Immune regulation of miR-30 on the *Mycobacterium tuberculosis*-induced TLR/MyD88 signaling pathway in THP-1 cells

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Abstract. The present study aimed to examine the expression of microRNA (miR)-30 family members in THP-1 human monocytes cells during *Mycobacterium tuberculosis* (MTB) H37Rv infection, and to investigate the role of miR-30 in the regulation of MTB-induced Toll-like receptor (TLR)/myeloid differentiation factor 88 (MyD88) activation and cytokine expression. The THP-1 cells were infected with MTB H37Rv and the expression of miR-30 family members was determined by reverse transcription-quantitative polymerase chain reaction analysis. In addition, miR-30a and miR-30e mimics were transfected into THP-1 cells to overexpress miR-30a and miR-30e. The expression of TLR2, TLR4 and MyD88 was determined by western blot analysis, and the expression of the cytokines tumor necrosis factor-α, interleukin (IL)-6, and IL-8 was determined using ELISA assays. A luciferase reporter assay was used to identify the target gene of miR-30a. MTB infection was demonstrated to significantly induce miR-30a and miR-30e expression in THP-1 cells in a time-dependent manner. Forced overexpression of miR-30a, but not miR-30e, exhibited an inhibitory effect on TLR/MyD88 activation and cytokine expression in the uninfected and MTB-infected THP-1 cells. The luciferase reporter assay demonstrated that miR-30a directly regulates the transcriptional activity of the MyD88 3′-untranslated region. In conclusion, the present study, to the best of our knowledge, is the first to demonstrate that miR-30a suppresses TLR/MyD88 activation and cytokine expression in THP-1 cells during MTB H37Rv infection, and that MyD88 is a direct target of miR-30a. The current study may aid in the development of novel therapeutic approaches for treating MTB.

Introduction

Tuberculosis (TB) is a chronic infectious disease caused by the *Mycobacterium tuberculosis* (MTB) bacterium. It may infect any part of the body, but most commonly occurs in the lungs (1). General signs and symptoms include fever, chills, night sweats, loss of appetite, weight loss, and fatigue (1). MTB is a leading cause of infection-associated mortality globally, with ~1/3 individuals globally having been infected with MTB (2-4). Approximately 8.8 million new cases of TB were diagnosed, and 1.20 or 1.45 million deaths occurred in 1990 and 2010, respectively, most of these occurring in developing countries (5). The pathogenic mechanism of MTB is complex and the emergence of drug resistance has led to difficulty in the control of TB (4). Although the bacillus Calmette-Guérin vaccine protects against TB in certain populations, it has limitations in disease control (6-10), as it is insufficient at protecting from adult pulmonary tuberculosis. Therefore, investigating effective novel methods to prevent and cure TB is of high priority worldwide.

MicroRNAs (miRNAs/miRs) are small (21-24 nucleotides) non-coding RNAs that participate in various physiological and pathological processes (11,12). miRNAs bind to partially complementary sequences in the 3′-untranslated region (3′-UTR) of target mRNAs, leading to degradation of the transcript or translational inhibition (13,14). Previous research has demonstrated that miRNAs are important regulators of the immune response that function at the post-transcriptional level (15). A number of miRNAs have been implicated in the pathogenesis of MTB infection, including miR-29, -147, -21 and -125b (16). Das et al (17) reported that the differential expression of miRNAs, including miR-30a and miR-30e, was observed between THP-1 human myeloid leukemia cells infected with MTB H37Rv and MTB H37Ra strains. Chen et al (18) suggested that miR-30a serves an important role in the elimination of intracellular MTB. However, the regulatory mechanism of miR-30 family induction in response to MTB infection remains unclear.

The present study evaluated and analyzed the expression of miR-30 family members in THP-1 cells during MTB H37Rv infection, and assessed the role of miR-30 in regulating MTB-induced Toll-like receptor (TLR)/myeloid differentiation factor 88 (MyD88) signaling pathway activation and cytokine expression.
Materials and methods

Cell culture and transfection. THP-1 human monocyte and HEK293 cells were purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (both Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C with 5% CO₂. For infection, the THP-1 cells were exposed to MTB H37Rv (multiplicity-of-infection, 1:10; ATCC, Manassas, VA, USA) at 37°C for 0, 6, 12 or 24 h. The miR-30a mimic (sense, UGUAAACAUCUCUGACUGGAAG; antisense, UCCAGUCGAGGAUUGUUACAU), miR-30e mimic (sense, UGUAACAUCCUGACUGGAAG; antisense, UCCAGUCAAAAGUUGAUUAUAU) and miR negative control (miR-NC) (UUGUUGACUGGAAG; antisense, UCCAGUCAAAAGUUGAUUAUAU) were synthesized by GenePharma Co., Ltd. (Shanghai, China). Cells were transfected with 100 nM miR-30a mimic, miR-30e mimic and miR-NC using Lipofectamine™ 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. After 4 h, the media was replaced and the cells were maintained in culture prior to analysis.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. The expression of miR-30a, miR-30b, miR-30c, miR-30d and miR-30e was analyzed by RT-qPCR. U6 gene was used as the control. Primers specific to miR-30a (forward, 5'-TGTAAACATCTCTGA CTGGAGG-3'; reverse, 5'-TGGTGTCTGGAGTCTG-3'), miR-30b (forward, 5'-TGTAAACATCTCTACATCGCTC-3'; reverse, 5'-TGGTGTCTGGAGTCTG-3'), miR-30c (forward, 5'-TGTAAACATCTCTACATCGCTC-3'; reverse, 5'-TGGTGTCTGGAGTCTG-3'), miR-30d (forward, 5'-TGTAAACATCTCTACATCGCTC-3'; reverse, 5'-TGGTGTCTGGAGTCTG-3'), miR-30e (forward, 5'-TGGTGTCTGGAGTCTG-3') was cloned into the pMIR-REPORT™ Wild-type Vector (Applied Biosystems; Thermo Fisher Scientific, Inc.) and cloned into pMIR-REPORT™ Mutant Vector. Total RNA was isolated from cells using an miRNeasy Mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol. Total RNA (1 µg) was reverse transcribed into cDNA using a RevertAid™ First Strand cDNA Synthesis kit (Fermentas; Thermo Fisher Scientific, Inc., Pittsburgh, PA, USA). The PCR reaction was performed on a 7300 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) using the SYBR-Green PCR Master mix (Applied Biosystems; Thermo Fisher Scientific, Inc.). qPCR was performed in a 25 µl reaction containing 12.5 µl 2x SYBR-Green Mix, 100 nM of forward and reverse primers and 1 µl cDNA. Thermocycling conditions for PCR were 5 min at 95°C followed by 40 cycles of 5 min at 95°C, 10 sec at 58°C and 10 sec at 72°C. Fold-changes in target mRNA expression were determined using the 2⁻ΔΔCq method (19).

Western blot analysis. Cells were harvested and lysed in RIPA lysis buffer (Sangon Biotech Co., Ltd., Shanghai, China). The total protein concentration was determined using a BCA Protein assay kit (Sangon Biotech Co., Ltd.) according to the manufacturer's protocol. Total protein (20 µg) was separated by SDS-PAGE using a 10% (wt/vol) gel and then transferred to polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked with 5% non-fat milk at 4°C overnight, followed by incubation with the following rabbit polyclonal primary antibodies at 37°C for 1 h: Anti-TLR2 (1:800; cat. no. 2229; Cell Signaling Technology, Inc., Danvers, MA, USA), anti-TLR4 (1:500; cat. no. sc-30002; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), anti-MyD88 (1:800; cat. no. 3699; Cell Signaling Technology, Inc.) and anti-MyD88 (1:800; cat. no. 3699; Cell Signaling Technology, Inc.). Following washing three times with Tris-buffered saline containing 0.1% Tween-20 (TBST) (10 min/wash), the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G secondary antibodies (1:10,000; cat. no. sc-2004; Santa Cruz Biotechnology, Inc.) at 37°C for 1 h. Protein bands were detected using a Pierce ECL western blotting substrate (Pierce; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The band intensity was quantified using Image J 1.48u software (National Institutes of Health, Bethesda, MD, USA) and the experiments were performed three times independently.

Cytokine quantification. The concentration of tumor necrosis factor (TNF-α), interleukin (IL)-6 and IL-8 in cell supernatants was determined using the Human TNF-α, IL-6 (cat. no. D6050) and IL-8 (cat. no. D8000C) Quantikine ELISA kits (R&D Systems, Inc., Minneapolis, MN, USA) according to the manufacturer's protocol. Briefly, the cells were harvested and centrifuged at 1,000 x g at room temperature for 5 min, then the cell supernatants were collected. Standard curves were generated from serial dilutions of recombinant cytokine standards in PBS. The detection range for TNF-α, IL-6 and IL-8 using these kits are 15.6-1,000, 3.1-300 and 31.2-2,000 pg/ml, respectively.

Luciferase reporter assay. The miRanda program (www.microrna.org/microrna/home.do) was used to identify the targets of miR-30a. Subsequently, the DNA fragment of MyD88 wild-type 3'-UTR or mutant 3'-UTR (NM_001172566.1) was cloned into the pMIR-REPORT™ miRNA Expression Reporter Vector (Applied Biosystems; Thermo Fisher Scientific, Inc.) to construct reporter plasmids. The HEK293 cells were co-transfected with 400 ng reporter plasmids and 100 nM miR-30a mimic or miR-NC using Lipofectamine™ 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The pRL-SV40 Renilla luciferase plasmid (Promega Corporation, Madison, WI, USA) was transfected as an internal control. The cells were harvested and lysed with Passive Lysis Buffer (Promega Corporation) 24 h after transfection. Subsequently, luciferase activity was measured using the Dual-Luciferase® Reporter Assay system (Promega Corporation) according to the manufacturer's protocol.

Statistical analysis. Statistical analysis was performed using SPSS software (version 19.0; IBM Corp., Armonk, NY, USA). All data are presented as the mean ± standard deviation of ≥3 independent experiments. The Student's t-test was used to compare the statistical significance of results between two groups, and one-way analysis of the variance followed by a Fisher's least significant difference test was used for the comparison of multiple groups. P<0.05 was considered to indicate a statistically significant difference.
Results

Expression of miR-30 family members in THP-1 cells infected with MTB. The expression of miR-30a, -30b, -30c, -30d and -30e in THP-1 cells was examined by RT-qPCR at 0, 6, 12 and 24 h following H37Rv infection (Fig. 1). The expression of miR-30a and miR-30e was significantly increased in H37Rv-infected THP-1 cells at 6, 12 and 24 h after infection compared with the uninfected THP-1 cells (all P<0.05) in a time-dependent manner. However, MTB infection had no significance effect on the expression of miR-30b, miR-30c or miR-30d in THP-1 cells.

Effect of miR-30a and miR-30e on TLR2, TLR4 and MyD88 expression in MTB-infected THP-1 cells. THP-1 cells were transfected with miR-30a mimic or miR-30e mimic, and were then infected with H37Rv for 24 h. As presented in Fig. 2, the expression of miR-30a and miR-30e was significantly increased in THP-1 cells following transfection with the mimics compared with the miR-NC-transfected control group (P<0.01; Fig. 2A and B, respectively).

The expression of TLR2, TLR4 and MyD88 protein in MTB-infected THP-1 cells following transfection with the miR-30a or miR-30e mimics was examined by western blot analysis. Compared with the control group, MTB infection led to significantly increased expression of TLR2, TLR4 and MyD88 in THP-1 cells (all P<0.05; Fig. 3). Transfection of the miR-30a mimic significantly suppressed the expression of TLR2, TLR4 and MyD88 in the uninfected and H37Rv-infected THP-1 cells compared with the control group and the MTB infected group, respectively (both P<0.05; Fig. 3). Transfection of miR-30e mimic did not exert any significant effects on TLR2, TLR4 and MyD88 expression in the uninfected or H37Rv-infected THP-1 cells (Fig. 3).

Effect of miR-30a and miR-30e on TNF-α, IL-6 and IL-8 expression in MTB-infected THP-1 cells. The expression of TNF-α, IL-6 and IL-8 in MTB-infected THP-1 cells following transfection with the miR-30a or miR-30e mimic was examined using an ELISA assay. As presented in Fig. 4, the expression of TNF-α, IL-6 and IL-8 was significantly increased in H37Rv-infected THP-1 cells compared with the control group (all P<0.01). In H37Rv infected cells, expression of TNF-α, IL-6 and IL-8 was significantly suppressed by transfection with the miR-30a mimic (P<0.01). Transfection of the miR-30a mimic also significantly inhibited TNF-α, IL-6 and IL-8 expression in uninfected THP-1 cells compared with the control group (P<0.05). The expression of TNF-α, IL-6 and IL-8 was not significantly altered in uninfected THP-1 cells following transfection with miR-30e.

MyD88 is a direct target of miR-30a. Using the miRanda program, it was identified that MyD88 is a direct target of miR-30a. The wild-type or mutant MyD88 3'-UTR was constructed and co-transfected with the miR-30a mimic or miR-NC into the HEK293 cells, and a dual-luciferase assay was performed. When compared with the control group, the miR-30a mimic significantly suppressed the luciferase activity of wild-type MyD88 (P<0.01); however, the miR-30a mimic had to effect on the luciferase activity of the mutant MyD88 (Fig. 5).

Discussion

The miR-30 family consists of five members, miR-30a-e. Previous studies have highlighted the importance of miR-30 in the development and progression of cancer (20-23). It has previously been demonstrated that miR-30 is associated with inflammatory responses (24-26). MTB infection may modulate immune responses by affecting the expression of miRNAs. A
previous study by Chen et al (18) revealed that the expression of miR-30a was induced in MTB-infected macrophages, and that this overexpression of miR-30a suppressed the ability of host cells to eradicate intracellular MTB. Anti-TB treatment of these cells led to the decreased expression of miR-30a (18). In the present study, H37Rv-infected THP-1 cells were used to investigate the effect of MTB infection on the expression of miR-30 family members. In agreement with previous findings, the current study demonstrated that MTB infection significantly induced miR-30a and miR-30e expression in THP-1 cells in a time-dependent manner. However, the expression of miR-30b, -30c and -30d was not altered following MTB infection. These findings indicate that miR-30a and miR-30e may interact with MTB in THP-1 cells. Subsequently, the regulatory mechanism of miR-30 induction in response to MTB infection was investigated.

TLRs are most potent inducers of inflammatory responses (27). Among the cell surface TLRs, TLR2 and TLR4

Figure 3. Expression of TLR2, TLR4 and MyD88 in MTB-infected THP-1 cells following transfection with miR-30a or miR-30e mimics assessed using western blot analysis. Quantification of expression of (A) TLR2, (B) TLR4 and (C) MyD88. (D) Western blot of TLR2, TLR4 and MyD88 expression. *P<0.05, †P<0.01 vs. CTRL. miR, microRNA; MTB, Mycobacterium tuberculosis; CTRL, control; TLR, Toll-like receptor; MyD88, myeloid differentiation factor 88; lane 1, CTRL; lane 2, MTB; lane 3, miR-30a; lane 4, miR-30a+MTB; lane 5, miR-30e; lane 6, miR-30e+MTB.

Figure 4. Expression of TNF-α, IL-6 and IL-8 in MTB-infected THP-1 cells following transfection with miR-30a or miR-30e mimics. ELISA analysis of (A) TNF-α, (B) IL-6 and (C) IL-8 levels. *P<0.05, †P<0.01 vs. CTRL. miR, microRNA; MTB, Mycobacterium tuberculosis; CTRL, control; TNF-α, tumor necrosis factor α; IL, interleukin.

Figure 5. MyD88 is a direct target of miR-30a. #P<0.01 vs. miR-NC. miR, microRNA; NC, negative control; MyD88, myeloid differentiation factor 88; WT, wild-type; MUT, mutant.

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have been the focus of research due to their unique capacity to identify extracellular and intracellular pathogens (28). TLR2 and TLR4 are able to trigger signal transduction by recruiting MyD88, which drives activation of the transcription factor nuclear factor-kB, leading to the expression of proinflammatory cytokines. It has been demonstrated that TLR2 and TLR4 may interact with MTB, and that they are associated with the immune response to MTB infection (29-31).

In the present study, an miR-30a or miR-30e mimic were transfected into THP-1 cells to investigate the effect of miR-30 on the TLR/Md88 signaling pathway. This revealed that miR-30a exhibits a significant inhibitory effect on TLR, MyD88 and cytokine expression in uninfected THP-1 cells. Furthermore, this effect was also observed in MTB-infected THP-1 cells. miR-30e did not exhibit a significant effect on TLR, MyD88 or cytokine expression in the untreated or MTB-infected THP-1 cells. These results indicate that miR-30a participates in MTB-induced immune responses via suppressing TLR/Md88 activation and cytokine expression.

To identify the molecules targeted by miR-30a in the TLR/Md88 signaling pathway, the present study used the miRanda program and identified that Md88 was a predicted target gene of miR-30a. The luciferase reporter assay demonstrated that miR-30a directly regulates the transcriptional activity of Md88 3’-UTR. These results suggest that miR-30a suppresses the TLR/Md88 signaling pathway by directly targeting Md88.

In conclusion, the current study presents, to the best of our knowledge, the first evidence that miR-30a suppresses TLR/Md88 activation and cytokine expression in THP-1 cells during MTB H37Rv infection, and that Md88 is a direct target of miR-30a. The present study highlights the role of miR-30a in the immune response against MTB in monocyes, and may aid the development of novel therapeutic approaches for treating MTB.

References
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