MicroRNA-502-3p promotes *Mycobacterium tuberculosis* survival in macrophages by modulating the inflammatory response by targeting ROCK1

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**Abstract.** Tuberculosis (TB) is caused by *Mycobacterium tuberculosis* (*M. tuberculosis*) infection and has the highest mortality rate of any single infectious disease worldwide. The aim of the present study was to investigate the function of microRNA (miR)-502-3p in *M. tuberculosis*-infected macrophages. The Gene Expression Omnibus database was used to analyze miR-502-3p expression in patients with TB and healthy individuals. THP-1 and RAW 264.7 cells were transfected with miR-502-3p mimic, miR-502-3p inhibitor, pcDNA3.1-ROCK1 or their negative controls. The expression levels of miR-502-3p and inflammatory cytokines were evaluated using reverse transcription-quantitative PCR. The colony-forming unit assay was performed to assess the survival of *M. tuberculosis* in macrophages, and Toll-like receptor (TLR)4/NF-κB signaling pathway-associated protein expression levels were detected by western blotting. The nuclear translocation of NF-κB p65 was detected via immunocytochemistry. TargetScan was used to predict the binding sites between miR-502-3p and ROCK1. The interaction between miR-502-3p and Rho-associated coiled-coil-forming protein kinase 1 (ROCK1) was confirmed using a dual-luciferase reporter assay; ROCK1 was demonstrated to be a direct target gene of miR-502-3p. Results from the present study demonstrated that miR-502-3p expression was significantly increased during *M. tuberculosis* infection in macrophages. Upregulation of miR-502-3p expression levels significantly enhanced the survival of intracellular *M. tuberculosis*. IL-6, TNF-α, and IL-1β mRNA expression levels were significantly upregulated during *M. tuberculosis* infection but were downregulated by miR-502-3p overexpression. Moreover, miR-502-3p mimics transfection significantly downregulated TLR4/NF-κB signaling pathway-associated protein expression and significantly reduced nuclear transcription of NF-κB in *M. tuberculosis*-infected macrophages. ROCK1 overexpression reversed the miR-502-3p inhibitory effect on cytokine production in *M. tuberculosis*-infected macrophages. In conclusion, miR-502-3p/ROCK1 may serve an anti-inflammatory role and may improve the survival of *M. tuberculosis* within macrophages, which may provide a promising therapeutic target for TB.

**Introduction**

Tuberculosis (TB) is caused by the etiological agent *Mycobacterium* (*M*.), which primarily affects the lungs (1). TB was responsible for ~1.5 million deaths in 2018 worldwide (2). *M. tuberculosis* parasitizes the macrophages of the host; it manipulates the host's defenses and consequently the immune response (3). Furthermore, *M. tuberculosis* can evade innate immunity to survive and replicate inside macrophages (4). Therefore, the development of therapeutics that prevent immune evasion is crucial.

MicroRNAs (miRNAs/miRs) are a class of non-coding RNAs (~22 nucleotides long) that serve a role in silencing target gene expression and are associated with immune signaling pathways (5,6). For example, miR-325-3p promotes *M. tuberculosis* survival by targeting ligand of numb-protein X1 and increasing the phosphorylation of STAT3 (7). Overexpression of miR-26a was reported to modulate the survival of *M. tuberculosis* in macrophages (8), miR-125a inactivates NF-κB signaling by targeting TNF receptor associated factor 6, attenuating inflammation and facilitating the survival of *M. tuberculosis* (9). Although the potential role of several miRNAs in *M. tuberculosis* infection has been examined, this field requires further investigation.

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Rho-associated coiled-coil-forming protein kinase 1 (ROCK1) is a downstream effector of RhoA; it acts as a ‘molecular switch’ in the activation of the monocyte pro-inflammatory response (10). Suppression of ROCK1 expression prevents NF-κB signaling in a variety of inflammatory diseases, such as cervical cancer, and is a hallmark of obstructive sleep apnea syndrome (11,12). A previous study has shown that Toll-like receptor (TLR)4 is involved in the regulation of pulmonary immune responses and recognition of M. tuberculosis (13). NF-κB is a downstream effector of the TLR4 signaling pathway and an important pro-inflammatory factor (14). Numerous inflammatory cytokines, including TNF-α, IL-1β and IL-6, regulate the TLR4/NF-κB pathway (15).

In the present study, upregulated miRNAs in patients with TB were identified using the Gene Expression Omnibus (GEO) datasets, GSE34608 and GSE116542. miR-502-3p was selected for further studies. The aim of the present study was to investigate the function of miR-502-3p in M. tuberculosis-infected macrophages.

Materials and methods

Bioinformatics. The GEO (https://www.ncbi.nlm.nih.gov/geo) database [GSE34608 (PMID: 22547807) and GSE116542] was used to analyze miR-502-3p expression in patients with TB and healthy individuals. In GSE116542, 11 patients with TB (8 men; 3 women; age range, 17-51 years) and 8 healthy individuals (4 men; 4 women; age range, 29-60 years) were collected for exosomal miRNAs extraction. TargetScan 7.2 (http://www.targetscan.org) was used to predict the binding sites between miR-502-3p and ROCK1.

Cell culture. The human leukemia monocytic THP-1 and the mouse macrophage-like RAW 264.7 cell lines were purchased from The Cell Bank of Type Culture Collection of The Chinese Academy of Sciences. Cells were maintained in an incubator (37°C; 5% CO₂; 70% relative humidity) in RPMI-1640 medium containing 10% fetal bovine serum (Hyclone; Cytiva).

Transfection. THP-1 and RAW 264.7 cells were plated on 12-well plates at a seeding density of 3x10⁴ cells/well and transfected with miR-502-3p mimic, miR-502-3p inhibitor, pcDNA3.1-ROCK1 (Shanghai GenePharma Co., Ltd.), or their negative controls (NCs), mimic NC, inhibitor NC and pcDNA3.1-NC (50 pg/well) at 37°C for 24 h, using Lipofectamine 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. pcDNA3.1-ROCK1 plasmid was synthesized by Guangzhou RiboBio Co., Ltd. for miR-502-3p experiments, NC was used as the internal reference gene. Bulge-loop™ miRNA RT-qPCR primer sets specific for miR-502-3p were designed by Guangzhou RiboBio Co., Ltd. for miR-502-3p. U6 was used as the internal control. Relative expression levels were analyzed using the 2⁻ΔΔCq method (16).

Colonization-forming unit (CFU) assay. Following transfection, cells (3x10⁴ cells/well) were infected with M. tuberculosis (MOI=10) for 48 h and lysed with 0.5% Triton X-100 at 25°C for 20 min. A 10-fold serial dilution method was used for quantitative culture of bacterial colonies. The cell lysate was diluted with Middlebrook 7H9 broth media and added to Middlebrook 7H10 agar plates (Beijing Solarbio Science & Technology Co., Ltd.) supplemented with 10% OADC. Plates were incubated at 37°C for 3 weeks before the colonies were quantified manually. A colony referred to the growth of bacteria on solid medium that could be identified by the naked eye.

Dual-luciferase reporter assay. TargetScan was used to predict the mRNAs that may have a target site of miR-502-3p. Wild-type (WT) and mutant (MUT) ROCK1 3'-untranslated regions (UTRs) were cloned into the firefly luciferase reporter plasmid psi-CHECK2 (Qiagen China Co., Ltd.) to synthesize the ROCK1-WT and ROCK1-Mut reporter plasmids. 293T cells (American Type Culture Collection) at 5×10⁴ cells/well in 24-well plates were co-transfected with WT or Mut ROCK1 3'-UTR reporter plasmids and miR-502-3p mimic or mimic NC using Lipofectamine™ 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) at 37°C. After 48 h transfection, luciferase activity was detected using a Dual-Luciferase Reporter Assay System (Promega Corporation), according to the manufacturer's protocol. Renilla luciferase activity was used for normalization.

Western blotting. Total protein was extracted from THP-1 and RAW 264.7 cells (5x10⁵ cells/well) using RIPA lysis buffer (Beyotime Institute of Biotechnology). The protein content was assessed using the BCA method. Total protein (50 μg protein/lane) was separated by SDS-PAGE at a 12% gel. The separated proteins were transferred onto a nitrocellulose membrane (Invitrogen; Thermo Fisher Scientific, Inc.). The membranes were blocked using 5% skimmed milk for 2 h at 25°C. The membranes were incubated with
infection, THP‑1 and RAW 264.7 cells. To further determine the potential role of miRNAs with markedly high expression in M. tuberculosis infected macrophages increased in a time‑dependent manner (Fig. 1C and D). The expression level of miR‑502‑3p in macrophages at 48 h post‑infection was >3‑fold higher than that in uninfected control (Fig. 1C). Moreover, there was a ~3‑fold increase in miR‑502‑3p expression in macrophages cells at a MOI of 10 for 48 h compared with uninfected cells (Fig. 1D). Macrophages infected with M. tuberculosis at an MOI of 10 for 48 h were used for subsequent experiments.

miR‑502‑3p facilitates M. tuberculosis survival in macrophages. To further determine the potential role of miR‑502‑3p in the cellular immune response during M. tuberculosis infection, THP‑1 and RAW 264.7 cells were transfected with either miR‑502‑3p mimic or inhibitor. miR‑502‑3p expression levels significantly increased following miR‑502‑3p mimic transfection compared with mimic NC, whereas comparisons between multiple groups were determined using one‑way ANOVA followed by Tukey's post‑hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

M. tuberculosis infection induces miR‑502‑3p expression in patients with TB. miRNAs with markedly high expression levels in TB compared with the healthy control group were selected by analyzing the GSE34608 and GSE16542 datasets from the GEO database. A total of seven miRNAs were identified as being the same between the datasets (Fig. 1A). The current study aimed to investigated ROCK1 in TB. TargetScan predicted the binding sites between miR‑502‑3p and ROCK1. Thus, miR‑502‑3p was chosen from the seven miRNAs. miR‑502‑3p expression levels were significantly elevated in patients with TB compared with healthy individuals in the GSE116542 database (Fig. 1B). miR‑502‑3p expression levels in M. tuberculosis‑infected macrophages increased in a time and dose‑dependent manner (Fig. 1C and D). The expression level of miR‑502‑3p in macrophages at 48 h post‑infection was >3‑fold higher than that in uninfected control (Fig. 1C).

Statistical analysis. All presented data were obtained from at least three independent experiments. Data are shown as the mean ± SD. Statistical comparisons between two groups were determined by Student's unpaired t‑test, whereas comparisons between multiple groups were determined using one‑way ANOVA followed by Tukey's post‑hoc test. P<0.05 was considered to indicate a statistically significant difference.

Table I. Primer sequences used in transfection.

| Gene          | Primer sequence (5′→3′) |
|---------------|-------------------------|
| miR‑502‑3p mimic | AAUGACCAGUGGCAAGAUCA    |
| Mimic NC      | UGACAACUCCUCAGGAGUAGA   |
| miR‑502‑3p inhibitor | UGAACCUUUGCCCCAGGUCAUU |
| Inhibitor NC  | UCUACACUUUUACAGGUGUUGA  |

miR, microRNA; NC, negative control.

Table II. Sequences of primers used for reverse transcription‑quantitative PCR.

| Gene          | Primer sequence (5′→3′) |
|---------------|-------------------------|
| miR‑502‑3p    | F: AACTCCAGCTGGGAATGCACCT GGGCAAGG |
|               | R: CTCAGCTGGGTGCTGAGCA |
| U6            | F: CTCCGTTCGACAGCA |
|               | R: AACCTTCAGGAATTCGAGT |
| IL‑6 (human)  | F: ACAACACGCGCCTCCCTACT TCC |
|               | R: CAAGTATCTCCAGAGACCATGTG |
| IL‑6 (mouse)  | F: GGGCTTGGATGAGATTCGAGAGAG |
|               | R: TCCCTACACAAGGGCTCGAC |
| TNF‑α (human) | F: GCCCTTCTTATCCTCCTGCTTG |
|               | R: GGCATTTGGGAACACTTCCA |
| TNF‑α (mouse) | F: TGACCCCCATATTCTGAC GCC |
|               | R: TTCAGGTCGATAGTCTGTC |
| IL‑1β (human) | F: GTGGCAATGAGAGATCTGCTGTC |
|               | R: GGTTGTCGAGATTCAGTCAGT |
| IL‑1β (mouse) | F: GAGCAAAGTTGGTGTTCCTCC R: AACAGCAGGAGACCATCAG |
| β‑actin (human)| F: CATGTACGGTTGATCCAGGCC |
|               | R: CTCTATTTGTCACAGGACAT |
| β‑actin (mouse)| F: GTTGCGGCACTTGTGAAGGGC |
|               | R: AGGTACACTTACCTGGTGCC |

F, forward; R, reverse; miR, microRNA.
transfection compared with inhibitor NC (Fig. 2A). The CFU assay demonstrated that miR-502-3p mimic transfection significantly increased the survival of *M. tuberculosis* from infected macrophages compared with mimic NC, whereas inhibition of miR-502-3p significantly reduced the intracellular growth of *M. tuberculosis* compared with inhibitor NC (Fig. 2B). The high number of *M. tuberculosis* colonies means that the phagocytosis of macrophages to *M. tuberculosis* is weakened and the survival of macrophages is reduced.

**miR-502-3p suppresses cytokine production in *M. tuberculosis*-infected macrophages.** *M. tuberculosis* infection promotes macrophages to produce cytokines (17). The effects of miR-502-3p on cytokines in *M. tuberculosis*-infected THP-1 and RAW 264.7 cells were investigated. The mRNA expression levels of IL-6, TNF-α and IL-1β were significantly induced in *M. tuberculosis*-infected macrophages. The mRNA expression levels of IL-6, TNF-α and IL-1β, were significantly reduced in *M. tuberculosis*-infected macrophages transfected with miR-502-3p mimic, compared with the *M. tuberculosis*-infected only group. Moreover, downregulation of miR-502-3p significantly increased the expression of IL-6, TNF-α and IL-1β, in *M. tuberculosis*-infected macrophages compared with the *M. tuberculosis*-infected only group (Fig. 3).

**miR-502-3p directly targets ROCK1.** Using TargetScan, ROCK1 was predicted to have a potential miR-502-3p target site in its 3'UTR (Fig. 4A). miR-502-3p mimic transfection significantly suppressed luciferase activity in 293T cells containing the ROCK1-WT vector compared with mimic NC, whereas no change in luciferase activity was observed in cells transfected with the ROCK1-Mut vector (Fig. 4B). Western blotting demonstrated that the protein expression levels of ROCK1 were significantly increased in *M. tuberculosis*-infected macrophages compared with the control. Compared with the *M. tuberculosis*-infected only group, overexpression of miR-502-3p significantly decreased...
ROCK1 protein expression levels, whereas knockdown of miR-502-3p significantly increased ROCK1 protein expression levels (Fig. 4C).

miR-502-3p regulates the TLR4/NF-κB signaling pathway in *M. tuberculosis*-infected macrophages. A previous study has shown that TLR-4/miR-125a/NF-κB signaling modulates the immune response to *M. tuberculosis* infection (9). To determine the mechanism by which miR-502-3p promoted *M. tuberculosis* survival in macrophages, the TLR4/NF-κB signaling pathway was examined. TLR4, p65 phosphorylation and IκBα phosphorylation were significantly increased in *M. tuberculosis*-infected THP-1 and RAW 264.7 cells. However, TLR4, p65 phosphorylation and IκBα phosphorylation were significantly reduced in *M. tuberculosis*-infected THP-1 and RAW 264.7 cells transfected with miR-502-3p mimic compared with the *M. tuberculosis*-infected only group. Moreover, the downregulation of miR-502-3p expression significantly increased TLR4 levels and the phosphorylation of p65 and IκBα compared with the *M. tuberculosis*-infected only group (Fig. 5). Furthermore,
compared with *M. tuberculosis*-infected only group, the expression level of p-p65 in nucleus was significantly reduced by miR-502-3p overexpression and significantly induced by miR-502-3p inhibition (Fig. 6).

**ROCK1 overexpression reverses the miR-502-3p inhibitory effect on cytokine production in *M. tuberculosis*-infected macrophages.** The effect of ROCK1 overexpression on cytokine production in miR-502-3p-overexpressing macrophages was investigated. ROCK1 overexpression was successfully achieved by transfecting THP-1 and RAW 264.7 cells with pcDNA3.1-ROCK1 (Fig. 7A). IL-6, TNF-α and IL-1β mRNA expression levels were increased in *M. tuberculosis*-infected THP-1 and RAW 264.7 cells. The miR-502-3p mimic increased and overexpression of ROCK1 decreased IL-6, TNF-α and IL-1β mRNA expression in *M. tuberculosis*-infected THP-1 and RAW 264.7 cells. Overexpression of ROCK1 partially reversed the inhibitory effects of miR-502-3p mimic on IL-6, TNF-α and IL-1β levels in *M. tuberculosis*-infected THP-1 and RAW 264.7 cells compared with the *M. tuberculosis* + miR-502-3p mimic group (Fig. 7B-D).

**Discussion**

miRNAs have been reported to serve important roles in the host response to intracellular *M. tuberculosis* (18-20). For example, increased miR-20a-3p expression was shown to regulate the host immune response to promote the growth of *M. tuberculosis* in human macrophages (21). In the present study, miR-502-3p expression was significantly upregulated in *M. tuberculosis*-infected THP-1 and RAW 264.7 cells. Furthermore, miR-502-3p overexpression significantly reduced the mRNA expression levels of inflammatory cytokines and the activation of NF-κB signaling through the targeting of ROCK1. miR-502-3p also serves a role in carcinomatosis, is associated with inflammation and its expression is significantly decreased following the buildup phase of venom immunotherapy (22-24). However, the role of miR-502-3p in the modulation of immune responses has not been extensively investigated.

The production of inflammatory cytokines by macrophages is regarded as a bactericidal pathway for *M. tuberculosis* infection (25). However, *M. tuberculosis* is able to resist these antimicrobial activities by establishing a niche for survival in macrophages (26). Previous studies have determined that miRNAs inhibit the expression of pro-inflammatory cytokines to generate niches favorable for mycobacterial survival (9,27,28). In the present study, it was considered that the detection of mRNA changes was more direct and convenient to reflect the level of inflammatory factors. The current study demonstrated that miR-502-3p overexpression significantly reduced pro-inflammatory cytokine mRNA expression levels and promoted mycobacterial intracellular survival.

Furthermore, previous studies have demonstrated that the association between miRNAs and mRNAs serves an important role during *M. tuberculosis* infection. For example, miR-21-5p enhances mycobacterial survival and weakens inflammatory
responses by targeting Bcl-2 and TLR4 (29). miR-144 regulates inflammatory cytokine secretion and ERK signaling by targeting tumor progression locus 2 in M. tuberculosis-infected macrophages (30). In the present study, miR-502-3p overexpression promoted mycobacterial survival in macrophages by directly targeting ROCK1 to evade the immune response via the ROCK1/TLR4/NF-κB pathway, suggesting that miR-502-3p may have a similar function as miR-21-5 during M. tuberculosis infection. Inhibition of ROCK1 alleviates lipopolysaccharide-induced inflammatory cytokine production and suppresses TLR4/NF-κB and ERK signaling pathways in corneal epithelial cells (31). Furthermore, it has been demonstrated that an M. tuberculosis nucleoside diphosphate kinase stimulates GTPase activity of RhoA, Rac1 and cell division control protein 42 (32). Therefore, the RhoA/ROCK signaling pathway may be important for M. tuberculosis-induced inflammatory responses. The regulatory function of miRNAs through TLRs and the NF-xB signaling pathway in M. tuberculosis infection has also recently been studied. For example, miR-148a inhibits TLR4-mediated NF-xB activation in
Figure 5. miR-502-3p regulates the TLR4/NF-κB signaling pathway in *M. tuberculosis*-infected macrophages. TLR4/NF-κB signaling pathway-associated proteins were assessed using western blotting. ***P<0.001 vs. control; **P<0.01 and ###P<0.001 vs. M.tb. miR, microRNA; TLR, toll-like receptor; M.tb, *Mycobacterium tuberculosis*; p, phosphorylated; NC, negative control.

Figure 6. miR-502-3p inhibits the expression of NF-κB p-p65 in nucleus. NF-κB p-p65 expression was evaluated by immunofluorescence staining. Scale bar, 50 µm. Untreated macrophages were used as the control. ***P<0.001 vs. control; **P<0.01 and ###P<0.001 vs. M.tb. miR, microRNA; M.tb, *Mycobacterium tuberculosis*; NC, negative control.
response to mycobacterial infection (33). The present study demonstrated that miR-502-3p regulated the host immune response to \textit{M. tuberculosis} by directly targeting ROCK1 to possibly attenuate TLR4-\(\text{NF-}\kappa\text{B}\) signaling pathway activation and consequent immune responses.

In conclusion, miR-502-3p promoted \textit{M. tuberculosis} survival in macrophages and reduced pro-inflammatory cytokine release in \textit{M. tuberculosis}-infected macrophages by targeting the ROCK1/TLR4/NF-\(\kappa\text{B}\) pathway. The expression levels of inflammatory cytokines were determined only via RT-qPCR, and the levels of these inflammatory factors were not assessed using ELISA. These results have provided the foundations for the future development of TB therapeutics.

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Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions

FL, YZ and ZD designed the study. YL, HY and PW performed the research and analyzed the data. FL and ZD confirmed the authenticity of all the raw data. YZ and PW wrote the paper and FL and ZD reviewed the article. All authors approved the final version of the manuscript.
Ethics approval and consent to participate
Not applicable.

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Not applicable.

Competing interests
The authors declare that they have no competing interests.

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