Int J Clin Exp Med 2016;9(5):8005-8013
www.ijcem.com /ISSN:1940-5901/IJCEM0019125

Original Article

MiR-128-3p regulates inflammatory response in LPS-stimulated macrophages through the TLR4-NF-κB pathway

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Received November 4, 2015; Accepted February 10, 2016; Epub May 15, 2016; Published May 30, 2016

Abstract: Atherosclerosis constitutes the leading contributor to morbidity and mortality in cardiovascular and cerebrovascular diseases. Inflammatory response is one of the crucial triggers for the development of atherosclerosis. Recently, microRNAs (miRNAs) have drawn more attention due to their prominent function on inflammatory process and lipid accumulation in cardiovascular disease. The aim of our study is to examine the role of miR-128-3p in lipopolysaccharide (LPS)-induced inflammatory response inmacrophages. We found that LPS induced miR-128-3p expression in macrophages in a time- and dose-dependent manner. Moreover, bioinformatic analysis showed that miR-128-3p regulated SASH1 expression by directly its 3'-UTR. Additionally, overexpression of miR-128-3p significantly abrogated the inflammatory cytokines secretion of IL-6 and increased IL-10 levels, the corresponding changes were also observed when silencing miR-128-3p expression, which was impeded by preconditioning with Toll like receptor 4 (TLR4) antibody or PDTC. Taken together, these results corroborated that miR-128-3p could negatively regulate LPS-induced lipid accumulation and inflammatory responses in macrophages by the TLR4-NF-κB pathway. Accordingly, our research will provide a prominent insight into how miR-128-3p reversely abrogates bacterial infection-induced pathological processes of atherosclerosis, indicating a promising therapeutic prospect for the prevention and treatment of atherosclerosis by miR-128-3p overexpression.

Keywords: Atherosclerosis, macrophages, miR-128-3p, SASH1, TLR4-NF-κB signaling

Introduction

Atherosclerosis, a chronic disease characterized by the accumulation of lipids and fibrous elements in the large arteries, constitutes the single most important contributor to coronary artery disease (CAD), a leading cause of morbidity and mortality worldwide [1]. Macrophages are believed to possess a pivotal function in inflammation during atherosclerosis progression and plaque destabilization [2, 3]. Risk factors of atherosclerosis, such as hyperlipidemia and hypertension, provoke vascular cells to produce cytokines. On the other hand, the expression of inflammatory genes promotes atherosclerosis. Nuclear factor-κB (NF-κB)-mediated vascular inflammation plays a critical role in the initiation and progression of atherosclerosis. In the vascular endothelium, NF-κB activation induces the expression of proinflammatory genes, including those encoding adhesion molecules, cytokines, and chemoattractant proteins that collectively play critical roles in the initiation and progression of atherosclerosis [4-6]. LPS has been gradually demonstrated to be associated with cardiovascular disease [7]. Importantly, LPS can induce macrophage inflammation response and secrete abundant pro-inflammatory cytokines, which aggravate the atherosclerosis progress and lead to the instability of vulnerable plaques.

MicroRNAs (miRNAs) are single-stranded, noncoding, small RNAs which regulate gene expression in post-transcriptional level by binding preferentially to the 3'-UTR of their target gene, destabilizing mRNAs and/or inhibiting translation [8]. Much work has revealed that miRNAs

may play a vital role in the regulation of atherosclerotic process, suggesting a feasible therapeutic option for cardiovascular diseases. Emerging evidences have demonstrated that miRNAs exert prominent roles in the inflammatory process and lipid accumulation in patients with coronary artery disease [9, 10]. For example, miR-147 can act as a negative feedback regulator for Toll like receptor 4 (TLR4)-induced inflammatory responses [11]. However, the function of miR-128-3p in the progress of atherosclerosis remains unknown.

SASH1 (SAM and SH3 domain containing 1) is a large protein with a predicted molecular mass of 137 kDa, and it was originally identified by expression profiling and in silico analysis as a gene that is downregulated in breast and other solid cancers, such as lung and thyroid [12]. Subsequently, SASH1 mRNA has been shown to be reduced in colon cancer and postoperative metastases in the liver. However, these studies are correlative and no direct evidence for SASH1 as a tumor suppressor has been described [13]. Previously, we showed in a large population based study that the expression of SASH1 in circulating monocytes was markedly increased in smokers when compared to nonsmokers and was also positively correlated with the number of carotid plaques, indicating a potential role of SASH1 in atherosclerosis [14, 15]. It has been reported that SASH1 expression is increased in atherosclerotic carotids in smokers and its silencing affects endothelial angiogenic functions [16]. SASH1 positively regulates signaling through TLR4 in human endothelial cells to increase activation of NF-κB and MAPKs, culminating in increased production of proinflammatory cytokines [17].

In the present study, we identified SASH1 as a direct target for miR-128-3p. Furthermore, we explored the effects of miR-128-3p on LPS-induced inflammation responses inmacrophages. In addition, the underlying mechanism involved in this process was also discussed.

Materials and methods

Cell culture

Mouse RAW 264.7 monocyte/macrophage-like cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA). Cells were cultured at 37°C under 5% CO_2 in DMEM supplemented with 10% FCS and 100 U/mL streptomycin-penicillin.

Transfection

Transfection of miR-128-3p inhibitor or miR-128-3p mimic (Promega) was conducted using the Lipofectamine 2000 reagent (Invitrogen, USA) according to the manufacturer's instruction. For luciferase reporter assay, cells (1×10^4) were seeded in triplicate in 48-well plates and allowed to settle for 24 h. One hundred nanograms of luciferase reporter plasmids or miR-128-3p inhibitor and miR-128-3p mimic, were transfected into cells using the Lipofectamine 2000 reagent according to the manufacturer's instruction. Luciferase signals were measured using the Dual Luciferase Reporter Assay Kit (Promega) according to a protocol provided by the manufacturer.

RNA extraction and quantitative real-time PCR

After stimulation with LPS, the total RNA from the cells was extracted using Trizol reagent (Invitrogen, Carlsbad, California, United States) according to the manufacturer's recommendations. RNA quality and concentration were determined using the Nanodrop 2000 system (Thermo Fisher Scientific, Wilmington, United States). Mature miR-128-3p and U6 levels were quantified with TaqMan miRNA assays (Applied Biosystems, Foster City, CA). SASH1 and β-actin mRNA levels were determined by qRT-PCR using the SYBR Green Master Mix on the HT 7500 System (Applied Biosystems). The relative expression levels of each gene were calculated and normalized using the $2^{-\Delta\Delta CT}$ method relative to U6 or β-actin. All of the reactions were run in triplicate.

Western blot analysis

Following rinses with PBS three times, the total protein extracts of RAW 246.7 cells were extracted using RIPA lysis buffer (Beyotime, Nantong, China), following the quantitative analysis of protein concentrations via the BCA assay (Pierce, Rockford, IL). For western blotting, the obtained protein was electrophoresed by SDS-polyacrylamide gel electrophoresis, and about 100 μg of protein was then transferred onto a polyvinylidene difluoride (PVDF) membrane in a semi-dry transblot apparatus. After incubation with buffer containing 5% non-

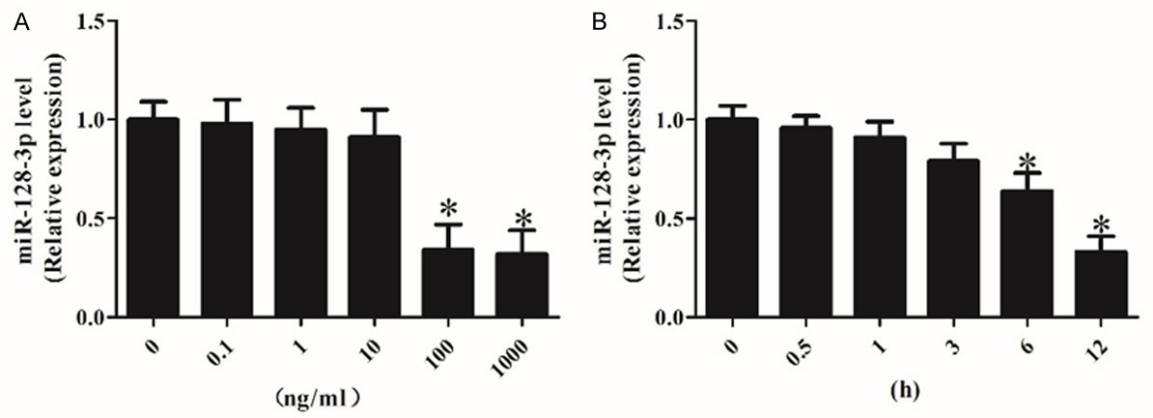

Figure 1. LPS down-regulates expression of miR-128-3p in macrophages. A. Macrophages were cultured in 6-well plate, after 80% confluence, cells were treated with different concentration of LPS for 12 h. B. Macrophages were treated with 100 ng/mL LPS for different times point as indicated. Quantitative real-time PCR showed that miR-128-3p expression was down-regulated, compared with that in the respective control group. *P < 0.05 vs. LPS-untreated group.

fat dry milk in Trisbuffered saline with Tween (TBST) at 4°C overnight, the PVDF membrane was cultured with anti-TLR4 and anti-p65 NF-κB antibodies for 1 h at 37°C to probe the targeted protein. Following washed three times with TBST, HRP conjugated secondary antibodies were added for 1 h. The LumiGLo reagent (KPL, Gaithersburg, MD) was used to visualize the bound antibodies. The protein expression levels were normalized by GAPDH.

Enzyme-linked immunosorbent assay (ELISA) assay

To analyze the levels of interleukin 6 (IL-6) and interleukin 10 (IL-10) in the transfected macrophages stimulated with LPS, the ELISA assay was introduced. Briefly, about 2×10^5 cells were seeded into 24 well plates and incubated at 4°C overnight. The transfected cells were stimulated with LPS for 24 h, and the concentrations of IL-6 and IL-10 in supernatants were measured using ELISA DuoSet Development systems according to the manufacturer's instructions (R&D Systems).

Luciferase reporter assay

Firefly luciferase reporter plasmid containing SASH1 3'-UTR (SASH1-WT) and mutated SASH1 3'-UTR (SASH1-MUT) were constructed. The cells plated in 24-well plate were co-transfected with miR-128-3p inhibitor or miR-128-3p mimic and NC and firefly luciferase reporter plasmid along with renilla luciferase control plasmid using lipofectamine 2000. After 24 h, luciferase activity was measured according to the manufacturer's instructions (Dual Luciferase Assay System; Promega, USA). Each measured firefly luciferase activity was normalized by the Renilla luciferase activity in the same well.

Statistical analysis

The data were presented as means ± SD from 3 independent experiments. SPSS 11.0 was used to analyze the data. The statistical significance between groups was determined using Student's t test. *P* value less than 0.05 was considered statistically significant.

Results

LPS down-regulates miR-128-3p expression in macrophages

LPS is known to be critical for the progress of atherosclerotic plaques [18]. To investigate whether miR-128-3p expression is altered during LPS-induced macrophages, macrophages were treated with different concentrations (0.1, 1, 10, 100 and 1000 ng/mL) of LPS for the indicated times. The expression level of miR-128-3p was detected by qRT-PCR. As shown in **Figure 1**, miR-128-3p expression was not affected by a LPS concentration of less than 10 ng/ml. However, an obvious downregulation of miR-128-3p mRNA levels was observed by 100 ng/ml LPS stimulation (**Figure 1A**). In addition,

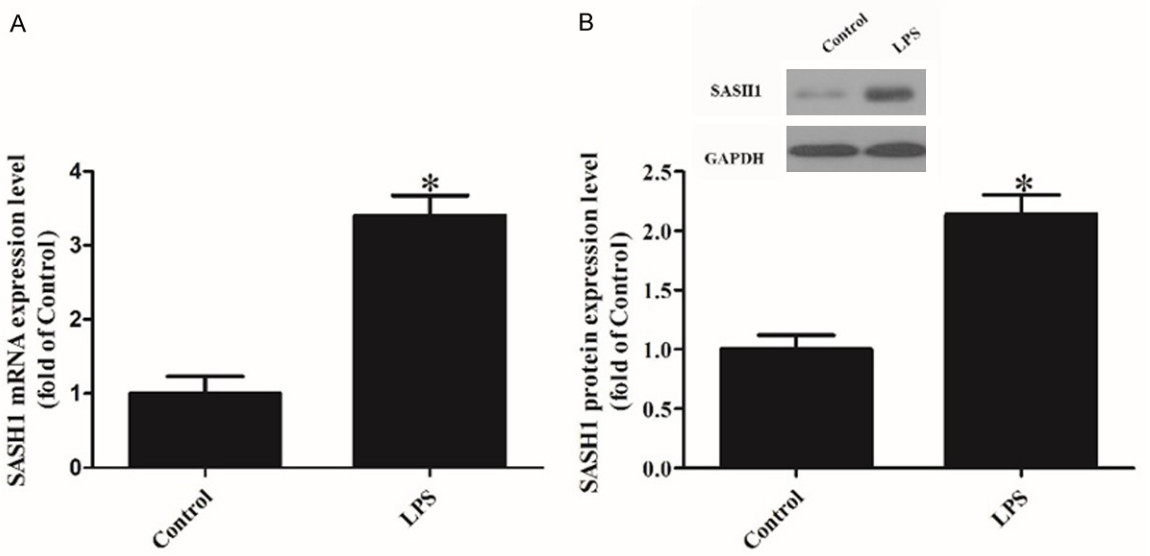

Figure 2. Expression of SASH1 was up-regulated during LPS-induced macrophages. A. The cells were cultured in 6-well plate, after 80% confluence, cells were treated with 100 ng/mL LPS for 12 h point as indicated. Quantitative real time PCR revealed that the expression levels of SASH1 were increased after LPS stimuli. B. SASH1 protein levels in macrophages were examined by western blotting and normalized with respect to GAPDH protein. *P < 0.05 vs. control.

we found a decrease in miR-128-3p expression after treatment with 100 ng/ml LPS for 12 h (**Figure 1B**). Taken together, these data show that LPS down-regulates miR-128-3p expression in a dose and time-dependent manner.

Expression of SASH1 is up-regulated during LPS-induced macrophages

To examine the possible link between miR-128-3p and SASH1, we also measured the expression of SASH1 at both mRNA and protein levels. Macrophages were stimulated for 12 h with LPS, and then qRT-PCR was performed using RNA isolated from control and treated cells. The results showed that SASH1 was significantly increased after LPS stimuli (**Figure 2A**). To determine whether the increase in SASH1 mRNA expression correlated with enhanced SASH1 protein expression, we performed western blotting and quantified SASH1 expression by densitometry. As shown in **Figure 2B**, treatment of macrophages with LPS led to increased expression of SASH1.

MiR-128-3p regulates SASH1 expression by targeting its 3'-UTR

As common regulators of TLR pathways, microRNAs play an essential role in TLRs homeostasis. In order to investigate miRs which could regulate the TLR4 pathway, we detected some miR expression and found miR-128-3p down-regulation after LPS stimulation in macrophages (**Figure 1**). We used the most commonly used bioinformatics prediction tools (TargetScan, PicTar, miRanda) and found SASH1 is one of the prediction targets of miR-128-3p (**Figure 3A**). To elucidate whether SASH1 is a direct target of miR-128-3p, we cloned the putative miR-128-3p binding site on SASH1 3'-UTR or its mutational sequences into luciferase reporter plasmid and co-transfected it with miR-128-3p mimics or miR-128-3p inhibitors into macrophages. Results showed that miR-128-3p overexpression significantly reduced the luciferase reporter activity by the SASH1 3'-UTR in a consistent manner, and inhibition of miR-128-3p had the opposite effect (**Figure 3B**). However, SASH1 3'-UTR luciferase reporter activity was unaffected by point mutations in the miR-18a-binding seed region. As shown in **Figure 3C**, overexpression of miR-128-3p decreased the mRNA and protein expression of SASH1 in macrophages, whereas miR-128-3p inhibitors increased SASH1 expression in macrophages. Taken together, these results indicate that SASH1 is a direct target of miR-128-3p in macrophages. MiR-128-3p regulates SASH1 expression by targeting specific binding sites in the 3'-UTR of SASH1.

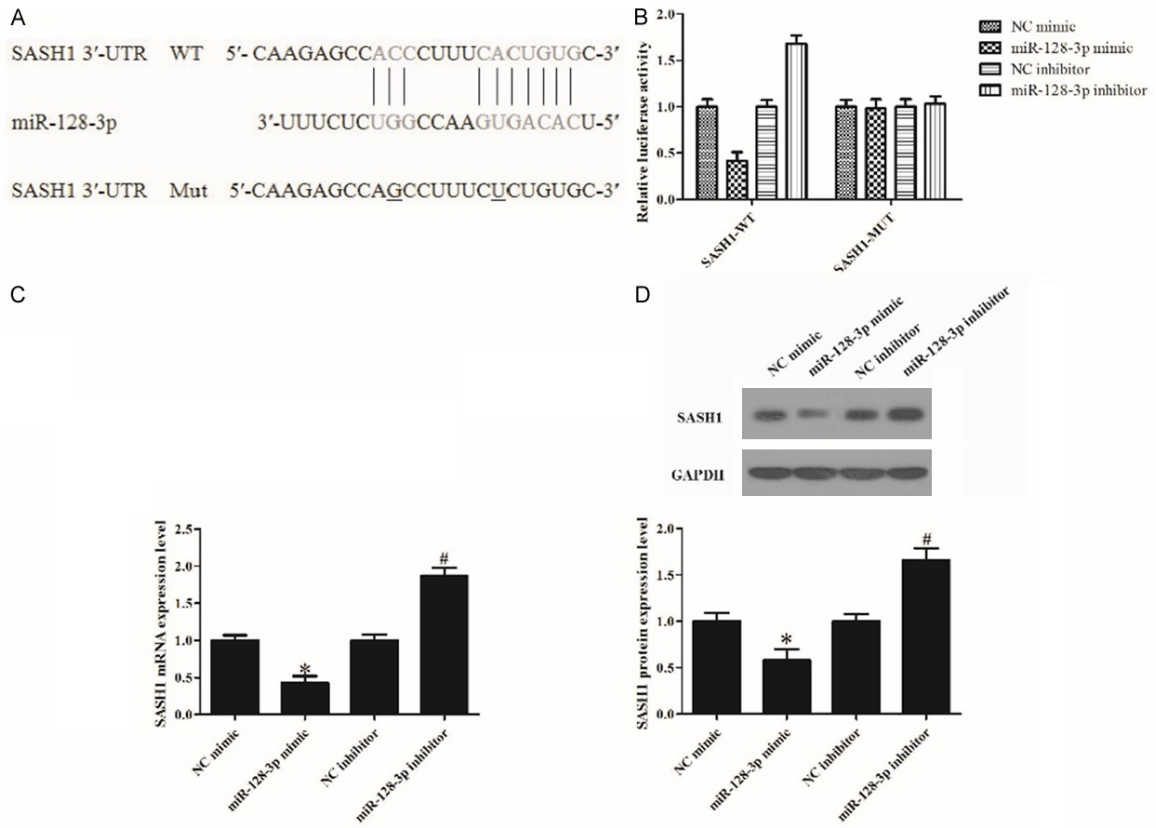

Figure 3. The effect of modulated miR-128-3p on SASH1 expression in macrophages. A. The binding site between the 3'-UTR of SASH1 and miR-495 was represented. Transfection of macrophages with miR-128-3p mimic or miR-128-3p inhibitor modified SASH1 expression. B. Activity of the luciferase reporter construct infected with miR-128-3p mimic or miR-128-3p inhibitor along with wild-type or site-directed mutations in the miR-128-3p target sites of the SASH1 3'-UTR. Luciferase activity was normalized with Renilla. Data shown are means ± SD of 3 independent experiments. C. SASH1 mRNA levels were measured by real-time PCR and normalized with respect to GAPDH. D. SASH1 protein levels were measured by western blotting with GAPDH as a loading control. Densitometry was performed and normalized with respect to GAPDH expression level. #P < 0.05 vs. NC-inhibitor, *P < 0.05 vs. NC-mimic.

miR-128-3p mediated the production of inflammatory cytokines by TLR4-NF-κB in LPS-induced macrophages

During the progression of atherosclerotic plaques, the release of critical pro-inflammatory cytokines such as IL-6, IL-12 and TNF-α, was considered to be pivotal. To further assess the effects of miR-128-3p on LPS-induced inflammatory response in macrophages, we assessed the inflammation cytokine levels of IL-6 and IL-10 by ELISA assay. As shown in **Figure 4A**, LPS dramatically induced the production of IL-6 levels, and this increase was significantly decreased by miR-128-3p mimic. Furthermore, the level of anti-inflammatory cytokine IL-10 was obviously enhanced compared to LPS-treated groups. Consistently, the inhibition of miR-128-3p expression with miR-128-3p inhibitor transfection notably augmented IL-6 levels (**Figure 4B**), as well as an obvious decrease in IL-10 levels (**Figure 4C**). Therefore, all of these results showed that miR-128-3p down-regulated the pro-inflammatory cytokine IL-6 levels and up-regulated the anti-inflammatory cytokine IL-10 levels, indicating an important function on inflammatory response in macrophage-stimulated by LPS.

The activation of TLR-4 induces the production of inflammation cytokines to regulate the immune responses, attenuated compared to control in LPS-stimulated macrophages (**Figure 4D**). Moreover, blocking the activation of TLR-4 downstream effector NF-κB with PDTC, IL-6 levels was significantly impended in LPS and miR-128-3p inhibitor treated groups. For IL-10, its expression was correspondingly increased

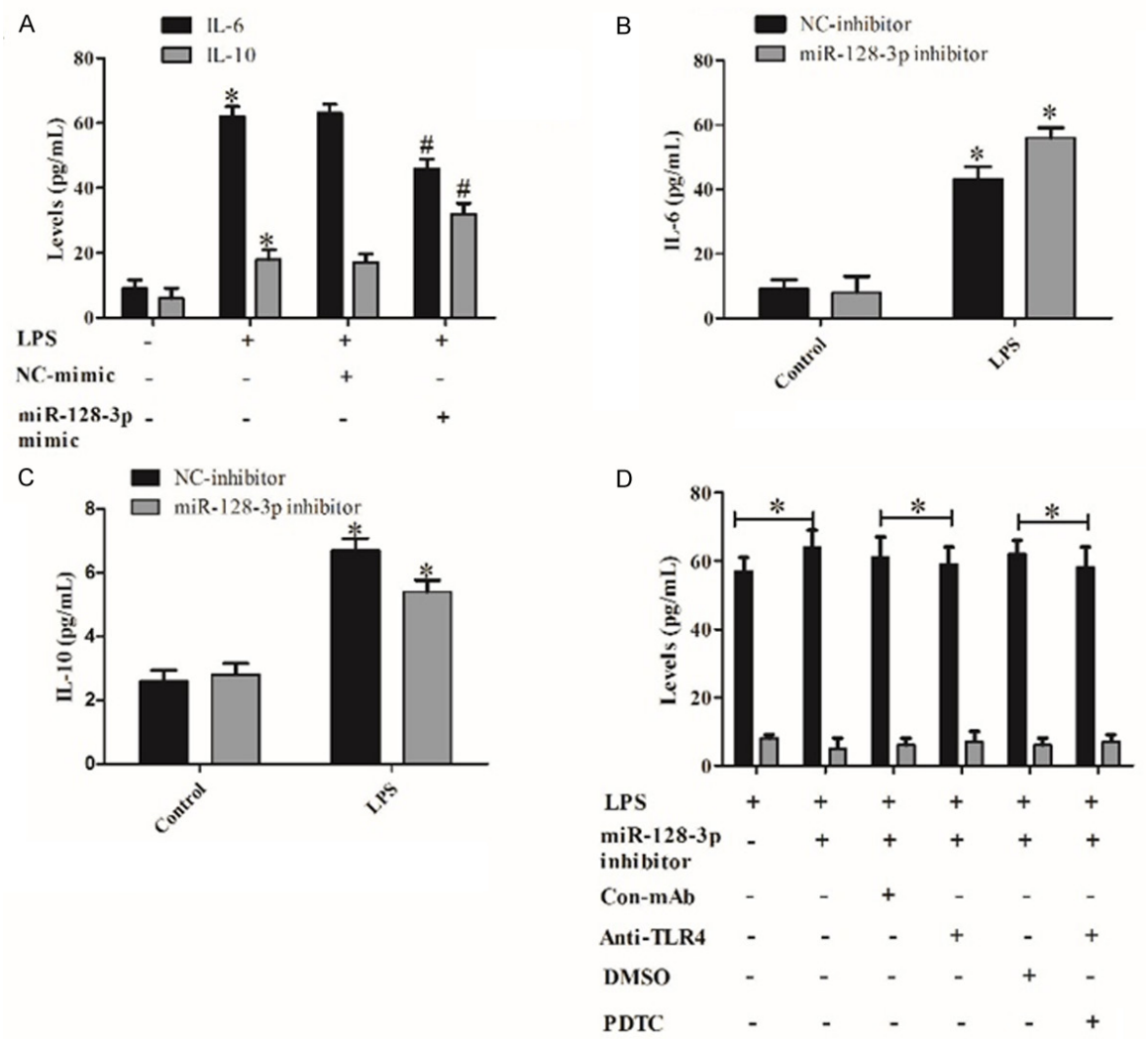

Figure 4. miR-128-3p regulated the secretion of inflammatory cytokines in LPS-induced macrophages. A. Following transfection with miR-128-3p, levels of IL-6 and IL-10 was detected by ELISA assay. *P < 0.05 vs. LPS-untreated group. #P < 0.05 vs. LPS + NC mimic group. B, C. The effect of miR-128-3p silencing on IL-6 and IL-10 levels in macrophages exposed to 100 ng/ml LPS. *P < 0.05 vs. control. D. Cells were pretreatment with anti-TLR4 antibody or NF-κB inhibitor PDTC for 4 h, and then LPS-induced IL-6 and IL-10 levels were detected in miR-128-3p inhibitor-transfected cells. *P < 0.05.

after preconditioning with TLR-4 specific antibody and NF-κB inhibitor PDTC, compared with LPS stimulation plus con-Amb and DMSO. Taken together, our data suggested that miR-128-3p could regulate inflammation response in macrophages stimulated by LPS via suppressing TLR-4-NF-κB signaling.

Discussion

Atherosclerosis, a chronic inflammatory disease, is the major cause of life-threatening complications such as myocardial infarction and stroke. Recently, numerous animal and cell experiments have focused on the miRNA profile in atherosclerotic processes, and an obvious upregulation of miR-21 has been demonstrated in atherosclerotic plaques [19, 20]. In this current study, we found that miR-128-3p levels were significantly increased, and can negatively regulate inflammation cytokine secretion in LPS-stimulated macrophages by TLR4 dependent signaling.

MiRNAs can participant in many physiological and pathological processes including cell proliferation, apoptosis, cardiovascular disease and inflammatory diseases [21]. MiRNAs have been

reported to regulate multiple important biological processes including metabolism and are potentially of use in atherosclerosis therapy. In the present study, we found that LPS dose and time-dependently induced the mRNA expression of miR-128-3p.

SASH1, a member of the SLY family of proteins, acts as a novel TLR4 signaling molecule that is expressed in microvascular endothelial cells. We next sought to identify direct target of miR-128-3p involved in atherosclerosis. We used the most commonly used bioinformatics prediction tools (TargetScan, PicTar, miRanda) and miR database to identify the target. We found that miR-128-3p directly regulated the expression of SASH1.

Growing evidence indicates that TLR4 plays a very important role in macrophage foam cells formation, indicating a critical roles of TLR4 in atherosclerosis via regulating lipid deposition [22, 23]. Increasing studies have shown that NF-κB signaling driven by toll-like receptors (TLRs) in monocytes and macrophages is an important contributor to acute and chronic inflammation, including atherosclerosis [24, 25]. The intracellular signaling cascades that are initiated by ligation of TLRs leads to activation of transcription factors such as NF-κB and IFN regulatory factor (IRF), resulting in production of proinflammatory cytokines [26]. The NF-κB signaling pathway centrally integrates multiple signal inputs in the pathogenesis of atherosclerosis, and emerging studies suggest that inhibition of NF-Kb in specific cell types may have divergent effects [27]. It is well established that inflammatory process evoked by LPS is mediated by the transmembrane receptor toll-like receptor 4 (TLR4) [28].

As a common receptor of LPS, TLR4 and its downstream signaling effector NF-κB are crucial for atherosclerotic plaque formation and coronary lesion progression [29]. LPS is known as a potent inducer of the inflammatory response. Therefore, we further analyzed the effect of miR-128-3p in LPS-triggered inflammation in macrophages. Following transfection with miR-128-3p, the levels of pro-inflammatory cytokine IL-6 was dramatically attenuated, accompany with an increase of anti-inflammatory cytokine IL-10. The corresponding changes of IL-6 and IL-10 were also confirmed when silencing miR-128-3p levels, indicating an important function of miR-128-3p on LPS induced macrophage inflammation.

As a key component of innate immune response, TLR4 possesses a pivotal role in the initiation and progression of atherosclerosis, and can regulate the inflammatory response in macrophages via its downstream NF-κB signaling [30, 31]. To further elucidate the underlying mechanism involved in miR-128-3p-mediated inflammation cytokines secretion, we blocked the activation of TLR4 and NF-κB. After blocking TLR4 expression, the increase in IL-6 and decrease in IL-10 was significantly mitigated in miR-128-3p-silencing cells. The similar changes in IL-6 and IL-10 were also corroborated when preconditioning with PDTC in miR-128-3p inhibitor-transfected macrophages. Together, these results told that miR-128-3p could regulate macrophage inflammation via the TLR4-NF-κB signaling pathway. However, the mechanism involved in miR-128-3p-induced inhibitory effect on TLR4-NF-κB is still unclear, which needs to be explored in our next plan.

In conclusion, our research investigated for a potential role of miR-128-3p in atherosclerosis. In this study, LPS induced the expression of miR-128-3p in a time- and dose- dependent manner. Further analysis manifested that miR-128-3p negatively regulates inflammatory responses in LPS-stimulated macrophages through the TLR4-NF-κB pathway, indicating a critical roles of miR-128-3p in the progression of atherosclerosis. Hence, the beneficial clinical effects of miR-128-3p overexpression in the prevention and treatment of atherosclerosis deserve further investigations.

Disclosure of conflict of interest

None.

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