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Research paper

Downregulated expression of miR-223 promotes Toll-like receptor-activated inflammatory responses in macrophages by targeting RhoB

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ABSTRACT

Toll-like receptors (TLRs) induced-inflammatory response must be tightly regulated to avoid impairment in host itself. Numerous factors have been identified in regulation of TLR-triggered inflammatory response. Among these, microRNAs (miRNAs) are small non-coding RNA molecules which have got much attention. MiR-223, which highly expresses in myeloid cells of the bone marrow, has reported to participate in kinds of inflammatory responses by targeting inflammasome sensor-NLRP3 to repress production of IL-6 and IL-1β, and thus attenuate inflammatory response. However, the function of miR-223 in TLRs-activated inflammatory response of macrophages is not clear. Here we found miR-223 expression is dramatically reduced in macrophages by TLR ligand stimulation (e.g. LPS, CpG and poly (I:C)). The down-regulated miR-223 leads to the increase in the RhoB expression, which induce the activation of NF-κB and MAPK signaling, promoting TNF-α, IL-6 and IL-1β production upon LPS stimulation. In addition, the histone deacetylase inhibitor trichostatin A increased miR-223 expression obviously in TLR-triggered macrophages, which in turn suppressed RhoB expression and downstream IL-6 production, suggesting that the inhibition of miR-223 by histone deacetylation may be involved in the regulation of TLR-activated inflammatory response. Herein, our findings suggest that miR-223-RhoB axis might be a novel target for the treatment of inflammatory diseases.

1. Introduction

Inflammation is a complex response to infection and tissue injury (Nathan, 2002). Macrophages are crucial mediators of the inflammatory response, and Toll-like receptors (TLRs) are the best-characterized inducers of acute inflammation, which lead to various pro-inflammatory cytokines and chemokines to limit infection (Medzhitov and Horng, 2009). Because the dysregulated inflammation can cause a variety of pathological conditions, including autoimmunity, septic shock, atherosclerosis and so on, so the inflammatory response must be tightly regulated (Foster et al., 2007). Numerous factors, including kinases, transcription factors, epigenetic modifiers and non-coding RNAs have been reported to take part in the regulation of TLRs-induced inflammatory response (Atianand and Fitzgerald, 2014; Kawai and Akira, 2010; Medzhitov and Horng, 2009; Stender and Glass, 2013; Zhang and Cao, 2016).

MicroRNAs (miRNAs) are a group of short, endogenous, single-stranded noncoding RNA molecules that regulate gene expression. They have important roles in cell proliferation and differentiation, development and apoptosis (Bartel, 2004; Iwakawa and Tomari, 2015). An increasing number of studies have revealed that miRNAs have critical functions in the regulation of both innate and adaptive immune responses (Gantier, 2010; Liston et al., 2010). MiR-223, which highly expressed in myeloid cells of the bone marrow, has reported to participate in kinds of inflammatory responses (Haneklaus et al., 2013), including intestinal inflammation (Neudecker et al., 2017), acute live failure (He et al., 2017), experimental autoimmune encephalomyelitis (EAE) (Ifergan et al., 2016) and intracerebral hemorrhage (ICH) (Yang et al., 2015), attracting much attention. In these inflammatory responses, miR-223 has reported to downregulate the production of IL-6 and IL-1β by targeting NLRP3, an inflammasome sensor, then attenuate inflammatory response, indicating miR-223 is a potential repressor of inflammation (Bauernfeind et al., 2012; Neudecker et al., 2017; Yang et al., 2015). However, the function of miR-223 in macrophages in TLR-induced inflammatory response is not clear.

In the present study, we found that miR-223 expression is reduced in macrophages by TLR ligand stimulation (e.g. LPS, CpG and poly (I:C)). Overexpression of miR-223 represses TNF-α, IL-6 and IL-1β upon TLR ligand stimulation. Moreover, we demonstrated that RhoB is a target of miR-223, and thus downstream signaling of NF-κB and MAPK is also regulated by miR-223. Our findings suggest that miR-223 might be a novel regulator of TLR-activated inflammatory response and miR-

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223-RhoB axis might be a target for the treatment of inflammatory diseases.

2. Material and methods

2.1. Mice and reagents

C57BL/6 mice (6–8wk) were obtained from Shanghai Laboratory Animal Center (Shanghai, China). Mice were kept and bred in pathogen-free conditions. All animal experiments were undertaken in accordance with Animal Care and Use Committee of Second Xiangya Hospital, Central South University. LPS (0111:B4) and poly(I:C) were from Sigma (St. Louis, MO, USA)., Phosphorothioate modified CpG ODN was synthesized by Sybersyn Co (Beijing, China). Abs specific to RhoB and horseradish peroxidase (HRP)-coupled secondary antibodies were from Santa Cruz Biotechnology (Dallas, TX, USA), Abs specific to GAPDH, phosphorylated IκBα, phosphorylated JNK, phosphorylated Erk, phosphorylated p38 were from Cell Signaling Technology (Danvers, MA, USA). TSA was from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Cell culture and transfection

RAW 264.7 cells were purchased from American Type Culture Collection (Manassas, VA) and cultured in DMEM containing 10% FBS (GIBCO). Primary peritoneal macrophages from C57BL/6 mice were maintained in DMEM supplemented with 10% FBS. MiR-223 mimics and inhibitor (Life Technologies Corporation, Shanghai, China) were used for the overexpression and inhibition of miR-223 in cells respectively. Cells were transfected with small RNAs using INTERFERin (Polyplus-Transfection SA, Illkirch, France) according to the manufacturer's instructions.

2.3. Measurement of miRNA and mRNA expressions

The total RNAs of each sample were extracted using mirVana miRNA Isolation Kit (Ambion Inc, Austin, Tex) following the manufacturer's instructions. The expression of miR-223 was quantitatively detected by using Taqman miRNA assay system (Applied Biosystems, Foster City, CA). The relative expression level of miRNA was normalized to that of internal control U6. The expression of mRNA was analyzed by the SYBR Green PCR Master Mix (Toyobo, Osaka, Japan). The transcription level of AK036748, pri-miR-223 and miR-223 was detected by real-time quantitative PCR (q-PCR) was performed using an ABI 7900HT thermocycler. Gene-specific primers were as follows:

AK036748 forward, 5′- TGATAGGAGGAAGGAACA-3′,
AK036748 reverse, 5′- AAAGAGGGAAGTGAACAA-3′;
pri-miR-223 forward, 5′- TCTGGCCATCTGCAGTGTCACG-3′,
pri-miR-223 reverse, 5′- CTGATAGGCATGAGCCACACTT-3′;
U6 forward, 5′- GCTTCGGCAGCACATATACTAAAAT-3′,
U6 reverse, 5′- CGCTTCACGAATTTGCGTGTCAT-3′;
IL-6 forward, 5′- CAGAAGGAGTGGCTAAGGACCA-3′,
IL-6 reverse, 5′- ACGCACTAGGTTTGCCGAGTAG-3′;
TNF-α forward, 5′- CCTTGTTGCCTCCTCTTTTGC-3′,
TNF-α reverse, 5′- TCAGTGATGTAGCGACAGCCTG-3′;
IL-1β forward, 5′- CCTGTGTTTTCCTCCTTGCCT-3′,
IL-1β reverse, 5′- GCCTAATGTCCCCTTGAATCAA-3′;
RhoB forward, 5′- CTGGCCCGCATGAAGCA-3′,
RhoB reverse, 5′- AGGCAGTCTGGTGGTGTCC-3′;
GAPDH forward, 5′- ATCTTCTTGTGCAGTGCCAGC-3′,
GAPDH reverse, 5′- ACTCCACGACATACTCAGCACC-3′.

2.4. Luciferase reporter assay

RAW264.7 cells were co-transfected with miR-223 mimics or inhibitors, pMIR-REPORT-RhoB-3′UTR luciferase reporter plasmid or empty pMIR-REPORT and pTK-RL plasmid. The full length RhoB 3′UTR was constructed by PCR-based amplification from peritoneal macrophages genomic DNA and cloned into pMIR-REPORT luciferase reporter. After 48 h, luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. The primers were as follows:

forward 5′-AAAGATCCTTTATTAAGCTTGGGACGCGTCCTGCCTCACGCCC
TTGCC-3′,
reverse 5′-CATAGGCCGGCATAGACGCGT TTGTCATCTGTCAGTTTATTTA AAAAAA −3′.

2.5. Cytokine detection

Cytokines TNF-α, IL-6, IL-1β in supernatants were measured using ELISA kits (R & D Systems) according to the manufacturer's instructions.

2.6. Treatment with TSA in macrophages

Cells were treated with TSA after transfected overnight with miR-223 inhibitor, 48 h later, LPS was added into cell cultures. After another 6 h, cells were harvested and for analyzing by q-PCR.

2.7. HDAC enzyme activity

HDAC enzyme activity was determined using HDAC Assay Kit (colorimetric) obtained from (Active Motif, Carlsbad, CA). HDAC assay was performed following the manufacturer's instructions measuring the color intensity at 405 nm.

2.8. Immunoblot analysis

Cells were lysed with cell lysis buffer (Cell Signaling Technology) supplemented with protease inhibitor cocktail. Protein concentrations in the extracts were measured by BCA assay (Pierce). Then proteins were separated by electrophoresis on 10% SDS-polyacrylamide gels and 5% polyacrylamide gels and transferred onto nitrocellulose membrane, blocked with 5% BSA in Tris-buffered saline with 0.05% Tween 20 (TBST) and incubated with indicated antibodies overnight. After three washes with TBST, the membrane was incubated with horseradish peroxidase-coupled secondary antibodies at room temperature for 1 h. After three times washing again with TBST, blots were detected by ECL chemiluminescence (Pierce, Rockford, IL, USA). Protein bands were quantified with ImageJ software (NIH, USA) using GAPDH as an internal control.

2.9. Statistical analysis

All the data were obtained from at least three independent experiments. The data were expressed as the mean values ± SD and the statistical significance between two groups was determined by the Student *t*-test. The P values < 0.05 were considered statistically significant.

3. Results

3.1. Stimulation of macrophages with LPS, CpG, or poly (I:C) significantly decreases the expression of miR-223

To determine the role of miR-223 in TLR-activated macrophages, we first measured its expression by qPCR analysis of macrophages treated with 0–1000 ng/mL LPS for up to 6 h. We found that miR-223 decreased in a time-dependent manner in cells treated with 100 ng/mL LPS, with miR-223 levels decreasing by 20% at 0.5 h and 50% at 1 h, compared with control cells (Fig. 1A). We also compared the effects of treatment with 10, 100, and 1000 ng/mL LPS for 6 h, and found that