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microRNA-124 negatively regulates TLR signaling in alveolar macrophages in response to mycobacterial infection

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ABSTRACT

The emerging roles of microRNAs (miRNAs) in regulating immune responses have attracted increasing attention in recent years; and the alveolar macrophages (AMs) are the main targets of mycobacterial infection, which play a pivotal role in the pathogenesis of *Mycobacterium tuberculosis* infection. However, the immunoregulatory role of miRNAs in AMs has not been fully demonstrated. In this study, we find that miR-124 is up-regulated in the peripheral leukocytes of patients with pulmonary tuberculosis; furthermore, the expression miR-124 can be induced upon *Mycobacterium bovis* Bacillus Calmette–Guerin (BCG) infection in both RAW264.7 AM cells *in vitro* and murine AMs *in vivo*. Mechanistically, miR-124 is able to modulate toll-like receptor (TLR) signaling activity in RAW264.7 cells in response to BCG infection. In this regard, multiple components of TLR signaling cascade, including the TLR6, myeloid differentiation factor 88 (MyD88), TNFR-associated factor 6 and tumor necrosis factor-α are directly targeted by miR-124. In addition, both overexpression of TLR signaling adaptor MyD88 and BCG infection are able to augment miR-124 transcription, while MyD88 expression silenced by small interfering RNA dramatically suppresses miR-124 expression in AMs *in vitro*. Moreover, the abundance of miR-124 transcript in murine AMs of MyD88 deficient mice is significantly less than that of their wild-type or heterozygous littermates; and the BCG infection fails to induce miR-124 expression in the lung of MyD88 deficient mouse. These results indicate a negative regulatory role of miR-124 in fine-tuning inflammatory response in AMs upon mycobacterial infection, in part through a mechanism by directly targeting TLR signaling.

1. Introduction

Tuberculosis (TB) is one of the world's leading infectious diseases with approximately 1.4 million deaths and 8.7 million new cases annually, which remains an enormous burden worldwide (WHO, 2013). *Mycobacterium tuberculosis* (Mtb) infection is the cause of TB. Mtb is an intracellular pathogen, which is capable of undergoing various genomic reprogramming events to subsequently prevent the immune system from completely eliminating latent infectious agents (Kaufmann et al., 2005).

Despite intensive efforts aimed to understand the mechanism of Mtb infection and host immune response have been made, the underlying mechanisms by which the interaction of a Mtb and its host cell remains incompletely understood (Russell, 2011). It has been well recognized that alveolar macrophages (AMs) are the main targets of Mtb infection, which play a dispensable role in the process of Mtb infection (Guilliams et al., 2013; Poirier and Av-Gay, 2012). As a subset of tissue-resident cells in pulmonary alveoli, AMs are able to activate inflammatory peripheral macrophages that migrate to the site of inflammation during processes of pathogenesis (Cho et al., 2011). It has been proved that apoptosis and necrosis of macrophages following an Mtb infection play a central role in the pathogenesis of TB (Wu et al., 2014). In this context, the Mtb infection triggers innate immune responses to induce the production of pro-inflammatory cytokines and chemokines, such as interleukin (IL)-6 and tumor necrosis factor-α (TNF-α), the TNF-α is then capable of subsequently inducing macrophages to

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produce other cytokines and chemokines, which in turn modulates macrophage apoptosis and necrosis (Zimmerman et al., 2013).

Mounting evidence has shown that microRNAs (miRNAs, or miRs) play important roles in the immunity by regulation of immune cell lineage commitment, differentiation, and maturation (Zhou et al., 2012; Zhu et al., 2013). Recently, increasing numbers of studies have demonstrated that miRNAs were able to regulate the immune responses of macrophages by targeting the toll-like receptor (TLR) signaling pathway (Liu et al., 2014; O'Neill et al., 2011; Xu et al., 2014). For instances, Liu et al. (2014) revealed that miR-146a could negatively regulate TNF-α in AMs upon *Mycobacterium bovis* Bacillus Calmette–Guerin (BCG) infection, in which miR-146a might through a mechanism by targeting TLR signaling TNFR-associated factor 6 (TRAF6) and IL-1R-associated kinase 1 (IRAK-1), subsequently inhibiting the production of inflammatory mediators (Taganov et al., 2006). Similarly, Xu et al. (2014) found that miR-149 was able to directly target myeloid differentiation primary response gene 88 (MyD88) and negatively regulates TLR-triggered inflammatory responses in murine macrophage RAW264.7 cells in response to BCG infection. Such negatively regulatory role of miRNA in immune cells was also demonstrated in other members of miRNAs (Garchow et al., 2011; Lu et al., 2011; Ma et al., 2014; Wei et al., 2013). Most recently, we found that BCG activated-TLR signaling was able to induce miR-124 expression, which in turn negatively regulated immune response in alveolar epithelial cells by targeting multiple components of TLR signaling cascade (Li et al., 2014; Ma et al., 2014). This study implies that miR-124 may also exerts an immunoregulatory role in alveolar epithelial cells in response to an exogenous stimulation, such as mycobacterial infection.

In light of the above findings, we therefore hypothesize that miR-124 plays a regulatory role in immune responses of AMs during mycobacterial infection. To this end, we first evaluated the miR-124 transcript in the peripheral leukocytes of patients with pulmonary TB, and in the AMs of mice infected with BCG; the biological function of miR-124 was further interrogated in murine macrophage RAW264.7 cells and AMs of MyD88 deficient mice in response to BCG infection. We found that the mycobacterial infection was able to provoke miR-124 expression in AMs where the miR-124 in turn alleviated the mycobacteria-trigged inflammatory responses through a mechanism in part by directly targeting multiple components, including the MyD88 of TLR signaling pathway.

2. Materials and methods

2.1. Human peripheral blood specimens

Peripheral blood specimens from patients diagnosed with pulmonary tuberculosis (6 males, aged from 20 to 89 years old; 6 females, aged from 17 to 69 years old) were consecutively collected at Tuberculosis Hospital of Ningxia (Yinchuan, China). Blood samples from age-matched Mtb infection negative healthy volunteers were collected from the outpatient clinic of the Affiliated Hospital of Ningxia Medical University (Yinchuan, China). The samples were used freshly for leukocyte isolation and substantial purification of small RNA using an RNAiso kit per manufacturer's recommendations (Takara, Dalian, China). All samples were collected under informed consent. The Human Research Ethic Committee at Ningxia University approved this study (NXU_HREC 20130507).

2.2. Animals and BCG infection

Female C57/BL6 mice with six to eight weeks of age were purchased from the Animal facility of Ningxia Medical University (Yinchuan, China). *Myd88* gene knockout (KO) mice were originally generated by RIKEN (Saitama, Japan) (Scanga et al., 2004), and were purchased from Institute of Animal Models at Nanjing University (Nanjing, China). The animals were housed in a special pathogen-free room and fed with food and water *ad libitum*. All experiments using animals were performed in accordance with the guidelines of the Chinese Council on Animal Care and approved by the Committee for Animal Care and Use of Ningxia University (NXU_CACU 20121219). Mice were randomly divided into two groups. The mice of control group were intranasally instilled with 40 μl of PBS; the animals of experimental group were instilled with 1×10^6 colony-forming units (CFU) of BCG in 40 μl of PBS. 48 h after the infection, animals were euthanized and the lung tissue, bronchoalveolar lavage (BAL) fluid and alveolar macrophages (AM) were harvested as described previously (Zhang et al., 2008). The lung tissues (AMs were removed) and AMs were used for small RNA preparation.

2.3. Cell culture and BCG infection

Murine macrophage RAW264.7 cells (Shanghai Institute of Biochemistry and Cell Biology, Shanghai, China). The cells were cultured and maintained at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air in DMEM medium (Invitrogen, Carlsbad, CA, United States) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. *M. bovis* BCG Beijing strain was purchased from the Center for Disease Control and Prevention (CCDC) of China (Beijing, China). The bacilli were grown and titrated as previously described (Lewin et al., 2003; Li et al., 2014; Ma et al., 2014; Wu et al., 2014). Aliquots of the stock were stored at −80 °C. RAW264.7 cells were infected with BCG for 6 h following a transfection at a multiplicity of infection (MOI) of 3 and incubated at 37 °C in a 5% CO_2, humidified air atmosphere for additional 24 h before they were harvested for analysis.

2.4. Synthesis of miRNA mimics and cell transfection

Based on the sequence of miR-124 in miRBase database (MIMAT0000422), miR-124 mimic (dsRNA oligonucleotides), negative control mimic (miR control) and miR-124 inhibitors (single-stranded chemically modified oligonucleotides) were synthesized at Ribobio Inc (Guangzhou, China) (Ma et al., 2014). The oligonucleotides were transfected into RAW264.7 cells using Lipofectamine 2000 reagents per manufacturer's instructions (Invitrogen, USA). For cells cultured in a 6-well plate, 20 μM of miR-124 mimic, miR control or miR-124 inhibitor was transfected. The transfection mixture was removed and 2 ml/well of fresh medium was added at 6 h post-transfection, the transfected cells were cultured for additional 24 h before they were harvested for analysis.

2.5. Quantitative reverse transcription-PCR (qRT-PCR)

Total RNAs of cells or tissues were isolated with an RNA Miniprep kit, and the small RNAs were further purified with an RNAiso kit per manufacturer's recommendations (Takara, Dalian, China). The stem-loop qRT-PCR for miR-124 was performed essentially as previous study (Ma et al., 2014). The sequences of RT-PCR primer sets are listed in Table 1. The β-actin or U6 promoter was included to normalize for sample loading and RNA abundance. Relative expression was calculated as previously described using real-time PCR efficiencies and the crossing point deviation of unknown sample *vs* control (Pfaffl, 2001). The specificity of the primer sets was determined by sequencing the product of each qRT-PCR reaction.