

Original Research

Down-regulation of miR-144 after *Mycobacterium tuberculosis* infection promotes inflammatory factor secretion from macrophages through the Tpl2/ERK pathway

H-Y. Liu*

Fourth Department of Internal Medicine, Tuberculosis control and Prevention Hospital of Shaanxi Province, Taiyi palace town, Xi'an 710100, Shaanxi, China

Abstract: Pulmonary tuberculosis is one of the deadliest human diseases and mainly occurs when the immune system is impaired. MicroRNAs (miRNAs) have a critical role in regulating innate and adaptive immunity. Based on previous reports that *Mycobacterium tuberculosis* (M.tb) can modulate host cell miRNA expression, this study aimed to investigate expression changes in miR-144 and miR-144 regulate macrophage function via targeting of tumor progression locus 2 (Tpl2, also named MAP3K8) and extracellular signal-regulated kinase (ERK) signaling. I examined the miRNA expression profile of M.tb-infected monocyte-derived macrophages (MDMs) by gene expression profiling and quantitative real-time PCR (qRT-PCR). miR-144 is obviously down-regulated in MDMs infected with M.tb and directly binds to the 3'-UTR of Tpl2, acting as a negative regulator. Moreover, inhibiting miR-144 or over-expression of Tpl2 can activate the ERK signaling pathway by inducing ERK1/2 phosphorylation. At the same time, TNF- α , IL-1 β and IL-6 secretion were significantly accelerated. Taken together, these results suggest that miR-144 is expressed at a low level in M.tb-infected MDMs and acts as a negative regulator for Tpl2 target, which is closely connected with ERK signaling that regulates inflammatory factor secretion.

Key words: *Mycobacterium tuberculosis* (M.tb), monocyte-derived macrophages (MDMs), miR-144, Tpl2 (MAP3K8), ERK.

Introduction

Pulmonary tuberculosis, caused by M.tb infection, is one of the most common chronic infectious diseases in the world. The world health organization (WHO) has reported that one third of people all over the world are infected with M.tb (1, 2). M.tb is the causative agent of tuberculosis and can survive and persist in the human host in the face of a robust immune response (3). Macrophages, as inherent immune cells, are among the frontline defenders against invading organisms, including bacterial infections such as M.tb.

miRNAs are an endogenous, non-coding and abundant class of small (18–25 nucleotides) RNAs that function as gene regulators (4). They generally act by mediating post-transcriptional regulators of gene expression or degradation of target mRNAs that have widespread effects on downstream pathways (5, 6). miRNAs specifically combine with the 3' untranslated regions (3'-UTRs) of target mRNAs to trigger degradation or translation inhibition, participating in the inflammation response, tumorigenesis, and other biological processes (7, 8). miRNAs have been recognized as a new class of genes involved in human tumorigenesis and have recently been shown to be diagnostic, prognostic and therapeutic biomarkers. Several studies have identified miRNAs (miR-146, miR-29a, miR-21, miR-34 etc.) that were significantly altered and have the potential to affect immune function in human macrophages and T cells of patients with tuberculosis (9-11). MiR-144, a broadly conserved miRNA was first shown to be an apoptosis suppressor in various tumor cell lines (12, 13). It was revealed that miR-144 can directly inhibit expression of the autophagy-related gene Atg4a and participate in the regulation of the autophagy process in RAW264.7 cells after Bacilli Calmette-Guerin (BCG)

stimulation (14). Manipulation of host miRNA expression may be another mechanism by which M.tb is able to subvert immune detection and persist intracellularly within macrophages (10, 15).

Tpl2 is a member of the mitogen activated protein kinase (MAPK) family of serine/ threonine kinases and mediates Toll-like receptor activation of ERK-1/2 MAP kinases (16). Tpl2 activation is currently understood to be induced downstream of various innate cellular receptors such as cytokine receptors, growth factors, death receptors and toll-like receptors (17, 18). ERK-1/2 induction has been shown to be critical for the transcription and production of the pro-inflammatory cytokines IL-1 β and TNF- α in monocytic cell lines and has also been implicated in prominent inflammatory diseases (19, 20). So I supposed that whether there was a miRNA to regulate inflammatory cytokines of macrophages through Tpl2 gene or ERK signaling.

Based on this research background, I analyzed possible immunoregulation mechanisms of miR-144 and the Tpl2/ERK pathway in M.tb-infected macrophages. It was showed in this study that infection of macrophages with M.tb results in low level expression of miR-144 and secretion of pro-inflammatory cytokines. miR-144 directly binds to the 3'-UTR of Tpl2, a key component in ERK signaling, to regulate the macrophage response to M.tb infection.

Received December 22, 2015; Accepted February 26, 2016; Published February 29, 2016

* **Corresponding author:** Hong-Yan Liu, Fourth Department of Internal Medicine, Tuberculosis control and Prevention Hospital of Shaanxi Province, Taiyi palace town, Xi'an 710100, Shaanxi, China. Email: hongyanliures@163.com

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Materials and Methods

Cell separation and culture

The study was approved by the ethics committee of institution (code 2014075) and informed consent was obtained from the participants. Blood was obtained from healthy, purified protein derivative-negative human volunteers to obtain MDMs as described in the literature (21, 22). Briefly, MDMs were isolated from blood by Fycoll-Paque plus density gradient centrifugation (Amersham Biosciences, Sweden) at 1800 rpm for 30 min. They were then cultured in RPMI 1640 medium (Carlsbad, CA, USA) supplemented with 20% (v/v) human serum albumin (Invitrogen, CA, USA) for 5 d at 37 °C in a 5% CO₂ atmosphere to allow the differentiation of monocytes into MDMs. MDMs were then collected and cultured for infection experiments. HEK293 cells were maintained in RPMI 1640 media supplemented with 10% (v/v) fetal bovine serum (FBS, Carlsbad, CA, USA) at 37 °C in a 5% CO₂ atmosphere.

M.tb infection of MDMs

MDMs were incubated with a M.tb H₃₇R_v (ATCC, Manassas, VA) suspension in cell culture plates at a multiplicity of infection of 5:1 in RPMI 1640 medium containing 0.4% human serum albumin for 4 h at 37 °C in a 5% CO₂ atmosphere after shaking to ensure equal dispersion of bacteria (23). In addition, I stimulated MDMs with paraformaldehyde (PFA)-killed M.tb. MDMs were resupplied with RPMI 1640 medium containing 5% human serum albumin for the subsequent experiments after washing with serum-free RPMI 1640 medium to remove excess bacteria. All experiments were completed in biosafety laboratory provided by tuberculosis control and prevention hospital of Shaanxi province where I worked.

NanoString nCounter miRNA expression profiling

RNA was isolated by the Trizol method (Invitrogen, CA, USA) and microRNA profiling analysis was performed by KangCheng Biosciences (Shanghai, China). Briefly, total RNA was extracted from 24 and 72 h M.tb-infected MDMs according to the Trizol reagent instructions (Invitrogen, CA, USA). The concentration of the RNA was determined using a ND-1000 microspectrophotometer (NanoDrop Technologies, Wilmington, DE). I prepared small RNAs according to the manufacturer's instructions by ligating a specific DNA tag (miR-tag) onto the 3' end of each mature miRNA with a bridge oligomer. Excess tags were removed by restriction digestion. Hybridization reactions were incubated at 64 °C for 18 h. The nCounter Digital Analyzer was used to count individual fluorescent barcodes and to quantify the target miRNA molecules present in each sample.

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted as described previously (24, 25). RNA quantity and quality were evaluated using a NanoDrop 2000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). mRNA expression was analyzed by real-time PCR using primers synthesized by the Shanghai Sangon Biological Engineering and Technology Service (Shanghai, China). Briefly, 20 µl

reactions containing 50 ng of total RNA, 10 µl of 2× SYBR Green PCR Master Mix, 6.25 U of AMV reverse transcriptase, 10 U of RNase inhibitor and 0.1 mM of primers were subjected to one cycle of 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s, 56 °C for 30 s and 72 °C for 45 s (Applied by Biosystems, CA, USA). The expression of genes in all groups was calculated using the 2^{-ΔΔCt} method (26). All experiments were repeated three times independently.

Transfection of MDMs

miR-144 mimic and inhibitor (Qiagen, Valencia, CA, USA) were used to overexpress and inhibit miR-144, respectively. A non-targeting scrambled small interfering RNA of Tpl2 (Tpl2-siRNA; Qiagen, Valencia, CA, USA) was used to silence Tpl2. MDMs were transfected with miR-144 mimic, inhibitor and Tpl2-siRNA using the Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol, together with the negative controls. Cells were then incubated at 37 °C in a 5% CO₂ atmosphere for 48 h.

Vector construction and luciferase reporter assays

The 3'-UTR of Tpl2 was PCR amplified from human genomic DNA, and cloned downstream of the firefly luciferase coding region of the pMIR-GLOTM Luciferase vector (Promega, CA, USA). The recombinant vector was named pMIR-Tpl2. Mutations in miR-144 binding sites were introduced by site-directed mutagenesis and the resulting vector was named pMIR-Tpl2-mut. Cells were seeded into 24-well plates and cotransfected with 200 ng of pMIR-Tpl2 or pMIR-Tpl2-mut vector and miR-144 mimic or control mimic, and the pRL-TK plasmid (Promega, Madison, WI) which is used as internal normalization. After 36 h, cells were harvested and lysed using the lysis buffer (Promega). The luciferase reporter gene assay was implemented using the Dual-Luciferase Reporter Assay System (Promega, Shanghai, China) according to the manufacturer's instructions.

Western blot analysis

MDMs were respectively transfected with miR-144 inhibitor, miR-144 mimic or Tpl2-siRNA (Qiagen) and stimulated with U0126 (Beyotime, Beijing, China), which can suppress ERK phosphorylation. Then MDMs were lysed in cell lysis buffer containing protease inhibitor (Shanghai, China). Protein concentrations were measured using the BCA protein assay kit (Thermo Scientific, Logan, UT, USA). Equal amounts of protein were separated on 12%–15% SDS-PAGE and transferred to a PVDF membrane (Millipore, USA). The membranes were incubated with primary antibodies, including anti-miR-144 (1/1000; Santa Cruz, CA, USA), anti-Tpl2 (1/1000; Santa Cruz, CA, USA), anti-ERK (Cell Signaling Technology, CA, USA) and anti-β-actin (Kangcheng, Shanghai, China) at 4 °C overnight. The membranes were then incubated with secondary antibodies for 1 h at room temperature. ImageJ software was used to analyze the protein band intensities.

Enzyme-linked immunosorbent assay (ELISA)

TNF-α, IL-6 and IL-1β production in cell supernatants was measured using ELISA kits (Boster, Wuhan,

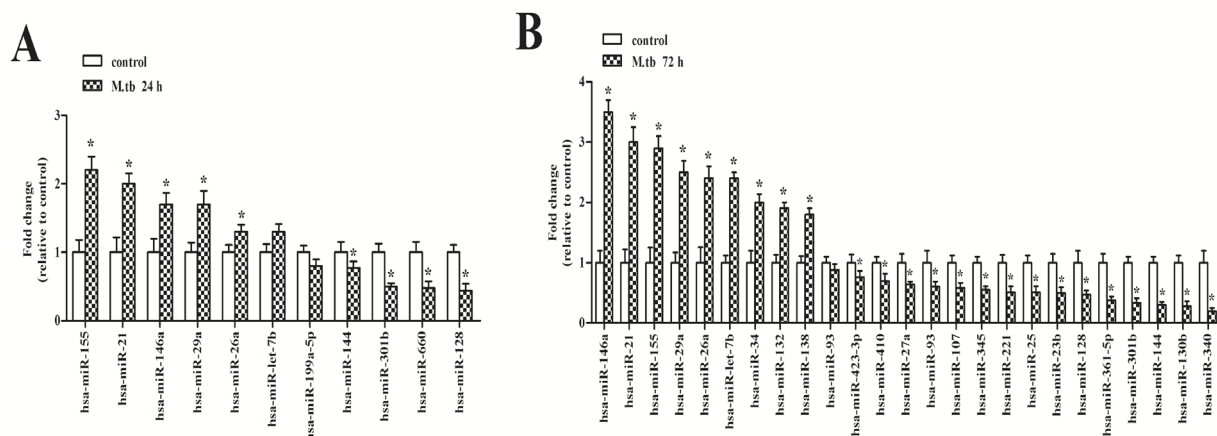


Figure 1. Human macrophage miRNAs altered by M.tb infection. Compared with the control group, miRNA expression changes in MDMs caused by M.tb infection after 24 h (A) and 72 h (B) were detected using the NanoString nCounter miRNA Expression Assay. * $P < 0.05$ versus control group.

China) according to the manufacturer's instructions.

Statistical analysis

All data were processed using SPSS13.0. For statistical analysis, quantitative data from at least three experiments were compared and are expressed as the mean \pm SD. Analysis of variance (ANOVA) was used for comparison among groups. $P < 0.05$ was considered to be statistically significant.

Results

Identification of miRNAs that are differentially expressed during M.tb infection

To identify the human macrophage miRNAs with altered expression during M.tb infection, I performed miRNA expression profiling in MDMs after infection with the virulent standardized strain M.tb H₃₇R_v using the NanoString nCounter miRNA Expression Assay. I chose 24 h and 72 h post infection time points based in the report of Bin Ni et al. that 24 h represented the phase of entry and acclimatization to the intracellular environment, and 72 h represented the phase of intracellular replication (23). At 24 h six miRNAs were significantly up-regulated, whereas five miRNAs were suppressed in response to M.tb infection, compared with the control group (Fig. 1A). At 72 h post infection, there were twenty-five differentially expressed miRNAs: nine were up-regulated and sixteen were distinctly down-regulated, relative to the control group (Fig. 1B). Notably, all eleven miRNAs altered at 24 h post infection were also changed at 72 h, with similar patterns. Taken together, these findings indicated that miRNAs might play a critical role in M.tb infection.

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miR-144 expression is increased in MDMs infected with M.tb

From recent reports and our miRNA expression profiling results it was clear that miR-144 expression was obviously reduced on M.tb infection. I further verified this using qRT-PCR and the data showed that miR-144 was markedly decreased in MDMs 24 and 72 h after M.tb infection (Fig. 2A). Furthermore, I detected Tpl2 protein expression by western blot and It was enhanced (Fig. 2B). Because 72 h post infection is the time at which M.tb is undergoing early rounds of replication in our model (23), we investigated whether replicating M.tb is required. Treatment of MDMs with PFA-fixed

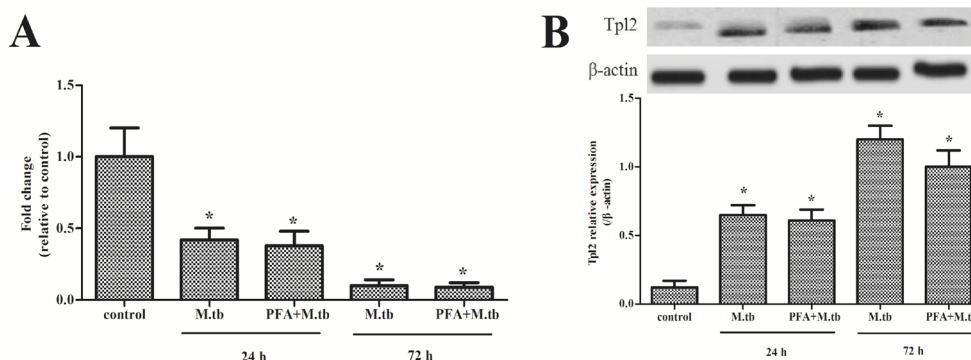


Figure 2. expression of miR-144 and Tpl2 during M.tb infection or treatment with PFA-killed M.tb. miR-144 expression was detected using qRT-PCR (A). Tpl2 expression was detected by western blot (B). The PFA+M.tb group contained MDMs treated with PFA-fixed M.tb, which is no longer able to replicate. The statistical analysis was based on three independent experiments. Data are represented as the mean \pm SD. ** $P < 0.01$ and * $P < 0.05$ versus control group.

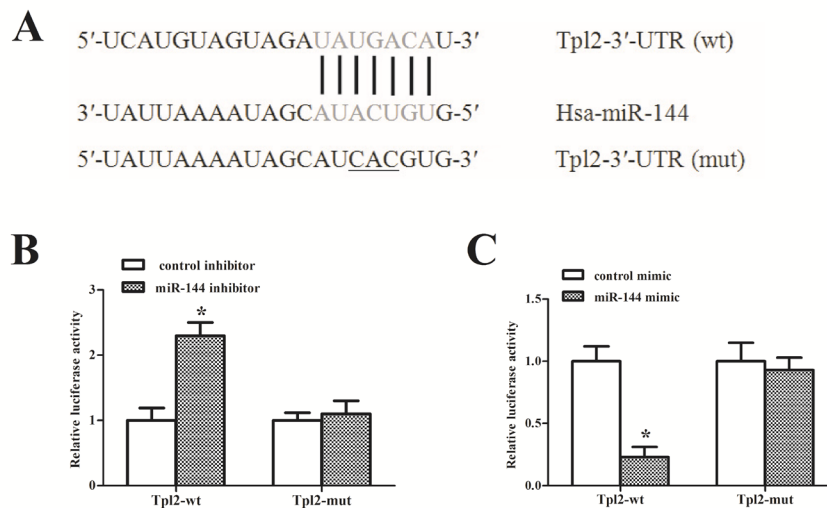


Figure 3. miR-144 directly targets the Tpl2 3'-UTR. (A) The miR-144 binding site was predicted in the 3'-UTR of Tpl2 mRNA. (B) Relative luciferase activity of HEK293 cells transfected with either the Tpl2-wt or the Tpl2-mut plasmid along with miR-144 mimic or miR-144 inhibitor was determined. Luciferase activity was normalized to the total protein amount in each group. Data are represented as the mean \pm SD. * P <0.05 versus control group.

M.tb, which can no longer replicate but possesses an intact cell wall structure, also significantly suppressed miR-144 expression and improved Tpl2 protein expression as shown in Fig. 2A and Fig. 2B. This suggested that M.tb cell-wall components were sufficient to inhibit miR-144 and increase Tpl2 expression in MDMs, consistent with previous studies (23).

miR-144 directly binds to the 3'-UTR of Tpl2

Using the bioinformatics software programs miR-base and Targetscan we identified Tpl2 as a putative target of miR-144 (Fig. 3A). To confirm whether miR-144 directly targeted the 3'-UTR of Tpl2 I constructed luciferase reporter vectors containing the wild type (wt) or mutated (mut) potential binding sequence in the Tpl2 3'-UTR. The wild type Tpl2 (Tpl2-wt) and mutant Tpl2 (Tpl2-mut) vectors were co-transfected with miR-144 mimic, miR-144 control mimic, miR-144 inhibitor or

control inhibitor into HEK293 cells. The results in Fig. 3B show that luciferase activity in Tpl2-wt and miR-144 inhibitor co-transfected cells was significantly enhanced, compared with control inhibitor co-transfected cells. Meanwhile, the Tpl2-wt and miR-144 mimic co-transfection cells were showed an obvious decrease in luciferase activity (Fig. 3C). Moreover, there was no significant difference in luciferase activity between cells co-transfected with Tpl2-mut with miR-144 mimic and Tpl2-mut with miR-144 inhibitor (Fig. 3B and 3C). These data provide evidence that miR-144 alters the Tpl2 protein level by directly targeting Tpl2 mRNA through binding sites in the Tpl2 3'-UTR.

Down-regulation of miR-144 increases Tpl2 expression

Tpl2 expression was noticeably increased in MDMs at 72 h post infection as shown in Fig. 4A and Fig. 4B.

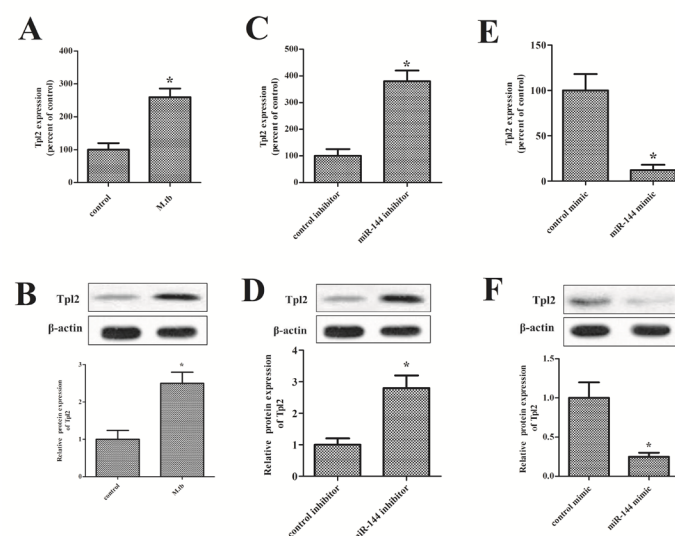


Figure 4. Down-regulation of miR-144 induces Tpl2 over-expression. Relative mRNA and protein expression of Tpl2 in MDMs by M.tb infection were determined by qRT-PCR (A) and western blot (B). Relative mRNA and protein expression of Tpl2 after transfected with miR-144 inhibitor (C, D) or mimic (E, F) were quantified. The statistical analysis was based on three independent experiments. Data are represented as the mean \pm SD. ** P <0.01 and * P <0.05 versus control group.

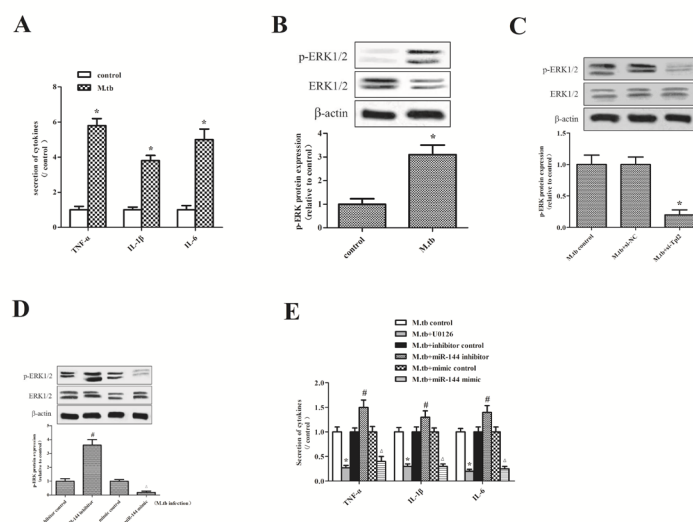


Figure 5. miR-144 regulates cytokine secretion from MDMs via the Tpl2-ERK pathway. Secretion of TNF- α , IL-1 β and IL-6 were detected in MDMs by ELISA after M.tb infection (A), or differentially co-transfected with miR-144 inhibitor and miR-144 mimic, or co-cultured with U0126 (E). Detection of p-ERK1/2 protein in MDMs with M.tb infection (B) or silencing Tpl2 gene(C), or respectively co-transfected with miR-144 inhibitor and miR-144 mimic (D). The statistical analysis was based on three independent experiments. Data are represented as the mean \pm SD. * P <0.05 versus control group.

In order to further verify whether Tpl2 is target gene of miR-144, I transfected cells with miR-144 inhibitor or mimic and measured the Tpl2 expression level. miR-144 inhibitor caused an obvious increase in mRNA and protein expression of Tpl2 (Fig. 4C and 4D), while the miR-144 mimic had the opposite effect (Fig. 4E and 4F), revealing an inverse correlation between Tpl2 and miR-144 expression.

miR-144 regulates cytokines secretion via the Tpl2-ERK1/2 pathway

Early studies have shown that ERK signaling can regulate the production of multiple cytokines in macrophages (19, 27). I detected the production of cytokines after infection with M.tb, and found that secretion of TNF- α , IL-1 β and IL-6 was especially increased (Fig. 5A). Numerous studies have reported that activation of ERK depends on Tpl2 in macrophages (19, 28). Tpl2 was silenced using Tpl2-siRNA in MDMs, and found that knockdown of Tpl2 repressed ERK1/2 phosphorylation (Fig. 5B) in comparison with control cells. Additionally, miR-144 inhibitor activated ERK1/2 phosphorylation and heightened TNF- α , IL-1 β , and IL-6 production; miR-144 mimic had the opposite effect (Fig. 5C and 5D). Furthermore, I explored the effect of ERK1/2 signaling on cytokine secretion in MDMs. As shown in Fig. 5D, U0126, an ERK inhibitor, restrained TNF- α , IL-1 β and IL-6 production. All these data suggested that cytokine secretion from M.tb-infected MDMs was due to up-regulation of Tpl2 and p-ERK1/2 protein expression.

Discussion

M.tb is an ancient organism and a host-adapted intracellular pathogen of macrophages (29). Host immunity against M.tb is complex and different innate and adaptive immune components are crucial for protection against the disease. Macrophages are the first line of defense against the infection, but M.tb can persist and

replicate within infected macrophages (30). A complex, evolutionally adaptive relationship exists between M.tb and the host during which M.tb evades and subverts the immune response mounted by the macrophage.

Recent years have witnessed a spate of investigation on the dysregulation and roles of miRNAs during bacterial infections (31), including M.tb (32, 33). Researchers have found that many miRNAs exhibit abnormal expression in the blood of TB patients (33). Several important miRNAs are potential therapeutic targets for M.tb infection, such as miR-146a, miR-21, and miR-155 among others (34, 35). To our knowledge, the function of miR-144 in human macrophages with M.tb infection has not been reported. This study emphasizes the effects of M.tb infection on macrophage miRNAs using a pure primary human macrophage model. I have identified 25 miRNAs differentially expressed in primary human macrophages during infection with M.tb, and miR-144 was markedly reduced. In addition, M.tb cell-wall components were sufficient to reduce miR-144 in MDMs.

Target gene prediction based on computational approaches reveals hundreds of genes that might be regulated by miR-144 including Tpl2, and almost all of them have not been validated. The MAP kinase Tpl2 has been previously identified as a host defense molecule against M.tb. It can switch inflammatory signals through diverse receptors in human macrophages, including cytokine receptors. It has been reported that Tpl2 plays a role in regulating the function of innate immune cells (36). Over-expression of Tpl2 in immune cells links the kinase with several crucial biochemical pathways. For example, Tpl2 over-expression in T cells led to the activation of nuclear factor of activated T cells and the production of interleukin-2 (IL-2), both of which are potent mediators of T-cell function and proliferation. I showed in this study that miR-144 directly binds to the 3'-UTR of Tpl2. Following infection of macrophages with M.tb, the Tpl2 gene was rapidly expressed and down-regulation of miR-144 also significantly elevated Tpl2 expression (16, 30).

Importantly, activation of Tpl2 has also been shown to be induced by pro-inflammatory cytokines during *Toxoplasma gondii* infection, and genetic ablation of Tpl2 caused mice to become more susceptible to the parasite (37). Recently it has been reported that Tpl2 can promote secretion of cytokines via ERK1/2 phosphorylation and is a signaling molecule that participates in the immune response (36, 38). ERK1/2 signaling has been shown to regulate the production of several cytokines, including IL-12, IL-10, and type I-IFN (39, 40). These pro-inflammatory factors can undermine the immune response to M.tb infection and lead to spreading of the infection (41). The data demonstrated that TNF- α , IL-1 β , IL-6 were significantly promoted and down-regulation of miR-144 significantly activated the Tpl2/ERK pathway to promote secretion of pro-inflammatory factors during M.tb infection. These findings are similar to results from previous reports suggesting that TPL-2/ERK was a positive regulator of TNF- α , IL-6, and IL-1 β following M.tb infection of macrophages. Therefore I examined the role of miR-144 in M.tb infection, providing a mechanism for the observations.

In summary, I showed that miR-144 in innate immune cells plays an important role in controlling intracellular bacterial infections caused by M.tb. miR-144 was a direct negative regulator of Tpl2 and played a crucial role in regulating inflammatory factors TNF- α , IL-1 β and IL-6 via targeting of the Tpl2/ERK pathway. Therapeutically, The findings raise the possibility of using miR-144 as a novel therapeutic agent to suppress the Tpl2/ERK signaling pathway in order to prevent pro-inflammatory factor secretion from macrophages infected with M.tb.

References

- Glaziou, P., K. Floyd and M. Raviglione. Global burden and epidemiology of tuberculosis. Clinics in chest medicine, 2009. 30(4): 621-636.
- Glaziou, P., D. Falzon, K. Floyd and M. Raviglione. Global epidemiology of tuberculosis. Semin Respir Crit Care Med, 2013. 34(1): 3-16.
- Flynn, J.L. Immunology of tuberculosis and implications in vaccine development. Tuberculosis, 2004. 84(1): 93-101.
- Croce, C.M. and G.A. Calin. miRNAs, cancer, and stem cell division. cell, 2005. 122(1): 6-7.
- box consensus AS uGGAAGAC, G. Micro RNAs are complementary to 3' UTR sequence motifs that mediate negative post-transcriptional regulation. Nature genetics, 2002. 30: 363.
- Filipowicz, W., S.N. Bhattacharyya and N. Sonenberg. Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? Nature Reviews Genetics, 2008. 9(2): 102-114.
- Ha, M. and V.N. Kim. Regulation of microRNA biogenesis. Nature reviews Molecular cell biology, 2014. 15(8): 509-524.
- Ott, C.E., J. Grünhagen, M. Jäger, D. Horbelt, S. Schwill, K. Kallenbach, et al. MicroRNAs differentially expressed in postnatal aortic development downregulate elastin via 3' UTR and coding-sequence binding sites. PLoS One, 2011. 6(1): e16250.
- Yi, Z., Y. Fu, R. Ji, R. Li and Z. Guan. Altered microRNA signatures in sputum of patients with active pulmonary tuberculosis. PLoS One, 2012. 7(8): e43184.
- Zhou, L., L. Guo, J. Tang, A. Zhang, X. Liu and G. Xu. miR-144 regulates BCG-and rapamycin-induced autophagy by targeting Atg4a in RAW264. 7 cells. Xi bao yu fen zi mian yi xue za zhi= Chinese journal of cellular and molecular immunology, 2015. 31(2): 163-167.
- Kleinstaub, K., K. Heesch, S. Schattling, M. Kohns, C. Sander-Julch, G. Walzl, et al. Decreased expression of miR-21, miR-26a, miR-29a, and miR-142-3p in CD4 (+) T cells and peripheral blood from tuberculosis patients. PloS one, 2013. 8(4): e61609.
- Guo, Y., L. Ying, Y. Tian, P. Yang, Y. Zhu, Z. Wang, et al. miR-144 downregulation increases bladder cancer cell proliferation by targeting EZH2 and regulating Wnt signaling. FEBS Journal, 2013. 280(18): 4531-4538.
- Zha, W., L. Cao, Y. Shen and M. Huang. Roles of Mir-144-ZFX pathway in growth regulation of non-small-cell lung cancer. PloS one, 2013. 8(9): e74175.
- Cai, Z.-G., S.-M. Zhang, Y. Zhang, Y.-Y. Zhou, H.-B. Wu and X.-P. Xu. MicroRNAs are dynamically regulated and play an important role in LPS-induced lung injury. Canadian journal of physiology and pharmacology, 2011. 90(1): 37-43.
- Zhang, C., L. Shu and A.-N.T. Kong. MicroRNAs: new Players in Cancer Prevention Targeting Nrf2, Oxidative Stress and Inflammatory Pathways. Current Pharmacology Reports, 2015. 1(1): 21-30.
- Watford, W., S. Rowley, M. Tarrio, S.-H. Lee, K. Hardwick, L. Boissel, et al. Analysis of Tpl2-deficient mice reveals functional redundancies in the regulation of IFN γ production by NK cells during MCMV infection (INM8P. 401). The Journal of Immunology, 2015. 194(1 Supplement):195.9-195.9.
- Richardson, E.T., S. Shukla, D.R. Sweet, P.A. Wearsch, P.N. Tschlis, W.H. Boom, et al. TLR2-dependent ERK signaling in Mycobacterium tuberculosis-infected macrophages drives anti-inflammatory responses and inhibits Th1 polarization of responding T cells. Infection and Immunity, 2015: IAI. 00135-15.
- López-Pelaéz, M., S. Fumagalli, C. Sanz, C. Herrero, S. Guerra, M. Fernandez, et al., Cot/tpl2-MKK1/2-Erk1/2 controls mTORC1-mediated mRNA translation in Toll-like receptor-activated macrophages. Molecular biology of the cell, 2012. 23(15): 2982-2992.
- Dumitru, C.D., J.D. Ceci, C. Tsatsanis, D. Kontoyiannis, K. Stamatakis, J.H. Lin, et al. TNF-alpha induction by LPS is regulated posttranscriptionally via a Tpl2/ERK-dependent pathway. Cell, 2000. 103(7): 1071-83.
- Sanz-Garcia, C., L.E. Nagy, M.A. Lasuncion, M. Fernandez and S. Alemany. Cot/tpl2 participates in the activation of macrophages by adiponectin. J Leukoc Biol, 2014. 95(6): 917-30.
- Schlesinger, L. Macrophage phagocytosis of virulent but not attenuated strains of Mycobacterium tuberculosis is mediated by mannose receptors in addition to complement receptors. The Journal of Immunology, 1993. 150(7): 2920-2930.
- Horwitz, M. and S. Silverstein. Influence of the Escherichia coli capsule on complement fixation and on phagocytosis and killing by human phagocytes. Journal of Clinical Investigation, 1980. 65(1): 82.
- Ni, B., M.V. Rajaram, W.P. Lafuse, M.B. Landes and L.S. Schlesinger. Mycobacterium tuberculosis Decreases Human Macrophage IFN- γ Responsiveness through miR-132 and miR-26a. The Journal of Immunology, 2014. 193(9): 4537-4547.
- Ogura, T., A. Tsuchiya, T. Minas and S. Mizuno. Methods of high integrity RNA extraction from cell/agarose construct. BMC Res Notes, 2015. 8: 644.
- Pavani, K.C., E.E. Baron, M. Faheem, A. Chaveiro and F.M. Da Silva. OPTIMISATION OF TOTAL RNA EXTRACTION FROM BOVINE OOCYTES AND EMBRYOS FOR GENE EXPRESSION STUDIES AND EFFECTS OF CRYOPROTECTANTS ON TOTAL RNA EXTRACTION. Tsitol Genet, 2015. 49(4): 25-34.
- Livak, K.J. and T.D. Schmittgen. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$

method. *methods*, 2001. 25(4): 402-408.

27. Waterfield, M.R., M. Zhang, L.P. Norman and S.C. Sun. NF-kappaB1/p105 regulates lipopolysaccharide-stimulated MAP kinase signaling by governing the stability and function of the Tpl2 kinase. *Mol Cell*, 2003. 11(3): 685-94.

28. McNab, F.W., J. Ewbank, R. Rajsbaum, E. Stavropoulos, A. Martirosyan, P.S. Redford, et al. TPL-2-ERK1/2 signaling promotes host resistance against intracellular bacterial infection by negative regulation of type I IFN production. *The Journal of Immunology*, 2013. 191(4): 1732-1743.

29. Schlesinger, L.S., C.G. Bellinger-Kawahara, N.R. Payne and M.A. Horwitz. Phagocytosis of *Mycobacterium tuberculosis* is mediated by human monocyte complement receptors and complement component C3. *The Journal of Immunology*, 1990. 144(7): 2771-2780.

30. Ni, B., C. Torres, M. Rajaram and L. Schlesinger. *Mycobacterium tuberculosis* infection decreases macrophage Tpl-2 protein levels over time which alters responses to TLR agonists (MPF4P. 730). *The Journal of Immunology*, 2015. 194(1 Supplement): 136.6-136.6.

31. Eulalio, A., L. Schulte and J. Vogel. The mammalian microRNA response to bacterial infections. *RNA biology*, 2012. 9(6): 742-750.

32. Singh, P.K., A.V. Singh and D.S. Chauhan. Current understanding on micro RNAs and its regulation in response to *Mycobacterial* infections. *J Biomed Sci*, 2013. 20: 14.

33. Yi, Z., Y. Fu, X. Wu, J. Li and F. Xu. Circulating microRNAs in patients with active pulmonary tuberculosis. *Journal of clinical microbiology*, 2011: 05459-11.

34. Spinelli, S.V., A. Diaz, L. D'Attilio, M.M. Marchesini, C. Bogue, M.L. Bay, et al. Altered microRNA expression levels in mononuclear cells of patients with pulmonary and pleural tuberculosis and

their relation with components of the immune response. *Molecular immunology*, 2013. 53(3): 265-269.

35. Meng, Q.-L., F. Liu, X.-Y. Yang, X.-M. Liu, X. Zhang, C. Zhang, et al. Identification of latent tuberculosis infection-related microRNAs in human U937 macrophages expressing *Mycobacterium tuberculosis* Hsp16. 3. *BMC microbiology*, 2014. 14(1): 37.

36. Roget, K., A. Ben-Addi, A. Mambole-Dema, T. Gantke, H.-T. Yang, J. Janzen, et al. IKK2 regulates TPL-2 activation of ERK-1/2 MAP kinases by direct phosphorylation of TPL-2 serine 400. *Molecular and cellular biology*, 2012: 01065-12.

37. Watford, W.T., B.D. Hissong, L.R. Durant, H. Yamane, L.M. Muul, Y. Kanno, et al. Tpl2 kinase regulates T cell interferon- γ production and host resistance to *Toxoplasma gondii*. *The Journal of experimental medicine*, 2008. 205(12): 2803-2812.

38. Ewbank, J. *Mycobacterium tuberculosis* induced transcription in macrophages: the role of TPL2/ERK signalling in the negative regulation of type I interferon production and implications for control of tuberculosis. 2012, University College London.

39. Agrawal, S., A. Agrawal, B. Doughty, A. Gerwitz, J. Blenis, T. Van Dyke, et al. Cutting edge: different Toll-like receptor agonists instruct dendritic cells to induce distinct Th responses via differential modulation of extracellular signal-regulated kinase-mitogen-activated protein kinase and c-Fos. *The Journal of Immunology*, 2003. 171(10): 4984-4989.

40. Dillon, S., A. Agrawal, T. Van Dyke, G. Landreth, L. McCauley, A. Koh, et al. A Toll-like receptor 2 ligand stimulates Th2 responses in vivo, via induction of extracellular signal-regulated kinase mitogen-activated protein kinase and c-Fos in dendritic cells. *The Journal of Immunology*, 2004. 172(8): 4733-4743.

41. Amdekar, Y. Tuberculosis-persistent threat to human health. *The Indian Journal of Pediatrics*, 2005. 72(4): 333-338.