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MicroRNA-98 negatively regulates IL-10 production and endotoxin tolerance in macrophages after LPS stimulation

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ABSTRACT

Interleukin 10 (IL-10) is a potent anti-inflammatory cytokine that is crucial for dampening the inflammatory response after pathogen invasion, and was found to be produced by macrophages after exposure to lipopolysaccharide (LPS). It remains unclear whether microRNA-mediated regulatory mechanism is involved in LPS-induced IL-10 production. Here we reported that miR-98 expression in macrophages significantly decreased following LPS stimulation. We also found that miR-98 targets the 3'untranslated region of IL-10 transcript. Overexpression of miR-98 inhibited TLR4-triggered IL-10 production and promoted COX-2 expression. We further demonstrated that miR-98 significantly mitigated the induction of endotoxin tolerance, suggesting that miR-98-mediated posttranscriptional control could potentially be involved in fine tuning the critical level of IL-10 production in endotoxin tolerance.

1. Introduction

Toll-like receptors (TLRs) play a critical role in innate immunity by recognizing a limited but highly conserved set of molecular structures produced by microorganisms (pathogen-associated molecular patterns, or PAMPs) [1]. TLR4 recognizes and binds to LPS to trigger a signaling cascade through the MyD88–dependent and/or MyD88-independent signaling pathway which eventually leads to activation of MAPK and NF-κB, resulting in the production of pro-inflammatory cytokines and factors (e.g., TNF-α, IL-1, IL-6 and COX-2). Later in the course of an innate antibacterial immune response, this burst of pro-inflammatory cytokines is followed by release of anti-inflammatory cytokines such as IL-10 and TGF-β that inhibit the pro-inflammatory immune response [2]. Although these LPS-induced robust and essential inflammatory reactions are indispensable for counteracting the growth and dissemination of gram-negative bacteria, this process needs to be tightly regulated in vivo. Uncontrolled inflammation leads to extensive tissue damage, sepsis syndrome, and even endotoxin shock [3]. IL-10 is a major anti-inflammatory cytokine that is crucial for dampening the inflammatory response after pathogen invasion and acts to protect the host from excessive inflammation [4].

MicroRNAs (miRNAs) are small RNA regulatory molecules (average 22 nucleotides long) that, when integrated into protein complexes known as RNA-induced silencing complexes, bind to partially complementary sequence in the 3′-untranslated regions of target mRNAs and thereby contribute to gene regulation by inhibiting translation and destabilizing transcripts [5]. miRNAs regulate the development and function of immune cells [6,7], and have pivotal roles in the regulation of both innate [8] and adaptive immune response [9]. miR-146a, miR-155, miR-125b and miR-21 were shown to inhibit the TLR-triggered inflammatory cytokines [8,10,11]. miR-98 and let-7 were reported to regulate cytokine-inducible Src homology 2-containing protein (CIS) protein expression via translational suppression in human cholangiocytes [12].

Regulation of IL-10 expression has been extensively studied at the transcriptional and posttranscriptional levels. Transcription factors IRF3, Sp1 and Sp3 have been found to regulate IL-10 transcription [13,14]. The AU-rich elements located in the 3′-UTR of these inflammatory mediators can be regulated by both miRNAs and RNA-binding proteins (RBPs). It has been shown that AU-rich elements in the 3′-UTR of mouse IL-10 lead to the degradation of

Abbreviations: TLR, toll-like receptors; LPS, lipopolysaccharide; MyD88, myeloid differentiation primary-response protein 88; IL, interleukin; TNF-α, tumor necrosis factor α; COX-2, cyclooxygenase-2; TGF-β, transforming growth factor β; TRIF, TIR-domain-containing adapter-inducing interferon β; TRAF3, TNF receptor-associated factor 3; PBMC, peripheral blood mononuclear cell; FBS, fetal bovine serum; IRF3, interferon regulatory factor 3

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its mRNA [15]. However, it remains unclear whether microRNA-mediated regulatory mechanism is involved in IL-10 production in TLR-triggered macrophages. In the present study, we reported that down-regulation of miR-98 after LPS exposure partially contributes to LPS-induced IL-10 production, which might be an important regulatory mechanism for controlling pro-inflammatory immune response and induction of endotoxin tolerance.

2. Materials and methods

2.1. Reagents

Lipopolysaccharide (LPS) was purchased from Sigma (St. Louis, MO). MiR-98 mimics and control mimics were obtained from GenePharma (Shanghai, China). Anti-COX-2, anti-β-actin and peroxidase (HRP)–labeled secondary antibody were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA).

2.2. Cell culture and transfection

HEK-293 and RAW 264.7 cell lines were obtained from American Type Culture Collection (Manassas, VA) and cultured in DMEM containing 10% FBS. 1×10^4 cells was seeded into 96-well plates and incubated overnight. JetSI-ENDO transfection reagents (Polyplus-transfection) were used for the cotransfection of plasmids and RNAs, according to the manufacture instructions.

2.3. 3′-UTR luciferase reporter assays

The wild type mouse IL-10 3′-UTR luciferase reporter vectors were constructed by amplifying the mouse IL-10 mRNA 3′-UTR and cloning it into Xbal site of PGL3-prooter vector (Promega). HEK-293 cells were cotransfected with 80 ng luciferase reporter plasmid, 40 ng thymidine kinase promoter-Renilla luciferase reporter plasmid, and the indicated miRNA mimics or controls (final concentration, 20 nM). After 24 h, luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacture instructions.

2.4. Detection of cytokine production

IL-10, IL-6 and TNF-α production in the cell supernatants were measured with ELISA Kits (ebioscience) according to the manufacturer's protocols.

2.5. RNA isolation and real-time quantitative PCR (qPCR)

Total RNA was extracted with TRIzol (Invitrogen). Real-time quantitative PCR, using SYBR Green detection chemistry, was performed on a 7500 Real-Time PCR System (Applied Biosystems).

2.6. RNA interference

The IL-10-specific siRNA were 5′-ACUGCUAACCGACUCCUUATT-3′ (sense) and 5′-UAAGGAGUCGGUUAGCAGUTT-3′ (antisense). The scrambled control RNA sequences were 5′-UUCUCCGAACGUGUCACGUTT-3′ (sense) and 5′-ACGUGACACGUUCGGAGAATT-3′ (antisense). siRNA duplexes were transfected into RAW 264.7 cells at a final concentration of 10 nM.

2.7. Western blot analysis

Total cell lysates were prepared and subjected to SDS/PAGE gel, transferred onto PVDF membrane, and blotted as we previously described [16].

2.8. Statistical analysis

All experiments were repeated three times. Data was presented as the mean ± S.D. Statistical significance was determined by Student's *t*-test, with values of $P < 0.05$ considered to be statistically significant.

3. Results

3.1. miR-98 is down-regulated in macrophages after LPS stimulation

Previous studies have demonstrated that LPS treatment augments the expression of several microRNAs such as miR-155 and miR-146, which have been implicated in LPS response and endotoxin shock models [3,17]. To investigate whether miR-98 is also a regulator of TLR-triggered signaling pathway, we analyzed the miR-98 expression in RAW 264.7 macrophages after LPS stimulation. As shown in Fig. 1A, miR-98 expression significantly decreased in LPS stimulated RAW 264.7 macrophage, and the down-regulation of miR-98 expression by LPS treatment was dose dependent. We also stimulated these cells with LPS over a time course. miR-98 levels rapidly decreased and reached the minimum in 8 h (Fig. 1B). These data demonstrated that miR-98 is constitutively expressed in RAW 264.7 macrophages, and LPS-generated signals negatively regulated its expression, suggesting that miR-98 may be involved in the regulation of TLR4 mediated signaling in macrophages.

3.2. IL-10 is a potential target of miR-98

To identify potential targets for miR-98, we searched for the miR-98 targets by multiple prediction algorithms (miRBase, PicTar,

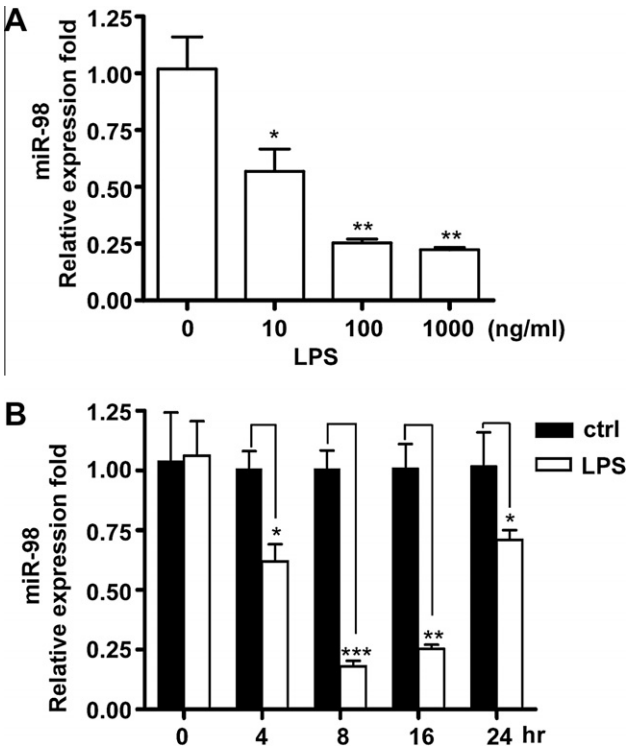

Fig. 1. LPS inhibits miR-98 expression in RAW 264.7 cells. (A) RAW 264.7 were treated with indicated dose of LPS for 24 h, the expression of miR-98 was measured by q-PCR and normalized to the expression of U6. (B) RAW 264.7 cells were stimulated with LPS (100 ng/ml) for indicated time, and miR-98 expression was measured. Data are the mean ± S.D. ($n = 4$) of one representative experiment. Similar results were obtained in three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

and TargetScan v.5.1). The different target prediction algorithms place varying emphases on seed sequence complementarity, heteroduplex free energy of binding, location and size of internal loops and hulges, and accessibility of the target site (as predicted by RNA binding). This analysis identified IL-10 as a potential miR-98 target (Fig. 2A). To certify the possibility that IL-10 was regulated post-transcriptionally by miR-98, we constructed reporter plasmids by amplifying the mouse IL-10 mRNA 3′-UTR and cloning into XbaI site of pGL3 vector. By cotransfection of the reporter plasmids and internal control pRL-TK-Renilla-luciferase plasmids with miR-98 mimics or the controls (scrambled oligonucleotide) in HEK-293 cells, we observed that miR-98 mimics markedly decreased the luciferase activity in cells transfected with the IL-10 3′-UTR vector compared with the controls. No change in luciferase activity was observed in the PGL3 control vector (Fig. 2B). We further examined the luciferase activity of IL-10 mRNA 3′-UTR reporter vector in RAW 264.7 macrophages. As shown in Fig. 2C–E, miR-98 mimics also markedly decreased the luciferase activity in RAW 264.7. LPS treatment significantly enhanced the luciferase activity, and this effect was abrogated by miR-98 mimics.

3.3. LPS-induced up-regulation of IL-10 production was abrogated by miR-98 mimics

To further examine the role of miR-98 in LPS-induced up-regulation of IL-10 in macrophages, IL-10 mRNA and protein levels were measured in macrophages after LPS stimulation by qRT-PCR and ELISA. As shown in Fig. 3A and B, LPS-induced IL-10 protein expression was significantly inhibited in macrophages transfected with miR-98, but LPS-induced IL-10 transcription was not affected. We further examined whether LPS-mediated down-regulation of miR-98 could affect the production of pro-inflammatory cytokines. TNF-α, IL-6 and COX-2 were measured in TLR4-triggered macrophages after 12 h. As shown in Fig. 3C–E, it showed no difference in TNF-α and IL-6 production in RAW 264.7 transfected with miR-98 mimics and controls. COX-2 protein level increased significantly in cells transfected with miR-98 mimics compared to controls. These results are consistent with previous observations that IL-10 inhibits LPS-induced COX-2 expression.

3.4. miR-98 significantly mitigates the induction of LPS desensitization

Considering the up-regulation of IL-10 acts as a hallmark of endotoxin-tolerant macrophages and IL-10 plays a key role in the regulatory network leading to LPS desensitization, we further investigated whether LPS-mediated down-regulation of miR-98 was involved in the induction of LPS hyporesponsiveness. RAW 264.7 cells were primed with 10 ng/ml LPS for 18 h, followed by washing with PBS, cells were incubated in fresh complete culture medium for 2 h before secondary LPS challenge (1, 10 μg/ml). Interestingly, RAW 264.7 cells transfected with miR-98 mimics produced significantly higher levels of TNF-α and IL-6 compared to the cells transfected with controls (scrambled oligonucleotide) in response to secondary LPS challenge as shown in Fig. 4A. Furthermore, we found cells transfected with miR-98 mimics produced significantly lower levels of IL-10 in both first and secondary LPS challenge (Fig. 4B). To further investigate whether overexpression of miR-98 mitigated the induction of LPS desensitization via

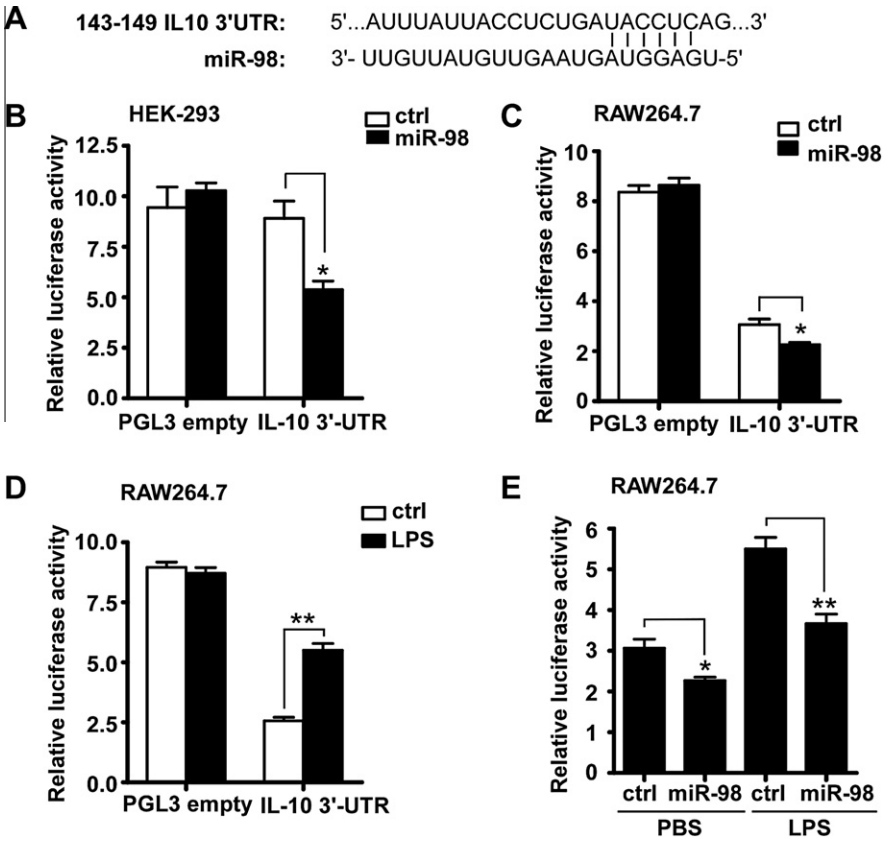

Fig. 2. IL-10 may be molecular targets of miR-98 posttranscriptional repression. (A) Shown is a sequence alignment of miR-98 and its target sites in 3′ UTRs of IL-10. 1×10^4 HEK-293 cells (B) or RAW 264.7 cells (C) were cotransfected as described in methods. After 24 h, firefly luciferase activity was measured and normalized by Renilla luciferase activity. (D) RAW 264.7 were transfected with indicated plasmids, 24 h later, cells were stimulated with LPS (1 μg/ml) for another 12 h, firefly luciferase activity was measured. (E) RAW 264.7 were cotransfected with indicated plasmids and miR-98 mimics. After 24 h, cells were stimulated with LPS (1 μg/ml) or PBS for another 12 h, then firefly luciferase activity was measured. Data are the mean ± S.D. ($n = 4$) of one representative experiment. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

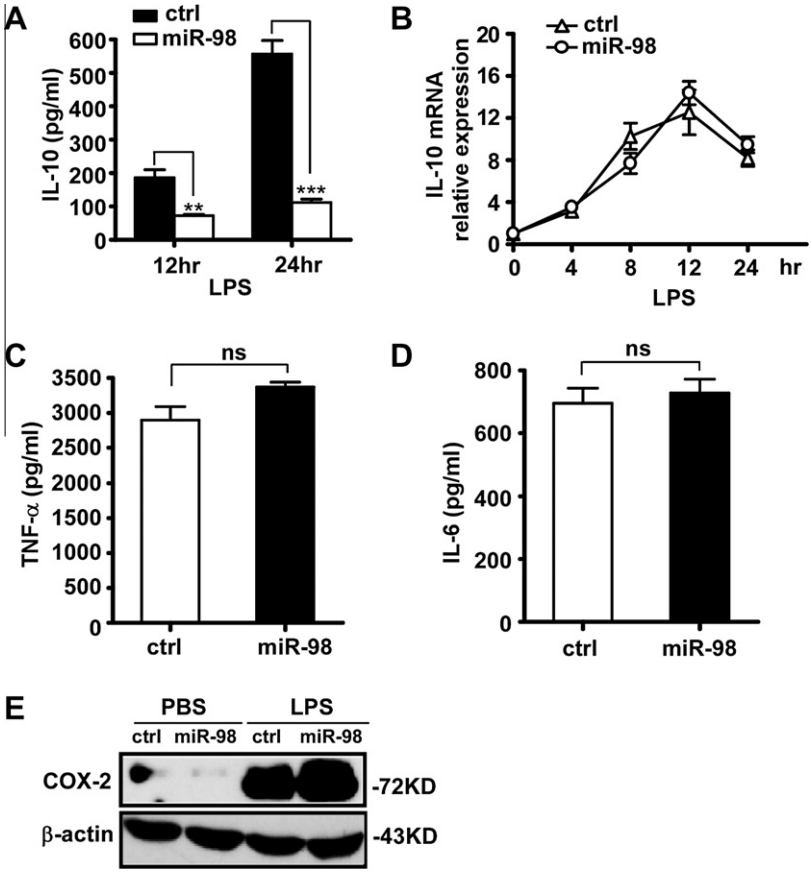

Fig. 3. miR-98 inhibits the IL-10 production in TLR4-triggered macrophages. RAW 264.7 cells were transfected with miR-98 mimics or controls at a final concentration of 20 nM. 24 h later, cells were stimulated with or without LPS (1 μg/ml). Supernatants were collected after 12 or 24 h to measure IL-10 (A), TNF-α (C), and IL-6 (D) by ELISA. (B) IL-10 mRNA was detected by qRT-PCR in RAW 264.7 stimulated by LPS (1 μg/ml) for indicated time. Data are the mean ± S.D. ($n = 4$) of one representative experiment. $^{**}P < 0.01$; $^{***}P < 0.001$. (E) COX-2 protein level was analyzed by Western blot. Representative of three independent experiments.

down-regulation of IL-10, we examined the effects of IL-10 knockdown on the induction of LPS desensitization. siRNA specific to mouse IL-10 dramatically inhibited LPS-induced mRNA and protein expression of IL-10 (Fig. 4C). As shown in Fig. 4D, silencing IL-10 by siRNA significantly mitigates the induction of LPS hyporesponsiveness. So, this result indicated that LPS-mediated inhibition of miR-98 expression is at least partially involved in the induction of LPS desensitization via up-regulation of IL-10.

4. Discussion

In our study, we detected a significant decrease of miR-98 expression in RAW 264.7 cells following LPS stimulation for up to 24 h. We also demonstrated that miR-98 targets the 3′-untranslated region of IL-10 transcripts, therefore, its down-regulation in response to LPS may be required for proper IL-10 production.

IL-10 are key mediators of the immune response during inflammation, stimulated by several endogenous and exogenous factors such as endotoxin (via Toll-like receptor 4), TNF-α, catecholamines, and cAMP-elevating drugs [18]. Activation of monocytes by LPS in the presence of neutralizing anti-IL-10 monoclonal antibodies resulted in the production of higher amounts of cytokines relative to LPS treatment alone, indicating that endogenously produced IL-10 inhibited the production of IL-1, IL-6, IL-8, TNF-α [19]. Accordingly, mice become more sensitive to LPS-induced shock by treatment with anti-IL-10 antibodies [20]. IL-10-deficient mice developed inflammatory bowel disease following colonization of the gut with particular microorganisms and showed exaggerated inflammatory responses to microbial challenge [21]. IL-10 also play a key role in endotoxin tolerance, which prevents severity of infections and endotoxin shock [22]. Upregulation of IL-10 through the TRIF pathway via TRAF3 is the hallmark of endotoxin-tolerant macrophages [23]. Moreover, incubation of PBMCs with IL-10 led to suppression of LPS-induced TNF-α production, and neutralization of IL-10 with blocking antibodies during pretreatment of macrophages with LPS dramatically mitigated induction of endotoxin tolerance [24]. Although LPS tolerance can be induced in IL-10-deficient mice, Berg et al. reported that while IL-l0 KO mice primed with 1 μg of LPS survived subsequent challenges of 5–10 μg of LPS, no survival could be seen when the mice were challenged with 25 μg of LPS. In contrast, wild type mice could resist and survive 10 fold higher doses of LPS. This phenomenon suggested that IL-10 may play an important but not essential role in induction of endotoxin tolerance [25].

Since IL-10 plays an important role in immune responses and a defect in its production has been attributed to certain inflammatory diseases, an understanding of the molecular mechanisms that regulate the expression of this cytokine is crucial [18]. Herein, we provide a new explanation characterizing the molecular mechanism responsible for IL-10 production after LPS-induced macrophage activation. We found that miR-98 expression levels and IL-10 secretion are inversely correlated. We further confirmed that IL-10 3′-UTR contains the miR-98 binding site. We also demonstrated that macrophages overexpressing miR-98 before LPS-induced endotoxin tolerance generated more IL-6 and TNF-α after a second LPS challenge, as compared with the mock control cells. The resistance to LPS induced endotoxin tolerance might be due to the down-regulation in IL-10 production which was induced

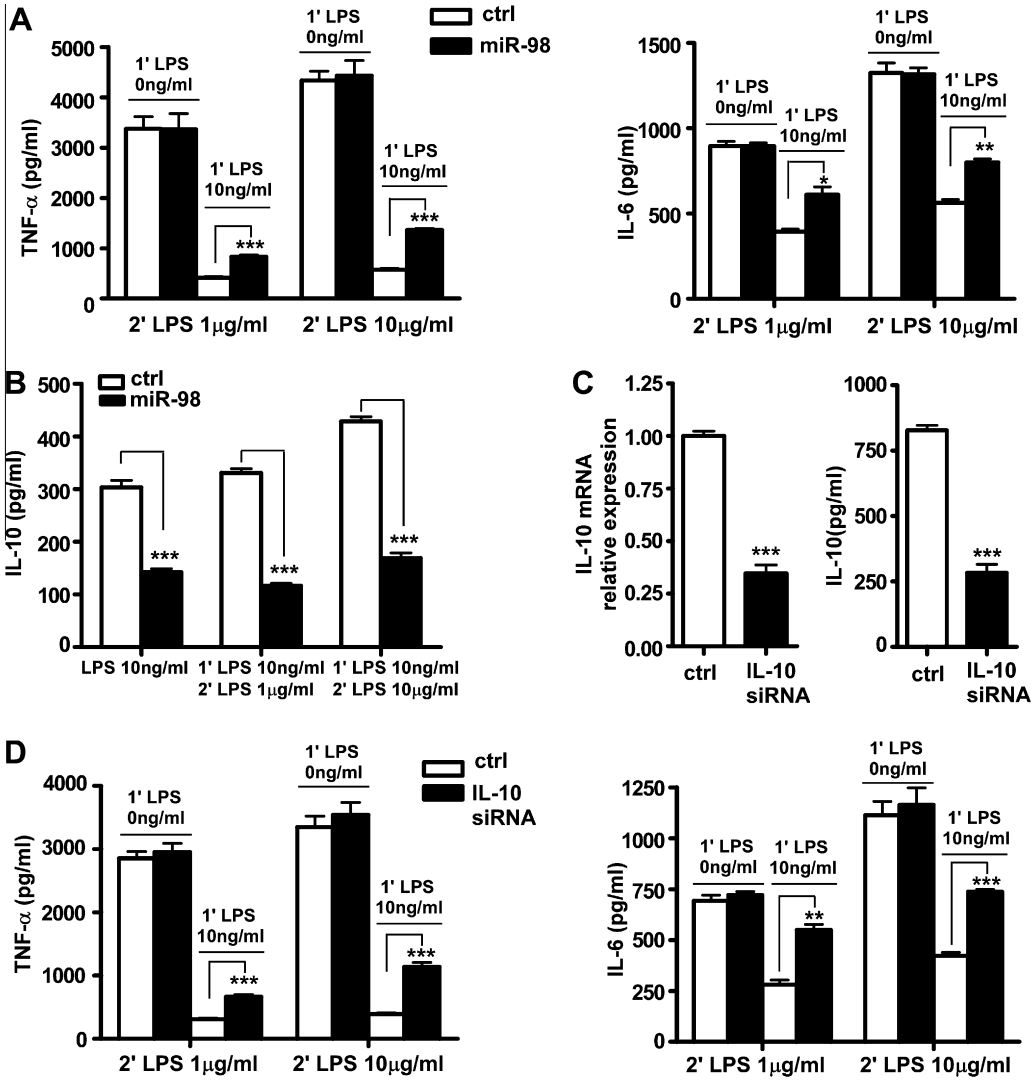

Fig. 4. Down-regulated miR-98 expression is partially involved in the induction of LPS desensitization. RAW 264.7 cells were transfected with miR-98 mimics at a final concentration of 20 nM. 24 h later, cells primed with 10 ng/ml LPS (1′LPS) continuously for 18 h were washed twice with PBS, cells were incubated in fresh complete culture medium for 2 h before challenged with high doses of LPS (2′LPS) for 5 h. Supernatants were collected 5 h after start of LPS challenge to measure TNF-α, IL-6 (A) and IL-10 (B) by ELISA. (C) RAW 264.7 cells were transfected with IL-10 siRNA or ctrl at a final concentration of 10 nM. 24 h later, cells stimulated with 1 μg/ml LPS for 24 h, IL-10 mRNA (left) and protein (right) were measured by qPCR and ELISA. (D) RAW 264.7 were transfected with IL-10 siRNA or ctrl. 24 h later, cells primed with 10 ng/ml LPS (1′LPS) continuously for 18 h were washed and incubated in fresh complete culture medium for 2 h before challenged with 10 μg/ml LPS (2′LPS) for 5 h. Supernatants were collected to measure TNF-α and IL-6 by ELISA. Data are the mean ± S.D. (n = 4) of one representative experiment from three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

by miR-98 overexpression. So, it suggested that miR-98 mediated posttranscriptional control could be involved in fine tuning the critical level of IL-10 expression in endotoxin tolerance.

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