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Enhanced immune responses in mice to combined immunization with *Mycobacterium tuberculosis* Ag85A and DDA/MPL

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The tuberculosis (TB) Bacillus Calmette-Guerin (BCG) vaccine, which is based on *Mycobacterium bovis*, has variable protective efficacy in humans; therefore, safe and effective vaccines are urgently required to prevent this disease. The antigen 85A from *Mycobacterium tuberculosis* (*M. tuberculosis*) is a prime target for immunity in the early phase of tuberculosis in various animal models. The purpose of this study was to confirm whether Ag85A could elicit protective immune responses in mice. Dimethyldioctadecylammonium (DDA), an adjuvant with Th1-promoting activities and monophosphoryl lipid A (MPL), an immunostimulatory component that has strong adjuvant activity for both cellular and humoral immune responses, has been used as the adjuvant to study the biological and immunological characteristics of tuberculosis proteins in the researches previously. The gene coding Ag85A (*fbpA*), was cloned into a pET-30a(+) prokaryotic expression vector, and the induced amino acid sequence corresponds to a 33 kDa protein, which was confirmed by mass spectrometry and western blots. Mice were subcutaneously immunized with Ag85A protein emulsified with DDA and MPL and the results showed that the 85A antigen, when combined with these adjuvants, elicited strong Ag85A-specific T-cell responses and humoral responses as compared with vaccination with BCG. Based on the fact that this vaccine combination induced strong antigen-specific immune responses, it is a prime candidate as a component of a future TB vaccine.

Key words: Tuberculosis, Ag85A, prokaryotic expression, cellular immune response, humoral response.

INTRODUCTION

Tuberculosis (TB) is one of the most serious diseases that afflict humans. About one third of the world's population is currently infected with *M. tuberculosis* (Armitige et al., 2000) and approximately 3 million people die from the TB caused by it each year. Bacillus Calmette-Guerin (BCG) is the only currently licensed

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vaccine against TB (Betts et al., 2012). Although this vaccine can induce high levels of protective immunity in animal models, its efficacy in humans differs (Magalhaes et al., 2008).

More and more researchers have concentrated on the study of TB vaccines recently; TB vaccines include viral-vectored (MVA85A, AERAS-402); protein/adjuvant (M72, Hybrid-1, Hyvac 4, H56); rBCG (VPM 1002); and DNA vaccines. However, no TB vaccine can induce stable and high levels of protective immunity in adult so far.

Some studies in murine and guinea pig models of TB have shown that culture filtrate antigens play a role as targets of protective immune responses (Pal and Horwitz, 1992; Roberts et al., 1995). The Ag85 complex antigens are important exocytosis antigens of various mycobacterial species including BCG (Launois et al., 1994; Fan et al., 2009).

This complex consists of three structurally related components, Ag85A, Ag85B (Borremans et al., 1989), and Ag85C. Ag85A is a major fraction of the Ag85 complex; in terms of its immunostimulatory properties, it is the most important component of *M. tuberculosis* and has been used in numerous vaccine preparations that that been shown to induce outstanding protective efficacy (Sugawara et al., 2007; Tanghe et al., 2000; Betts et al., 2012).

The Ag85A protein of *M. bovis* BCG or *M. tb* are actually identical; it has many T-cell-epitopes and B-cell-epitopes, one of which was synthesized as the $Ag85A_{261-280}$ polypetide in this text.

When combined with adjuvant, Ag85 complex antigens can induce immunological responses sufficient for protective immunity to occur. Adjuvants can activate immune cells such as macrophages and enhance their ability to present antigen (Korsholm et al., 2007). Adjuvants can also enlarge the surface area of an antigen, and prolong its retention time, thus allowing it to remain in contact with the lymphatic system for longer than when the antigen is used on its own. Different adjuvants induce different types of immune responses. For example, aluminum salts induce Th2 differentiation and humoral immunity (Korsholm et al., 2009). To induce Th1 responses, monophosphoryl lipid A (MPL) formulated with DDA adjuvant is used. DDA can enhance antigen uptake and antigen presentation to T cells, and stimulate dendritic cells (DCs) through Toll-like receptors. Recent studies show that DDA efficacy can be enhanced by adding immunostimulatory components such as MPL (Brandt et al., 2000).

In this study, we expressed the Ag85A protein of *M. tuberculosis* in a prokaryotic expression system. The recombinant Ag85A protein was used in combination with DDA and MPL adjuvants to immunize C57BL/6 mice and investigated its immunological characteristics in such immunized mice.

Our results may make a significant contribution to the further investigation of Ag85A as a subunit vaccine against TB.

MATERIALS AND METHODS

Bacterial strains and vectors

Escherichia coli DH5α and BL21 (DE3) strains were grown in Luria-Bertani medium and used for gene cloning and expression, respectively. *M. bovi*s BCG, DH5α, BL21(DE3), and pET-30a(+) were kept by our laboratory, whereas the pMD20-T vector was purchased from TaKaRa (Dalian, China).

Experimental animals

Six-week-old female C57BL/6 mice were purchased from VITAL RIVER (Beijing, China). The mice were housed, handled and immunized at the Animal Biosafety facilities and all procedures were approved by the institutional animal experimental committee.

Construction of the pET-30a(+)-*fbpA* recombinant plasmid

The *M.tb* vaccine BCG genome was used to design primers for PCR amplification of the *fbpA* gene. The nucleotide sequences of the two primers used for PCR amplification of *fbpA* gene are as follows: forward primer, 5-AAGCGGATCCATGTTTTCCCGGCCGGGCTTG-3, and reverse primer, 5-AGTCGAATTCTGTTCGGAGCTAGGCGCCCTGGG-3. *Bam*HI and *Eco*RI restriction endonuclease sites were incorporated into the forward and reverse primers, respectively. The 891 bp *fbpA* amplicon, purified using TaKaRa mini columns according to the manufacturer's instructions, was ligated to the pMD20-T vector using T4 DNA ligase, and then transformed into DH5α competent cells. The recombinant plasmid, which we called pMD20-T-*fbpA*, was sequenced by a commercial company (genscript,Nanjing,China) after construct verification by PCR and restriction enzyme digestion.

pET-30a (+) plasmid DNA and pMD20-T-*fbpA* were digested with *Bam*HI and *Eco*RI. Prior to ligation, the linearized plasmid DNA and insert DNA were gel purified using a TaKaRa Gel extraction kit. Ligation of *fbpA* to pET-30a(+) was performed using T4 DNA ligase and the ligation products were transformed into *E. coli* DH5a cells. The transformants were screened by *Bam*HI and *Eco*RI plasmid digestion and Ag85a-recombinants were confirmed by DNA sequencing.

The pET-30a-*fbpA* plasmid, purified from an overnight culture of recombinant *E. coli* DH5a cells, was transformed into *E. coli* BL21(DE3) competent cells. LB plates containing 50 μg/ml kanamycin were used to screen for *E. coli* pET-30a-*fbpA*-positive BL21(DE3) colonies. The Ag85A protein was expressed in the same host cells by addition of 0.5 mmol/L of isopropyl-β-D-thiogalactopyranoside (IPTG), and purified by His-Bind Purified Kit (Novagen, Germany). The concentration of purified Ag85A protein was up to 7 mg/ml and the purity was 98%.

MALDI-TOF mass spectrometry (MS)

Recombinant proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12% gels. The gel containing the induced Ag85A protein was sent for secondary mass spectrometry using the MALDI-TOF-MS protein spectrum analysis technique (CISCO, Nanjng,China).

SDS-PAGE and Western blotting

Following induction of the recombinant protein, cell lysates separated by SDS-PAGE on 12% gels were analyzed by Western blotting. Total cellular proteins were transferred onto a nitrocellulose

Table 1. The immunizations of mice.

Group	The first immunity	The second immunity
Group 1	PBS+DDA-MPL	PBS+DDA-MPL
Group 2	DDA-MPL+Ag85A protein	DDA-MPL+Ag85A protein
Group 3	Ag85A protein	Ag85A protein
Group 4	BCG	BCG
Group 5	BCG	DDA-MPL+Ag85A protein

membrane. The membrane was saturated with 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS) and then incubated with an anti-BCG mouse polyclonal antibody, followed by incubation with a goat anti-rabbit HRP-conjugated antibody diluted 1/5000 in phosphate-buffered saline containing 0.05% Tween 20, pH 7.4 (PBST). Immunoblots prepared in this manner were developed as described previously (Xie et al., 2002).

Mouse immunizations

Twenty five 6-week-old female C57BL/6 mice were randomly divided into the 5 groups: (1) the DDA-MPL+phosphate-buffered saline (PBS) group, (2) the DDA-MPL+Ag85A protein group, (3) the Ag85A protein group, (4) the BCG group and, (5) the BCG+DDA-MPL+Ag85A protein booster-immunized group. Mice were immunized two times at 2-weeks intervals subcutaneously with inoculum containing either 100 µg DDA and 25 µg MPL, 25 µg Ag85A protein, 1×10^6 colony-forming units (CFUs) BCG, or 25 µg Ag85A protein emulsified in DDA and MPL in a volume of 0.2 ml. Mice in the first group were immunized with DDA and MPL adjuvants, while mice in the second group were immunized subcutaneously with Ag85A emulsified in 100 µg of DDA adjuvant and 25 µg of MPL. Group 3 mice were immunized with Ag85A protein alone, whereas group 4 mice were immunized subcutaneously with BCG at a dose of 1×10^6 colony-forming units (CFUs). Group 5 mice were immunized with BCG at the first immunity, and then immunized with Ag85A at the second immunity (Table 1). All mice were humanely killed, dissected, and their spleens and T lymphocytes removed for immunological analysis at seven to nine days after the secondary immunization. Concurrently, serum samples were obtained from the mice for antibody detection.

Cytokine measurements

Spleen cells (2×10^7 white blood cells/ml) from five mice per group were tested individually for their cytokine responses to the $Ag85A_{261-280}$ polypeptide and the PPD of *M. tuberculosis*. 48 h later, the supernatants from at least three separate wells of a 96-well cell culture plate were pooled and stored frozen at −20°C until required. Experiments were performed at least three times, and data from one representative experiment are reported. Mouse IFN-γ and IL-4 enzyme-linked immunosorbent assay (ELISA) kits (BD Biosciences, New York, New Jersey) were used to determine the IFN-γ and IL-4 expression levels of the spleen cell suspensions, following the manufacturer's instructions.

Antigen-specific IgG assays

Antigen-specific IgG antibodies responses against the purified Ag85A protein in the serum of mice from different group at 7-9 days after the second immunization were measured by ELISA. ELISA plates were coated overnight at 4°C with 10 µg of *M. tuberculosis* PPD. PPD are the purified protein derivative of *M. tuberculosis* which contains Ag85A protein. The plates were blocked with 200 µl/well of PBS containing 1% BSA for 2 h at 37°C and washed three times with PBS containing 0.05% Tween 20. Individual serum samples were added to the wells in a serial two-fold dilution series (beginning at a 1/50 dilution), incubated for 2 h at 37°C, washed, and then 100 µl/well of horseradish peroxidase-conjugated goat anti-mouse IgG (diluted at 1/10000) was added. Plates were incubated for 1 h at 37°C, washed, and then developed with 3, 3′ 5, 5′-tetramethylbenzidine substrate. Reactions were stopped by addition of 50 µl/well of 2 M H_2SO4 and the plates were read on an ELISA plate reader at 450 nm. Sera from the group of mice treated with PBS were used as the negative control (N). Sera with P/N values that were ≥ 2.1 were considered positive. We also peformed IgG2b and IgG1 assay with the method as above.

T cell proliferation assays

T lymphocytes were washed in fresh RPMI 1640 medium (Hyclone, Beijing,China) and cultured (4×10^5 cells/well) in flat-bottom 96-well plates (Roche Products, Switzerland) in 100 µl of RPMI 1640 medium supplemented with 10% FBS and a 1% solution of a broad-spectrum antibiotic-antimycotic mixture, and then incubated with the Ag85A or medium (control) at 37°C in a humidified 5% CO_2 incubator. The Ag85A polypeptide titer was determined to obtain its optimal concentration for use in the proliferation assays (that is 10 µg/ml). All stimulants were plated in triplicate and incubated for 72 h. All subsequent steps followed the manufacturer's instructions of the Cell Proliferation ELISA BrdU colorimetric kit (Roche). The mean counts for the cultures performed in triplicate and the stimulation index (SI) were obtained for each stimulant. The SI was the ratio of the mean counts in the presence of Ag85A protein to the means counts of the medium alone.

Statistical analysis

A two-way ANOVA was used to compare differences between groups and differences were considered statistically significant when the P value was less than 0.05.

RESULTS

Construction of an expression vector containing the *fbpA* gene

To obtain recombinant Ag85A protein, the *fbpA* gene was cloned into the *E. coli* expression vector pET-30a(+), which contains a C-terminal His-tag sequence to facilitate recombinant protein purification. Genomic DNA isolated from the BCG laboratory strain was used for PCR amplification of the *fbpA* gene. Agarose gel (1%) electrophoretic analysis of the PCR-amplified product revealed

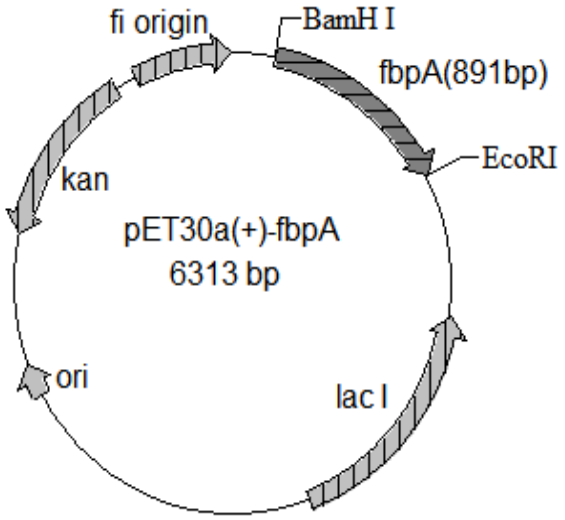

Figure 1. Construction of recombinant plasmids. The gene encoding Ag85A protein (*fbpA*) cloning from *M.bovis* genomic DNA was inserted into the pET30a(+), resulting in the recombination of plasmid pET30a-*fbpA*.

the presence of a single band of approximately 900 bp in size that was almost equal to the expected size (891 bp) of the *fbpA* gene. The PCR-amplified product was confirmed by nucleotide sequencing to be the *fbpA* gene.

During PCR amplification, *Bam*HI and *Eco*RI sites, which were incorporated into the PCR product containing the *fbpA* gene, were used to ligate the gel purified PCR product to a pMD20-T(+) plasmid containing an ampicillin resistance gene.

Following PCR and restriction digestion analysis, the recombinant plasmid, pMD20-T-*fbpA*, was commercially sequenced. After verifying that the correct sequence was present in pMD20-T-*fbpA*, the purified *fbpA* fragment was ligated to pET-30a(+) (Figure 1) and used to transform *E. coli* BL21(DE3) cells for hetero-logous expression of the protein.

The recombinant plasmids were analyzed for expression of recombinant Ag85A protein following induction with 0.5 mM IPTG. The intracellular localization of the Ag85A protein after lysis of the cells indicated that it was retained in the cytosol as an insoluble protein in the form of inclusion bodies (Figure 2a).

Identification of Ag85A protein

Because the Ag85A protein has a mass of only 33 kDa, the band could be clearly visualized by SDS-PAGE. The Ag85A protein was column-purified via its His-tag sequence under denaturing conditions. SDS-PAGE analysis of the purified samples revealed a single band with a molecular mass of about 33 kDa (Figure 2c). Western blots revealed that the recombinant Ag85A protein was specifically recognized by a mouse anti-BCG polyclonal antibody (Figure 2b).

After determining the molecular weight of the purified protein by SDS PAGE, the band was excised from the gel for analysis by MALDI-TOF-MS, a technique in which proteins with very small differences in mass can be separated. The results of the MALDI-TOF-MS analysis of the Ag85A protein is shown in Figure 3a, and the secondary mass spectrometry analysis in Figure 3b, c, d. The results confirm that the 32695 Da recombinant protein (isoelectric point of 6.04), was that of *M. tuberculosis* Ag85A.

Antibody responses to the Ag85A protein

According to the result of ELISA, mice vaccinated with DDA-MPL produced no antigen-specific antibodies. The BCG+Ag85A vaccine induced the highest IgG-specific response to PPD. The DDA-MPL+Ag85A-treated group and the BCG vaccinated group also exhibited strong IgG responses, while the Ag85A-immunized mice produced weak IgG responses. The serum antibody titer in the mice of group 2 was 1:12800 (Figure 4a). These results show that the Ag85A protein could induce production of Ag85A-specific humoral immune responses in the mice .What's more, the antibody titer of IgG2b was higher than IgG1 in group 2, which indicated that Ag85A protein had a tendency to induce Th1 type responses (Figure 4b).

Proliferative responses to mycobacterial antigens

To determine T cell reactivity to the mycobacterial antigens tested, lymphoproliferative responses were measured in the cells from the immunized mice. As shown in Figure 5, compared with the adjuvant-immunized group and the Ag85A-immunized group, the group immunized with DDA-MPL+Ag85A protein, BCG, and the booster-immunized group produced better proliferative responses (SI>2.1) upon stimulation with the synthetic Ag85A protein. Moreover, the splenocyte SI value for the DDA-MPL+Ag85A group is greater than that of the BCG group, thus indicating that the Ag85A protein induced a stronger immunological effect than that of BCG ($P<0.05$).

Cytokine profile in response to recombinant mycobacterial antigens

Splenocytes isolated from the immunized mice were stimulated with culture medium (CM), the synthetic Ag85A polypeptide, or PPD (Figure 6). Splenocytes from the DDA-MPL+PBS control group and the Ag85A group produced very low levels of IFN-γ over the whole experimental period, whether stimulated with CM, Ag85A

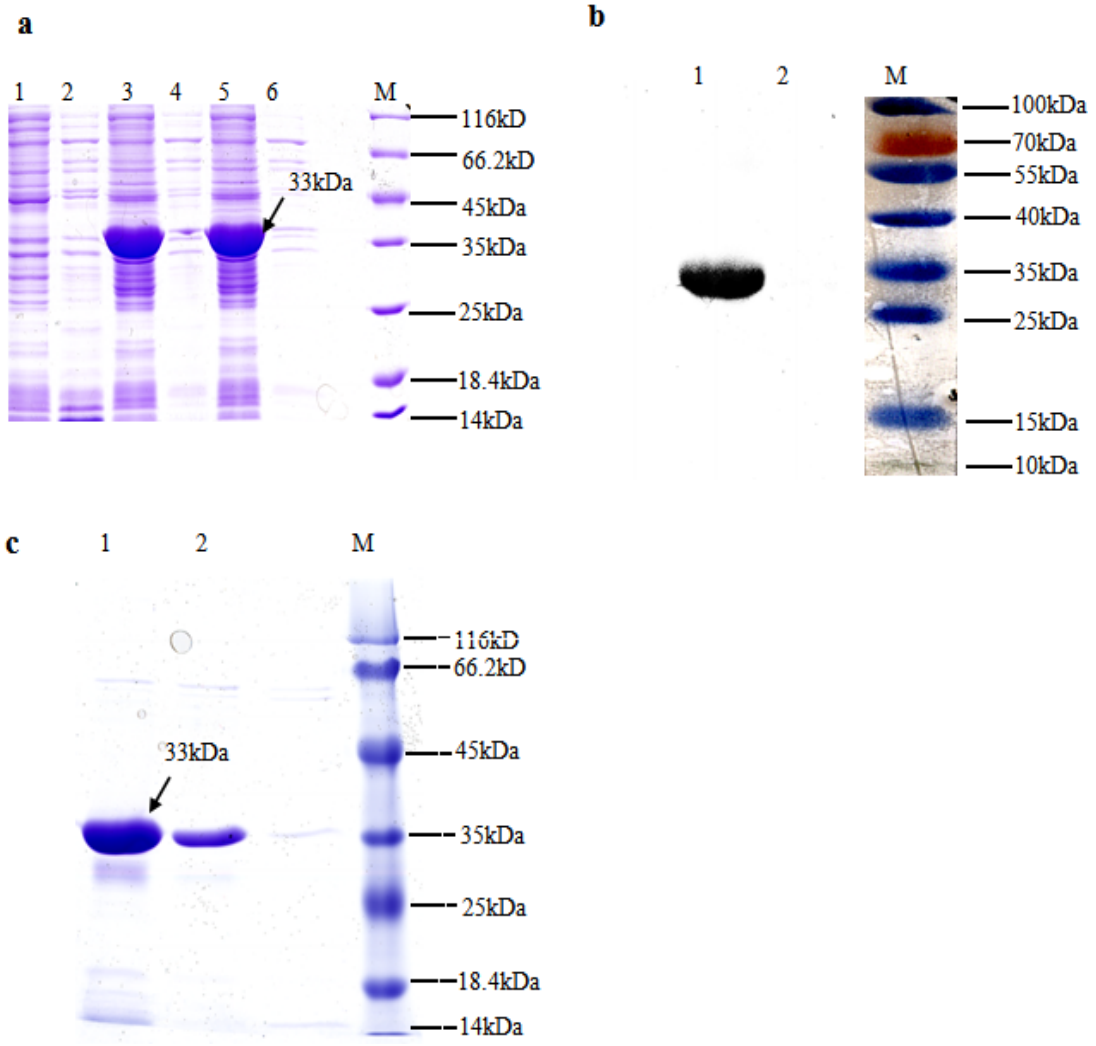

Figure 2. Expression and identification of Ag85A protein. *E.coli* BL21(DE3) harboring pET30a-fbpA was cultured with IPTG. The expression (a, c) and identification (b) of Ag85A protein was confirmed by SDS-PAGE (a, c) and Westren blotting (b). Lane a1, the precipitation of *E.coli* haboring pET30a(+); Lane a2, the supernatant of *E.coli* haoboring pET30a(+); lane a3,a5, the precipitantion of *E.coli* haboring pET30a-fbpA; lane a4,a6, the supernatant of *E.coli* haboring pET30a-fbpA; lane aM, protein marker; lane b1, the precipitantion of *E.coli* haboring pET30a-fbpA; lane b2, the supernatant of *E.coli* haboring pET30a-fbpA; lane bM, protein marker; Lane c1, c2, the purified Ag85A protein; Lane cM, protein marker.

polypeptide, or PPD. When stimulated with Ag85A polypeptide, IFN-γ levels increased significantly in the DDA-MPL+Ag85A group, the BCG group, and the booster-immunized group ($P<0.001$). The highest IFN-γ levels were observed in the booster-immunized group when stimulated with Ag85A polypeptide; these levels were much higher than those of the Ag85A and adjuvant groups ($P<0.001$). However, there were no significant differences in the IFN-γ levels between the DDA-MPL+Ag85A group and the booster immunized group. All of the groups produced lower amounts of IL-4 compared with IFN-γ regardless of whether they were stimulated with CM, Ag85A polypeptide, or PPD. Although the highest IL-4 levels were seen in the booster-immunized group, IL-4 production was lower than 200 pg/ml in all groups. Therefore, the Ag85A protein had a tendency to induce Th1 type responses in the mice, which was in accordance with the result of IgG2b and IgG1 assay.

DISCUSSION

About one third of the world's population is currently infected with *M. tuberculosis* and it is estimated that 2 million people die from TB annually (Romano et al., 2006). Vaccination is the most cost-effective strategy for TB control and an effective vaccine could eventually eliminate the disease. Currently, the only licensed

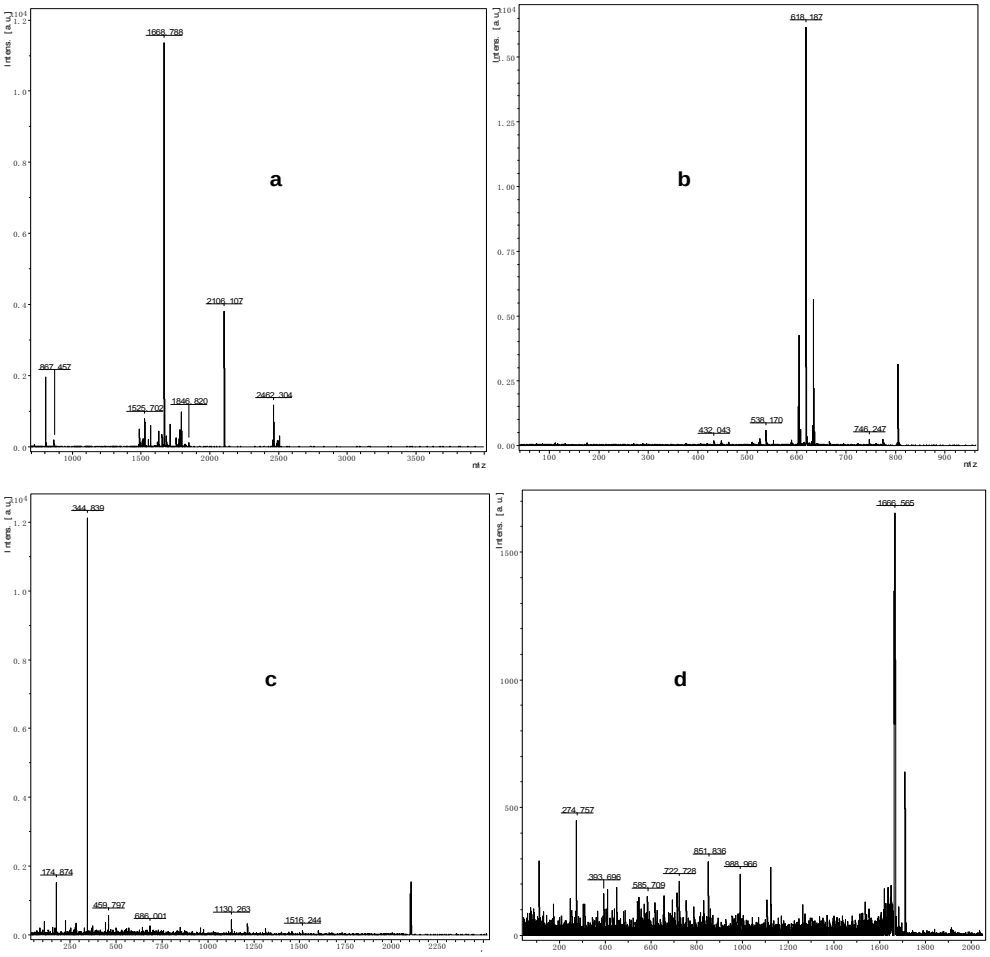

Figure 3. The result of MALDI-TOF-MS mass spectrometry. **a,** The level of mass spectrometry of Ag85A protein. **b,c,d,** The secondary mass spectrometry of Ag85A protein.

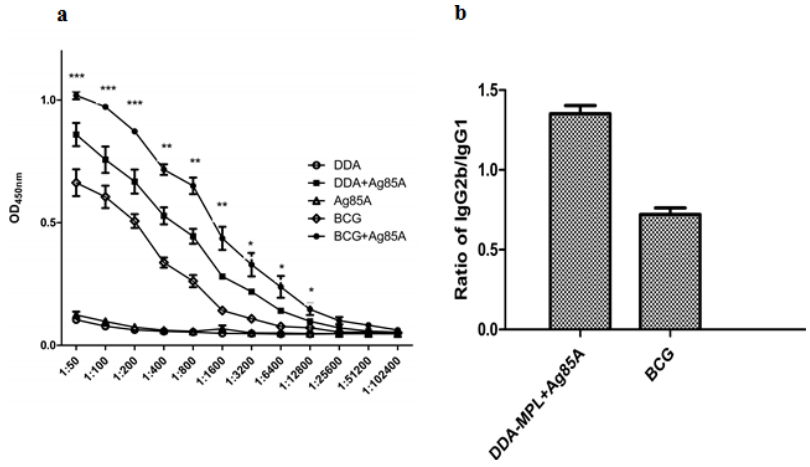

Figure 4. Antigen-specific total IgG production and the ratio of IgG2b/IgG1. C57BL/6 mice were vaccinated with different vaccines. Control groups were immunized with BCG or DDA. 8 days after the second immunization, the animals were bled and sera were obtained. PPD (10 μg/ml) were used for detection of IgG, IgG2b, IgG1 in each groups by enzyme-linked immunosorbent assay (ELISA). Detection of anti-Ag85A IgG levels in DDA-MPL+Ag85A was compared to those of other groups (a), the ratio of IgG2b/IgG1 was calculated by the titre of IgG2b and IgG1 (b).

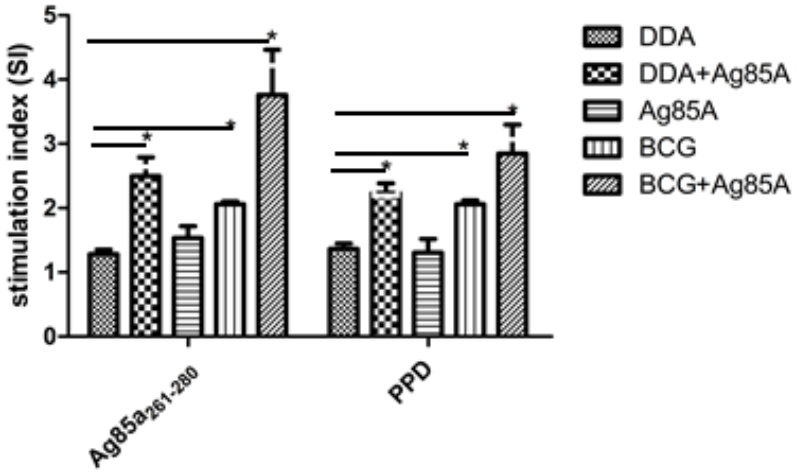

Figure 5. Lymphocyte proliferation in response to Ag85A polypeptide and PPD. Splenocytes isolated from all groups were cultured in the presence of Ag85A polypeptide and PPD(10 µg/ml) for 72 h. Results are expressed as stimulation index (SI) mean of triplicate cultures.

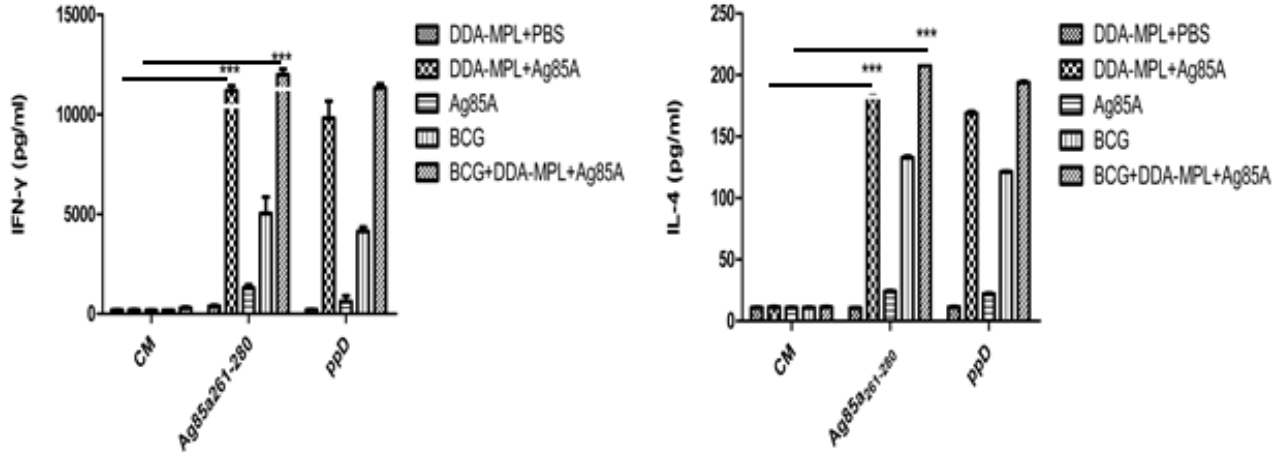

Figure 6. IFN-γ (a) and IL-4 (b) levels released by splenocytes stimulated with Ag85A polypeptide and PPD after 48 h of culture. Splenocytes were prepared and pooled from five mice per group 8 days after the last vaccination and were then stimulated in vitro with Ag85A polypeptide, PPD, and CM. The level of IFN-γ and IL-4 after 48 h incubation was analyzed. The experiments were repeated three times with similar results, and the data from one representative experiment is shown. The results are expressed as the mean (SD) amount of IFN-γ and IL-4 (pg/ml) of each group. A P value less than 0.05 was considered to be significant and is presented as follows: *p < 0.05, **p < 0.01, ***p < 0.001.

vaccine against TB for use in humans is BCG, which has been in use since 1921 (Spencer et al., 2012); however, its efficacy is highly variable, and particularly so for protection against adult pulmonary TB. Therefore, there is an urgent need for a vaccine with better protection against adult pulmonary disease than that afforded by BCG.

M. tuberculosis secreted proteins, as opposed to cytoplasmic proteins (Fan et al., 2009), have generally been considered as strong candidates for a vaccine. Thus, much research has been focused on *M. tuberculosis* secretory antigens. Ag85A and Ag85B are the major secreted proteins in *M. tuberculosis* culture filtrates; both belong to the antigen 85 (Ag85) complex. As a major fraction of this complex, Ag85A is the most essential component involved in *M. tuberculosis* immunostimulation (Lozes et al., 1997). Recently, different gene transfer systems such as modified vaccinia

virus Ankara (Dai et al., 2013), rBCG (Rahman et al., 2012), and plasmid DNA (Tanghe et al., 2000) have been used to express Ag85A. However, compared with these systems, the Ag85A protein (with adjuvant) has the advantage that it can generate antigen-specific immune responses that are enhanced by boosting, thus offering the potential for a safe, effective and targeted vaccine against TB.

Ag85A is usually emulsified with adjuvants that enhance its ability to induce protective immunity. The adjuvants used for enhancing the immunogenicity and protective efficacy of Ag85A are DDA, CpG7909. Studies show that CpG7909 is able to enhance the immunological effects of Ag85A (Hu et al., 2013), but it does not confer significant protective efficacy against infection with *M. tuberculosis*. In contrast, Brandt's research has shown that DDA-MPL can induce similarly high levels of immunity as BCG (Brandt et al., 2000). DDA can enhance antigen uptake, antigen presentation to T cells, and stimulate DCs through Toll-like receptors. MPL can stimulate macrophages to release cytokines and enhance antigen uptake, as well as stimulating antigen processing and presentation. Recent studies show that the effect of DDA can be enhanced by the addition of MPL.

Recently, Ag85A has been expressed in different gene transfer systems. One study (Wang et al., 2012) using a recombinant BCG strain over expressing the Ag85A antigen showed that there was no significant difference between the rBCG::85A protein and the BCG group. Additionally, lower levels of IFN-γ were detected in splenocyte cultures derived from C57BL/6 mice vaccinated with the Influenza A virus expressing Ag85A, and the IFN-γ levels of the vaccinated mice were below 200pg/ml (Dai et al., 2012). Furthermore, it was found that the relative mRNA expression level of a DNA vaccine expressing Ag85A protein was lower than that obtained by BCG (Lu et al., 2011). In our study, we used the Ag85A protein emulsified with DDA-MPL adjuvants to immunized C57BL/6 mice subcutaneously, and found that IFN-γ secretion was higher than BCG, while IFN-γ production was >12000 pg/ml. The IFN-γ levels in the booster group were higher than those of the DDA-MPL+Ag85A group. Thus, the Ag85A protein, when expressed in the prokaryotic expression system used herein, induced higher levels of IFN-γ than did previously used gene transfer systems. At the same time, IL-4 levels in all of the groups of mice were relatively low. Taken together, these results indicate that Ag85A can induce Th1-type cellular immune responses in vaccinated mice. Owing to the characteristic of tuberculosis, cellular immunity, especially Th1-type cellular immunity plays a major role during the process of resisting tuberculosis. Therefore, the ability of inducing Th1-type cellular immunity may confer Ag85A protein the protective immunity for TB. Moreover, the lymphoproliferative assay shows that the Ag85a polypeptide can increase splenocyte proliferation *in vitro* (after the second immunity).

Compared with the 1:2000 serum antibody titer obtained from vaccination with plasmid DNA (Lu et al., 2011), the ELISA result in this study showed that the Ag85A protein induced a stronger humoral immune response, which was greatly depended on the B-cell epitopes of Ag85A protein. What's more, apart from the induction of strong cell-mediated immunity responses, DDA and MPL also could stimulate the host humoral immune response. The result of IgG2b and IgG1 assay indicated that Ag85A protein had a tendency to induce Th1 type response which was in accordance with the cytokine measurement assay.

Previous studies have shown that repeated vaccination with BCG may be detrimental to protection (Rodrigues et al., 2005); therefore, booster vaccinations with heterologous antigen are likely to be used to enhance the specific immunity primed by BCG (Kaufmann, 2005). Consequently, we used the Ag85A protein to boost the immunity of mice previously immunized with BCG. The results suggest that irrespective of whether it was a humoral or a cellular immune response, the booster group exhibited a stronger immune response than did the DDA-MPL+Ag85A group. Overall, the Ag85A protein expressed in the prokaryotic expression system had similar immunogenicity and immune reactivity as the natural protein, making it worth considering as a vaccine candidate. Because the Ag85A protein is only the antigen molecule of *M. tuberculosis*, it is likely to be safer than live vaccines (such as rBCG) for the immune-compromised people (for example, AIDS patients). Furthermore, the Ag85A protein and adjuvant can be combined to eliminate irrelevant or immune-suppressive components of the whole bacterium, which might enable it to induce a stronger immune response than that of the whole bacterium. Therefore, we consider that our current study serves as an important step for future investigation of a subunit vaccine against TB.

Conflict of Interests

The authors have not declared any conflict of interests.

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