

Effect of recombinant Mce4A protein of *Mycobacterium bovis* on expression of TNF- α , iNOS, IL-6, and IL-12 in bovine alveolar macrophages

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Abstract The pathogenesis of tuberculosis-causing *Mycobacterium bovis* is largely due to its ability to enter and survive in alveolar macrophages. Its mechanism of entry, mediated by proteins encoded by mammalian cell entry (mce) genes, is important for its pathogenesis. Here we focussed on the role of the Mce4A protein in the pathogenesis of *M. bovis* in cattle. Cell livability decreased in a dosage-dependent manner when Mce4A proteins were used to stimulate alveolar macrophages, which suggested that the recombinant Mce4A protein significantly inhibited alveolar macrophage activity. To test whether Mce4A modulates the gene expression profile of alveolar macrophages, alveolar macrophages were stimulated by Mce4A protein and other proteins/ligands (such as MtbPPD, MbPPD, and BCG), followed by real-time RT-PCR assay for the mRNA expression level of TNF- α , iNOS, IL-6, and IL-12. The results showed that the expression of TNF- α , iNOS, and IL-6 in alveolar macrophages was up-regulated by stimulation with the recombinant Mce4A protein of *M. bovis*; in contrast, expression of IL-12 was unaffected.

MbPPD and BCG up-regulated the mRNA expression of TNF- α , iNOS, IL-6, and IL-12 ($P < 0.05$), whereas MtbPPD stimulated the mRNA expression of TNF- α , IL-6, and IL-12 with no effect on iNOS. This study suggests that Mce4A proteins may induce the body's inflammation response to *M. bovis* and therefore may play an important role in the immune response.

Keywords *Mycobacterium bovis* · Mce4A protein · Bovine alveolar macrophage · Real-time RT-PCR

Introduction

In 1992, *Mycobacterium tuberculosis* (*M. tuberculosis*) which causes the disease of tuberculosis (TB) began its comeback as a major world-wide public health problem. By 2002, one-third of the world's population was infected with TB, which now causes more deaths each year than any other infectious bacterium [1, 2]. In addition, more than 50 million cattle are infected with *M. bovis*, the causative agent of bovine tuberculosis (BTB) [3]. In China, the active lung tuberculosis affects about 4.5 million people mostly caused by *M. tuberculosis* infection and 5 ~ 10% by *M. bovis* infection [4], which is highly related to *M. tuberculosis*.

Mycobacterium bovis is a classic intracellular bacterium, locates in macrophages of the host, and multiply intracellularly and primarily in macrophages. *M. tuberculosis* causes serious disease as a result of its ability to enter and survive in human macrophages [5, 6]. Thus, understanding its mechanism of entry into human macrophages would help researchers develop new drugs or vaccines to control TB.

Mycobacterium tuberculosis enters human macrophages by a process that involves its mammalian cell entry (mce)

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genes [7]. This fact was illustrated in an experiment in which non-pathogenic *E. coli* containing a cloned *mce* locus from *M. tuberculosis* gained the ability to enter mammalian cells and survive inside the macrophage [7]. In another experiment, a mutant with a disrupted mycobacterium cell entry gene from Bacille Calmette Guerin (BCG) in *M. bovis*, exhibited a reduced invasiveness for epithelial cells [8].

There are four *mce* operons in the *M. tuberculosis* genome, coding for putatively exported proteins [9], and sharing similarities in sequences and organizations. Previous studies revealed that *mce* proteins played important roles in pathogenesis and immune response. Knock-out mutants with modifications of *M. tuberculosis* *mce1*, *mce2*, and *mce3* operons attenuated *M. tuberculosis* virulence [10]. The *mce2* and *mce3* mutants induced a higher level of immune protection in Balb/c mice than wild type by lessening tissue damage (pneumonia) and lowering colony-forming units (CFU) [10]. In addition, *mce1* virulence operons were obtained from phagocytized bacilli from *M. bovis* during infection of cultured primary human macrophages [11]. *M. tuberculosis* *mce* operons were expressed differentially under different culture conditions and during different infection stages [12, 13]. *Mce1* and *Mce3* proteins are expressed during natural infection in humans, as identified by TB patients' antibodies [14, 15]. *Mce* genes are conserved in all members of the *M. tuberculosis* complex, except that the *mce3* gene is absent in *M. bovis* and in a subgroup of *M. africanum*, *M. microti*, and *M. pinnipedii* [16, 17].

We hypothesized that *mce* proteins may directly interact with the host macrophages and modulate alveolar macrophage function and cytokine gene expression profile. To test this hypothesis, we first initiated the present study of the *Mce4A* protein, a putatively secreted protein and a member of the *mce* protein family in *M. bovis* (<http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nucleotide&val=31791177>). To investigate the interaction of *mce* proteins with alveolar macrophages, the *Mce4A* gene from *M. bovis* was cloned, and expressed in *E. coli*, followed by purification. Bovine alveolar macrophages stimulated by recombinant *Mce4A* protein displayed inhibited macrophage activity, and their cytokine expression profiles regulated by *Mce4A* protein were also detected.

Materials and methods

Mycobacterial cultures

Bacille Calmette Guerin (BCG, Beijing strain, Chinese center for disease control and prevention, CDC, China) and *M. bovis* strain 93006 (China Veterinary Culture Collection

Center, CVCC, China) were grown at 37°C with shaking in Middlebrook 7H9 broth containing 10% albumin dextrose catalase supplement (Difco, West Molesey, UK).

Cloning, expression, and purification of *Mce4A*

DNA from *M. bovis* was extracted according to the cetyltrimethylammonium bromide (CTAB) method [18]. The open reading frame (ORF) of the *Mce4A* gene (Mb3529c, 1,128 bp) was PCR-amplified from the genomic DNA of *M. bovis*. Primer sequences of *Mce4A* were 5'-atatgtaccgcagtcgctgacttatcttctgt-3' (forward) and 5'-ttaagagctctcagaagtcgtcccggtccg-3' (reverse). PCR conditions were as follows: 5 min at 98°C, 32 cycles of 1 min at 94°C, 1 min at 62°C, 1.5 min at 72°C, and a 10-min extension period at 72°C. The PCR fragments were purified by E. Z. N. A.[®] Gel Extraction Kit (Omega Bio-tek, Doraville, GA, USA), digested with *Kpn* I and *Sac* I, and cloned into *Kpn* I and *Sac* I sites of the expression vector pET30a(+) with six histidine tags (Invitrogen, Carlsbad, CA, USA). The generated construct was further transformed into the BL21(DE3) strain of *E. coli*. The clones were confirmed by digestion of *Kpn* I and *Sac* I and sequencing. The expressed His-tagged recombinant protein was purified with nitrilotriacetic acid (Ni-NTA) agarose columns (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Renaturation was performed by dialysis of purified protein gradually in 20 mM Tris-HCl buffer, pH 7.4, which contained successively decreasing urea concentrations (8 M, 6 M, 4 M, 2 M, and 0 M, separately) and 100 mM NaCl, 4 M arginine, 1 mM PMSF, 1 mM GSH, and 0.1 mM GSSG. All proteins were concentrated by concentrator (Millipore, Massachusetts, USA) and dialyzed with phosphate buffered saline (PBS), then proteins in the dialyzed PBS buffer (without 4 M arginine and urea) were collected and identified by SDS-PAGE and Western blot analysis. The concentration of protein was analyzed by spectrophotometer (BioPhotometer, Eppendorf, Germany) and the data were analyzed using AlphaImager 2200 software (Alpha Innotech, California, USA).

Western blot assay

Refolded *Mce4A* protein (0.4 mg ml⁻¹) was boiled and electrophoresed on a 12% acrylamide gel with SDS. After electrophoresis, the proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Roche Applied Science, Indianapolis, IN, USA). After incubation in blocking buffer (Tris-buffered-saline with 5% lyophilized skim milk), the membrane was incubated in TBST buffer (Tris-buffered-saline with 0.2% Tween), Anti-His-Tag Monoclonal Antibody (New England Biolabs (Beijing)

LTD;1:2000 dilution) for 1 h at room temperature. The membrane was then washed five times with TBST for 5 min each before peroxidase-conjugated goat anti-mouse IgG (New England Biolabs (Beijing) LTD, 1:2000 dilution) was added. After washing five times with TBST for 5 min each, the membrane was stained with DAB (3,3'-Diaminobenzidine) (Roche Applied Science) and scanned to collect image data.

Isolation of alveolar macrophages

Alveolar macrophages were isolated by lung lavage. Briefly, the lungs and trachea were removed from freshly slaughtered Chinese Simmental cattle (two-year-old, male) and lavaged with approximately 2 l of Dulbecco's modified Eagle's medium (DMEM) (Invitrogen). The lavage fluid was strained through sterile gauze to remove tissue. The strained fluid was washed three times in DMEM, and the recovered cells were plated at 1×10^6 cells ml^{-1} in DMEM containing 10% fetal bovine serum (FBS) and fungizone. Non-adherent T cells were removed 2 h later and fresh medium was added. The cells at this step were >96% macrophages as assessed by α -naphthyl acetate esterase staining. Alveolar macrophage cells were cultured in DMEM culture medium for 24 h (5% CO_2 at 37°C).

Effect of Mce4A on viability of alveolar macrophages

The cultured macrophages (1×10^5 ml^{-1}) for 24 h, the cells were stimulated for 24 h and 48 h by the Mce4A protein (50, 100, 200, 400 pg ml^{-1}), each protein sample was repeated for four times in this experiment. For a control, we used cultured macrophages (1×10^5 ml^{-1}) with addition of PBS buffer in the same volume without Mce4A protein. After the 24 h or 48 h stimulation period, 20 μl MTT [3, (4,5 dimethylthiazol-2yl)-2, 5 diphenyl-tetrazolium bromide] was added to each sample. After 4 h, dimethyl sulfoxide (DMSO) was added and the sample was kept at 37°C for 10 min. Then each sample was observed under an optical microscope, and the absorbance were measured at 490 nm.

RNA extraction and detection of the mRNA expression of TNF- α , iNOS, IL-6 and IL-12p40 and by real-time RT-PCR

The alveolar macrophage cells were cultured in DMEM culture medium for 24 h (5% CO_2 at 37°C), stimulated by the proteins for 48 h, Mce4A protein, purified protein derivative of *M. tuberculosis* (MtbPPD, a gift from Chinese center for disease control and prevention, CDC, China), purified protein derivative of *M. bovis* (MbPPD, a gift from

China Veterinary Culture Collection Center, CVCC, China) and BCG (1×10^6 cfu ml^{-1}) with a final protein concentration of 400 pg ml^{-1} . The alveolar macrophage cells were maintained at 37°C for another 48 h before scrape harvesting and counting. All these experiments were repeated three times.

The culture medium was discarded, then RNA-Solv reagent (Omega Bio-tek, Doraville, GA, USA) was added. RNA was extracted according to the manufacturer's instructions and then treated with DNAase I (Takara, Kyoto, Japan). The DNAase-treated total RNA (0.5 μg) was reverse transcribed with oligo(dT)₁₈ primer using the ImProm-IITM Reverse Transcription System (Promega, Madison, WI, USA) for cDNA synthesis.

Real-time PCR assay was performed as previously described [19] using the DNA Engine OpticonTM2 Continuous Fluorescence Detection System and DyNAmoTM SYBR[®] Green qPCR kit (MJ Research Inc., Waltham, MA, USA). The expression levels of the genes for IL-6, and IL-12 p40, iNOS, and TNF- α were then measured by real-time quantitative PCR using the DyNAmoTM SYBR[®] Green qPCR kit (MJ Research, Waltham, USA) on a DNA Engine Opticon TM2 fluorescence detection system (MJ Research Inc.). The sequences of the PCR primers were 5'-gagggaaatcaggaaatgt-3' (forward) and 5'-ttctccagcaggtcagtggt-3' (reverse) for IL-6, with a size of 121 bp; 5'-catcaggacatcatcaaac-3' (forward) and 5'-aacgcagggaagaagtagga-3' (reverse) for IL-12 p40, with a size of 135 bp; 5'-cagccccctccagtcagtgga-3' (forward) and 5'-gactattcccgtgctgccc-3' (reverse) for iNOS, with a size of 71 bp; 5'-cctgctgacgggtttacct-3' (forward) and 5'-atggcagacaggatgtgacc-3' (reverse) for TNF- α , with a size of 142 bp; and 5'-catcgcaatgagcgggtcc-3' (forward) and 5'-ccgtgttgccgtagagggtcc-3' (reverse) for PCR of the housekeeping gene β -actin, with an amplification size of 145 bp.

Each PCR was carried out in a 25 μl reaction volume containing 12.5 μl DyNAmoTM SYBR[®] Green qPCR mix and 0.5 μl primer pair at 20 pmol μl^{-1} each for cytokine genes or β -actin as control and 2 μl cDNA. The expression level of each cytokine gene was obtained by normalizing the quantity of cytokine transcripts to that of β -actin transcripts using a relative standard curve method. The amplified cDNA fragments of both cytokine genes and β -actin genes were cloned and the standard curves of the cycle threshold (C_t) values were obtained from amplification of serial dilutions (10 – 10^4 copies/well) of the purified recombinant plasmids. For each experimental sample, the amounts of cytokine and β -actin mRNA were determined from the respective standard curves, and the quantity of cytokine mRNA was divided by that of β -actin mRNA to obtain a normalized value for cytokine gene expression. All samples were analyzed in triplicate.

Statistical analysis

The distribution of each data set was analyzed with a Kolmogorov–Smirnov normality test and then a one-way ANOVA test by SPSS (Statistical Package for the Social Sciences, version 13.0 for Windows; SPSS Inc., Chicago, IL, USA) was used to determine if different cytokine mRNA expression levels from different treatments were significantly different. Data are shown as mean \pm SEM (standard error of the mean) and differences with $P < 0.05$ are considered to be significant.

Results

Cloning, expression, and purification of the Mce4A gene

The ORF of the Mce4A gene in *M. bovis* is 1128 bp. It encodes 376 amino acids, with cysteines at sites 289 and 342 that can form a disulfide bond and 5 glycosylation sites at 97, 161, 231, 242, and 335 (Supplementary Fig. 1 and Supplementary Fig. 2). In this study, a PCR-amplified Mce4A gene was cloned into *E. coli*, and the expressed protein from *E. coli* was analyzed by SDS-PAGE (Fig. 1A). A protein with a molecular weight of 43 kDa was detected and its expression efficiency was 52%. When the expression of Mce4A protein was induced by IPTG at concentrations of 0.2, 0.4, 0.6, 0.8, and 1 mM, SDS-PAGE results showed that the expression variance was not distinct and that 0.2 mM was the optimum induction concentration. The recombinant protein was purified using a Ni-NTA agarose system and the protein was present as a single band at about 43 kDa (Fig. 1B). The purification efficiency was about 96%.

Western blot assay

Anti-His-Tag monoclonal antibody was used to identify the purified protein from *E. coli* in Western blot analysis (Fig. 2). The data show that the Mce4A protein from *M. bovis* was successfully expressed in *E. coli* and thus could be used in further biological analysis.

Effect of the Mce4A protein on viability of alveolar macrophages

Mce4A protein at different concentrations was added to the culture of alveolar macrophages to test its effect on macrophage viability. MTT analysis at OD₄₉₀ was performed 24 and 48 h after treatment. For 24 h treatments with Mce4A protein at concentrations of 50, 100, 200, and 400 pg ml⁻¹, the viabilities of alveolar macrophages were 111.1%,

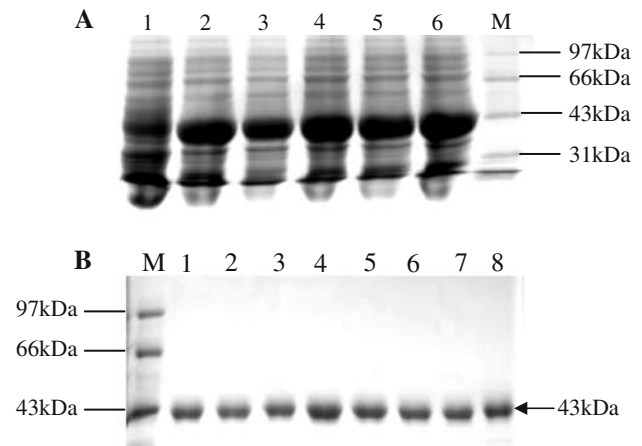


Fig. 1 (A) SDS-PAGE analysis of proteins expressed by *E. coli*. Lane 1: Mce4A protein expression in vector pET30a in *E. coli* strain BL21, without IPTG induction, as control; Lanes 2–6: pET30a-Mce4A expression in BL21 cultured for 6 h with IPTG at 0.2 mM, 0.4 mM, 0.6 mM, 0.8 mM, and 1 mM, respectively. Lane M: Molecular weight protein marker. Using Alphamager 2200, a protein with mass molecular weight of 43 kDa was detected and its expression efficiency was 52%. (B) SDS-PAGE analysis of purified protein. Lane M: Molecular weight protein marker. Lanes 1–8, proteins eluted at pH 4.0 in different elution collecting fractions. Lane 1 ~ 3, the initial elution fractions, lane 4 ~ 6, the elution peak fractions, lane 7 ~ 8, the last elution fractions. The protein appeared as a single band at about 43 kDa. The purification efficiency was about 96%

123.9%, 117%, and 100%, respectively, compared to the control (Fig. 3). For 48-h treatments, the macrophage viability was 76.7%, 65.7%, 48.7%, and 47.7%, respectively, compared to the control (Fig. 3). Inhibition of macrophages activity was significant at all protein concentrations ($P < 0.05$). These results indicated that Mce4A can inhibit the viability of alveolar macrophages after 48-h stimulation.

mRNA expression of TNF- α , iNOS, IL-6 and IL-12p40 assayed by real-time RT-PCR

The mRNA expression levels of cytokines were analyzed to test if Mce4A protein had effect on cytokine expression

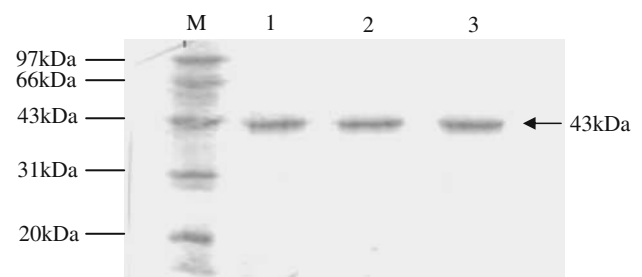


Fig. 2 Western blot assay of recombinant protein. Lane M: Molecular weight protein marker. Lanes 1–3: Expression product of the recombinant plasmid pET30a-Mce4A in *E. coli* strain BL21. Anti-His-Tag monoclonal antibody was used to identify the purified protein from *E. coli* in Western blot analysis

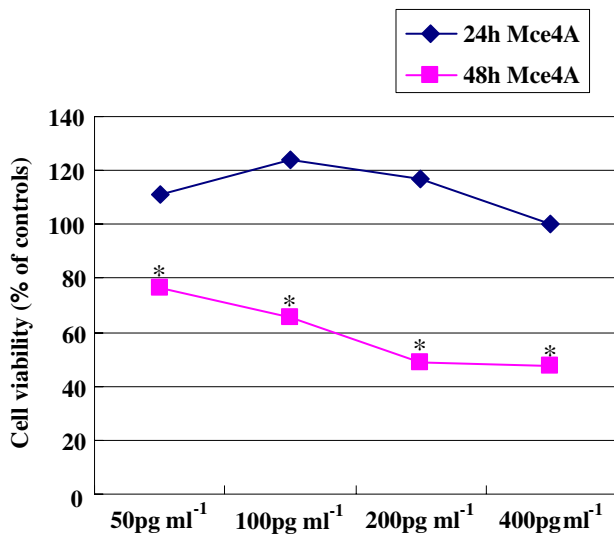


Fig. 3 Alveolar macrophages viability analysis after stimulating by different proteins with various doses. Alveolar macrophages viability analysis after stimulated by Mce4A proteins at different concentrations for 24 h and 48 h. Cell viability was assessed by MTT. Mce4A protein (400 pg ml⁻¹) cannot inhibit activity of alveolar macrophages for 24 h at $P > 0.05$. Various dose proteins inhibit alveolar macrophages viability for 48 h at $P < 0.05$. Alveolar macrophages viabilities were present as mean percentages of the untreated controls. Each treatment was repeated three times and alveolar macrophages numbers were measured four times. *, statistically significant comparing to values of control group ($P < 0.05$)

in alveolar macrophages. mRNA transcripts of TNF- α , iNOS, IL-6, IL-12, and β -actin genes from alveolar macrophages were amplified by RT-PCR and the products were confirmed to be specific by sequencing of the products (data not shown). The relative copies of all of the genes from all of the treatments are shown in Table 1. Our data showed that expression of TNF- α , iNOS, and IL-6 in alveolar macrophage was up-regulated by stimulation with the recombinant Mce4A protein of *M. bovis*; in contrast, expression of IL-12 was unaffected. MbPPD and BCG significantly up-regulated the mRNA expression of TNF- α , iNOS, IL-6, and IL-12 ($P < 0.05$). MtbPPD increased the expression level of TNF- α , IL-6, and IL-12 ($P < 0.05$) with no effect on iNOS.

Discussion

The pathogenesis of *M. tuberculosis* is largely due to its ability to enter and survive in human macrophages. Mycobacterial proteins, including MceA, which are coded by the mce genes, play very important roles in the pathogen's entry and survival in the host cell [7]. The alveolar macrophage, also called the scavenger of the body, kills and clears pathogens and heterogeneous materials via phagocytosis and initiation of inflammation response.

When an animal is infected with *M. bovis*, alveolar macrophage is the major primary target for *M. bovis* invasion. Previous studies revealed the existence of mycobacterial virulence determinants that modulated the apoptotic and/or necrosis responses to intracellular infection and supported the hypothesis that macrophage apoptosis/necrosis contributed to innate host defense in tuberculosis [20, 21]. Our current study demonstrates that exposure to Mce4A proteins inhibits the viability of alveolar macrophages and hence may influence the macrophage phagocytization function and the ability to kill *M. bovis*. The mechanism of macrophage viability change upon the exposure to Mce4A is intriguing and may be related to the apoptotic/necrosis effect of intracellular infection described above, which merits further investigation to determine if Mce4A is one of such virulence determinants. Alveolar macrophages act as antigen presenters and secret various kinds of cytokines and tumor necrosis factors to mediate the inflammation response. The microbicidal activity of macrophages in the inflammatory milieu has been related to the production of a large number of cytokines. Therefore, our results also suggested that the Mce4A proteins might have substantial impact on host cell immune response by influencing the cytokine production of macrophage.

Tumor necrosis factor alpha (TNF- α) plays an important role in the host's immune response to infection with *M. bovis* [22, 23]. It possesses multifunctional activities and is one of the most important pro-inflammatory and pro-immune cytokines [24]. TNF- α is essential for the formation of protective tuberculosis granulomas, and it regulates the

Table 1 mRNA assay of expression IL-6, IL-12, TNF- α , and iNOS after stimulation by Mce4A protein, MtbPPD, MbPPD, and BCG

	Treatment groups				
	Control	MtbPPD	MbPPD	BCG	Mce4A
INOS (M \pm S.E.M; n = 3)	0.39 \pm 0.008	0.38 \pm 0.014	0.70 \pm 0.031*	0.78 \pm 0.017*	0.64 \pm 0.026*
TNF- α (M \pm S.E.M; n = 3)	0.11 \pm 0.010	1.68 \pm 0.194*	2.02 \pm 0.144*	3.54 \pm 0.397*	1.63 \pm 0.165*
IL-12 (M \pm S.E.M; n = 3)	0.08 \pm 0.004	0.20 \pm 0.004*	0.27 \pm 0.007*	0.11 \pm 0.007	0.13 \pm 0.004
IL-6 (M \pm S.E.M; n = 3)	0.08 \pm 0.000	0.16 \pm 0.001*	0.39 \pm 0.005*	0.28 \pm 0.008*	0.56 \pm 0.007*

The quantity of cytokine transcripts was normalized to that of β -actin measured from the same sample and expressed as the fold changes over that of the respective controls. * $P < 0.05$ versus respective control values

expression of other cytokines that contribute to a protective immune response [25]. Our data revealed that Mce4A could up-regulate the mRNA expression of TNF- α in alveolar macrophages, which is consistent with the report by Shimono et al. in which a strain of *M. tuberculosis* with disrupted *mce1* operon could down-regulate the expression of TNF- α , IL-6, and monocyte chemo-attractant protein 1 of macrophages and resulted in decreased and diminished tubercular nodes [10, 26]. It has been observed by Xue et al. that Mce1A protein, a homolog of Mce4A protein, induces a proinflammatory response in macrophages [27]. In consistent with this observation, Mce4A protein may also induce a proinflammatory response in alveolar macrophages.

Nitric oxide (NO) plays a major role in the pulmonary host-defense mechanism and is implicated in bacteriostatic as well as bactericidal processes [28, 29]. In inflammatory responses, NO is produced by the inducible form of NO synthase (iNOS), which is present mostly in inflammatory cells such as macrophages [30–32]. Our data demonstrated that Mce4A proteins could promote the secretion of iNOS, which in turn would speed up the production of NO, previously shown to be bacteriostatic to *M. tuberculosis* in vitro, MtbPPD had no effect on the mRNA expression of iNOS in alveolar macrophages, which is probably because these cells are not susceptible to *M. tuberculosis* infection.

Interleukin-6 is a pleiotropic cytokine that is produced by a variety of cells, including macrophages, T cells, endothelial cells, and fibroblasts, and also exerts its effects on multiple cell types [33–36], including playing a role in the initiation of T cell activation [37], and supporting effector T cell proliferation in vivo by suppressing regulatory T cells [38]. Our results show that Mce4A proteins can up-regulate the IL-6 mRNA expression level of alveolar macrophages, which suggests that Mce4A proteins can induce the inflammation response and immune response of the body by up-regulating IL-6 expression of macrophage. MtbPPD, MbPPD, and BCG also enhanced the IL-6 mRNA expression level of the macrophage, which is consistent with previous reports [39].

In a summary, our current study demonstrated that *M. bovis* Mce4A protein could affect the viability of macrophage and enhance the mRNA expression levels of IL-6, TNF- α , and iNOS with no effect on IL-12. Therefore, this study suggests that Mce4A protein may be one of the virulent factors which directly interact with macrophage and may induce the body's inflammation response to *M. bovis* and also can play an important role in the host immune response.

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