luciferase gene and eventually results in a decreased luciferase activity.

In Vivo Cardiac Function Assessment

Briefly, LV ejection fraction (EF), LV fractional shortening (FS), maximal positive velocity of left ventricular pressure (LVdp/dt max) and maximal negative

velocity of left ventricular pressure (LV$dp/dt$ min) of mice were assessed as previously described (Tang 2016; Fang 2018).

RNA Immunoprecipitation (RIP) Assay

RIP assay was performed using Magna RIP Kit (Millipore, Bedford, MA) and Ago2 antibody (Sigma-Aldrich, Shanghai, China) and IgG (Sigma-Aldrich, Shanghai, China) according to the manufacturer's instructions. Cells were lysed using RIP lysis buffer and cell lysate was incubated with magnetic beads conjugated to human anti-Ago2 or anti-TRAF6 antibody (Millipore) or isotype-matched control antibody (Millipore). Ago2 or anti-TRAF6 antibody was used to coprecipitate potential substances and IgG antibody was used as a negative control. The enrichment degrees of MIAT and miR-330-5p were measured by qRT-PCR analysis.

Statistical Analysis

All data were expressed as mean ± SD. Student's t-test was used to compare the differences between two groups. Comparison on multi-groups was analyzed using one-way ANOVA. The statistical analysis was performed on GraphPad Prism 6 (Graph-Pad, CA, USA). $P < 0.05$ was considered as a statistical significance. All data were obtained from at least three independent experiments.

Results

MIAT was Upregulated While miR-330-5p was Downregulated in LPS-Induced Sepsis

The involvement of lncRNA MIAT in the modulation of inflammation response and oxidative stress during the LPS-induced sepsis progression was examined. Changes of myocardial function in control and sepsis mice were examined. As Fig. 1a–d demonstrates, significantly lower EF (%), FS (%), LV$dp/dt$ max and LV$dp/dt$ min values were observed in septic mice compared with that in control. Time and dose curve of LPS injection demonstrated that in the heart tissue of mice injected with LPS for 12 h, the level of MIAT and cytokines (IL-1β, IL-6, TNF-α) were upregulated but the expression of miR-330-5p was downregulated with the increase of LPS dose (5, 10, 15 or 20 mg/kg), and the effect was most significant after administrated with 15 mg/kg LPS for 12 h (Supplementary information-Fig. 1). Results also showed strongly elevated levels of inflammatory cytokine TNF-α, IL-6 and IL-1β in the serum samples collected from LPS-induced septic mice compared to that in control (Fig. 1e–g, $P < 0.001$). A significant threefold increase of MIAT expression and a onefold decrease of miR-330-5p were observed in heart tissue from septic mice compared with normal control (Fig. 1h and i). The qRT-PCR test confirmed that relative mRNA expression of above mentioned cytokines was significantly upregulated in mice heart tissue from septic mice compared to that in Controls (Fig. 1j,
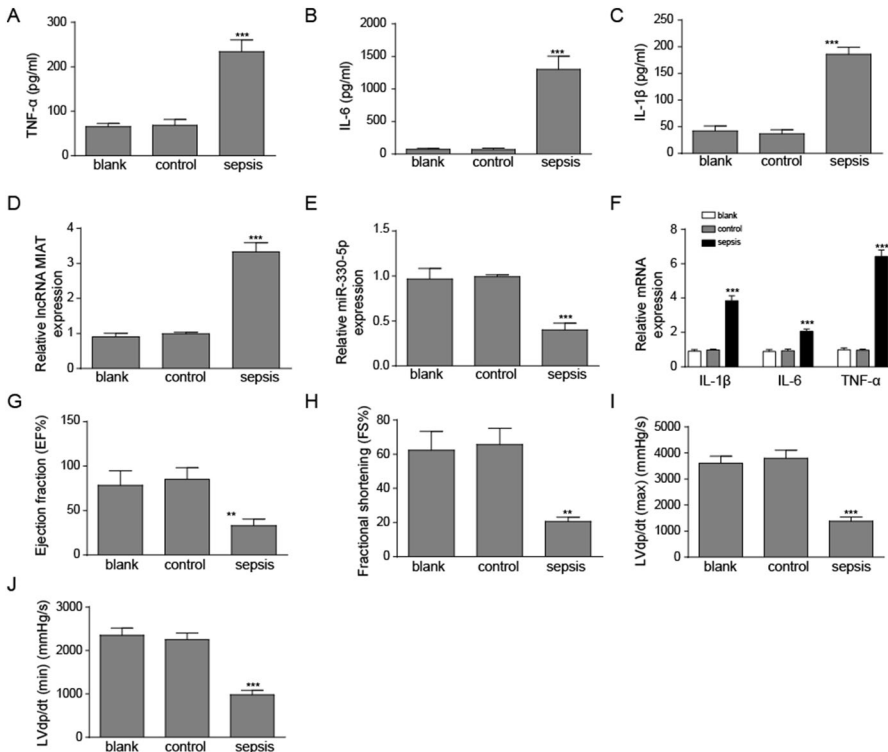
Fig. 1 MIAT was upregulated while miR-330-5p was downregulated in LPS-induced sepsis. **a–d** The ejection fraction (EF%), fractional shortening (FS%), maximal positive velocity of left ventricular pressure (LVd*P*/d*t* max), maximal negative velocity of left ventricular pressure (LVd*P*/d*t* min) were determined. **e–g** Sera secretion of inflammatory cytokines TNF-α, IL-6 and IL-1β in blank group, control mice and sepsis mice were detected using ELISA assay. **h–j**: the expression level of lncRNA MIAT, miR-330-5p and inflammatory cytokines (TNF-α, IL-6 and IL-1β mRNA) in blank group, control mice and sepsis mice were determined using qRT-PCR. ***P < 0.001. Data are presented as the means ± SD (*n* = 6). All tests were performed in three independent biological and technical replicates

P < 0.001). The results demonstrated an increased lncRNA MIAT but a decreased miR-330-5p level in LPS-induced sepsis at the in vivo level.

LncRNA MIAT Knockdown Inhibited the Expression of Inflammatory Cytokines

To assess the roles of lncRNA MIAT on modulating inflammation response after LPS (1 μg/ml) treatment of murine HL-1 atrial myocytes, the expression level of MIAT, miR-330-5p and inflammatory cytokines (TNF-α, IL-6 and IL-1β mRNA) were measured using qRT-PCR. The same amount of saline treatment was used as a control. As shown in Fig. 2a, b, expression of lncRNA MIAT was significantly upregulated while the expression of miR-330-5p was downregulated in HL-1 cells treated with LPS compared to that in control. After transfection of si-MIAT, the level of lncRNA MIAT was decreased in HL-1 cells (Fig. 2c). A significant increased
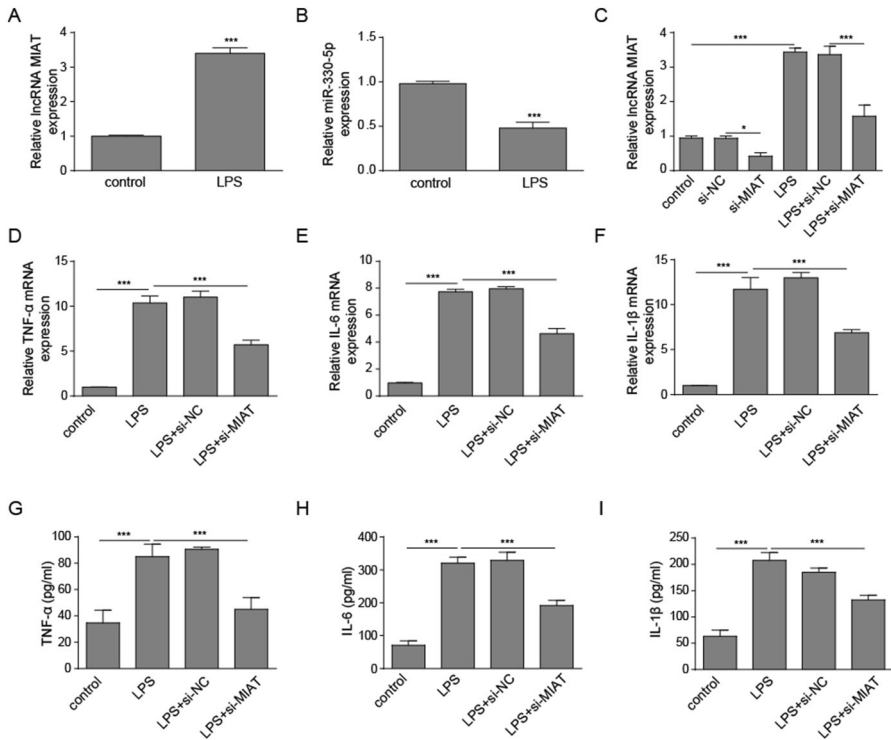
Fig. 2 LncRNA MIAT knockdown inhibited the expression of inflammatory cytokines. **a**, **b** The level of lncRNA MIAT and miR-330-5p were detected using qRT-PCR in LPS-induced HL-1 cells or normal controls. **c** qRT-PCR analysis was used to evaluate the expression of lncRNA MIAT in HL-1 cells. **d–f** qRT-PCR analysis was used to evaluated the relative mRNA level of inflammatory cytokines (TNF-α, IL-6 and IL-1β) in LPS-induced HL-1 cells with si-MIAT or si-NC. **g–i** The expression level of inflammatory cytokines (TNF-α, IL-6 and IL-1β) in HL-1 cells were detected using ELISA assay. ***$P < 0.001$. Data are presented as the means ± SD. All tests were performed in three independent biological and technical replicates

mRNA level of TNF-α, IL-6 and IL-1β was also observed in LPS groups compare with control groups (Fig. 2d–f). Notably, lncRNA MIAT depletion was corresponding to obviously lower mRNA expressions of TNF-α, IL-6 and IL-1β compared to that in LPS+si-NC groups (Fig. 2 D-F). The change of TNF-α, IL-6 and IL-1β protein levels were consistent with those of mRNA (Fig. 2g–i). The results suggested MIAT knockdown could ameliorate the inflammation responses induced by LPS in HL-1 atrial myocytes.

LncRNA MIAT Knockdown Alleviated LPS-Induced Oxidative Stress in HL-1 Cells

The roles of lncRNA MIAT in the LPS-induced oxidative stress were further evaluated in HL-1 cells. As shown in Fig. 3a and b, LPS treatment was corresponding to a distinct increase of green fluorescence signals in HL-1 cells compared with normal
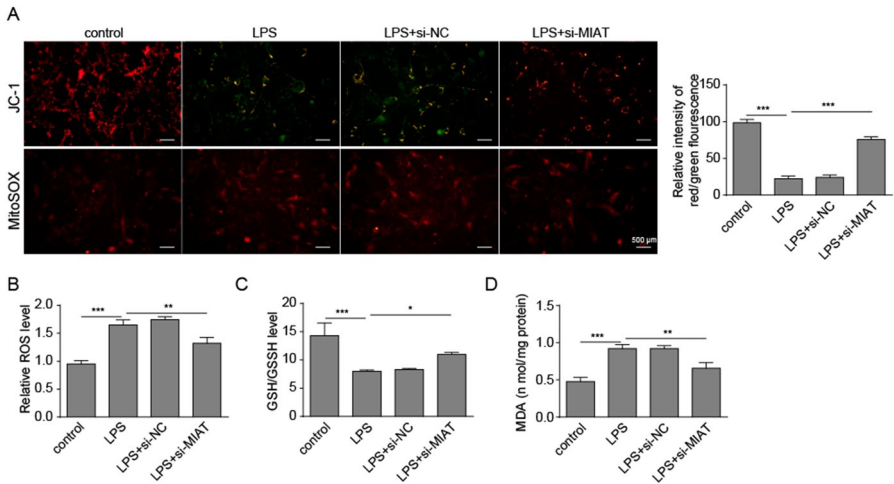
Fig. 3 LncRNA MIAT knockdown alleviated LPS-induced oxidative stress in HL-1 cells. After transfected with si-MIAT or si-NC, HL-1 cells were stimulated by LPS. **a** Confocal images of HL-1 cells stained with JC-1 and MitoSOX. Green fluorescence refers to JC-1 stained mitochondrial plasma with low JC-1 concentration and loss of mitochondrial membrane potential. Red fluorescence refers to JC-1 stained mitochondrial plasma with a high JC-1 concentration. Mitochondrial ROS was measured by MitoSOX. **b** Quantification of relative mitochondrial membrane potential (MMP). **c** Quantification of relative intracellular ROS level. **d** Reduced (GSH)/oxidized (GSSG) ratios. **e** Lipid peroxidation (MDA) level. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Data are presented as the means ± SD. All tests were performed in three independent biological and technical replicates (Color figure online)

control, indicating a depolarization in mitochondrial membrane potential (MMP) in LPS-stimulated HL-1 cells. While lncRNA MIAT knockdown restored the LPS-induced membrane potential decrease compared to that in cells that transfected with si-NC. As shown in Fig. 3a and c, mitochondrial ROS was significantly increased in LPS-stimulated HL-1 cells compared with normal control, while the ROS production was obviously suppressed in LPS-stimulated HL-1 cells that transfected with si-MIAT. Besides, compared to normal control, decreased GSH/GSSH ratio along with increased MDA level was observed in HL-1 cells after LPS treatment (Fig. 3d, e). For LPS-stimulated HL-1 cells, lncRNA MIAT knockdown significantly caused an increase in GSH/GSSH ratio but an obvious decrease in MDA level compared with si-NC group. These results suggested MIAT was associated with elevate oxidative stress in LPS treated HL-1 atrial myocytes.

LncRNA MIAT Directly Targeted miR-330-5p to Suppress NF-κB Signaling

To evaluate the effects of lncRNA MIAT on NF-κB signaling, the protein levels of TRAF6 and p-NF-κB (p-p65) were measured by western blot. As Fig. 4 A shows, obviously elevated levels of TRAF6 and p-p65/p65 were observed in LPS-stimulated group compared with normal control, while both were lowered by the transfection of si-MIAT than that in si-NC. Also, though MIAT was upregulated in
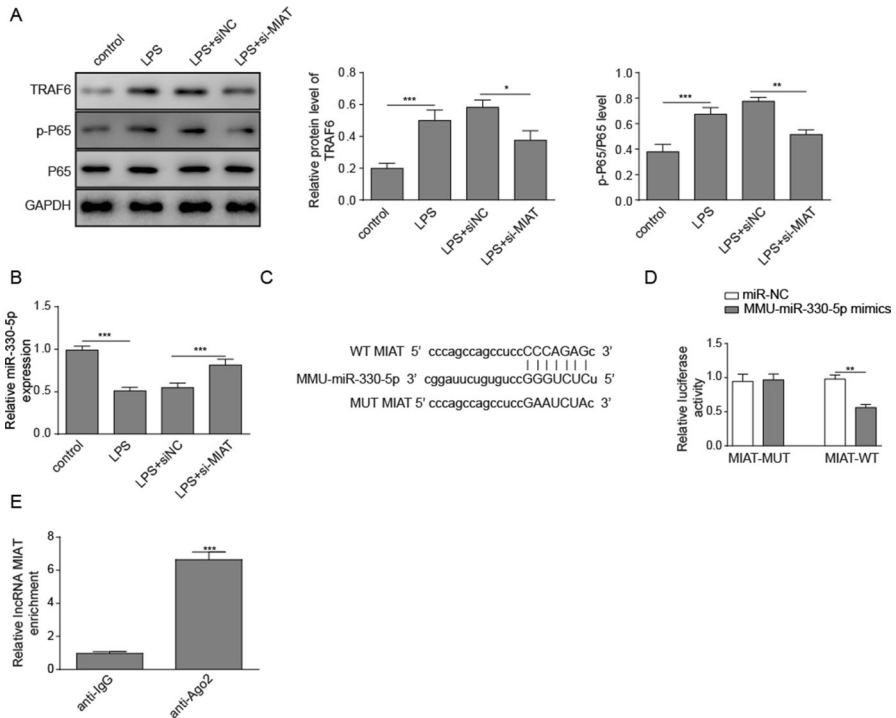
Fig. 4 LncRNA MIAT directly targeted miR-330-5p to suppress NF-κB signaling. **a** The protein levels of TRAF6, p-p65 and p65 were determined using western blot. GAPDH was used as an internal control. The grey degree analysis was quantitatively demonstrated in the right. **b** The relative expression level of miR-330-5p was determined using qRT-PCR. **c** Schematic of the predicted miR-330-5p binding site of lncRNA MIAT. **d** Dual-luciferase reporter assay was applied to evaluate relative luciferase activity. **e** RIP assay using IgG or Ago2 antibody was used to investigate the possible interaction between MIAT and miR-330-5p. Anti-IgG treatment group acted as a negative control. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ Data are presented as the means ± SD. All tests were performed in three independent biological and technical replicates

LPS-induced cells, the addition of NF-κB inhibitor JSH-23 did not change the level of MIAT obviously, suggesting the change on MIAT level was induced by LPS treatment and MIAT could be the upstream of NF-κB activation (Supplementary information-Fig. 2A). The role of lncRNA MIAT knockdown in miR-330-5p expression level was evaluated as well using qRT-PCR. MiR-330-5p was significantly down-regulated in LPS-stimulated HL-1 cells compared with normal control; however, the expression of miR-330-5p was distinctly reversed by the transfection of si-MIAT than that in si-NC (Fig. 4b). Dual-luciferase reporter assay was further conducted to identify the interaction between lncRNA MIAT and miR-330-5p. MIAT was predicted to be the potential target of miR-330-5p based on bioinformatics analysis. The putative binding site of miR-330-5p to MIAT is illustrated in Fig. 4c. As shown in Fig. 4d, a significant decrease in luciferase activity of MIAT-WT by the overexpression of miR-330-5p, whereas this effect was not obviously detected in

MIAT-MUT. Furthermore, RNA immunoprecipitation assay demonstrated MIAT were much more enriched by the antibody against Ago2 than IgG (Fig. 4e), which confirmed MIAT may act as a ceRNA to sponge miR-330-5p.

Overexpression of miR-330-5p Inhibited Inflammation Response and Alleviated LPS-Induced Oxidative Stress in HL-1 cells

To explore the roles of miR-330-5p in the regulation of inflammation response and oxidative stress during the septic cardiomyopathy progress, miR-330-5p was overexpressed in HL-1 cells and cells were then treated with LPS. Figure 5a–d show that miR-330-5p was downregulated while inflammatory cytokines (TNF-α, IL-6, IL-1β) were markedly upregulated in LPS-stimulated HL-1 compared with that in normal control. The miR-330-5p was validated to be overexpressed after the induction of miR-330-5p mimics in cells with or without LPS (Fig. 5a). After the LPS treatment, the overexpressed miR-330-5p was corresponding to the significantly lower mRNA expressions of above mentioned inflammatory cytokines in HL-1 compared with that transfected with mimic NC (Fig. 5b–d). Under the same condition, the protein levels of TNF-α, IL-6 and IL-1β were also decreased by miR-330-5p overexpression based on ELISA analysis (Fig. 5e–g). Additionally, loss of MMP and elevated mitochondrial ROS level were observed in LPS-stimulated HL-1 cells compared with that in normal control (Fig. 5h–j). The overexpression of miR-330-5p inhibited depolarization in MMP and was corresponding to a decreased ROS level compared with that in LPS-stimulated HL-1 cells transfected with mimic NC. As shown in Fig. 5k, the decreased GSH/GSSH ratio after LPS treatment was restored by the overexpression of miR-330-5p. Also, the increase in the level of lipid peroxidation marker MDA after LPS treatment was declined by the overexpression of miR-330-5p under the same condition (Fig. 5l). Collectively, the current results suggested anti-inflammatory and anti-oxidative properties of miR-330-5p overexpression in LPS-induced cardiac injury.

MiR-330-5p Suppressed NF-κB signaling via Targeting TRAF6

In order to investigate the effects of miR-330-5p on NF-κB signaling and its underlying mechanism, HL-1 cells were transfected with miR-330-5p mimics or mimic NC, followed by LPS treatment. As shown in Fig. 6a, protein levels of TRAF6 and phosphorylated p65 (p-p65) subunit dramatically elevated after LPS treatment compared with normal control, while both levels were downregulated by the transfection of miR-330-5p mimics compared with mimic NC. Notably, the expression level of miR-330-5p in HL-1 significantly decreased after the LPS treatment, while the inhibition of NF-κB did not cause obvious change on miR-330-5p expression level under LPS treatment, suggesting miR-330-5p might be the upstream of NF-κB signaling (Supplementary information-Fig. 2B). Dual-luciferase reporter assay was further conducted to examine the interaction between miR-330-5p and TRAF6. TRAF6 was predicted to be the potential target of miR-330-5p via bioinformatics analysis. The putative binding site of miR-330-5p in the 3′-UTR of TRAF6 is
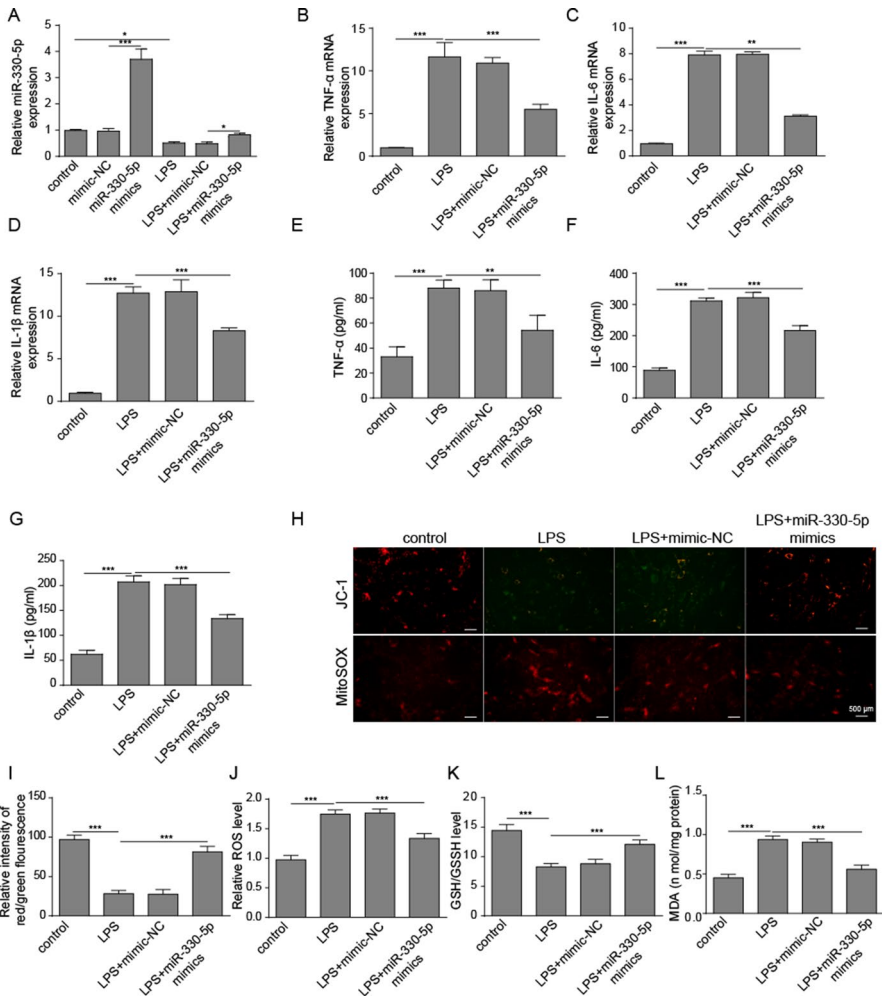
Fig. 5 The effects of miR-330-5p on the expression of inflammatory cytokines and LPS-induced oxidative stress in murine HL-1 myocyte. After transfected with miR-330-5p mimic or mimic NC, HL-1 cells were stimulated with or without LPS. **a–d** The level of miR-330-5p, TNF-α, IL-6 and IL-1β were determined by qRT-PCR. **e–g** The expression of inflammatory cytokines TNF-α, IL-6 and IL-1β in HL-1 cells were detected using ELISA assay. **h** Confocal images of HL-1 cells stained with JC-1 and Mito-SOX. **i** Quantification of relative mitochondrial membrane potential (MMP). **j** Quantification of relative mitochondrial ROS level. **k** Reduced (GSH)/oxidized (GSSG) ratios. **l** Lipid peroxidation (MDA) level. $**P < 0.01$, $***P < 0.001$. Data are presented as the means ± SD. All tests were performed in three independent biological and technical replicates

illustrated in Fig. 6b. Luciferase reporter vectors containing the predicted wild type or mutant binding sites of miR-330-5p in the 3′-UTR of TRAF6 were co-transfected with miR-330-5p mimics or mimic NC into the HL-1 cells. As Fig. 6c demonstrates, the overexpression of miR-330-5p significantly declined the luciferase activity of TRAF6-WT at both positions. Contrarily, no obvious change on luciferase activity
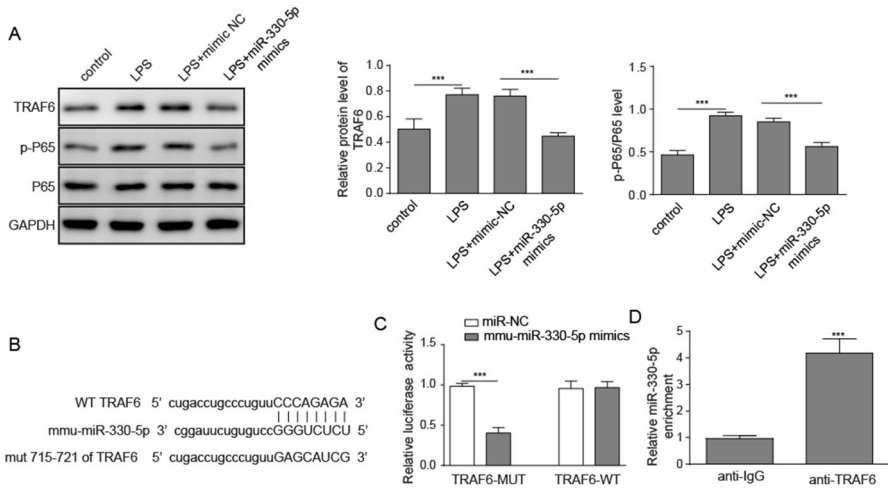
Fig. 6 MiR-330-5p suppressed NF-κB signaling via targeting TRAF6. **a** The protein levels of TRAF6, p-p65 and p65 were determined using western blot. GAPDH was used as an internal control. The grey degree analysis was quantitatively demonstrated in the right. **b** Schematic of the predicted mmu-miR-330-5p binding site at 3′-UTR of TRAF6 (Position 1298–1305). **c** Dual-luciferase reporter assay was applied to evaluate relative luciferase activity. **d** RIP assay was used to investigate the possible interaction between TRAF6 and miR-330-5p. Anti-IgG treatment group acted as a negative control. ***$P < 0.001$. Data are presented as the means ± SD. All tests were performed in three independent biological and technical replicates

was observed in TRAF6-MUT. RIP assay further confirmed miR-330-5p were substantially enriched by the antibody against TRAF6 compared to control antibody IgG (Fig. 6d), indicating the direct binding between TRAF6 and miR-330-5p.

Discussion

The current study was aimed at investigating the role of lncRNA MIAT in the modulation of inflammation responses and oxidative stress during the septic cardiomyopathy pathogenesis. At in vivo level, our results demonstrated that lncRNA MIAT was upregulated, whereas miR-330-5p was downregulated in the heart tissue from septic mice. The levels of serous inflammatory cytokine TNF-α, IL-6 and IL-1β were found to rise significantly in septic mice. Furthermore, in vitro experiments conducted on HL-1 murine atrial myocyte revealed that lncRNA MIAT promotes the activation of TRAF6/NF-κB signaling via targeting miR-330-5p, which eventually mediates the release of inflammatory cytokines and the intracellular oxidative stress.

Sepsis is the dominant cause of death in the intensive care units worldwide (Zou 2014). Evidence showed that myocardial dysfunction occurred during the course of sepsis has contributed to a high mortality of septic shock clinically (Rudiger and Singer 2007). Notably, the main reasons for septic cardiomyopathy included the excessive release of inflammatory cytokines and enhanced oxidative stress by the

incidence of sepsis (Oliveira et al. 2017; Kalbitz et al. 2016; Montini et al. 2016). It has been well known that lncRNAs play functional roles in multiple biology processes, especially in inflammatory responses in sepsis-related diseases (Yang 2013; Pearson and Jones 2016). For instance, lncRNA HOTAIR facilitates TNF-α production in cardiomyocytes from LPS-induced septic mice via activating NF-κB signaling (Wu 2016); lncRNA H19 regulates miR-874 expression in LPS-induced sepsis (Fang 2018) and lncRNA Lethe is involved in sepsis by negatively regulating NF-κB (Rapicavoli 2013). Myocardial dysfunction is a fatal complication that can develop in the patients who suffer from sever sepsis. LncRNA MIAT has come to our interest in light of its upregulated expression in sepsis-related myocardial diseases (Meydan et al. 2018). Mounting studies have suggested the role of MIAT in regulating inflammatory cytokines including IL-1, IL-18 and TNF-α (Meydan et al. 2018; Yan 2015). Besides, the interaction between MIAT and several miRNAs such as miR-150-5p and miR-93 has been reported previously, which demonstrated its functional roles in multiple disease pathogenesis including diabetes and EOC (Li 2018; Shao 2018; Yan 2015). However, the underlying molecular mechanism by which MIAT affects the progress of septic cardiomyopathy has not been fully elucidated. To our best knowledge, the present study reported for the first time the molecular mechanism by which MIAT regulated the release of inflammatory cytokines and oxidative stress in septic cardiomyopathy.

Moreover, the current study identified miR-330-5p directly targeted TRAF6 to alleviate sepsis-induced inflammation response and oxidative stress in cardiomyocytes, which is a novel finding. It has been demonstrated earlier that miR-330-5p targeted against MIAT expression in human EOC cells (Shao 2018). Our findings demonstrated MIAT can directly bind to miR-330-5p in murine cardiomyocytes, which extended the previous findings to septic cardiomyopathy. Additionally, this study confirmed that miR-330-5p targeted TRAF6 to modulate inflammation response and intracellular oxidative stress in septic cardiomyopathy by suppressing NF-κB signaling. TRAF6 acts as a signal transducer in the activation of NF-κB signaling by binding to its downstream signaling molecules, and is a key mediator for diverse signaling pathways (Lee 2016). Previous studies reported that TRAF6-mediated activation of NF-κB signaling was elevated in ox-LDL-induced mast cells and patients with acute coronary syndrome (Meng et al. 2013; Lin and An 2017), indicating its role in modulating inflammation response in cardiovascular dysfunction. The present study demonstrated a statistically significant inverse association between the expression of miR-330-5p and TRAF6 in HL-1 cells, indicating a biological function of miR-330-5p-TRAF6 complex in cardiomyocytes. Evidence has shown the upregulation of miR-330-5p can ameliorate oxidative stress and inflammation response in ox-LDL-stimulated human macrophages (Liu et al. 2019). Our findings demonstrated a direct inhibitory effect of overexpressing miR-330-5p on TRAF6 expression and the NF-κB signaling, along with its repressive role in modulating sepsis-induced oxidative stress and inflammation response in septic cardiomyopathy.

In conclusion, our study revealed that lncRNA MIAT targets against miR-330-5p to activate TRAF6/NF-κB axis, which could further promote inflammation and oxidative stress of sepsis-induced cardiac injury. Current findings suggest the upregulation of MIAT and downregulation of miR-330-5p could be the response reactions of

cardiomyocyte after LPS stimulation, and MIAT knockdown or miR-330-5p overexpression can ameliorate LPS-induced inflammation responses and oxidative stress in cardiomyocyte. It is also a novel finding that miR-330-5p can target TRAF6, which subsequently mediates the regulation of NF-κB signaling to play a protective role in sepsis-induced cardiac injury. However, limitations still exist in this study as the current molecular mechanisms are restrained to in vitro level. Further in vivo experiment in would be required to validate this result. Also, as mentioned before this study was conducted in animal model, experiments based on human tissues are necessary to extend our findings to clinical applications. As lncRNAs are involved in the regulation of multiple signaling pathways, other downstream miRNAs or mRNAs of MIAT will be considered as the targets in future studies.

Compliance with Ethical Standards

Conflict of interest The authors of this study declared no conflict of interests.

Ethics Approval All animal experiments were approved by the Institutional Animal Care and Use Committee of the Shanghai Sixth People's Hospital East.

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