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Mycobacterium marinum down-regulates miR-148a in macrophages in an EsxA-dependent manner

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ABSTRACT

As a key virulence factor of *Mycobacterium tuberculosis*, EsxA is not only involved in phagosome rupture, but also functions in stimulation of immune responses in macrophages. Here, we report thatmiR-148a is down-regulated in the macrophages infected with *Mycobacterium marinum* (Mm). Using the knockout strain MmΔEsxA/B, recombinant EsxA, EsxB and EsxA/B heterodimer proteins, we provide evidence that down-regulation of miR-148ais dependent on EsxA, and up-regulation of miR-148a reduces Mm intracellular survival. Moreover, up-regulation of miR-148a down-regulates the pro-inflammatory cytokines (e.g. TNF-α and IL-1β) and the TLR4-mediated NF-κB activation. Together, miR-148a may function as an anti-inflammation modulator in responses to mycobacterial infection. Regulation of miR-148a may provide a novel venue in development of therapies in tuberculosis.

1. Introduction

Pulmonary tuberculosis (TB) is caused by the bacterial pathogen *Mycobacterium tuberculosis* (Mtb). It is a global health problem that accounts for over 8 million new cases and 1.5 million death annually [1–4]. Mtb establishes infection after being inhaled into the alveolar cavity, where it is internalized into alveolar macrophages [5,6], which are believed to be the first line of immune defense against bacterial infection and also act the cellular hosts for Mtb [7,8]. Although alveolar macrophages are one of important components for Mtb control [3], the Mtb-macrophage interaction remains to be further elucidated. Current studies have suggested that within the phagosomes of alveolar macrophages, Mtb inhibits phagosome-lysosome fusion, penetrate the phagosome membranes, and progressively translocates to the cytosol for replicating and cell-to-cell spreading [9–14]. The ability of Mtb to penetrate the phagosome membrane is dependent on the Esx-1-secreted virulence factors, EsxA or 6-kDa early-secreted antigenic target (ESAT-6) and EsxB or 10-kDa culture filtrate protein (CFP-10), presumably through the membrane-lytic activity of the Esx-1 virulence factors [15–17]. Using liposome as a model membrane system, we have shown that EsxA, but not EsxB, undergoes significant conformational change and disrupts liposomal membranes at acidic pH [18]. EsxA inserts into lipid membranes with its two α-helixes forming a membrane-spanning domain [19]. We have also found that the orthologous EsxA from non-pathogenic *Mycobacterium smegmatis* (Ms), which shares over 76% homology with EsxA from Mtb, is inactive in membrane interaction [18,20]. All the findings suggest that the membrane-permeabilizing activity of EsxA is a major determinant for virulence phenotypes of mycobacterial species [20,21]. Most recently, we have presented evidence that a single-residue mutation Q5K of EsxA significantly reduced the membrane-permeabilizing activity and attenuated mycobacterial virulence in macrophages and in zebra fish embryos through inhibiting mycobacterial cytosolic translocation [22].

Not only functioning as membrane-permeabilizing virulence factors, ESX-1 system has also been heavily investigated as a modulator of macrophage function by inhibiting macrophage activation and promoting inflammasome activation [23]. For instance, EsxA has been shown to inhibit TLR4-mediated MAPK activation and NF-κB activation through the binding of the C-terminal 20 amino acid of EsxA to TLR2 in macrophage [24]. Moreover, Mtb and Mm induced potent TNF-α and IL-1β production in macrophage through NLR-mediated inflammasome activation in an ESX-1-dependent manner [25–28]. Further, the recombinant EsxA has been shown to directly activate inflammasome to produce IL-1β in macrophage [29–31].

MicroRNAs (miRNAs), a small, non-coding RNAs of about 21 nucleotides, regulate expression of multiple protein-encoding genes in the

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immune system via targeting mRNAs for degradation or translational repression [32,33]. There is growing evidence that multiple miRNAs, such as miR-26a, miR-23a-5p and miR-146a, are reportedly involved in the regulation of host immunity during Mtb infection, and the differential expression of miRNA in TB patients may help to distinguish active TB from healthy individuals or latent TB [34–36]. Interestingly, several studies have suggested that Mtb infection causes up-regulation of miR-155 and down-regulation of miR-let7f in an EsxA-dependent manner [37–40], which implicates potential therapeutic value for TB treatment through modulating miRNAs. Recently, the miR-148 family has drawn our attention. The members of miR-148 family, including miR-148a-3p, have been intensively investigated as important immune regulators in cancer research as well as bacterial and viral infections [41–43]. Moreover, a recent study of chlamydial infection in mice has shown that the virulent chlamydial strain, but not attenuated strain, significantly down-regulated miR-148a-3p, resulting in up-regulation of pro-inflammatory cytokines, such as TNF-α and IL-1β, leading to pathology [44]. Therefore, we hypothesize that miR-148a-3p plays a role in mycobacterial infection by acting as a negative regulator of pro-inflammatory response. In the present study, we have found that miR-148a is down-regulated in the macrophages infected by *Mycobacterium marinum* in an EsxA-dependent manner and up-regulation of miR148a decreases production of proinflammatory cytokines and inhibits mycobacterial intracellular survival.

2. Materials and methods

2.1. Protein expression and purification

The plasmid pMyNT was a generous gift from Dr. Matthias Wilmanns, which contains the native operon of Mtb EsxB:EsxA with a His_6-tag at the N-terminus of EsxB [45–47]. The plasmid pMyNT was transformed into *M. smegmatis* by electroporation at 2.5 kV. The bacterial colonies were grown in 7H9 plates supplemented with 10% OADC, 0.5% glycerol and 100 μg/mL of hygromycin. A single colony was picked and pre-cultured in 50 mL of 7H9 medium supplemented with 10% OADC, 0.5% glycerol, 0.05% Tween 80 and 100 μg/mL of hygromycin with shaking at 37 °C. The pre-culture was transferred to 1 L of 7H9 medium supplemented with 0.2% glycerol, 0.05% Tween80, 0.2% glucose and 100 μg/mL of hygromycin to a final OD_{600} = 0.05 with shaking at 37 °C. Protein expression was induced at OD_{600} = 1.5 by adding acetamide (33 mM final concentration) for 48 h, and the cells were harvested by centrifugation. The pellet was lysed by sonication in the soluble buffer (20 mM TrisHCl, 150 mM NaCl, 10 mM imidazole, pH 7.4), and the supernatant was collected by centrifugation at 15,200 × *g* for 1 h at 4 °C. The supernatant was loaded onto a Ni^{2+}-charged Sepharose column pre-equilibrated with the soluble buffer. The bound proteins were eluted from the column by a linear gradient (10–100%) of the elution buffer (20 mM TrisHCl, 150 mM NaCl, 500 mM imidazole, pH 7.4). The eluted heterodimer was further clarified through a gel filtration chromatography using a Sephadex 75 column in the gel filtration buffer (20 mM TrisHCl, 100 mM NaCl, pH 7.3). The EsxA/B heterodimer was separated by 6 M guanidine. The solution was then concentrated to 1 mL and injected into a 5 mL His-trap column, which was pre-equilibrated with 25 mM TrisHCl, 150 mM NaCl, 10 mM imidazole, 6 M guanidine. The His-tagged EsxB protein was bound to the column, while EsxA flew through the column. The EsxA was collected and extensively dialyzed in 20 mM TrisHCl, 100 mM NaCl, pH 7.3 and stored at −80 °C. The EsxB was eluted from the column by a linear gradient (10–100%) of the elution buffer (20 mM TrisHCl, 150 mM NaCl, 500 mM imidazole, pH 7.4). The eluted EsxB protein is dialyzed in 20 mM TrisHCl, 100 mM NaCl, pH 7.3 and stored at −80 °C.

2.2. Mm infection of macrophages

RAW264.7 macrophage cells (ATCC) were cultured in DMEM medium supplemented with 10% fetal bovine serum in 5% CO_2 at 37 °C. The Mm wild type strain and the mutant strain with deletion of EsxA/B (MmΔEsxA/B) are maintained in the laboratory as previously described [22]. Mycobacteria were grown in Middlebrook 7H9 liquid medium supplemented with 10% OADC at 37 °C. RAW264.7 cells were infected with Mm or (MmΔEsxA/B) at MOI of 5 and 10, respectively. At 6 and 12 h of post-infection, the cells were collected and subjected to subsequent analysis, such as qRT-PCR and Western blot.

2.3. qRT-PCR

Total RNA, including miRNA, was extracted with TRIzol reagent (Invitrogen) according to the manufacture instruction. For the expression of miRNA, a commercial TaqMan™ MicroRNA Reverse Transcription Kit (Invitrogen) was used. The relative expression of miRNA was normalized to U6 by the $2^{-\Delta\Delta Ct}$ cycle threshold method.

For the mRNA expression of TNF-α and IL-1β, the qRT-PCR was performed using the SYBR® Select Master Mix kit (Invitrogen) and standard protocols on the Step One Real-Time PCR System (Applied Biosystems, USA). The relative gene expression levels were normalized by GAPDH through the $2^{-\Delta\Delta Ct}$ comparative approach. The primers used for the studies were listed in the Table 1.

2.4. ELISA

The supernatants of the cells were harvested to detect the expression of IL-1β and TNF-α using ELISA kits (BioLegend, USA) following the corporation's instructions. Finally, the absorbance value was measured at 450 nm using automatic enzyme standard instrument (Thermo,

Table 1
Primers used for qPCR.

Name	Sequence (5′ → 3′): forward and reverse	GenBank accession No.	Product size (bp)
TNF-α	CTTCTCATTCCTGCTTGTG ACTTGGTGGTTTGCTACG	NM_013693.3	198
IL-1β	CCTGGGCTGTCCTGATGAGAGTCCACGGGAAAGACACAGGTA	NM_008361.4	131
GAPDH	CAATGTGTCCGTCGTGGATCTGTCCTCAGTGTAGCCCAAGATG	NM_001289726.1	124

miRNA	Primer names	Sequences
mmu-miR-148a-3p	Stem-loop	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGACAAAGTT
	Forward	GCCGAGTCAGTGCACTACAG
	Reverse	CTCAACTGGTGTCGTGGA
U6	Stem-loop	AACGCTTCACGAATTTGCGT
	Forward	CTCGCTTCGGCAGCACA
	Reverse	AACGCTTCACGAATTTGCGT

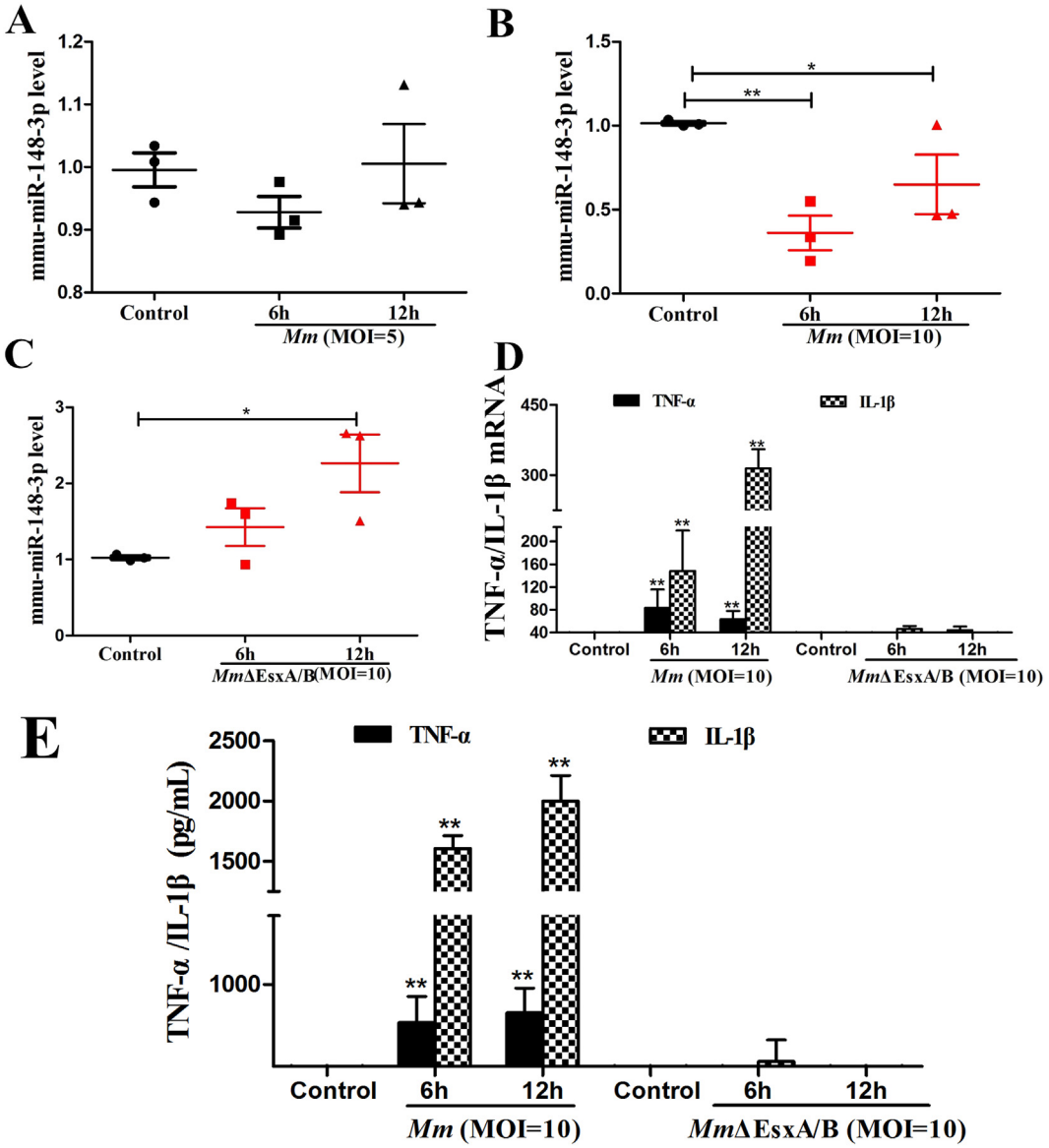

Fig. 1. Mm infection down-regulated miR-148a and up-regulated pro-inflammatory cytokines in an EsxA/B dependent manner. A, B. RAW264.7 cells were infected with Mm at MOI of 5 and 10, respectively. At 6 and 12 h of post infection, the expression level of miR-148a was determined by qRT-PCR assay. C. RAW264.7 cells were infected by MmΔEsxA/B at MOI of 10. The miR-148a expression at 6 and 12 h post infection was analyzed by qRT-PCR. D. The mRNA expression of TNF-α and IL-1β was measured by qRT-PCR in RAW264.7 cells infected by Mm or MmΔEsxA/B at 6 and 12 h post infection. The RNA expression in the uninfected cells was set as control. E. The protein level of TNF-α and IL-1β from RAW264.7 was measured by ELISA. All the data above represent the mean ± S.E.M of three independent experiments. $*p < 0.05$ and $**p < 0.01$.

USA).

2.5. Cell viability assay

The miR-148a mimic and inhibitor were purchased from Invitrogen. The effects of miR-148a mimic and inhibitor on RAW264.7 cell viability were determined by the Methyl thiazolyl tetrazolium (MTT) assay (Thermo Fisher). Briefly, the RAW264.7 cells were transfected with miR-148a mimic (50 nM and 100 nM) or inhibitor (100 nM and 150 nM) using lipofectamine 3000 according to the manufacture instruction. At 24 h of post-transfection, MTT solution was added and incubated for 4 h. Precipitates were then dissolved with dimethyl sulfoxide and optical density was measured at 570 nm with a microplate reader.

2.6. Western blot analysis

The cellular total protein was obtained by a lysis solution containing the phosphatase repressor. The protein concentration was determined by BCA kit (Pierce). Then, samples with the same amount of protein were applied to 10% SDS-PAGE gel electrophoresis, followed by transferring to PVDF membrane. After being placed in the blocking buffer (5% skim milk), the membrane was incubated with corresponding primary antibodies overnight at 4 °C. Then, the membrane was incubated with secondary antibody for 1 h at room temperature. The protein expression was determined by Chemiluminescence System. β-actin was used as a loading control.

2.7. Colony-forming unit (CFU) assay

RAW264.7 cells were transiently transfected with miR-148a mimic

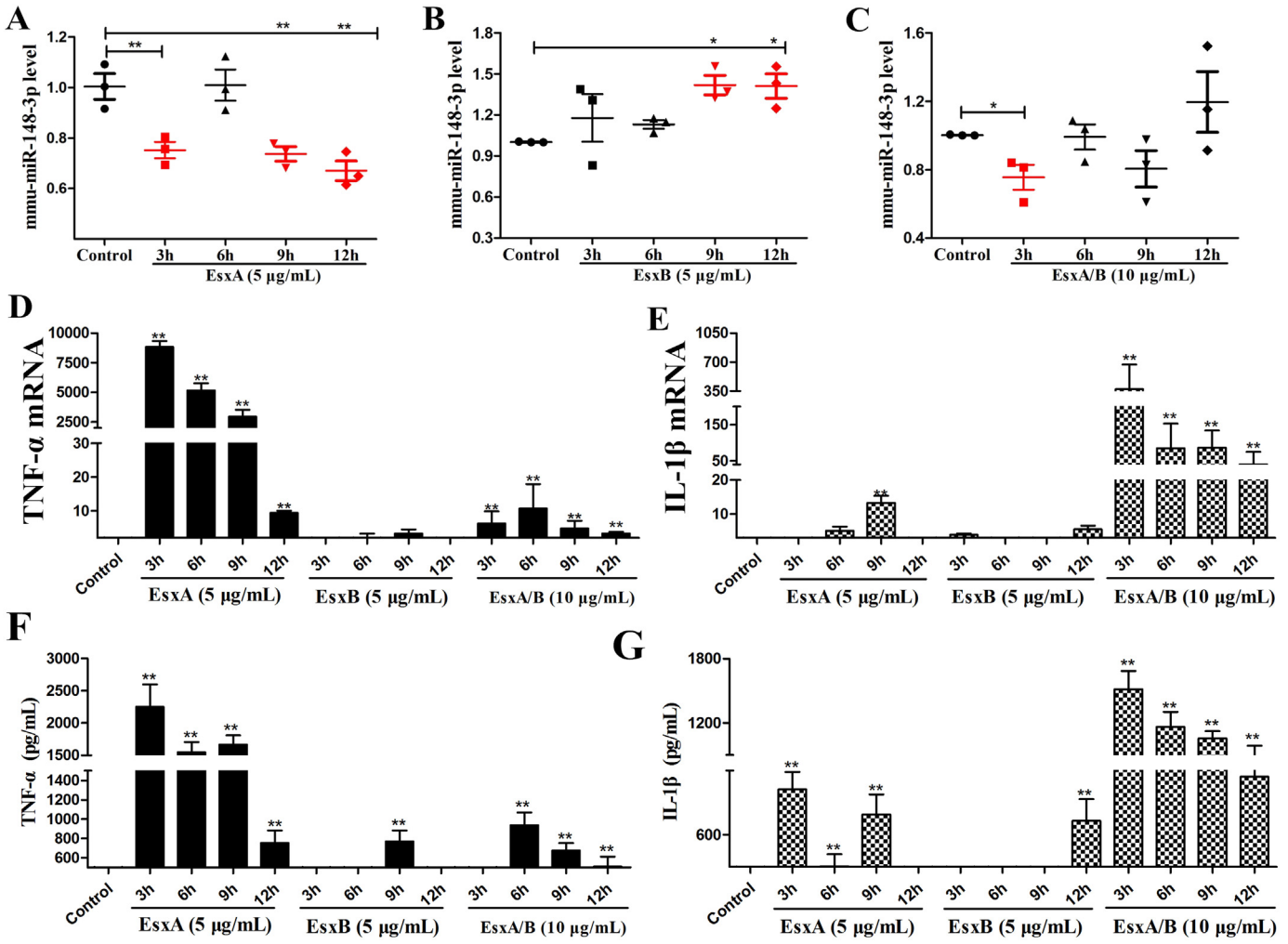

Fig. 2. The recombinant EsxA and EsxB proteins had differential effects on the expression of miR-148a and pro-inflammatory factors. A–C: RAW264.7 cells were incubated with 5 μg/mL of the purified proteins EsxA, EsxB and 10 μg/mL of EsxA/B heterodimer, respectively. At 3, 6, 9 and 12 h of incubation, the miR-148a expression was detected by qRT-PCR. The untreated cells were used as controls. D–E. The mRNA level of TNF-α and IL-1β was determined by qRT-PCR. F–G. The protein level of TNF-α and IL-1β was measured by ELISA. All the data above represent the mean ± S.E.M of three independent experiments. $^{*}p < 0.05$ and $^{**}p < 0.01$.

or inhibitor for 24 h as described above, which was followed by Mm infection at MOI of 10. At 6 h of post-infection, the infected cells were lysed with 0.05% Triton X-100 for 10 min. Ten-fold serial dilution method was used for quantitative culture of bacterial colonies. Aliquots of each dilution were plated on Middlebrook 7H9 medium supplemented with 10% OADC and incubated at 37 °C for one week. Finally, the colonies were counted and CFU was calculated using standard procedures.

2.8. Statistical analysis

Statistical data were represented as the mean ± S.E.M of three independent experiments. Data were analyzed using Student's *t*-test or one-way analysis of variance (ANOVA). *p-values* ≤ 0.05 were considered a statistically significant difference.

3. Results

3.1. Mm infection in macrophages down-regulated miR148a in an EsxA/B-dependent manner

Since miR-148a has shown strong immunomodulatory effects on bacterial infection [48–50], we tested how this miRNA responded to Mm infection in macrophages. RAW264.7 cells were infected with Mm at MOI of 5 and 10, respectively. At 6 and 12 h of post-infection, the expression of miR-148a was measured by qRT-PCR (Fig. 1A, B). The results show that miR-148a was apparently down-regulated by Mm infection, especially at 6 h post-infection with MOI of 10. Interestingly, infection with the knockout strain MmΔEsxA/B up-regulated miR-148a (Fig. 1C), suggesting that miR-148a responds to mycobacterial infection in an EsxA/B-dependent manner. Next, we tested the effects of mycobacterial infection on the pro-inflammatory response of macrophages. Since miR-148a has been shown to negatively regulate the pro-inflammatory factors (e.g. TNF-α and IL-1β), we predict that both TNF-α and IL-1β will be up-regulated by Mm infection in an EsxA/B-dependent manner. As expected, compared to MmΔEsxA/B, Mm infection significantly up-regulated TNF-α and IL-1β in both mRNA and protein levels (Fig. 1D, E). In fact, this result is consistent with the previous reports that Mtb and Mm induced potent TNF-α and IL-1β production in macrophages in a ESX-1-dependent manner [25–28]. We also notice the differential responses between TNF-α and IL-1β in terms of the responding times and strength (Fig. 1D, E), indicating that TNF-α responds earlier, while IL-1β has a delayed but stronger response.

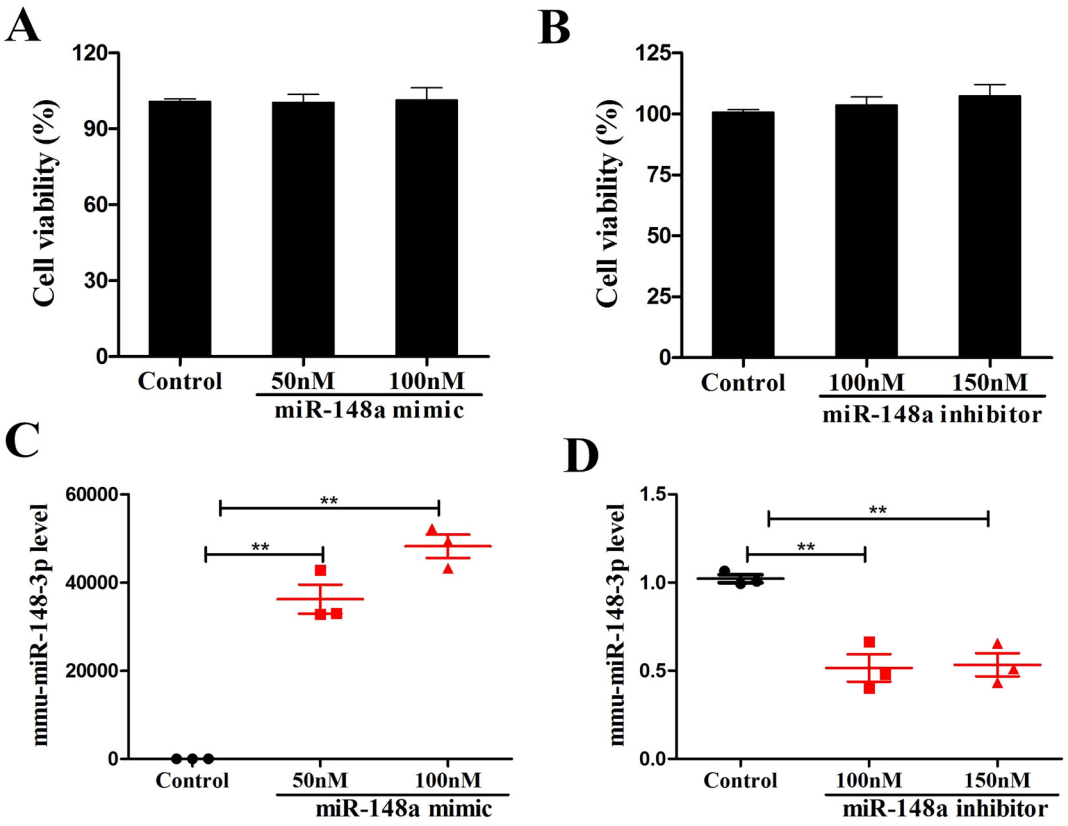

Fig. 3. The miR-148a mimic and inhibitor up- and down-regulated endogenous miR-148a expression, respectively. A-B. RAW264.7 cells were transiently transfected with miR-148a mimic (final concentration 50 and 100 nM) or inhibitor (final concentration 100 and 150 nM) for 24 h. The viability of the cells treated with miR-148a mimic or inhibitor was detected using the MTT assay. C–D. The expression of miR148a was measured by qRT-PCR. The cells with mock transfection were set as control. All the data above represent the mean ± S.E.M of three independent experiments.*$p < 0.05$ and **$p < 0.01$.

3.2. Effects of ESX-1 virulence factors on miR-148a expression in RAW264.7 cells

In order to test the effects of ESX-1 virulence factor on expression of miR-148a, RAW264.7 cells were treated with the recombinant proteins EsxA, EsxB and EsxA/B heterodimer, respectively. It is worth of mentioning that the proteins used in this study were expressed and purified from *Mycobacterium smegmatis* without using the detergent ASB-14, which was believed to cause artifactual cell lysis [51]. The expression of miR-148a was markedly reduced by EsxA protein treatment at 3, 9 and 12 h of post-treatment, while there was no significant change at 6 h of post-treatment (Fig. 2A). On the contrary, the expression of miR-148a was increased in the EsxB-treated macrophages, especially at 9 and 12 h of post-treatment (Fig. 2B). Interestingly, in the EsxA/B heterodimer-treated cells, miR-148a expression was significantly reduced at 3 h of post-treatment and then remained similar levels as the control (Fig. 2C). Consistent to the data of miR-148a expression, EsxA markedly up-regulated TNF-α expression both in mRNA and protein levels with 3 h of post-treatment reaching the highest levels, while EsxB failed to induce TNF-α expression (Fig. 2D, F). The EsxA/B heterodimer induced a moderate increase of TNF-α in both mRNA and protein levels with 6 h of post-treatment reaching the highest point (Fig. 2D, F). Surprisingly, however, IL-1β induction shows a different pattern than TNF-α. The EsxA/B heterodimer induced the strongest IL-1β expression both in mRNA and protein levels at 3 h of post-treatment, while EsxA only induced a modest level of increase in IL-1β expression, but it is still significantly higher than EsxB (Fig. 2E, G). The data suggest that EsxA prefers to stimulate TNF-α over IL-1β, while the heterodimer prefers to stimulate IL-1β over TNF-α, which is more closely related to the mycobacterial infection (Fig. 1D).

3.3. Modulating miR-148a expression using miRNA mimic and inhibitor

To modulate miR-148a expression, miR-148a mimic (50 nM and 100 nM, respectively) or inhibitor (100 nM and 150 nM, respectively) were transiently transfected into RAW264.7 cells. As expected, miR-148a mimic up-regulated the miR-148a expression in a dose-dependent manner with ~ 40,000–50,000 folds of increase (Fig. 3C), while miR-148a inhibitor down-regulated miR-148a expression by ~50% (Fig. 3D). At the indicated concentrations, transfection of miR-148a mimic and inhibitor did not affect cell viability (Fig. 3A, B).

Up-regulation of miR-148a down-regulated TNF-α and IL-1β and reduced mycobacterial intracellular survival.

RAW264.7 cells were transfected with miR-148a mimic for 24 h, followed by 6 h of exposure to Mm (MOI = 10), and the pro-inflammatory cytokines TNF-α and IL-1β were determined by qRT-PCR and ELISA. As expected, both TNF-α and IL-1β were down-regulated by miR-148a mimic, suggesting thatmiR-148a negatively regulates TNF-α and IL-1β (Fig. 4A–D). Next, we tested the effect of miR-148a on mycobacterial intracellular survival. As expected, mycobacterial intracellular survival was increased by miR-148a inhibitor and decreased by miR-148a mimic (Fig. 4E), suggesting that miR-148a plays a role in inhibiting mycobacterial intracellular survival.

3.4. Effects of miR-148a on the TLR4/NF-κB signaling pathway in Mm-infected macrophage

As one of the major receptors for pathogen associated molecular pattern, TLR4 is involved in innate immune response in many bacterial infections [52]. An earlier study has shown that EsxA inhibits the TLR4-mediated MAPK/NF-κB activation through the binding of the C-terminal 20 amino acids of EsxA to TLR2 in macrophages [24]. Thus, we

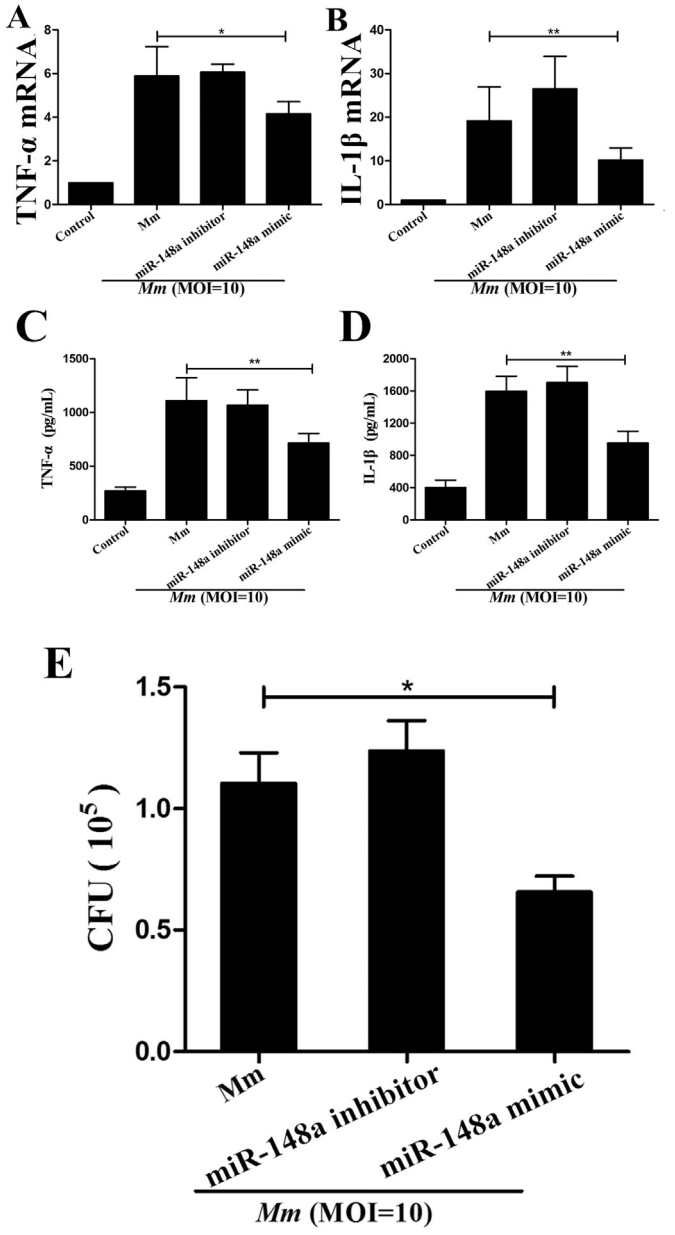

Fig. 4. Effects of miR-148a mimic and inhibitor on the expression of pro-inflammatory cytokines and mycobacterial survival in the *Mm*-infected cells. RAW264.7 cells were transfected with miR-148a mimic (50 nM) or miR-148a inhibitor (100 nM) for 24 h, which was followed by infection with Mm at 10 MOI for 6 h. A–B. The mRNA expression of TNF-α and IL-1β was detected by qRT-PCR. C–D. The protein expression of TNF-α and IL-1β was measured by ELISA. E. The Mm intracellular survival was determined by CFU assay. All the data above represent the mean ± S.E.M of three independent experiments. $^{*}p < 0.05$ and $^{**}p < 0.01$.

hypothesize that up- or down-regulation of miR-148a will affect the TLR4/NF-κB signaling pathway. The effects of miR-148a on the expressions of TLR4 in the Mm-infected RAW264.7 cells were detected by Western blot (Figs. 5 and 6). The results showed that Mm infection markedly increased the expression of TLR4, while over-expression of the miR-148a mimic inhibited it, indicating that miR-148a down-regulates TLR4 upon Mm infection.

Consistent to the results in Fig. 5, the down-stream factors of TLR4 in the NF-κB pathway, p65 and IκBα were activated upon Mm infection, as evidenced by the increase of phosphorylation, while over-expression of the miR-148a mimic down-regulated the phosphorylation of p65 and IκBα (Fig. 6).

4. Discussion

ESX-1/EsxA system has long been known as a modulator of immune responses in macrophages and other phagocytes, featured by inhibiting macrophage activation, activating inflammasome and inducing pro-inflammatory factors TNF-α and IL-1β, etc. [23–31]. However, the mechanism of EsxA-mediated immune response is not completely understood. In recent years, numerous miRNAs have been shown to play an important role in host immune responses to Mtb infection [34–36,53–55]. To date, two miRNAs, miR-155 and miR-1et7f, have been reported to respond to mycobacterial infection in an EsxA-dependent manner with miR-155 being up-regulated and miR-let7 being down-regulated [39,40]. Here, we report a new miRNA, miR-148a, which is down-regulated in mycobacterial infection in an EsxA-dependent manner. Up-regulation of this miRNA inhibits production of TNF-α and IL-1β, mycobacterial intracellular survival and TLR4/NF-κB signaling pathway, suggesting that miR-148a is a negative regulator of proinflammatory response in macrophages during mycobacterial infection. Similarly, an earlier study has shown that the virulent chlamydial strain, but not attenuated strain, significantly down-regulated miR-148a, resulting in up-regulation of pro-inflammatory cytokines, such as TNF-α and IL-1β, leading to pathology [44], indicating that miR-148 may be a common negative regulator of inflammatory responses for intracellular bacterial pathogens.

Production of pro-inflammation cytokines by macrophages is believed to be a common response to bacterial infection, which is usually regarded as a defense mechanism of controlling the bacterial infection. But inflammation, especially overly activated inflammation, disrupts cellular signaling and causes extensive tissue damages, which becomes the niches for pathogen invasion and proliferation. Recent studies have shown that the pro-inflammatory cytokines are double-edged sword in tuberculosis [56]. The inflammatory responses are regulated by both the host and the mycobacterium during different stages of infection, resulting in either inhibiting or promoting mycobacterial growth [57–60]. Therefore, a homeostatic state of pro- and anti-inflammation interplayed by both the host and mycobacterium is the key for the outcome of infection [56,61]. As discussed above, a collection of previous studies [23–31] and the present study suggest the virulence factor ESX-1/EsxA overly activates pro-inflammatory cytokines (e.g. TNF-α and IL-1β) to generate a niches favorable for mycobacterial growth. Thus, down-regulation of the pro-inflammatory cytokines through up-regulation of MiR-148a inhibits mycobacterial intracellular survival (Fig. 4).

The role of the recombinant EsxA protein in stimulating immune responses in macrophages and other cell types has been well documented [29–31]. However, a recent publication has brought up a concern that the contamination of ASB-14, a cytolytic detergent used in EsxA purification, may cause artifacts in the study of EsxA-induced immune responses [51]. Therefore, in the present study we purified the detergent-free EsxA, EsxB and the EsxA/B heterodimer from *Mycobacterium smegmatis* and used them to challenge the macrophages. The proteins expressed in *M. smegmatis* carry mycobacteria-specific post-translational modifications (e.g. N-α-acetylation of EsxA), which are more physiological relevant than the proteins produced in *E. coli*. Interestingly, EsxA, EsxB and the heterodimer show different specificity in stimulation of TNF-α and IL-1β (Fig. 2D-G). Overall, EsxB is apparently inactive in stimulation of immune response, while both EsxA and the heterodimer induced a strong response. EsxA has a preference for TNF-α over IL-1β, while the heterodimer has a preference for IL-1β over TNF-α, which is consistent to the results of Mm infection (Fig. 1D–E), suggesting that the heterodimer is more closely relevant to mycobacterial infection. It was for the first time that we tested the effects of the EsxA/B heterodimer purified from *M. smegmatis* on the immune responses in macrophages.

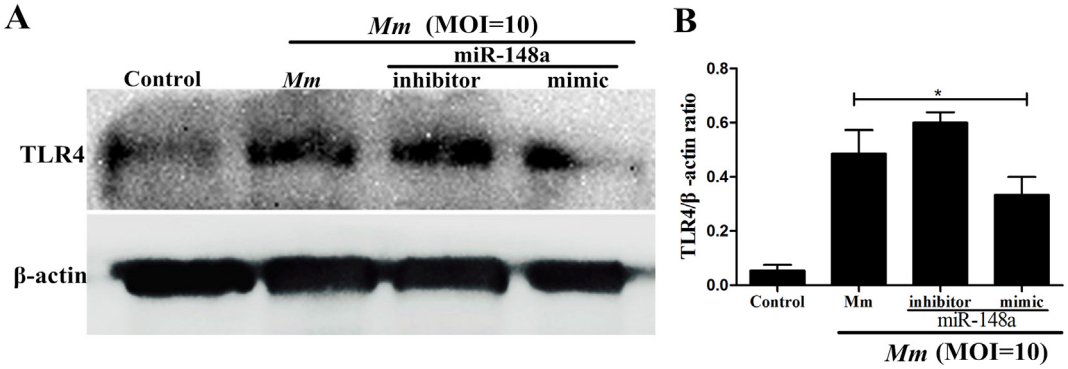

Fig. 5. miR-148a negatively regulated TLR4 expression in the *Mm*-infected cells. RAW264.7 cells were transfected with miR-148a mimic (50 nM) or miR-148a inhibitor (100 nM) for 24 h, which was followed by infection with Mm at 10 MOI for 6 h. The expression of TLR4 was detected by Western blot and shown in A. β-actin was used as an internal control. The relative expression was calculated and shown in B. All the data above represent the mean ± S.E.M of three independent experiments. $*p < 0.05$ and $**p < 0.01$.

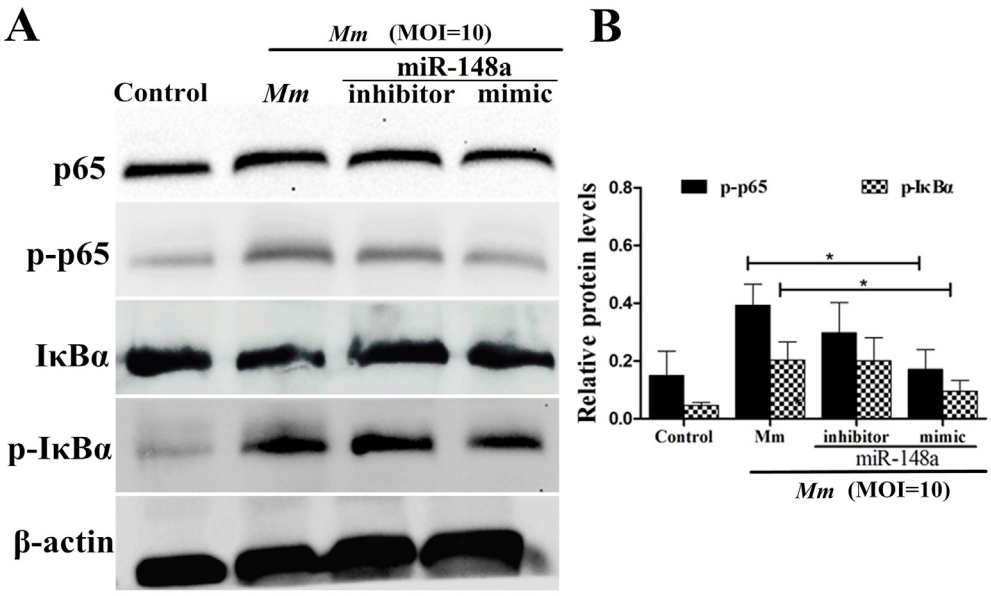

Fig. 6. Effects of miR-148a on the NF-κB activation in the *Mm*-infected cells. RAW264.7 cells were transfected with miR-148a mimic (50 nM) or miR-148a inhibitor (100 nM) for 24 h, which was followed by infection with Mm at 10 MOI for 6 h. The expression of p65, p-p65, IκBα and p-IκBα was detected by Western blot and shown in A. β-actin was used as an internal control. Relative expression of p-p65 and p-IκBα was calculated and shown in B. All the data above represent the mean ± S.E.M of three independent experiments. $*p < 0.05$ and $**p < 0.01$.

In conclusion, the present study has found that Mm infection down-regulates miR-148a in an EsxA-dependent manner. Up-regulation of miR-148a suppresses the expression of IL-1β and TNF-α, inhibits the Mm intracellular survival as well as TLR4-mediated NF-κB activation. The study provides a novel venue for the EsxA-mediated immune response and may benefit future development of new therapies against tuberculosis.

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The content is solely the responsibilty of the authors and does not necessarily represent the official views of the National Institutes of Health.

Conflicts of interests

The authors declare that they have no conflicts of interest.

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