

# Changed immune and miRNA response in RAW264.7 cells infected with cell wall deficient mycobacterium tuberculosis

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**Abstract.** Cell wall deficient (CWD) forms of *Mycobacterium tuberculosis* (Mtb) confers a marked resistance to immune system of the host. However, there is limit data on the effect of intracellular CWD-Mtb infection on macrophages. In the study, effects of CWD-Mtb on cell viability, cytokine response and miRNA expression of macrophages were analyzed. Cell viability was reduced, levels of interleukin-1 $\alpha$  (IL-1 $\alpha$ ), IL-1 $\beta$ , IL-6, IL-10 and interferon- $\gamma$  (IFN- $\gamma$ ) were also significantly changed after infection of RAW264.7 cells with CWD-Mtb. A total of 105 miRNAs were deregulated between CWD-Mtb and wild Mtb group, and among them, miR-29b was upregulated in CWD-Mtb group. Downregulation of miR-29b resulted in significant elevation level of IFN- $\gamma$  mRNA. Involved signaling pathways of potential target genes of differentially expressed miRNAs mainly focused on T cell receptor signaling pathway, MAPK signaling pathway, neurotrophin signaling pathway, and regulation of actin cytoskeleton. Taken together, the results showed that cytokine production of CWD-Mtb infected macrophages was altered and many miRNAs were involved in regulation of macrophage response to CWD-Mtb infection, which probably determined the differential outcome following different phenotype Mtb infection. These findings open up a new and interesting avenue for an improved understanding of pathogenesis of CWD-Mtb.

## Introduction

Cell wall deficient (CWD or L) forms of *Mycobacterium tuberculosis* (Mtb), characterized by loss of cell wall components, are usually considered as a product of interaction between wild Mtb and defence system of host. As a result, CWD-forms of Mtb confers a marked resistance of mycobacteria to immune system of host and antibiotics (1). CWD-forms of Mtb have been found in specimens from 42% patients with freshly or previously diagnosed pulmonary TB, and of all bacteria, CWD-forms of Mtb predominate *in vivo* (2). CWD-forms of Mtb are responsible for chronic and persistent tuberculosis (TB) infection that constitutes one of the major hurdles in control of TB (3,4). Unfortunately, over the last several decades, investigations on CWD-Mtb have been ignored and overlooked. Recently, interest in CWD-Mtb has been reborn due to its significant role in chronic and persistent TB disease. Despite long history of TB research, underlying pathogenic mechanism by which CWD-forms of Mtb counteract the bactericidal activities and survive in host cells still remains obscure. Macrophages, the main targets of mycobacterial infection, play an important role in the pathogenesis of Mtb infection. Different Mtb strains could induce macrophage to produce distinct responses (5,6). Many studies have explored the interaction between macrophages and Mtb with cell wall (wild Mtb) (7-9). However, there is limited data on the effect of intracellular CWD-Mtb infection on macrophages.

MicroRNAs (miRNAs) are crucial biological regulators that bind complementarily to 3'-untranslated regions (3'-UTRs) of target mRNAs leading to mRNA cleavage or translational inhibition (10). The emerging roles of miRNAs in regulating immune responses have attracted increasing attention in recent years and miRNAs play a major role in the prognosis of infectious diseases (11,12). Many studies have shown that regulation of specific miRNAs during mycobacterial infection can either enhance immune response or facilitate pathogen immune evasion. For instance, miRNA-21 could impair anti-mycobacterial responses by targeting interleukin-12 (IL-12) and Bcl-2 (13); miR-124 played a negative regulatory role in fine-tuning inflammatory response in macrophages upon mycobacterial infection (14); miR-223 was critical for the control of TB by regulating leukocyte chemotaxis (15); miR-155 could

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enhance survival of Mtb in macrophages (16). These results suggest that miRNAs play important roles in TB infectious disease. However, the immunoregulatory role of miRNAs in CWD-Mtb infection is still poorly understood.

The present study was carried out to determine the effects of intracellular CWD-Mtb infection on cell viability and immune response of macrophages, and further elucidate the potential role of miRNA in macrophage response to CWD-Mtb infection.

## Materials and methods

**Preparation of CWD-Mtb.** Mtb strain H37Rv (wild Mtb with cell wall) was grown in Middlebrook 7H9 broth supplemented with 10% OADC enrichment (BD Biosciences, San Jose, CA, USA), 0.5% glycerol and 0.05% Tween-80. Wild Mtb was used to prepare CWD-forms of Mtb by nutrient starvation method as described by Markova *et al* (1). Mid-log phase suspension of H37Rv culture was pelleted, resuspended in DMEM medium plus 10% fetal bovine serum (FBS) and HEPES, vortexed for 2 min and sonicated for 5 min. Following sonication, bacteria were dispersed by aspiration with needle. Dispersed bacterial suspension was then allowed to stand in a vertical position for 5 min and top portion of suspension was used for subsequent infection experiment. CWD-Mtb was examined and confirmed by Ziehl-Neelsen staining and electron microscopy.

**Cell culture and infection.** One day prior to infection, RAW264.7 cells were cultured in DMEM medium supplemented with 10% heat-inactivated FBS (complete medium) under standard conditions (37°C and 5% CO<sub>2</sub> humidity). Twenty-four hours later, cells at ~80% confluence were infected with either CWD-Mtb or wild Mtb at a multiplicity of infection (MOI) of 10 bacteria/cell. At 4 h post-infection, cells were carefully washed 3 times with fresh DMEM medium to remove extracellular bacteria and further cultured in pre-warmed complete medium for subsequent analysis.

**Methyl thiazolyl tetrazolium (MTT) assay and intracellular bacterial growth.** RAW264.7 cells were infected with either CWD-Mtb or wild Mtb as mentioned above. Effect of intracellular Mtb infection on cell viability was determined by MTT assay. Briefly, culture medium was discarded at 4, 12 or 24 h post infection and cells were then incubated with MTT solution for 2 h. Precipitates were then dissolved with dimethyl sulfoxide and optical density (OD) at 570 nm was measured using a microplate reader. Data were collected in triplicate for each sample. Infected cells from at least three parallel wells were harvested and lysed by adding 1% Triton X-100 at 24 h time-point. Cell lysates were serially diluted and seeded on Middlebrook 7H10 solid plates for wild Mtb growth and Middlebrook 7H9 semisolid plates (both from BD Difco, Franklin Lakes, NJ, USA) for CWD-Mtb growth in, at least, duplicates. Colony forming units (CFUs) were enumerated after 30 days for wild Mtb and 5 days for CWD-Mtb, respectively. Three separate biological replicates were performed.

**Cytokine analysis.** Culture supernatants were collected from either wild Mtb or CWD-Mtb infected cells at 24 h post-infection and immediately stored in liquid nitrogen. Levels of

IL-1 $\alpha$ , IL-1 $\beta$ , IL-6, IL-10, TNF- $\alpha$  and interferon- $\gamma$  (IFN- $\gamma$ ) in culture supernatants were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits (BioSource, Nivelles, Belgium) according to the manufacturer's instructions. For each group, three separate biological replicates were performed. All culture supernatants were measured in triplicate (technical replicates) and results from technical replicates were combined.

**miRNA expression and data processing.** Cells were harvested at 24 h post-infection and stored in liquid nitrogen until RNA was extracted. Total RNA was isolated from harvested cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and further purified with a miRNeasy mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. RNA quantity and quality were assessed using a NanoDrop Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and electrophoresis. After having passed RNA quantity and quality measurement, each sample was labeled using miRCURY™ Hy3™ Power labeling kit and hybridized on mouse miRCURY™ LNA array (v.16.0) (Exiqon, Vedbaek, Denmark) according to the manufacturer's protocol. Three arrays were used for each group. Following hybridization, microarray slides were washed and scanned using an Axon GenePix 4000B microarray scanner (Axon Instruments, Foster City, CA, USA). GenePix Pro 6.0 software (Axon Instruments) was used to analyze acquired array images. After quantile normalization of raw data, fold change filtering method was used to select miRNA with at least a 2-fold change for further statistical analysis. U6 (U6 small nuclear RNA) was used as an internal control. Statistical significance was determined by false discovery rate (FDR) <0.05 to identify differentially expressed miRNAs. Hierarchical clustering was performed based on Pearson's correlation for differentially expressed miRNAs.

**RT-qPCR analysis.** Some deregulated miRNAs were randomly selected from among those showing the greatest fold changes for confirmation by real-time reverse transcription quantitative PCR (RT-qPCR). Total RNA (200 ng for each sample) was used for the synthesis of first stranded cDNA in 20  $\mu$ l RT reaction containing 15 nM miRNA-specific primers (available on request), 1X RT buffer, 0.25 mM each of dNTPs, 2 U/ $\mu$ l reverse transcriptase and 0.6 U/ $\mu$ l RNase inhibitor. Obtained 2.5 ng cDNA for each sample was then used for PCR analysis using a GeneAmp PCR system 9700 (Applied Biosystems, Perkin-Elmer, Foster City, CA, USA) in 10  $\mu$ l reaction including 1X master mix and 0.5  $\mu$ M each primer. Normalized, relative gene expression was calculated using standard 2<sup>- $\Delta\Delta$ Ct</sup> method. Data are presented as fold change in gene expression normalized to level of internal control U6. Each qPCR reaction was run three independent times, with technical triplicates in each reaction.

**Correlation between miR-29b level and IFN- $\gamma$  expression in CWD-Mtb infected RAW264.7 cells.** miR-29b mimics, miR-29b inhibitors and nonsense RNA controls were purchased from GenePharma (Shanghai, China). Briefly, RAW264.7 cells were seeded in triplicate in 24-well plates at a concentration of 1x10<sup>5</sup> cells/well in 100  $\mu$ l complete medium.

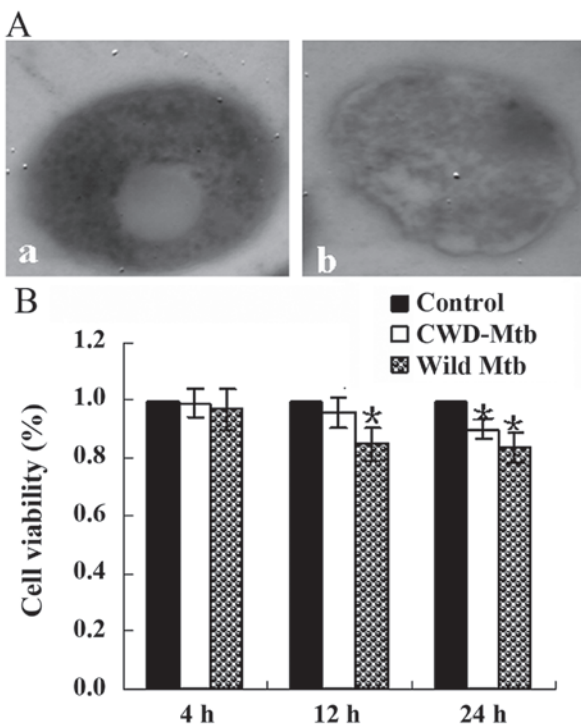


Figure 1. Electron microscopy images and methyl thiazolyl tetrazolium (MTT) assay. (A) Transmission electron microscopy of ultrathin sections of *Mycobacterium tuberculosis* (Mtb) H37Rv with (a) cell wall and (b) cell wall deficient (CWD)-Mtb. CWD-Mtb was covered only by a 'unit' cytoplasmic membrane. Bars, 400.00 nm. (B) Measurement of cell viability of either CWD-Mtb or wild Mtb infected as well as non-infected RAW264.7 cells using MTT assay. Results are shown as means  $\pm$  SD of 3 individual experiments. \* $P < 0.05$  vs. non-infected control.

At 4 h post-infection with CWD-Mtb, culture medium was removed and cells were then transiently transfected with miR-29b mimics, miR-29b inhibitors or nonsense RNA controls in equimolar amounts with HiPerFect transfection reagent (Qiagen) according to the manufacturer's protocols. Cells transfected with only transfection reagent were used as mock control. Culture medium was replaced with fresh complete DMEM medium at 6 h post-transfection and cells were then harvested after transfection for 24 h. RT-qPCR was used to determine IFN- $\gamma$  mRNA expression. Each experiment was repeated three separate times.

**Kyoto encyclopedia of genes and genomes pathway analysis based on miRNA expression profile.** Target genes of deregulated miRNAs were predicted by TargetScan (<http://www.targetscan.org/>) and pathway analysis was used to find significant pathways of differential genes according to Kyoto encyclopedia of genes and genomes (KEGG).

**Statistical analysis.** Data are presented as means  $\pm$  standard deviation (SD) of three independent experiments. One-way ANOVA analysis or t-test was used for statistical analysis.  $P < 0.05$  was considered statistically significant.

## Results

**Cell viability and intracellular bacterial growth.** CWD-forms of Mtb were induced from wild Mtb by nutrient starvation

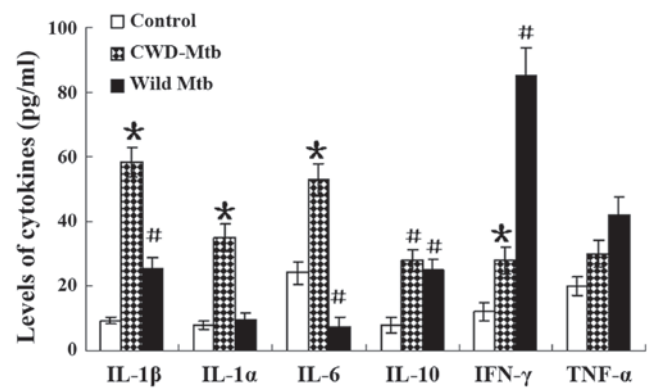
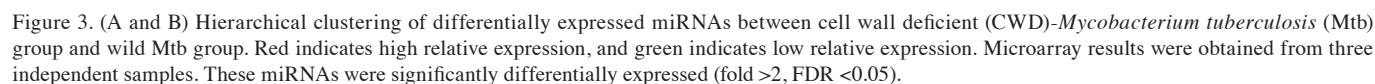


Figure 2. Determination of cytokines production with enzyme-linked immunosorbent assay (ELISA). Culture supernatants of RAW264.7 cells were harvested after 24 h post-infection. Levels of cytokines in culture supernatants were determined by ELISA. Data were presented as means  $\pm$  SD from three independent experiments. \* $P < 0.05$  compared with wild *Mycobacterium tuberculosis* (Mtb) group as well as non-infected control; # $P < 0.05$  compared with non-infected control.

stress, which showed non-acid-fast staining under light microscopy and was bound only by a single unit membrane under electron microscopy (Fig. 1A). Effect of CWD-Mtb on cell viability during intracellular infection of macrophage was detected by MTT assay. As shown in Fig. 1B, compared with non-infected cell controls, cell viability of wild Mtb infected cells was significantly reduced at 12 and 24 h post-infection, while cell viability of CWD-Mtb infected cells was obviously decreased only at 24 h post-infection. However, there was no significant difference between wild Mtb group and CWD-Mtb group at 12 or 24 h post-infection. Data also showed that there was no significant difference in intracellular bacterial growth between CWD-Mtb group and normal Mtb group at 24 h post-infection (data not shown).

**Measurement of cytokine production.** Cytokines have a critical role in regulating innate and adaptive immunity and changes in the pattern of cytokine production clearly contribute to TB progression (17,18). Different mycobacteria strains could induce macrophages to produce distinct pattern of cytokines (19). To study cytokine response of macrophages to intracellular CWD-Mtb infection, levels of IL-1 $\alpha$ , IL-1 $\beta$ , IL-6, IL-10, TNF- $\alpha$  and IFN- $\gamma$  in supernatants of infected cells were evaluated by ELISA. Levels of IL-1 $\alpha$ , IL-1 $\beta$ , IL-6 and IFN- $\gamma$  were changed in CWD-Mtb group compared with wild Mtb group as well as non-infected control; IL-10 was only altered in CWD-Mtb group compared with wild Mtb group. However, there was no obvious difference for TNF- $\alpha$  level between the three groups (Fig. 2).

**miRNA expression.** Roles of miRNA have been highlighted in TB infection recently (13-16). To investigate whether miRNAs were involved in the response of macrophages to intracellular CWD-Mtb infection, total RNA was isolated from infected cells and miRNA expression was determined. Compared to control, downregulated 43 miRNAs and upregulated 28 miRNAs were observed in both CWD-Mtb group and wild Mtb group, simultaneously (Fig. 3A). A total of 105 miRNAs were deregulated between CWD-Mtb group and wild Mtb





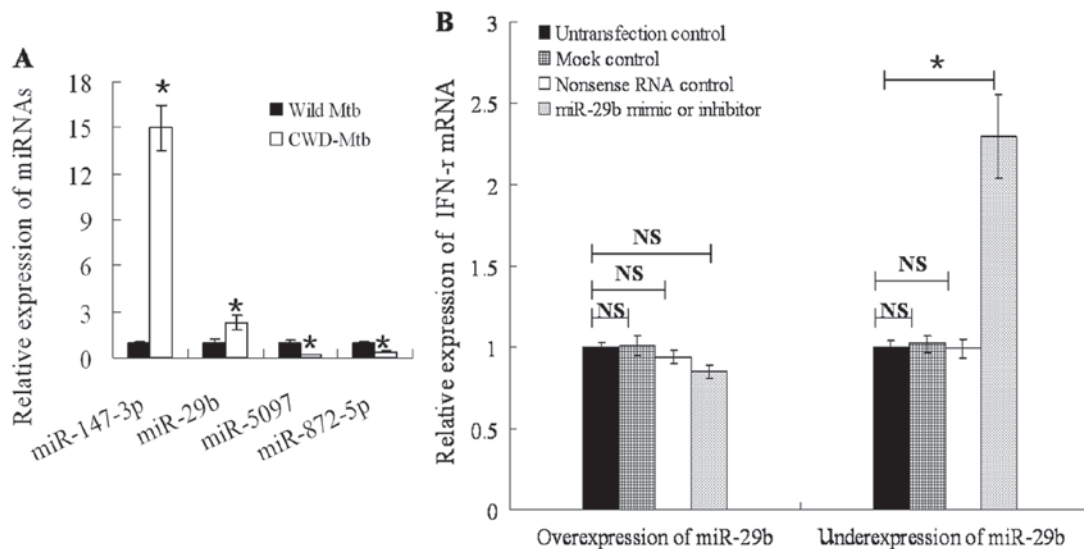


Figure 4. RT-qPCR analysis. (A) Validation of microarray data. Four miRNAs were randomly selected to confirm the microarray data. After normalization to U6 level, data are presented as mean  $\pm$  SD and obtained average value for each miRNA was used for statistics. \* $P < 0.05$  compared with wild *Mycobacterium tuberculosis* (Mtb) group. (B) Effects of regulation of miR-29b expression on interferon- $\gamma$  (IFN- $\gamma$ ) mRNA level in RAW264.7 cells. Downregulation of miR-29b significantly increased IFN- $\gamma$  mRNA level. Upregulation of miR-29b failed to obviously reduce expression of IFN- $\gamma$  mRNA, but there was a trend toward decrease in its level. All experiments were performed in triplicate. \* $P < 0.05$  was considered statistically significant. NS, no significance.

group, with 63 miRNAs increased and 42 miRNAs decreased in CWD-Mtb group compared with wild Mtb group (Fig. 3B).

**Validation of microarray results by RT-qPCR.** Four deregulated miRNAs, including miR-468-3p, miR-23a-5p, miR-340-3p and miR-29b, were randomly selected to validate the correctness and confidence of microarray data. Results showed that miR-147-3p and miR-29b were increased, while miR-5097 and miR-872-5p were decreased in CWD-Mtb group in comparison with wild Mtb group (Fig. 4A). The data were consistent with the microarray results.

**Effects of modulating miR-29b level on IFN- $\gamma$  mRNA expression.** Growing literature shows that cellular miRNAs play important roles in host-pathogen interaction by regulating cytokine production (20-22). It has been reported that IFN- $\gamma$  is a direct target of miR-29b (23,24). Our data showed that miR-29b was increased in CWD-Mtb infected RAW264.7 cells with a concomitant decrease in IFN- $\gamma$  level. To further validate the relationship between miR-29b and IFN- $\gamma$  during intracellular CWD-Mtb infection, we studied the effects of regulation of miR-29b expression on IFN- $\gamma$  level in CWD-Mtb infected RAW264.7 cells. Upregulation or downregulation of miR-29b was achieved by transfection of CWD-Mtb infected RAW264.7 cells with miR-29b mimics or miR-29b inhibitors, respectively. Downregulation of miR-29b resulted in significant increase in IFN- $\gamma$  mRNA. Overexpression of miR-29b failed to lead to obvious decrease in IFN- $\gamma$  mRNA, but there was a trend toward reduction (Fig. 4B).

**KEGG pathway analysis of deregulated miRNAs.** To identify the most significant candidates and investigate cellular function, involved signaling pathways of potential target genes of deregulated miRNAs were analyzed. Results showed that a wide variety of cellular processes were featured significantly in signaling pathways. Potential targets of overexpressed

miRNAs in CWD-Mtb vs. wild Mtb group were mainly involved in T cell receptor signaling pathway, pathways in cancer, ABC transporters, bacterial invasion of epithelial cells, while potential targets of downregulated miRNAs in CWD-Mtb vs. wild Mtb group were mainly involved in pathways in cancer, MAPK signaling pathway, neurotrophin signaling pathway, regulation of actin cytoskeleton, long-term potentiation (Fig. 5).

## Discussion

There are many unsolved questions of CWD-forms of Mtb concerning their regulatory mechanisms leading to their survival (25). This study demonstrated that, cell viability was significantly reduced at 12 and 24 h post wild Mtb infection, by contrast, it was obviously decreased only at 24 h post CWD-Mtb infection, which indicated that CWD-Mtb exerted less inhibitory effect on cell viability at earlier stage than wild Mtb. Macrophage can secrete pro-inflammatory cytokines, such as TNF- $\alpha$  and IFN- $\gamma$ , in order to mediate control of infection with Mtb. Different Mtb strains can induce macrophages to produce different pattern of cytokines (26). We determined the effect of intracellular CWD-Mtb infection on cytokine production in macrophages. The standards for selecting IL-1 $\alpha$ , IL-1 $\beta$ , IL-6, IL-10, TNF- $\alpha$  and IFN- $\gamma$  in the study was that the four cytokines play important roles in TB infection. IL-1 $\alpha$ , IL-1 $\beta$ , IL-6 and IL-10 lead to macrophage deactivation (27-30), whereas TNF- $\alpha$  and IFN- $\gamma$  are the central mediators of macrophage activation and inhibit intracellular Mtb growth in macrophages (31,32). Our results showed that levels of IL-1 $\beta$  as well as IL-6 were increased, and IFN- $\gamma$  was decreased, while TNF- $\alpha$  was not altered in CWD-Mtb group compared with those in wild Mtb group.

In general, intricate regulation of various eukaryotic/host genes is crucial in the development of infectious diseases (33,34). Based on the critical role in regulating innate and adaptive

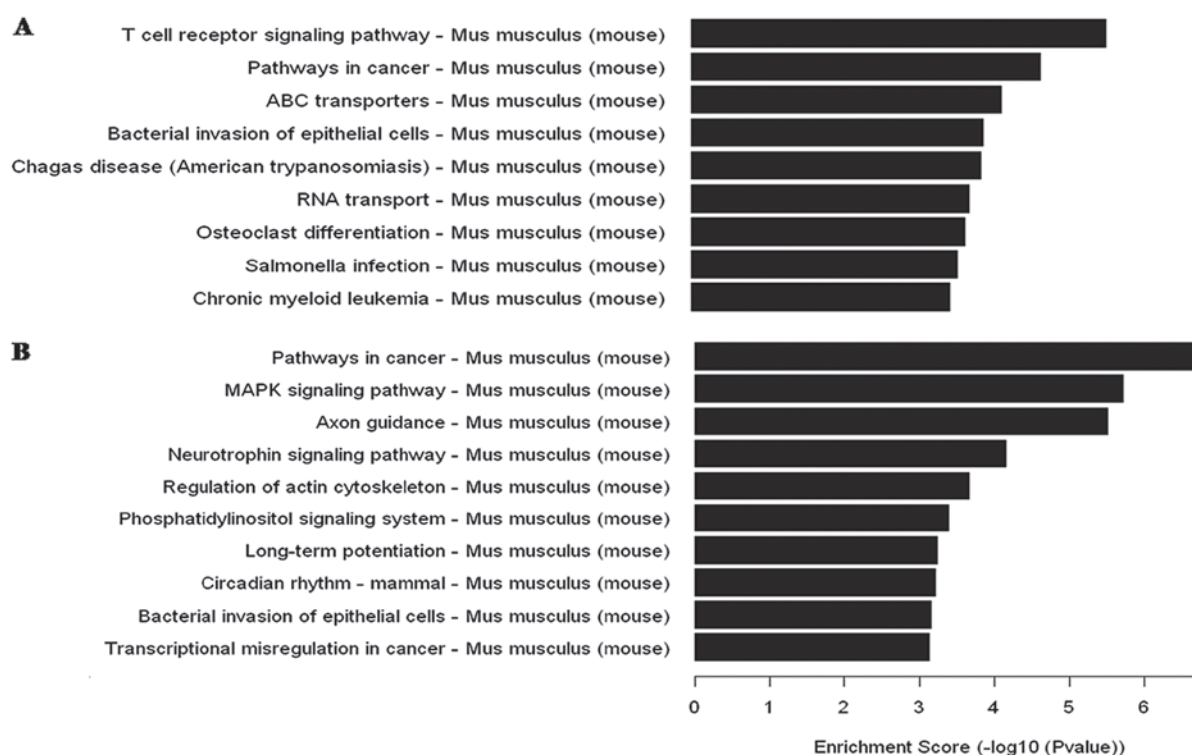


Figure 5. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis based on (A) upregulated miRNAs and (B) downregulated miRNA targeted genes in cell wall deficient (CWD)-Mtb group vs. wild Mtb group. Enrichment Score value equals  $-\log_{10}$  (P-value);  $P < 0.05$  was considered statistically significant.

immunity, we investigated the effects of intracellular CWD-Mtb infection on miRNA expression in macrophages. Our data showed that miR-147-3p (increased 69-fold) was the most over-expressed miRNA, miR-5097 (decreased 10-fold) was the most downregulated miRNA in CWD-Mtb group compared with wild Mtb group. Among 105 differentially expressed miRNAs, many miRNAs have been found to be associated with diseases: miR-147-3p was deregulated in virus infection (35), miR-5097 was altered in renal cell carcinoma (36), miR-183-5p was involved in ovarian cancer development (37), miR-468-3p was found in association with gastric cancer (38), miR-338-3p participated in the regulation of apoptosis-associated tyrosine kinases and miR-93-5p was deregulated in head and neck cancer (39,40). Levels of miRNAs mentioned above were also altered in our study. Previously, the roles of these changed miRNAs in TB infection were not clear, which suggested an additional model of action for these deregulated miRNAs. Several deregulated miRNAs in the study have been reported with involvement in TB infection: miR-191-5p was one of latent TB infection-related miRNAs in macrophages (41); miR-7 and miR-7-5p were upregulated in active TB disease (15,42); miR-720 was increased in latent TB infection group compared with healthy controls. In the study, miR-7b-3p was overexpressed, while miR-191-5p and miR-720 were downregulated in CWD-Mtb group compared with those in wild Mtb group. The data suggested that the above miRNAs may be involved in Mtb-macrophage interactions.

miRNAs play important roles in host-pathogen interaction by regulating cytokine production. Many studies showed that let-7 family members, such as let-7i, let-7g, let-7b, let-7c, and let-7d were deregulated in TB disease (14,15,43). It was reported that let-7 mediated regulation of IL-10 by directly targeting IL-10 (20,43). In the study, compared to control and wild

Mtb group, many let-7 members and IL-10 were deregulated in CWD-Mtb group, which indicated that let-7 members may play important roles in Mtb infection. Our data showed that miR-29b was increased in CWD-Mtb infected cells compared with control as well as wild Mtb infected cells. It was reported that miR-29 was increased in serum, sputum and CD4<sup>+</sup> T cells from active TB patients (14,15,44), which suggested that miR-29 plays important roles in TB infection. It has been demonstrated that IFN- $\gamma$  is associated with latent TB infection and is a direct target of miR-29b (23,24,45,46). Our data showed that an increased miR-29b level was concomitant with a decreased IFN- $\gamma$  expression in CWD-Mtb group compared with wild Mtb group. To investigate the roles of miR-29b in CWD-Mtb infection, we detected the effects of regulation of miR-29b level on IFN- $\gamma$  expression during intracellular CWD-Mtb infection. Results showed that downregulation of miR-29b resulted in obvious increase in IFN- $\gamma$  level and upregulation of miR-29b failed to decrease IFN- $\gamma$  level, whereas there was a strong trend toward decrease. These data showed that intracellular CWD-Mtb infection could induce distinct immune response by miRNA-mediated gene regulation, which may confer CWD-Mtb an important survival advantage.

Functions of most differentially expressed miRNAs are still largely unknown, such as miR-669e-5p, miR-1839-3p, miR-3068-5p, miR-467e-5p and miR-674-3p, but, we can be sure that miRNAs play important roles in the response of macrophages to intracellular CWD-Mtb infection. Based on reported and predicted target genes of the deregulated miRNAs, KEGG pathway analysis was applied to identify enriched pathways involved in intracellular CWD-Mtb infection. Results showed that many signaling pathways were involved in response to CWD-Mtb infection and T cell receptor signaling pathway,

MAPK signaling pathway, neurotrophin signaling pathway, regulation of actin cytoskeleton were abundant among the significantly enriched ones.

In conclusion, levels of some cytokines and miRNAs were deregulated in CWD-Mtb infected cells, and intracellular CWD-Mtb infection could elicit different immune response of macrophages by miRNA-mediated gene regulation, which probably determined the differential outcome following different phenotype Mtb infection. These findings open up a new and interesting avenue for an improved understanding of pathogenesis of CWD-Mtb. However, it is difficult to synthesize the results to reach a definitive conclusion based on this single study. Further studies are required to confirm our present findings.

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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

ZY and YF participated in bacteria culture, cell infection, microarray and bioinformatics analysis. ZY and KG participated in PCR, modulating analysis and ELISA. RL performed total RNA preparation, quality check and modulating analysis. YF conceived of the study and drafted the manuscript. All authors read and approved the final manuscript.

### Ethics approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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