Inhibition of Autophagy by MiR-30A Induced by Mycobacteria tuberculosis as a Possible Mechanism of Immune Escape in Human Macrophages

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SUMMARY: The regulatory mechanism of miRNA induction in response to Mycobacterium tuberculosis (MTB) infection has not been clearly established. Autophagy has recently been identified as an effective way to control intracellular survival of MTB. In the present study, we demonstrate a novel role of miR-30A in the negative regulation of the autophagy-mediated anti-MTB response. We found that overexpression of miR-30A suppresses the elimination of intracellular MTB through the inhibition of autophagy. Furthermore, there was a negative correlation between concentrations of miR-30A and beclin-1 in MTB positive patients and miR-30A expression decreased after anti-TB treatment. Our results indicate that miR-30A plays a key role in immune response against MTB and, therefore, may serve as a potential target for future treatments of tuberculosis infection.

Mycobacterium tuberculosis (MTB) is a human pathogenic bacterium, which infects over one third of the world’s population and causes more death than any other infectious agent (1). Macrophages play a pivotal role in innate immune responses against a wide range of pathogens, including MTB. Some mechanisms that allow macrophages to limit the growth of mycobacteria have been elucidated; however, MTB arrests normal phagosome maturation, avoids fusion with lysosomes, and renders the intraphagosomal environment more compatible with bacterial survival and replication. Thus, a critical balance between the activity of macrophages and MTB interactions could be instrumental in determining the outcome of infection. During co-existence with the host, mycobacteria form a complex network within macrophages to precisely regulate the immune response (2).

Autophagy is an evolutionarily conserved process, which is involved in maintaining cytoplasmic homeostasis by degrading damaged organelles or misfolded proteins (3). The autophagic cascade is initiated by the engulfment of cytoplasmic cargo by an autophagosome, which then fuses with a late endosome to form the autolysosome, exposing the inner compartment to lysosomal hydrolases for degradation (4). Recently, a number of studies suggested that autophagy may link multiple steps of intracellular MTB clearance (5,6).

As a class of small non-coding RNAs, microRNAs are highly conserved between different eukaryotic species. They function as key regulators of gene expression at the post-transcriptional level by targeting mRNAs for translational repression or degradation (7). Given that many autophagy-related genes are targeted by various microRNAs, it can be concluded that microRNAs play a critical role in autophagy regulation. In our previous report, we have identified non-overlapping signatures of a small number of miRNAs that were aberrantly expressed in MTB infected THP-1 cells and found that the expression level of miR-30A was significantly upregulated (8). In this study, we focused on the regulatory role of miR-30A in autophagy triggered by MTB infection in human macrophage.

The in vitro infection model was established according to our previous report (8). Briefly, THP-1 cells were seeded at a density of 5 × 10⁶ cells/flask and grown to 80% confluence. THP-1 cells were allowed to grow for 2d at 37°C in 5% CO₂ and then phorbol myristic acid acetate was added to a final concentration of 250 ng/ml. THP-1 monolayers containing approximately 10⁷ cells were incubated with 1 × 10⁶ bacteria (MOI of 10) for 4 h and washed with RPMI 1640 to remove any extracellular bacteria. This infection model was monitored for morphological changes and the release of TNF-α, as measured by the DuoSet ELISA Development System (R&D). Alveolar macrophages (AMs) from bronchoalveolar lavage (BAL) of 65 pulmonary tuberculosis (PTB) patients (45 smear-positive and 20 smear-negative) and 15 volunteers were collected from the Hospital of No. 309 of Chinese People’s Liberation Army (PLA), as described in our previous report (8) and used for RNA extraction. During the 8-week long treatment, all patients received a short course of 4 anti-tuberculosis drugs, which were administered daily at standard doses (isoniazid, 300 mg; rifampin, 600 mg; pyrazinamide, 1.6 g; and ethambutol, 1.2 g), under the directly observed treatment, short-course strategy, as proposed by the World Health Organization. Drugs were administered in the morning, approximately 30 to 60 min before breakfast. After the termination of treatment, AM miR-30A levels were determined and conventional clinical examinations were carried out.

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synthesized from GenePharma (Shanghai, P.R. China) products. Transfections were performed using Lipofectamine 2000 (Invitrogen). Cells were transfected with 50 nM miRNA mimics, inhibitors, or scrambled miR-control for 24 h.

Total RNA was extracted from cells using Trizol reagent (Invitrogen) according to the manufacturer’s protocol. qRT-PCR analyses for miRNAs were performed by using TaqMan miRNA assays (Ambion) in an iQ5 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). Reverse transcription reactions were performed using the following parameters: 16°C, 30 min; 42°C, 30 min; and 84°C, 5 min. PCR reactions were performed using the following parameters: 95°C for 2 min followed by 40 cycles of 95°C for 15 s and 60°C for 30 s. U6 small nuclear RNA was used as an endogenous control for data normalization. Relative expression was calculated using the comparative threshold cycle method. qRT-PCR analyses for Beclin-1 and β-actin mRNA molecules were performed using PrimeScript RT-PCR kits (Takara, Shiga, Japan). The level of β-actin mRNA was used as an internal control.

For western blotting, cells were washed with ice-cold phosphate-buffered saline and then lysed with protein lysate (Pierce, Rockford, IL, USA). After centrifugation at 5,000 × g for 15 min at 4°C, the protein concentration was measured with a bicinchoninic acid protein assay kit (Pierce). Fifty microgram aliquots of lysates were loaded on a sodium dodecylsulfate polyacrylamide 10% gradient gel and transferred to a polyvinylidene difluoride membrane. The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline, pH 7.4, containing 0.05% Tween 20, and were incubated with primary antibodies (1:200; Santa Cruz, Delaware Avenue, CA, USA) and horseradish peroxidase-conjugated secondary antibodies (1:5000; Santa Cruz) according to the manufacturer’s instructions. The protein of interest was visualized using an enhanced chemiluminescence Western blotting substrate (Pierce) and Chemidoc XRS Gel Documentation System (BioRad, Guangzhou, China). LC3 and beclin-1 antibodies were purchased from Abcam (Cambridge, MA, USA).

The results are expressed as means ± SD from at least 3 separate experiments performed in triplicate. The differences between groups were determined by a two-tailed Student’s t-test using SPSS software (Armonk, NY, USA). P values of less than 0.05 were considered to indicate statistically significant changes. The chi-square test or Fisher’s exact test were used to analyze the relationship between miR-30A expression and the clinicopathological features. All the experiments were carried out according to the principles of Helsinki

Fig. 1. Relationship between miR-30A expression and the survival of intracellular MTB in THP-1 cells. (A and B) THP-1 cells were infected H37Rv at an MOI of 10 for the indicated time points (A) or at indicated MOIs for 24 h (B). (C and D) THP-1 cells were transiently transfected with miR-30A mimic (C) or miR-30A inhibitor (D), and then challenged with MTB at an MOI of 10, and intracellular MTB viability was determined by CFU counting at certain time point. *P<0.05; **P<0.01.
Declaration. The Ethics Committee of the PLA hospital No. 309 approved this study.

Using H37Rv infected THP-1 cells, we found that miR-30A expression was increased after an MTB challenge in a time (Fig. 1A) and dose-dependent manner (Fig. 1B). The same tendency was also observed after BCG and H37Ra challenges (data not shown). To determine the role of miR-30A during MTB infection, we next examined its effects on bacterial survival using the colony-forming unit (CFU) assay. THP-1 cells were transiently transfected with a miR-30A mimic or inhibitor, and then challenged with H37Rv at MOI of 10. Our results showed that the treatment with miR-30A increased the survival of intracellular MTB compared to the survival in the control group in a time-dependent manner (Fig. 1C). Furthermore, the transfection with a miR-30A inhibitor led to the opposite outcome (Fig. 1D). These results indicated that miR-30A could suppress the ability of host cells to kill intracellular MTB.

It has been reported that autophagy promotes the killing of MTB within infected cells (9). On the other hand, miR-30A potently inhibits autophagy by selectively

Fig. 2. miR-30A promote MTB clearance by decreasing autophagy in THP-1 cells. THP-1 cells were transfected with (A) miR-30A mimics or (B) miR-30A inhibitor for 24 h followed by H37Rv infection (MOI = 10 : 1), and mRNA and protein level of Beclin-1 were determined (C). THP-1 cells were treated with H37Rv for 4 h in the presence of Baf A1, miR-30A mimics, and miR-30A inhibitors, and the LC3-II/GAPDH level was determined. Cells were pretreated with Rapamycin or 3-MA, and then transfected with (D) miR-30A mimics or (E) miR-30A inhibitor for 24 h followed by H37Rv infection (MOI = 10 : 1), and intracellular H37Rv viability was determined by CFU counting at 24 h. **P<0.05, ***P<0.01.
downregulating beclin-1 and ATG5 expression (10). Therefore, we examined whether miR-30A suppresses autophagy after MTB infection. We found that mRNA expression and protein level of the autophagy related gene beclin-1 was significantly up-regulated during MTB infection compared to its expression levels in uninfected control cells, whereas miR-30A mimics could block this upregulation (Fig. 2A). The miR-30A inhibitor induced a small increase in beclin-1 expression (Fig. 2B), which was probably because miR-30A concentration was already relatively augmented by MTB infection. In consistence with the observed beclin-1 density, similar results were observed when the LC3-II/GAPDH ratio was determined. Furthermore, bafilomycin A1 (Baf A1) challenge resulted in a further increase of the LC3-II/GAPDH ratio, indicating that the autophagic flux was unobstructed (Fig. 2C). Collectively, these data demonstrated that miR-30A inhibited the autophagic response to MTB infection.

It has been shown previously that inhibition of beclin-1 expression by a miR-30A mimic blunted the activation of autophagy induced by rapamycin (11). In order to explore whether miR-30A enhances the survival of intracellular MTB by suppressing of autophagy, we pretreated THP-1 cells with a miR-30A mimic or inhibitor, and either left the cells untreated or pretreated them with 3-methyladenine (3-MA, autophagy inhibitor) or rapamycin (autophagy enhancer) followed by the exposure to H37Rv for 24 h. We found that rapamycin blocked the production of miR-30A and enhanced the survival of intracellular MTB, whereas 3-MA had no obvious effect (Fig. 2D). As expected, the opposite result was observed in THP-1 cells treated with a miR-30A inhibitor (Fig. 2E). These observations indicated that inhibition of autophagy by miR-30A negatively affected the killing of intracellular MTB.

Then, we analyzed the relationship between miR-30A concentration and clinical parameters in human AMs. To this end, we compared miR-30A levels measured by quantitative PCR in AMs of 45 smear-positive PTB patients, 20 smear-negative PTB patients, and 15 healthy volunteers (Fig. 3A). Although miR-30A levels

![Graph A](image1)

![Graph B](image2)

![Graph C](image3)

Fig. 3. Correlation of miR-30A with Clinical value in PTB patients. (A) differential miR-30A expression in BAL of individuals. (B) differential miR-30A expression in BAL between smear positive, smear negative, and control groups. (C) differential miR-30A expression in BAL in 17 patients before and after anti-tuberculosis treatment.
exhibited a significant variability between individuals, we found that the mean miR-30A concentration was 3.13-fold and 2.01-fold higher in smear-positive and smear-negative groups compared to the mean miR-30A level in healthy volunteers (Fig. 3B, \( P < 0.01 \)). We also found that miR-30A expression correlated with the degree of smear-positive and the degree of radiological extent of disease (data not shown). We next followed up 17 patients who were smear-positive before anti-TB treatment and showed a significant reduction of MTB infection after anti-TB treatment (15 exhibited smear conversion and 2 had a complete reduction). Ten of these patients (58.8\%) exhibited a significantly decreased level of miR-30A (Fig. 3C). These results indicated that miR-30A levels could discriminate between active tuberculosis and healthy condition and reflect of efficiency of anti-tuberculosis treatment.

We have also tested several AM samples from pneumonia and lung cancer patients and found variable enhancements of miR-30A levels with significant inter-group differences between MTB, pneumonia, and lung cancer cohort. It suggested that the enhancement of miR-30A levels was not specific for tuberculosis.

The capacity of MTB to survive and cause disease strongly correlates with its ability to escape immune defense mechanisms. In particular, MTB has a remarkable ability to survive within the hostile environment of the macrophage. Autophagy has been recently demonstrated as a previously unrecognized mechanism for killing pathogenic bacteria. In our earlier report, using microarrays we found that miR-30A, an autophagy related microRNA, was upregulated by infection. We report here that miR-30A acts as a negative regulator of autophagy in MTB infected macrophages and that H37Rv triggered increase in miR-30A levels may play an important role in mediating the escape of MTB from killing by macrophages due to inhibited autophagic pathways. Our conclusions are supported by the following observations: (i) miR-30A was upregulated during MTB infection in THP-1 cell line and human BAL AMs; (ii) exogenous miR-30A simultaneously suppressed autophagy and bacterial elimination; (iii) induction of autophagy by rapamycin promoted intracellular killing of MTB. However, given that background miR-30A levels were low and only twice higher in MTB infected cells compared to miR-30A concentration in non-stimulated cells, we believe that the enhancement of endogenous miR-30A expression induced by MTB cannot be the principal reason of the inhibition of autophagy and subsequent immune escape. Most probably, dynamics of miR-30A expression may be a part of a complex mechanism, which is regulated by many autophagy related molecules. Converging on a potential immune escape mechanism, our findings may help to uncover previously unknown signaling pathways related to the host response to MTB infection and define novel targets for drug development.

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**Conflict of interest** None to declare.

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