

IL-6 induced lncRNA MALAT1 enhances TNF- α expression in LPS-induced septic cardiomyocytes via activation of SAA3

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Abstract. – OBJECTIVE: This study aimed to explore the expression of MALAT1 and its correlation with TNF- α production in lipopolysaccharide (LPS)-induced septic cardiomyocytes. Then, the effect of metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) on LPS-induced cardiomyocyte apoptosis is further studied.

MATERIALS AND METHODS: The hub genes in cell response to LPS treatments was analyzed by using Affymetrix gene profiling data downloaded from GEO dataset (GSE3140). Mice model of sepsis was induced by intraperitoneal injection of LPS. HL-1 cells were used as the *in vitro* cell model. MALAT1 and serum amyloid antigen 3 (SAA3) expression were measured by the qRT-PCR analysis. IL-6, TNF- α , and SAA3 concentrations were quantified by the ELISA assay. Flow cytometric analysis and TUNEL assay were performed to detect cell apoptosis.

RESULTS: IL-6 is a hub gene in cell response to LPS treatment and induces MALAT1 upregulation in cardiomyocytes. MALAT1 siRNA had an inhibitive effect, while MALAT1 overexpression showed enhancing effect on LPS induced TNF- α elevation. HL-1 cells treated with LPS had significantly elevated SAA3 expression. Inhibition of SAA during LPS treatment significantly reduced the TNF- α expression, while the addition of apo SAA significantly abrogated the suppressive effect of MALAT1 siRNA on TNF- α expression. HL-1 cells transfected with MALAT1 siRNA were less susceptible to LPS-induced cell apoptosis and with a lower apoptosis rate than the control group.

CONCLUSIONS: IL-6 induced MALAT1 upregulation in cardiomyocytes in response to LPS treatment. MALAT1 can enhance TNF- α expression at least partly via SAA3 in LPS-treated car-

diomyocytes. MALAT1 upregulation is a mechanism of cardiomyocyte death in response to the LPS stimulation.

Key Words:

MALAT1, TNF- α , Lipopolysaccharide, Sepsis, SAA3.

Introduction

Lipopolysaccharide (LPS), a Gram-negative bacterial cell wall component, is a potent inducer of acute sepsis, which is characterized as a systematic inflammatory response^{1,2}. Currently, sepsis is still a major cause of death in intensive care units across the world^{3,4}. Sepsis-induced myocardial dysfunction is a complication of severe sepsis that indicates poor prognosis⁴. Now it is clear that LPS-stimulated production of cytokines, such as tumor necrosis factor-alpha (TNF- α), interleukin-6 (IL-6), IL-10 and IFN- γ can induce apoptosis of cardiomyocytes^{3,5,6}. However, the mechanism underlying the LPS-induced upregulation of cytokines is not well elucidated.

Some studies^{7,8} suggest that long non-coding RNAs (lncRNAs) are also involved in the pathological progress of sepsis. For example, one recent study⁹ observed that the lncRNA HOTAIR expression was significantly upregulated in cardiomyocytes from sepsis mice, which enhanced the TNF- α production via activating the NF- κ B pathway. The metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) is a lncRNA

that may regulate the expression of inflammatory mediators, such as TNF- α and IL-6 in endothelial cells^{10,11}. MALAT1 can also regulate the expression of serum amyloid antigen 3 (SAA3), an inflammatory ligand that can stimulate IL-6 and TNF- α production^{10,12,13}. These findings implicate a role of MALAT1 as an inflammation regulator. Another recent study¹⁴ reported that MALAT1 is significantly upregulated in cardiac tissue of diabetic rats, while MALAT1 inhibition could improve left ventricular systolic function partly via attenuating diabetes-induced myocardial inflammation. However, how MALAT1 upregulation is induced is still not clear.

In this work, we firstly investigated the association between IL-6 and MALAT1 expression in cardiomyocytes. Also, we hypothesized that MALAT1 might act as an inflammation regulator in cardiac tissues and might be associated with LPS-induced production of cytokines and cardiomyocyte apoptosis. Therefore, we further studied the correlation between MALAT1 and TNF- α production in LPS-induced cardiac sepsis in mice model and in *in vitro* cardiomyocyte model. Then, the effect of MALAT1 on LPS-induced cardiomyocyte apoptosis is also studied.

Materials and Methods

Microarray Data and Protein-Protein Interaction (PPI) Analysis

Affymetrix gene profiling that compared transcriptional gene profile in human adult peripheral blood mononuclear cells in response to 100 ng/ml LPS was retrieved from GEO dataset with Accession Number: GSE3140. The raw data file of the microarray was reanalyzed using Morpheus (<https://software.broadinstitute.org/morpheus/>). The top 40 upregulated genes were loaded into the Search Tool for the Retrieval of Interacting Genes (STRING) (<http://string-db.org/>) database for analysis. To ensure the validity of the network, only the experimentally validated interactions with a high confidence score ≥ 0.70 were included.

Mouse Model of LPS-induced Sepsis

The animal experiments were approved by the Experimental Animal Ethics Committee of WeiFang Medical University. Male C57B6/L mice were purchased from the Laboratory Animal Research Center of WeiFang Medical University and were housed under the specific pathogen free (SPF) facilities. The mice at the age of 8-10 week were

randomly divided into experiment group (n=6) and control group (n=6). In the experimental group, the mice received intraperitoneal injection of 5 mg/kg LPS (*Escherichia coli* LPS serotype 0111:B4; Sigma-Aldrich, Saint Louis, MO, USA), while in the control group, all mice received intraperitoneal injection of the same amount of saline solution. 12 hours after the injection, blood samples were collected for the ELISA assay of TNF- α protein; then, the mice were sacrificed to get the left ventricular (LV) cardiomyocytes. The cells were further used to quantitative real-time PCR (qRT-PCR).

Cell Culture

Murine HL-1 cardiomyocytes were kindly provided by William Claycomb (Louisiana State University, USA)¹⁵ and were maintained in Claycomb medium (51800C, Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 0.1 mM noradrenaline, 100 U/mL penicillin, and 100 U/mL streptomycin in a cell incubator with humidified atmosphere and 5% CO₂ at 37°C. For LPS or IL-6 treatment, the cells reached 60-70% confluence in six-well plates were treated with LPS dissolved in phosphate-buffered saline (PBS) (0.5 μ g/ml) or IL-6 dissolved in PBS (1 ng/ml or 10 ng/ml) for 12 hours.

Plasmid Preparation and Cell Treatment

The human MALAT1 cDNA (Gene ID: 378938) was amplified by RT-PCR with the RNA extracted from HL-1 cells, and then cloned into pLV4 vector according to the method introduced in one previous study and was named as pLV4-MALAT1. The lentiviral particles were produced by co-transfecting expression vector pLV4-MALAT1 with viral particle packaging helper vector into 293T cells. The HL-1 cells were infected with the viral particles with the presence of Polybrene.

Small interference RNA (siRNA) targeting MALAT1, SAA as well as the corresponding negative controls were designed and synthesized by RiboBio (Guangzhou, China). HL-1 cells reached 50% confluence in six-well plates were transfected with siRNAs (50 nM) by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. Then cells were cultured for an additional 48 hours before subjected to LPS treatment.

For apo SAA treatment, HL-1 cells after transfection of MALAT1 siRNA were cultured in the presence or absence of 5 μ g/ml apo SAA (Peprotech, Rocky Hill, NJ, USA) for 48 hours before subjected to LPS treatment.

QRT-PCR Assay

Total RNAs in the cell samples were extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Then, the first strand cDNA was synthesized using the PrimeScript[®] RT reagent kit (TaKaRa, Dalian, Liaoning, China). MALAT1 and SAA3 level was then measured using gene-specific primers, MALAT1: forward, 5'-GACGGAGGTTGAGATGAAGC-3', reverse, 5'-ATTTCGGGGCTCTGTAGTCCT-3'; SAA3: forward, 5'-TGCCATCATTCTTTGCATCTTGA-3', reverse, 5'-CCGTGAACTTCTGAACAGCCT-3' and Power SYBR Green PCR Master Mix in an ABI Prism 7500 system (Applied Biosystems, Foster City, CA, USA). GAPDH served as the endogenous control. The expression change was calculated using $2^{-\Delta\Delta CT}$ method.

ELISA Assay

IL-6, TNF- α and SAA3 concentration in the blood samples and the cultured supernatant were quantified by using the mouse IL-6 ELISA KIT, TNF- α ELISA Kit (Boster, Wuhan, China) and the mouse SAA3 ELISA Kit (Cusabio, Wuhan, China) according to the manufacturer's instructions.

Flow Cytometric Analysis

After LPS treatment, cells were harvested by trypsinization and washed with PBS. The cells were stained using the annexin V/propidium iodide (PI) apoptosis detection kit (Yeasen, Shanghai, China) according to the manufacturer's instruction. The apoptotic cells were detected with a flow cytometer, using Cell Quest Pro software (FACScalibur, BD Biosciences, San Jose, CA, USA).

TUNEL Assay

The cardiomyocyte apoptosis was measured using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) (Roche Applied Science, Mannheim, Germany) according to the manufacturer's protocol. The cardiomyocytes were identified by simultaneous immunostaining with the anti-sarcomeric α -actinin monoclonal antibody (mAb) (ab9465, Abcam, Cambridge, UK). The nuclei were counterstained with Hoechst 33342 (Sigma-Aldrich, St. Louis, MO, USA). The stained cells were visualized using an Olympus IX73 (Tokyo, Japan) fluorescent microscope. The percentage of TUNEL-positive cells was determined by counting at least 200 cells in 5 randomly selected fields.

Statistical Analysis

The data are reported as the mean \pm standard deviation. The comparison between groups was performed using the Student's *t*-test. $p < 0.05$ was considered as statistically significant.

Results***IL-6 is a Hub Gene in Cell Response to LPS-induced Sepsis and is Associated with MALAT1 Upregulation***

Previous studies^{16,17} reported that septic shock is associated with significantly upregulated TNF- α and IL-6, which are the major triggers of LPS-induced septic pathogenesis. By reviewing the Affymetrix microarray data (GSE3140) and performing PPI analysis, we found that IL-6 might be a hub controller in the most significantly upregulated genes in cell response to LPS treatment (Figure 1A-B). In a mouse model of LPS-induced sepsis, ELISA assay confirmed significantly elevated plasma TNF- α and IL-6 levels in the septic mice (Figure 1C-D). Also, we observed that MALAT1 expression in the cardiac tissue from sepsis mice was about four times higher than that from the control group (Figure 1E).

IL-6 Induces MALAT1 Upregulation in Cardiomyocytes

By using HL-1 cells as LPS-induced septic model *in vitro*, we also observed upregulation of TNF- α , IL-6 and MALAT1 after LPS treatment (Figure 2A-C). Notably, we also observed that IL-6 treatment induced MALAT1 upregulation in HL-1 cells in a dose-dependent manner (Figure 2D).

MALAT1 Modulates TNF- α Expression via SAA3 in LPS-treated Cardiomyocytes

Then, we further studied whether MALAT1 has a direct regulative effect on TNF- α expression. HL-1 cells transfected with MALAT1 siRNA had about 60% inhibition of TNF- α elevation induced by LPS treatment (Figure 3A). In contrast, HL-1 cells infected with pLV4-MALAT1 increased significantly the LPS-induced TNF- α elevation (Figure 3B). Previous works^{12,13} reported that SAA3 is an inflammatory ligand that can stimulate IL-6 and TNF- α production through NF- κ B and p38 mitogen-activated protein kinase signaling pathway. Furthermore, MALAT1 can stimulate SAA3 expression in endothelial cells¹⁰ and mice liver cells¹⁸. Therefore, we hypothesized that MALAT1 might exert regulative effect on TNF- α

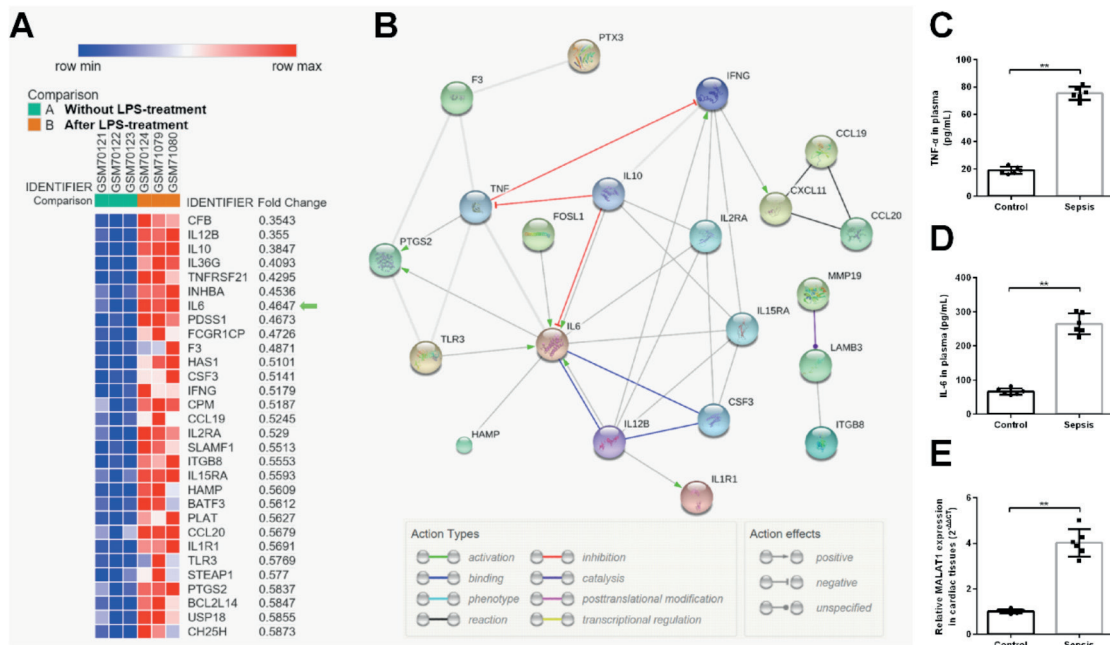


Figure 1. IL-6 is a hub gene in cardiomyocytes in response to LPS-induced sepsis. **A**, Heat map of the 40 up-regulated genes in human adult peripheral blood mononuclear cells with or without LPS-treatment (100 ng/ml). Data was retrieved from the GEO Dataset (Accession No. GSE3140). **B**, The protein-protein interaction (PPI) network of the 40 up-regulated genes analyzed by using the (STRING) (<http://string-db.org/>) database. Only experimentally validated interactions with a high confidence score ≥ 0.70 were included. **C-D**, ELISA assay of TNF- α (**C**) and IL-6 (**D**) expression in blood samples from mice (n=6) with or without LPS-induced sepsis. **E**, QRT-PCR analysis of MALAT1 expression in cardiac tissues from mice (n=6) with or without LPS-induced sepsis. ** $p < 0.01$.

expression via SAA3 in cardiomyocytes. QRT-PCR and ELISA assay showed that HL-1 cells treated with LPS had increased significantly the SAA3 expression (Figure 4A-B). HL-1 cells with enforced MALAT1 expression also had increased significantly the SAA3 expression at both mRNA and protein levels (Figure 4C-D). The inhibition of SAA during LPS treatment significantly reduced TNF- α expression (Figure 4E). To investigate whether the suppressive effect of MALAT1 siRNA on LPS-induced TNF- α upregulation requires SAA, MALAT1 silencing, and apo SAA treatment were performed simultaneously in HL-1 cells during LPS treatment. The addition of apo SAA significantly abrogated the suppressive effect of MALAT1 siRNA on LPS induced TNF- α expression (Figure 4F). These results suggest that MALAT1 could modulate the TNF- α expression via SAA3 in LPS-treated cardiomyocytes.

Knockdown of MALAT1 Partly Protects Cardiomyocytes from LPS-induced Apoptosis

To investigate whether MALAT1 participates in LPS-induced apoptosis in cardiomyocytes,

HL-1 cells were firstly transfected with MALAT1 siRNA (Figure 5A). Both flow cytometric analysis (Figure 5B-C) and TUNEL staining (Figure 5D-E) showed that the HL-1 cells transfected with MALAT1 siRNA were less susceptible to LPS-induced cell apoptosis, with a lower apoptosis rate than the control group transfected with scramble siRNA (Figure 5B-D). These findings suggest that MALAT1 upregulation contributed to the cardiac cell death in response to LPS stimulation.

Discussion

MALAT1 was initially discovered as a tumor-associated lncRNA, which is mainly involved in splicing and epigenetic regulation of gene expression¹⁹. Recent studies^{11,20} reported that MALAT1 might also act as a regulator of inflammation in kidney and cardiovascular diseases. In diabetic retinopathy (DR) models, MALAT1 knockdown could significantly ameliorate DR in terms of pericyte loss, capillary degeneration, microvascular leakage, and retinal inflammation¹¹. In cardiac tissue of diabetic rats, MALAT1 is si-

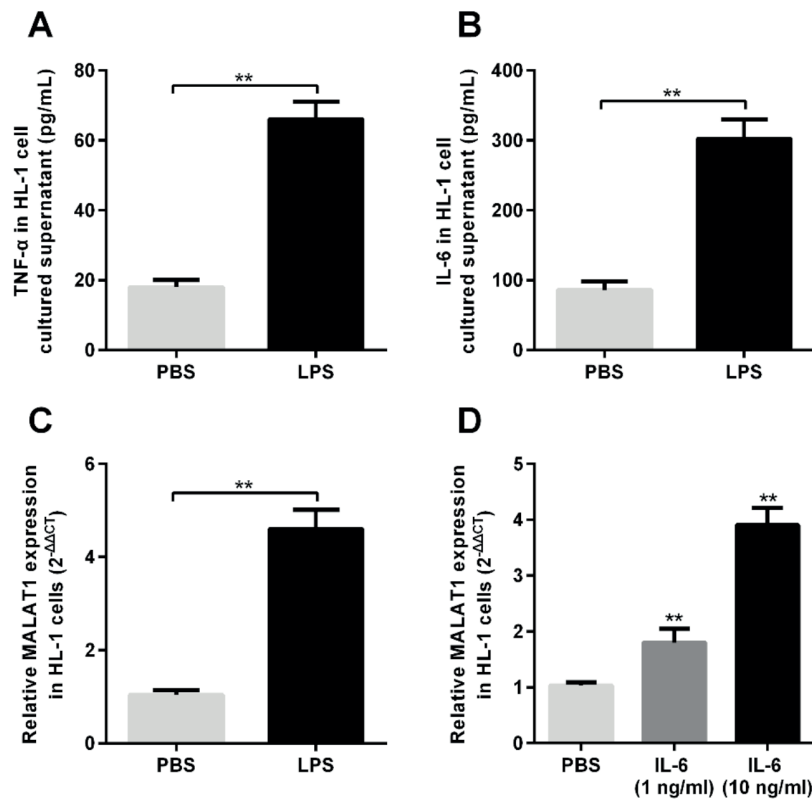


Figure 2. IL-6 induces MALAT1 upregulation in cardiomyocytes. *A-B*. ELISA assay of TNF- α (A) and IL-6 (B) levels in cultured supernatant of HL-1 cells (D) after treatment with LPS or PBS. *C-D*. QRT-PCR analysis of MALAT1 expression in HL-1 cells after treatment with LPS or PBS (C) or after treatment with LPS or IL-6 (D) ** $p < 0.01$.

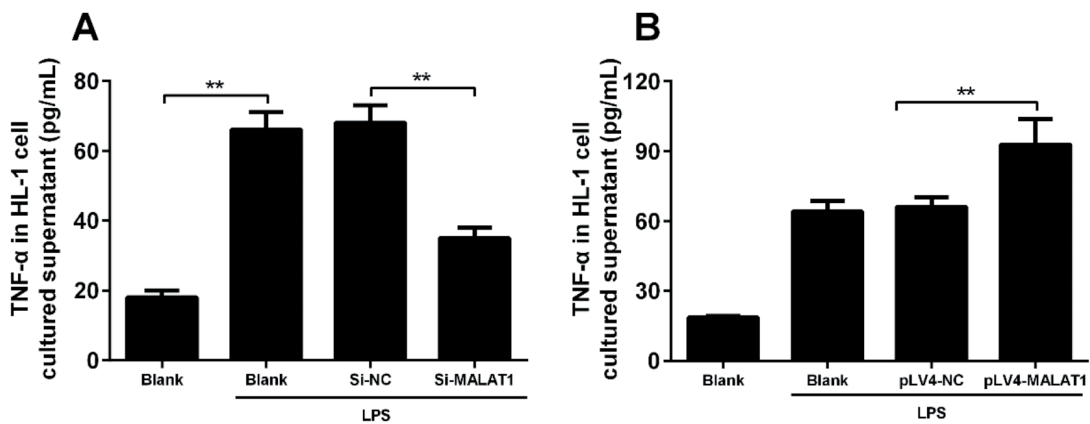


Figure 3. MALAT1 knockdown impairs LPS-induced TNF- α production. *A-B*. ELISA assay of TNF- α expression in cultured supernatant of HL-1 cells transfected with MALAT1 siRNA or the negative control (A) or transfected with pLV4-MALAT1 or the negative control (B) after treatment with LPS or PBS. ** $p < 0.01$.

gnificantly upregulated and MALAT1 inhibition could improve left ventricular systolic function partly via attenuating diabetes-induced myocardial inflammation¹⁴. Another recent paper²¹ re-

ported that MALAT1 is upregulated significantly in the peripheral blood of patients with acute myocardial infarction. These findings suggest that MALAT1 might act as an inflammation regulator

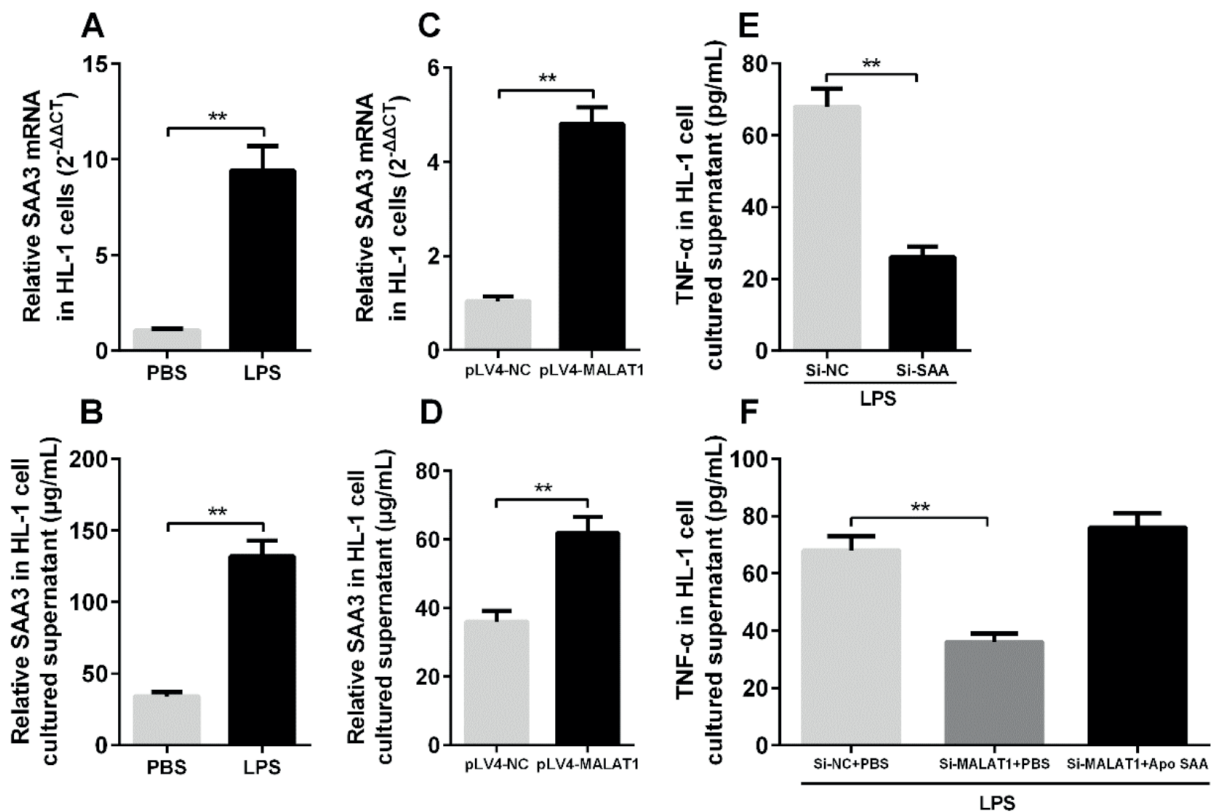


Figure 4. MALAT1 modulates TNF- α expression via SAA3 in LPS-treated cardiomyocytes. **A-B**, QRT-PCR analysis of SAA3 mRNA expression in HL-1 cells (A) and ELISA assay of SAA3 expression in cultured supernatant of HL-1 cells (B) after treatment with LPS or PBS. **C-D**, QRT-PCR analysis of SAA3 mRNA expression in HL-1 cells (C) and ELISA assay of SAA3 expression in cultured supernatant of HL-1 cells (D) after infection of pLV-MALAT1 or the negative control. **E**, ELISA assay of TNF- α expression in cultured supernatant of HL-1 cells with or without transfection of SAA siRNA after treatment with LPS. **F**, ELISA assay of TNF- α expression in cultured supernatant of HL-1 cells with or without stimulation of apo SAA after treatment with LPS. ** $p < 0.01$.

in the cardiac tissues. Till to date, there is no study that investigated the involvement of MALAT1 in LPS-induced inflammatory responses and how it plays a role in LPS-induced cardiomyocyte apoptosis. In this report, we firstly investigated MALAT1 expression in cardiac tissue of LPS induced septic mice and cells. The results showed that LPS-induced MALAT1 upregulation might be a result of IL-6 upregulation. Besides, previous studies^{10,11} reported that MALAT1 could enhance the expression of inflammatory mediators, such as TNF- α and IL-6 in endothelial cells. Therefore, we suggested that there might be possible feedback regulation between IL-6 and MALAT1 in septic cardiac tissues. However, this hypothesis needs further experimental validation.

In this study, we also observed that MALAT1 knockdown could suppress LPS-induced TNF- α production. TNF- α stimulated by LPS has been

demonstrated as one important inducer of myocardial dysfunction²². TNF- α doesn't only exerts negative inotropic effects on cardiac performance, but also induces cardiomyocyte apoptosis²³. The anti-TNF- α therapy shows a protective effect on the cardiac function in sepsis models^{23,24}. Considering the important role of TNF- α in LPS-induced myocardial dysfunction, we decided to investigate further the association between MALAT1 and TNF- α . Recent papers showed that MALAT1 could induce upregulation of SAA3, which belongs to the serum amyloid A family of the major acute-phase proteins with regulative effect on inflammation²⁵. The SAA upregulation is also closely related to the inflammatory responses in cardiovascular diseases^{26,27}. The elevated plasma SAA level is associated with an increased cardiovascular risk and predicts worse prognosis in patients with acute coro-

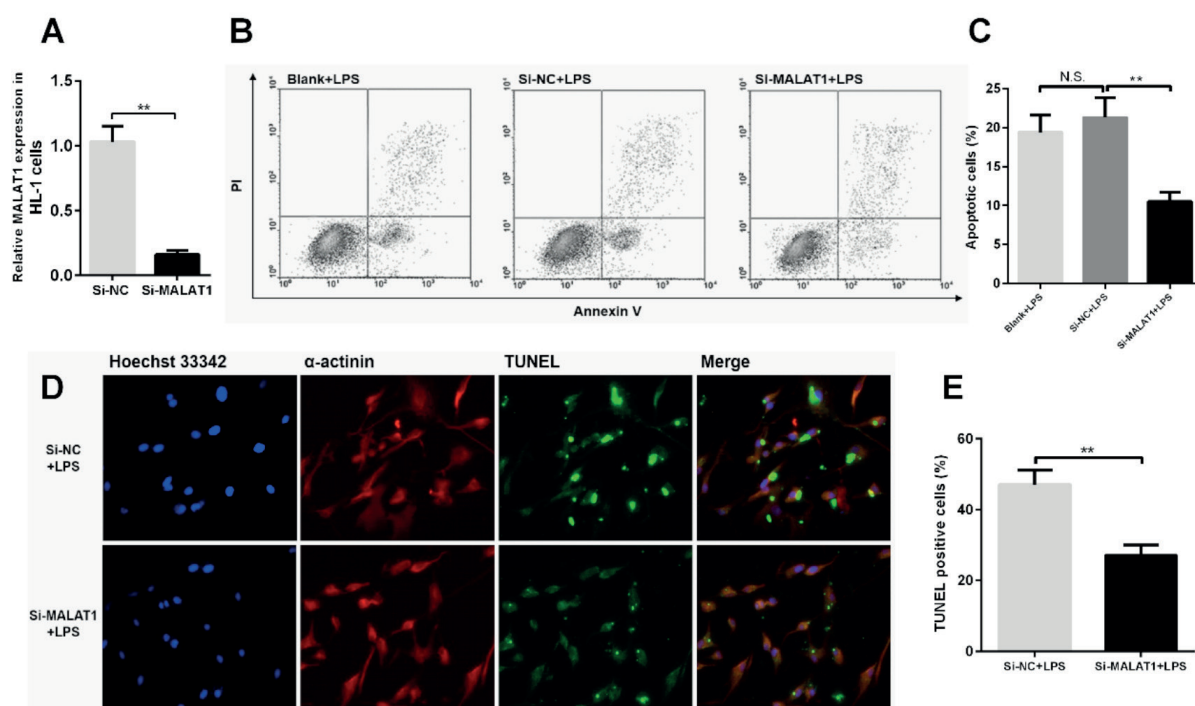


Figure 5. Knockdown of MALAT1 partly protects cardiomyocytes from LPS-induced apoptosis. **A**, QRT-PCR analysis of MALAT1 expression in HL-1 cells after transfection of MALAT1 siRNA or the negative control. **B-C**, Representative images (B) and quantitation (C) of flow cytometric analysis of apoptotic HL-1 cells with or without transfection of MALAT1 siRNA after treatment of LPS (0.5 $\mu\text{g/ml}$) for 12 hours. **D-E**, Representative images (D) and quantitation (E) of TUNEL-staining of apoptotic HL-1 cells with or without transfection of MALAT1 siRNA after treatment of LPS (0.5 $\mu\text{g/ml}$) for 12 hours. Blue: Hoechst33342; Red: α -actinin; Green: TUNEL. ** $p < 0.01$.

nary artery disease (CAD)²⁸. Functionally, SAA3 can stimulate IL-6 and TNF- α production through NF- κB and p38 mitogen-activated protein kinase signaling pathway^{12,13}. Therefore, we stated that MALAT1 might regulate LPS-induced TNF- α expression via SAA3 in cardiomyocyte. By performing QRT-PCR and ELISA, we found that HL-1 cells treated with LPS had elevated significantly the SAA3 expression. HL-1 cells with MALAT1 overexpression also had increased the SAA3 expression. The inhibition of SAA during LPS treatment significantly reduced the TNF- α expression, while the addition of apo SAA significantly abrogated the suppressive effect of MALAT1 siRNA on the TNF- α expression induced by LPS. These results suggest that MALAT1 can modulate TNF- α expression at least partly via SAA3 in LPS-treated cardiomyocytes.

Since we confirmed the regulative effect of MALAT1 on TNF- α production in cardiomyocytes, we decided to study further its regulative effect on LPS-induced cardiomyocyte apoptosis. By performing flow cytometric analysis and TUNEL

staining, we demonstrated that HL-1 cells transfected with MALAT1 siRNA were less susceptible to LPS-induced cell apoptosis, with a lower apoptosis rate than the control group transfected with scramble siRNA. Therefore, we infer that IL-6 induced MALAT1 upregulation is a mechanism of the cardiomyocyte death in response to the LPS stimulation.

Conclusions

IL-6 induced MALAT1 upregulation in cardiomyocytes in response to LPS treatment. MALAT1 can enhance TNF- α expression at least partly via SAA3 in LPS-treated cardiomyocytes. MALAT1 upregulation is a mechanism of the cardiomyocyte death in response to the LPS stimulation.

Conflict of interest

The authors declare no conflicts of interest.

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