

## Original Article

# ***Mycobacterium tuberculosis* heat-shock protein 16.3 activates macrophages probably through TLR4/MyD88 pathway**

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**Abstract:** Tuberculosis is a chronic disease caused by *Mycobacterium tuberculosis* (Mtb), and many previous studies indicated that Hsp16.3 may play an important role in long-term survival of Mtb within macrophages. However, the underlying mechanism of Hsp16.3 in protecting of Mtb is not clear. In this study, we constructed a prokaryotic expression vector, pET28a-Mtb Hsp16.3, which induced the expression of Mtb Hsp16.3 and purified the fusion protein through nickel-affinity chromatography. Subsequently, qPCR and EIA assays were used to assess the expression of TNF- $\alpha$  and IL-10 in RAW264.7 cells after treated with Mtb Hsp16.3 *in vitro*. Finally, we evaluated the impact of small interference RNA-mediated TLR4 silencing on Mtb Hsp16.3-induced macrophage activation, and further confirmed its molecular mechanism. We found that Mtb Hsp16.3 was successfully purified and could activate macrophages by enhancing the levels of TNF- $\alpha$  and IL-10 *in vitro*. In addition, knockdown of TLR4 via using siTLR4 could obviously reduce TNF- $\alpha$  and IL-10 expression and significantly increase the levels of IL-12 and IL-4. Moreover, Mtb Hsp16.3 could enhance the activation of MYD88-NF- $\kappa$ B signaling in RAW264.7 cells in a concentration dependent manner. Our data suggests that Mtb Hsp16.3 could activate macrophage through TLR4-NF- $\kappa$ B signaling pathway, providing a basis for further studies on the biological activity of Mtb Hsp16.3 and its role in the pathogenesis of TB.

**Keywords:** Tuberculosis, Mtb Hsp16.3, macrophage, TLR4

## Introduction

Tuberculosis (TB) is a communicable disease that remains a major global health concern worldwide. Based on the report of WHO, the number of new TB patients has reached about 9.6 million in China during 2014, approximately 10% of the total number of new patients each year in the world [1]. This chronic disease is caused by *Mycobacterium tuberculosis* (Mtb), a typical intracellular parasite that can survive for long periods within macrophages.

It has been widely recognized that macrophage is one kind of specialized antigen-presenting cells and plays a vital role in the anti-bacterial process. In addition to inducing apoptosis, macrophages form phagolysosomes and present Mtb antigens to initiate the adaptive immune response. Moreover, In case of drug-resistant Mtb, the macrophages generate nitrogen oxides to destroy the parasite [2]. Several

studies have shown that Mtb could evade the macrophage-mediated immune killing effect through a variety of molecular mechanisms, and then enter a latent phase for long-term survival after being engulfed by macrophages. These related mechanisms mainly include blocking lysosome formation, neutralization of the acid environment within the phagolysosome, inhibition of Mtb antigen presentation and inflammatory cytokine secretion [3].

Toll-like receptors (TLRs) are an important class of pattern-recognition receptors, mainly expressed on the surface of monocyte/macrophages and dendritic cells. Previous studies have shown that most of the Mtb bacterial proteins could activate the TLR-signaling pathway and initiate immune response. When macrophages engulf Mtb, specific Mtb antigens interact with TLRs to activate its downstream signaling with or without the involvement of Myeloid differentiation factor 88 (MyD88), and then triggers an

immediate inflammatory reaction [4]. Recently, it has been identified that TLR2 and TLR4 implicated in Mtb-induced macrophage activation. Previous studies have shown that lipoarabinomannan (LAM), lipomannan (LM), and phosphatidyl-myo-inositol mannoside (PIM) are recognized by TLR2, while 19-kDa lipoproteins of Mtb may also activate macrophages via TLR2 [5-7]. It has reported that MyD88-knockout mice are more susceptible to Mtb infection *In vivo* experiments [8]. Besides MyD88, cooperation between TLR2- and TLR4-dependent mediated signals also play an important role in macrophage apoptosis induced by Mtb and the TLR4-mediated signaling has key role in the maintenance of the balance between apoptotic vs. necrotic cell death induced by macrophage infection with Mtb [9]. Moreover, a previous study has shown that Mycobacteria-infected macrophages are poor responders to interferon-gamma (IFN-gamma), resulting in decreased expression of IFN-gamma-induced genes by multiple pathways including TLR2 and non-TLRs-mediated signaling [10]. In a word, Mtb could affect the function of macrophages by interfere with the TLR-signaling pathway, this mechanism may could explain the phenomenon that Mtb can survive for a long period within the macrophages.

Heat-shock proteins (HSPs) are a group of highly conserved structural proteins that bring about quick, short-term changes to cells in response to stress, thus helping to restore normal cellular structure and function. Hsp16.3, a member of HSPs, has been shown to function as an ATP-dependent chaperone. It is encoded by Mtb *hspX* (*acr1*, *rv2031c*) gene and contains 144 amino acids, with a relative molecular weight of approximately 16.3 kDa. This protein forms oligomeric complexes to exert its protective effect on cells and has benefits for the stabilization of cell structure and function, which contributes to Mtb survival within the host macrophage [11]. Many groups have shown that the Hsp16.3 play an important role in Mtb surviving within macrophages. Mtb did not survive in an anoxic environment when Hsp16.3 was knocked down [12, 13]; additionally, Wayne et al. found that Hsp16.3 was highly expressed during Mtb dormancy, while Yuan et al. found that Hsp16.3 was highly expressed during latency and not the logarithmic growth phase. Using 2-dimensional electrophoresis, Starck et al. analyzed Mtb Hsp16.3 cultivated

in absence of oxygen and found that this protein showed the highest increase in expression, among a total of 50 proteins showing increased expression [14]. Abulimiti et al. found that Mtb-produced methylthioamide could protect Mtb from the oxynitride secreted by macrophages via reversible sulfonation and desulfonation [15]. In addition, Cunningham et al. found that the cell walls of Mtb were thickened in an anoxic environment, and increased expression of Hsp16.3 afforded long-term survival during the latency period [16]. These previous studies indicated that Hsp16.3 may play an important role in long-term survival of Mtb within macrophages by regulating the activity of host immune cells. However, the underlying mechanism of Hsp16.3 in protecting of Mtb is not clear, in the present study, the fusion protein of His-Hsp16.3 has expressed and purified through constructed prokaryotic expression vector, and then we identify its function and molecular mechanism in macrophage activation.

## Materials and methods

### Experimental procedures

**Cloning, expression, and purification of Mtb Hsp16.3:** Hsp16.3 gene was amplified by PCR using Mtb genomic DNA (gDNA) as template, and the primers were as follow: p1: 5'-CGCGGATCCGCCACCACCCTTCCCGTTCA (BamHI)-3' and p2: 5'-CCCAAGCTTTCAGTTGGTGGACGGAT (HindIII)-3'. PCR products and plasmid pET28a were cleaved with BamHI and HindIII enzymes and ligated using T4 ligase. The recombinant plasmid pET28a-Hsp16.3 was transformed into *Escherichia coli* BL21 (DE3) cells and induced with 1 mM isopropyl  $\beta$ -D-1-thiogalactopyranoside. The cells were harvested and lysed by sonication. The supernatant and pellet were collected and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The supernatant was harvested to collect recombinant Mtb Hsp16.3 purified by Promega V1320 kit (Promega, USA).

**Macrophage stimulation assay:** The RAW264.7 cell line was maintained in RPMI-1640 medium (Gibco, Brazil) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin, and 100  $\mu$ g/mL streptomycin at 37°C in a humidified incubator with 5% CO<sub>2</sub>. When the cells reached 80% confluence, the cells were harvested by trypsin digestion and transferred to a 24-well plate (1  $\times$  10<sup>5</sup> cells/

**Table 1.** List of primers used for amplification

| Name               | Target                   | Sequence (5'-3' direction) |
|--------------------|--------------------------|----------------------------|
| GAPDH (F1)         | qRT-PCR internal control | GAGCCAAACGGGTCATCATCT      |
| GAPDH (R2)         | qRT-PCR internal control | GAGGGGCCATCCACAGTCTT       |
| TNF- $\alpha$ (F3) | qRT-PCR amplification    | CAGGGGCCACACGCTCTTC        |
| TNF- $\alpha$ (R4) | qRT-PCR amplification    | TTTGTGAGTGTGAGGGTCTGG      |
| IL-10 (F5)         | qRT-PCR amplification    | TACAGCCGGGAAGACAATAA       |
| IL-10 (R6)         | qRT-PCR amplification    | AGGAGTCGGTTAGCAGTATG       |
| IL-4 (F7)          | qRT-PCR amplification    | AAGAGGACACTGGAGCAA         |
| IL-4 (R8)          | qRT-PCR amplification    | AGACATCCCGAAGGTTC          |
| IL-12 (F9)         | qRT-PCR amplification    | CCTCTTCATCCTTACCCC         |
| IL-12 (R10)        | qRT-PCR amplification    | CACATTGGTCTTTCCACGCA       |

mL). After 12 h, the cells were divided into 6 groups and each group was treated with different concentrations of Mtb Hsp16.3 protein for 24 to 96 h.

**Real-time PCR analysis of TNF- $\alpha$ , IL-10, IL-4, and IL-12:** Total RNA was extracted from the above-mentioned cell groups by using TRIzol, and approximately 2  $\mu$ g total RNA was reverse-transcribed using PrimeScript RT reagent Kit (TaKaRa, Japan). Real-time quantitative PCR was performed to measure the mRNA levels of each group. The primers used for PCR amplification are listed in **Table 1**. The reaction mixes were prepared using SYBR Green (TaKaRa, Japan), and PCR conditions were as follow: denaturation at 94°C for 30 s, annealing at 55°C for 45 s, and extension at 72°C for 1 min. After 30 cycles, fluorescence was measured after each 0.2°C increase to obtain the melting and standard curves. Gene expression levels were quantified using the C1000™ Thermal cycler (BIO-RAD, USA), and the results were expressed at a relative mRNA level, analyzed using the comparative threshold cycle ( $2^{-\Delta CT}$ ) method with GAPDH as the reference gene.

**Cytokine enzyme immunoassay (EIA):** The levels of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin (IL)-10 were measured by 2-site sandwich EIA (eBioscience, CA). Standard curve for these cytokines was obtained using the recombinant standard proteins provided by the manufacturer.

**TLR4 siRNA:** TLR4-targeting siRNA (sense 5'-CCAAUCUAGAGCACUUGGAtt-3') was purchased from RIBO (China) and used according to the manufacturer instructions. RAW264.7 transfection was carried out using Lipofectamine

2000 (Invitrogen, New York). Macrophages were seeded at a density of  $2 \times 10^6$  cells per well in a 12-well plate and were incubated for an additional 24 h. The transfected cells were activated with 100 ng/mL Mtb Hsp16.3 for 48 h. IL-10 and TNF- $\alpha$  levels were measured by EIA and real-time PCR.

**Measurement of MyD88, NF- $\kappa$ B, and NF- $\kappa$ B by western blotting**

RAW264.7 cells were treated with different concentrations of Mtb Hsp16.3 for 24 to 96 h at 37°C. RIPA lysis buffer (Beyotime, China) was used to lyse the cells from each of the above-mentioned groups, and the lysate was centrifuged at 12,000 g for 10 min to obtain the proteins in the supernatant. Equal quantities of the extract were separated by 12% SDS-PAGE and following electrophoretic transfer, the nitrocellulose membranes were incubated with antibodies to either MyD88, nuclear factor (NF)- $\kappa$ B, or NF- $\kappa$ B (Abcam, UK). Membranes were washed and incubated with appropriate secondary antibodies conjugated to horseradish peroxidase (Abcam, UK). Bound enzyme was detected by enhanced chemiluminescence as per the manufacturer protocol (GE Healthcare, US).

### Statistical analysis

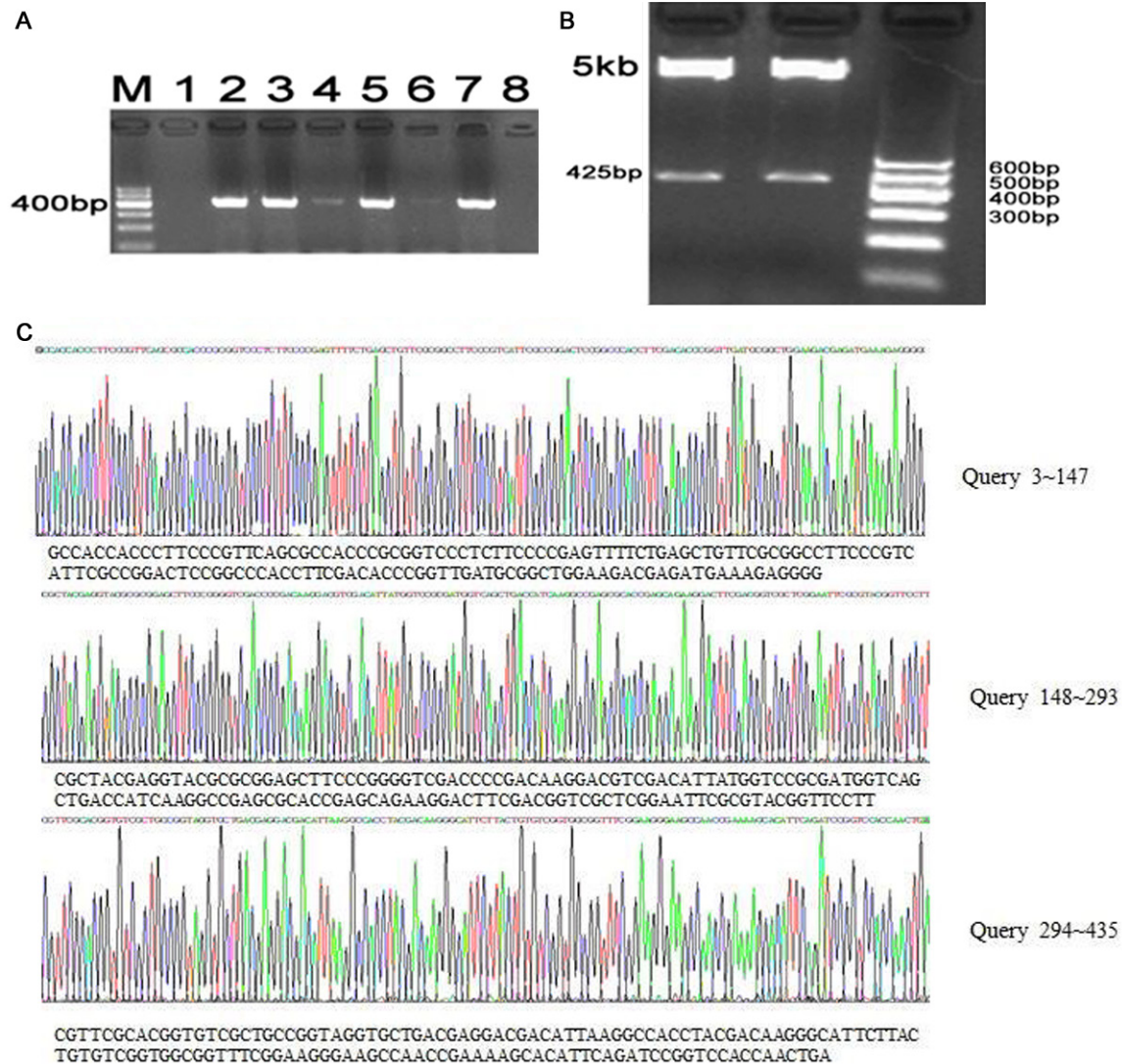
Data are shown as mean  $\pm$  S.D. of one representative experiment from at least three independent experiments. Analysis of the data was performed by SPSS v16.0 software. Data were assessed using Mann-Whitney U test, where  $P < 0.05$  were considered statistically significant.

## Results

### Construction and identification of the prokaryotic expression vector pET28a-Mtb Hsp16.3

In this study, the Mtb Hsp16.3 gene was ligated into the prokaryotic expression vector pET28a and then the recombinant plasmid pET28a-Mtb Hsp16.3 was transformed into the *Escherichia coli* DH5 $\alpha$  cells, and 8 positive colonies were selected for specific PCR amplification. After PCR amplification, the target band was





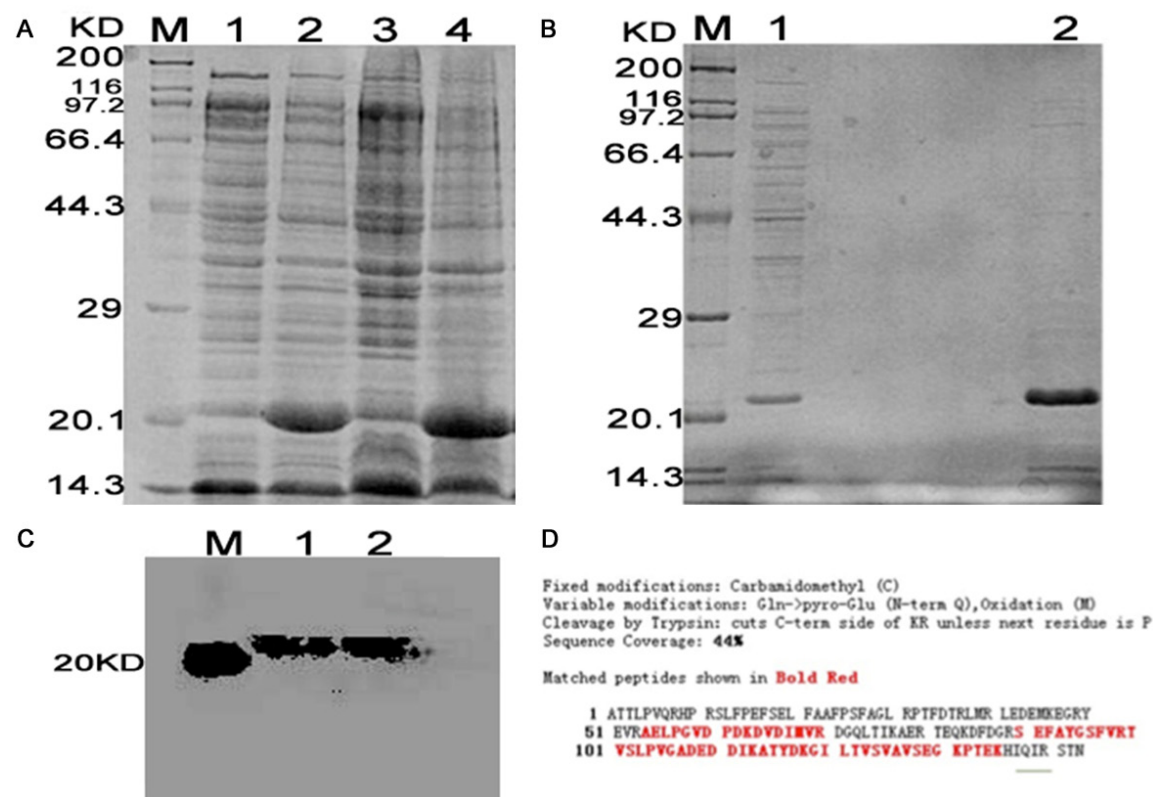
**Figure 1.** Construction and identification of the prokaryotic expression vector pET28a-Mtb Hsp16.3. A. Identification of the prokaryotic expression vector pET28a-Mtb Hsp16.3 by colony PCR (M: 600-bp marker, 1-8: positive colonies after transformation with recombinant plasmid); B. Identification of pET28a-Mtb Hsp16.3 via *Bam*HI and *Hind*III double enzyme digestion (M: 600-bp marker); C. DNA sequencing result of the recombinant plasmid.

observed at approximately 425 bp for the 2<sup>nd</sup>, 3<sup>rd</sup>, 5<sup>th</sup>, and 7<sup>th</sup> positive colonies (**Figure 1A**), and 2 colonies selected from these PCR-positive colonies were cultured in Luria-Bertani medium supplemented with Kanamycin (Kan+) for plasmid extraction after 8 to 12 h. The extracted recombinant plasmids were identified via *Bam*HI and *Hind*III double-enzyme digestion. As shown in (**Figure 1B**), the target bands were observed at approximately 425 and 5000 bp after the double-enzyme digestion, indicating that the recombinant plasmid pET28a-Mtb Hsp16.3 was successfully constructed. The plasmid was identified by double-enzyme digestion, and then was sent to Beijing Genome Institute (BGI) for DNA sequencing. The DNA

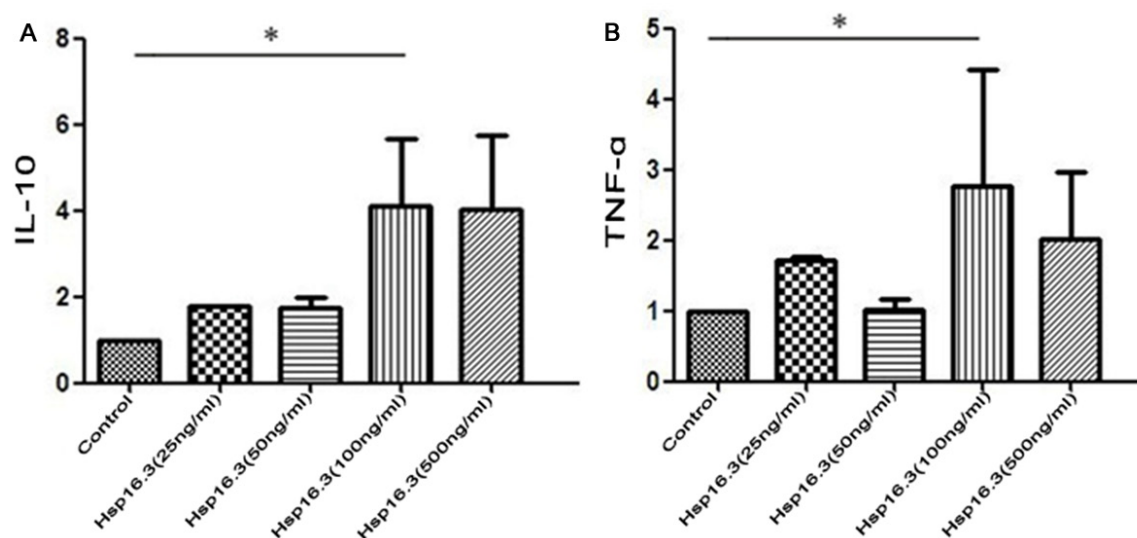
sequencing result indicated that the Hsp16.3 sequence in the recombinant plasmid was consistent with the its CDS region in the NCBI database (**Figure 1C**).

#### Expression, purification, and determination of the Mtb Hsp16.3 protein

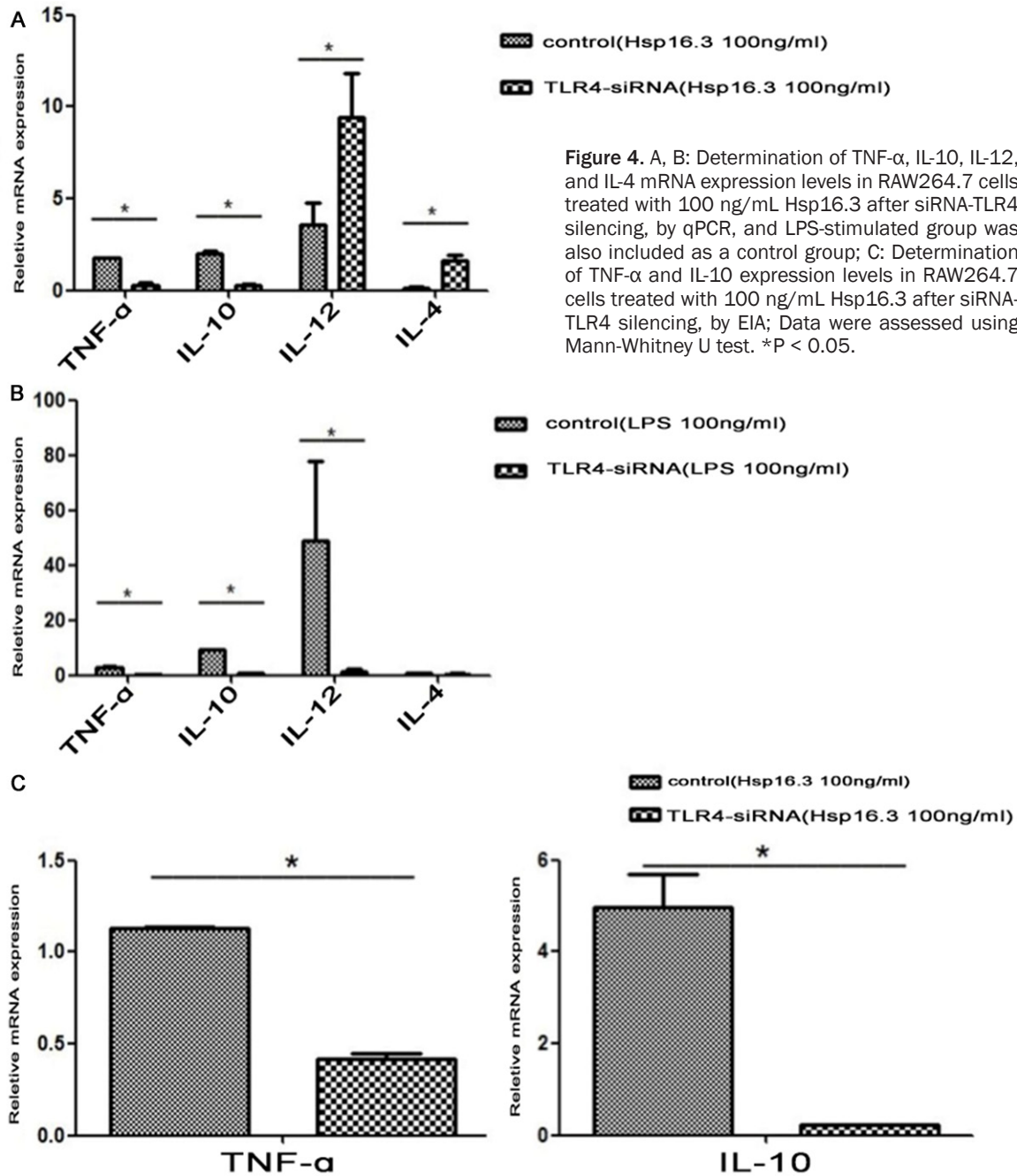
The protein target band could be observed at about 20 kDa after induction by IPTG (**Figure 2A**). After purified by nickel column, the concentration of Interest protein was 80 ug/ml measured by the BCA assay (**Figure 2B**). The expressed protein were confirmed by western blot by using sheep antibodies against Hsp16.3 and His-tag, the fusion protein band of pET28a-



**Figure 2.** Expression, purification, and identification of the Mtb Hsp16.3 protein. A. SDS-PAGE result of protein expression after induction by IPTG (M: protein marker; 1: pET28a-Hsp16.3 in the supernatant of non-induced cells; 2: pET28a-Hsp16.3 in the supernatant of induced cells; 3: Pet28a-Hsp16.3 in the pellet of non-induced cells; 4: pET28a-Hsp16.3 in the pellet of induced cells); B. Purification of Mtb Hsp16.3 protein (M: Mr protein marker; 1: the flow-through of nickel column; 2: nickel column-purified protein); C. Western blot of Mtb Hsp16.3 protein (M: Mr protein marker; 1, 2: His-Hsp16.3 fusion protein); D. MS/MS identification.



**Figure 3.** Determination of TNF-α and IL-10 mRNA levels in RAW264.7 cells after treatment with Hsp16.3 for 48 h by qPCR; data were assessed using Mann-Whitney U test. \* $P < 0.05$ .



Hsp16.3 was detected at  $M_r$  of about 20,000 (Figure 2C). The purified proteins were subjected to tandem mass spectrometry (MS/MS) identification and the amino acid sequence of the selected protein was consistent with that of the Mtb Hsp16.3 protein in NCBI database (Figure 2D).

#### Real-time PCR analysis of TNF- $\alpha$ and IL-10 expression in the cells

qRT-PCR was used to determine the mRNA levels of TNF- $\alpha$  and IL-10 in RAW264.7 cells after

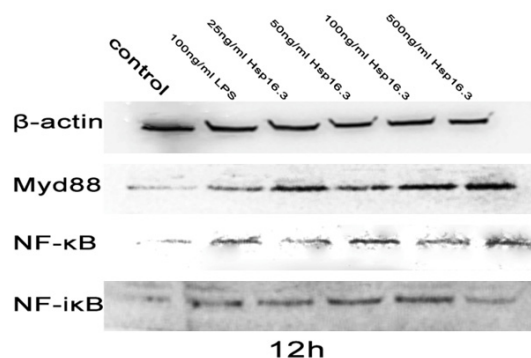
treated with different concentrations of Mtb Hsp16.3 protein. The mRNA levels of TNF- $\alpha$  and IL-10 in RAW264.7 cells were significantly increased (\* $P < 0.05$ ) after treatment with 100 ng/mL Mtb Hsp16.3 for 48 h (Figure 3).

#### Determination of TNF- $\alpha$ and IL-10 expression levels in each group after siRNA-TLR4 silencing by qPCR and EIA

Levels of TNF- $\alpha$ , IL-10, IL-12, and IL-4 were determined by qRT-PCR and EIA in RAW264.7



## MTB HSP16.3 activates macrophages by TLR4/MyD88 pathway



**Figure 5.** Determination of MyD88, NF-κB, and NF-κB protein expression levels in each experimental group by western blot.

cells after using small interference RNA-mediated TLR4 silencing (**Figure 4**). Compared with the control cells, a decrease in the levels of TNF-α and IL-10 and an increase in the levels of IL-12 and IL-4 were observed in TLR4-silenced cells (\*P < 0.05).

### *Determination of protein expression levels of MyD88, NF-κB, and NF-κB in each experimental group by western blot*

To determine whether macrophage activation in response to stimulation with Mtb HSP16.3 proceeds through MyD88 dependent pathways, we detected the levels of MyD88, NF-κB, and NF-κB by western blot. MYD88-NF-κB signal transduction was enhanced in RAW264.7 cells after treated with different concentrations of Mtb Hsp16.3 for 12 h (**Figure 5**), which indicates that Mtb Hsp16.3 might induced the inflammatory response by RAW264.7 cells via TLR4 in the early phase.

### Discussion

The host macrophage provides a platform for Mtb-antigen presentation to natural killer cells as well as long-term survival of Mtb. It has been widely recognized that macrophage is participated in the innate and adaptive immune response to infection. For instance, IFN-γ-induced and -activated macrophages induce the classical Th1 response, which initiates a direct killing response to intracellular pathogens such as Mtb. The Th2 response could result in an antibody-mediated immune response to specific antigens with large quantities of cytokines such as IL-10 [17, 18].

Hsp16.3 is reported to be a promising candidate for TB vaccines owing to the induction of Th1-type T cell immunity and Hsp16.3-mediated protection against tuberculosis depends on its chaperoning of a mycobacterial molecule [19]. Haile et al. have reported that Hsp16.3 was expressed at aerobic conditions and restricted oxygen supply, and was high expressed during slow growing period [20]. Moreover, Hu Y et al. found that Hsp16.3 knock-out in *Mycobacterium tuberculosis* leads to shorter antibiotic treatment and lower relapse rate in a mouse model [21]. These data indicated that Hsp16.3 might contribute to survival and growth for Mtb in host macrophages. However, very little is known about how Hsp16.3 elicits host immune responses. Thus, we wanted to explore the possible biological effects of Hsp16.3 in the present study.

The type of macrophage activation is an important factor to determine Th1- or Th2-type immune response. The macrophages contribute to the regulation of the strength and type of immune response, where a Th1-type immune response exerts a bactericidal effect. Our results show that the TNF-α and IL-10 levels were significantly increased in Mtb Hsp16.3 stimulated RAW264.7 cells (**Figure 3**), suggesting that Mtb Hsp16.3 affected macrophage function by stimulating the secretion of TNF-α, IL-10. TNF-α is necessary for the activation of macrophages, and is a pro-inflammatory cytokine, involved in the early inflammatory response. Classical activation of macrophages results in the upregulated expression of the surface molecules MHC II and B-7 and enhanced antigen presentation and killing ability; IL-10 causes downregulation of MHC class II molecules, mannose receptors, and scavenger receptors, and reduced macrophage phagocytosis of antigens, thereby inhibiting the function of macrophages [22]. Therefore, Mtb Hsp16.3 protein presumably induces macrophage differentiation via classical activation.

Previous studies suggest that the interaction between *M. tuberculosis* and the various TLRs is complex, and it appears that distinct mycobacterial components may interact with different members of the TLR family [23-25]. TLR2 and TLR4 have been widely conformed its activation effect in Mtb-induced macrophage [5-7, 9]. In this study, we found that the down-regulation of RAW264.7 cell-surface TLR4 mol-

ecules by exposure to Mtb Hsp16.3 (100 ng/mL) for 48 h resulted in a significant reduction in TNF- $\alpha$  and IL-10 expression levels, while IL-12 and IL-4 expression levels were significantly increased (**Figure 4**). These data indicates that except TLR4 ligand, other receptors may also be involved in Hsp16.3 MTB-mediated macrophage activation.

To extend our study, we investigated the TLR4 downstream adaptor molecules in Mtb HSP-induced signaling. Compared to the control group, Mtb Hsp16.3 (100 ng/mL) stimulation group and lipopolysaccharide (LPS) (100 ng/mL) positive-control group showed a higher expression of MyD88 and NF- $\kappa$ B (**Figure 5**), suggesting that Mtb Hsp16.3 may interact with TLR4 to activate the NF- $\kappa$ B-MyD88 signal pathway and stimulate macrophages to secrete inflammatory cytokines.

Conclusively, the results in this study have shown that Mtb Hsp16.3 may play an important role in Mtb-induced activation of macrophages via regulating TLR4/NF- $\kappa$ B/MyD88 pathway. Our findings might provide the basis for further exploration of the potential role of Hsp16.3 in tuberculosis immune defense.

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# Disclosure of conflict of interest

None.

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