

Full Length Research Paper

MPB64 DNA vaccine with immunogenicity and efficacy against tuberculosis

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Tuberculosis is a serious infectious disease caused by *Mycobacterium tuberculosis*. DNA vaccination is an advanced technique for protecting human bodies from infectious diseases including tuberculosis by injecting exogenous gene engineering DNA into the body to produce an immunological response. In this study, we examined the immunogenicity and protective efficacy of DNA vaccine (pCDNA-MPB64) expressing MPB64 protein and its booster effects in mice for controlling tuberculosis. The results showed that MPB64 DNA vaccine led to a dramatic augmentation of humoral and cellular responses. All these suggested that MPB64 DNA vaccines is an ideal vaccine and may be further developed as a useful method to prevent tuberculosis.

Key words: Tuberculosis, DNA vaccines, MPB64, recombination plasmid, antibody titer.

INTRODUCTION

Tuberculosis is a common, serious and sometimes lethal infectious disease caused by *Mycobacterium tuberculosis*. Tuberculosis usually attacks the lungs, while sometimes it also attacks other organs of human body such as bone, ovary and stomach (Sala et al., 2011). The main distribution of tuberculosis is located in many Asian and African countries. The most common vaccine against tuberculosis currently is Bacillus Calmette-Guérin (BCG), prepared from a strain of the attenuated or weakened live bovine tuberculosis bacillus. However, the protection effect of BCG sometimes is not certain, sometimes the immune results are not well as expected (Rouanet et al., 2010). What is more, BCG could interfere the test of

purified protein derivative (PPD, also known as the Mantoux screening test), a diagnostic tool for tuberculosis, making the PPD test very confused (Kernodle et al., 2010). Moreover, it makes the routine quarantine more difficult after vaccinated with BCG since natural infection and artificial immunity would be undistinguished. And the safety of BCG is still need to be improved. So it is very important to discover the new vaccine for the diagnosis and prevention of the tuberculosis.

DNA vaccines are one of the ideal vaccines. They are third generation vaccines, made up of a plasmid containing exogenous genetically engineered DNA in order to produce antigens (Kaufmann et al., 2011; Martín et al., 2011). DNA vaccines elicit a quick and ideal immunological response when highly active expression vectors used and thus result in an ideal protection for human bodies (Hawkrigde et al., 2011). No risk for

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infection, long-term persistence of immunogen and focused immune response are the advantages of new DNA vaccines compared with the other traditional vaccines (Frantz et al., 2011; Okada et al., 2011; Liang et al., 2011). What's more, ease of development, production, storage and shipping, together with cost-effectiveness, are also the advantages of new DNA vaccines (Hanif et al., 2010). Thus, it is important to design a new DNA vaccine to better prevent tuberculosis. This is the main task and purpose of this research.

MATERIALS AND METHODS

Construction of recombinant vector pCDNA-MPB64

Genes coding MPB64 was amplified by PCR with primers and the genomic DNA of *M. tuberculosis* H37Rv as the template. The PCR products were first digested with *Bam*HI and *Eco*RI and then cloned into the corresponding sites eukaryotic expression vector pCDNA, resulting in recombinant plasmids named pCDNA-MPB64, respectively. The correctness of recombinant plasmids was confirmed by DNA sequencing and enzyme digestion. Plasmids pCDNA-MPB64 for DNA immunization were transformed into competent *Escherichia coli* DH5 α , and endotoxin-free plasmid DNA was purified using the Qiagen Plasmid Giga kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The purity of the recombinant vector was detected by ultraviolet spectrophotometer and evaluated according to OD260/OD280.

Transfection of BHK-21 Cells and detection

BHK-21(ATCC) cells were transfected with pCDNA-MPB64 and pCDNA by Lipofectamine 2000 (Invitrogen, USA). Cells at 90 to 95% confluence were transfected with 5 μ g vectors. After 6 h exposure, the normal culture medium, RPMI 1640 supplanted with 15% horse serum (Gibco, USA) was added into cells for another 18h-42h. RT-PCR and Western blot were used to detect the effect of transfection. Oligonucleotide primers of MPB64 (Up 5' TTGCTCTGTTGTTCCGGGTGTCGC 3' Down 5'AAGGCCCTTGACGTGGTCTGTTG 3') were synthesized by Takara Company. After reverse transcription, cDNA was used as the templates for PCR (PE, 9600) and PCR product was detected for the gene expression. Total protein extracted (15 μ g) from the cells was boiled at 100°C with loading buffer for 5 min, and then subjected to 12.5% SDS-PAGE. After electrophoresis, transfer was performed at 70 V 2 h at 4°C. After blocking in 5% Nonfat milk for 1 h, membranes were incubated overnight at 4°C with primary antibody (rabbit polyclonal IgG, Millipore) and secondary antibody (Goat Anti-Rabbit IgG, QIAGEN) for 1 h at room temperature in blocking solution. Photos were taken with film exposures. Beta-actin was used as control gene both in RT-PCR and Western blot.

Animal immunization

Specific pathogen-free (SPF) 6- 8- week-old, male C57BL/6 mice (Lab Animal, Chengdu, China) were bred in cages on the animal feeding cabinet in a bio-safety level 3 laboratory. Mice received free access to food and water throughout the study. The research protocol was reviewed and approved by Sichuan University Committees on Bio-safety and Animal Care and Use Committee of China. Mice were randomly divided into (20 mice in each group): non-vaccinated control, vector control pCDNA, pCDNA-MPB64, BCG. Pasmid DNA (100 μ g) was injected intramuscularly in the

same area, and immunization was repeated thrice with 2-week intervals.

Detection of antibody titer in serum in immunized mice

Blood were collected 3 weeks after immunization from mice tail. Serum were separated and stored at -20°C. ELISA was used to detect the antibody titer in serum. MPB64 was diluted to a final concentration of 10 μ g/ml in coating buffer. 100 μ l solution were transferred to each well of a high affinity, protein-binding ELISA plate and incubated at 4°C overnight. Solution was flicked off and washed 3 min/ time \times 3 times with PBST, and blocked using 100 μ l of 1% BSA in each well and incubated at 37°C for 1 h. Solution was flicked off and washed 3 min/ time \times 3 times with TPBS, Serum were diluted to desired concentrations(1:50, 1:100, 1:150, 1:200, 1:250...) in Blocking Solution and 100 μ l were added per well to the ELISA plate and incubated at room temperature for 2 h. Solution was flicked off and washed 3 min/time \times 3 times with TPBS. The HRP-labeled rabbit anti mouse IgG was diluted (1:500, Invitrogen, USA) in Blocking Solution. 100 μ l of diluted antibody were added to each well and incubated at 37°C for 2 h. Solution was flicked off and washed 3 min/time \times 3 times with PBS/Tween. 100 μ l of substrate solution was added and incubated at room temperature for 30 min avoiding light. 50 μ l of suspending solution were added and the optical density (OD) for each well were recorded with a microplate reader (Ray Biotech, Inc) set λ to 492 nm.

Detection of IL-2 in immunized mice

To detect IL-2 in serum of mice after immune vaccine, ELISA was conducted by using assay kit. The mice IL-2 enzyme-linked immunosorbent assay (ELISA) kit (Promega, USA) is an in vitro ELISA for the quantitative measurement of rats IL-2 in serum, plasma, cell culture supernatants and urine. This assay employs an antibody specific for rats IL-2 coated on a 96-well plate. Standards and samples are pipetted into the wells and mice IL-2 present in a sample is bound to the wells by the immobilized antibody. The wells are washed, and then biotinylated anti-mice IL-2 antibody was added. After washing away unbound biotinylated antibody, HRP-conjugated streptavidin is pipetted to the wells. The wells are again washed, a TMB substrate solution is added to the wells and color develops in proportion to the amount of rats IL-2 bound. The stop solution changes the color from blue to yellow, and the intensity of the color is measured at 450 nm (Ray Biotech, Inc).

Statistical analysis

All values were expressed as mean \pm SD. The results were evaluated by one-way ANOVA and Tukey's multiple comparison tests. Statistically significant differences between groups were defined as $p < 0.05$. Calculations were performed with SPSS 12.0 software (SPSS, Chicago, USA).

RESULTS

Construction and identification of expressing plasmid

The gene of MPB64 was first amplified by PCR and *M. tuberculosis* H37Rv genomic DNA as the template. The purified PCR productions were digested with *Bam*HI and

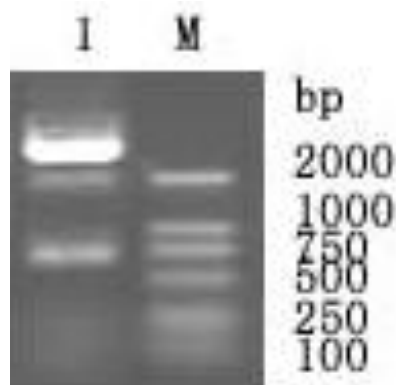


Figure 1. Construction of recombinant plasmids. Line 1: Product of pCDNA-MPB64 digested by enzyme; M:DNA Marker/DL2000.

EcoRI and separately cloned into the plasmid and pCDNA predigested with the same restriction enzymes, in order to construct eukaryotic expression plasmid pCDNA-MPB64. The positive recombination expressing plasmids were confirmed by analysis of the base pairs of plasmid, enzyme digest and PCR amplification. The pCDNA linear section and 620 bp insert section were generated after digestion of recombination expressing plasmids with BamH I and *EcoR* I (Figure 1). The same base pairs of DNA were generated after PCR amplification. All these indicated that the recombination expressing plasmid pCDNA-MPB64, which could translate MPB64 protein, was constructed successfully.

Result of detection for BHK-21 cells transfected with vectors

As shown in Figure 2, the gene expression of MPB64 was detected by RT-PCR. Positive result could be detected in BHK-21 translated with recombination expressing plasmid pCDNA-MPB64, while negative result could be detected in BHK-21 translated with pCDNA as control vector. As shown in Figure 3, the protein expression of MPB64 was detected by Western blot. Positive result could be detected in BHK-21 translated with recombination expressing plasmid pCDNA-MPB64, while negative result could be detected in BHK-21 translated with pCDNA as control vector. This means the MPB64 was expressed in BHK-21 translated with recombination expressing plasmid pCDNA-MPB64.

Result of antibody titer detection in mice serum

As shown in Figure 4, the antibody titer in the serum was obviously increased significantly with the increased immune days. The antibody titer in the serum was

obviously increased significantly in compare with control group, and pCDNA-MPB64 group and BCG group had no significantly difference, which showed that MPB64 vaccine could induce strong humoral immunity in vaccinated mice.

Expression of IL-2 in serum.

To determine cell-mediated immune responses in the serum, immunized mice were prepared 2 weeks after the last DNA vaccination. The expression levels of IL-2 in serum of vaccinated mice were determined by ELISA. As shown in Figure 5, IL-2 response increased in all groups except the vector control group. Mice vaccinated with BCG induced the highest levels of IL-2 responses in the serum. Moreover, MPB64 group were significantly higher than control group ($P < 0.05$), and MPB64 group and BCG group had no significantly difference. These results showed that MPB64 could cause cell-mediated immune responses.

DISCUSSION

Tuberculosis (TB) is a common and sometimes lethal infectious disease caused by various strains of *Mycobacterium tuberculosis* (MTB). The main pathogenic bacteria of TB, MTB, are a small aerobic non-motile bacillus (G^+). MTB could withstand weak disinfectants and survive in a dry state for several weeks (Sarkar et al., 2011). Tuberculosis usually attacks the lungs through the air but can also affect other body organs such as bone, stomach and ovary. Most infections in humans are asymptomatic and latent, however, without proper treatment, some infected person would be dead finally. Strains of tuberculosis with antibiotic resistance, sometimes even with multi-drug-resistant are growing. Prevention of TB relies on screening programs, early detection and vaccination, among which *Bacillus Calmette-Guérin* vaccine (BCG) is most often used (Amaral et al., 2011).

Vaccines are one of the greatest achievements of modern medicine. It is one of the most effective methods to prevent zymosis including tuberculosis. DNA vaccination is a new technique for protecting the human body from epidemic diseases by injecting genetically engineered DNA, also called foreign DNA, into bodies to produce immunological response. To get the best immune response, highly active expression vectors are required (Mkrtichyan et al., 2008). These expression plasmids usually consist of a strong promoter to drive the *in vivo* transcription and translation of the target gene. They could elicit and stimulate the body to produce certain antibody to protect itself. These new vaccines have a number of advantages over conventional vaccines, including the ability to induce a wider range of immune response types, no risk for infection, long-term persistence of immunogen and focused immune

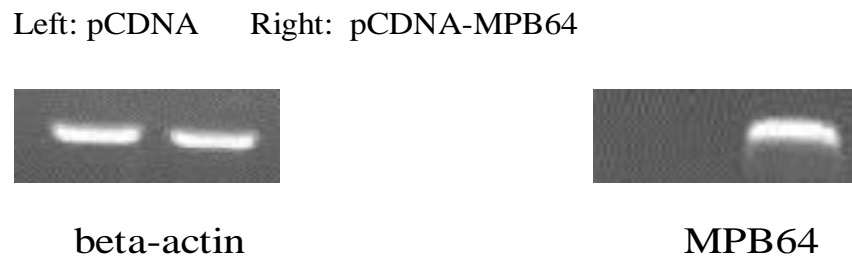


Figure 2. Gene assay for BHK-21 cell transfected with pCDNA and pCDNA-MPB64.

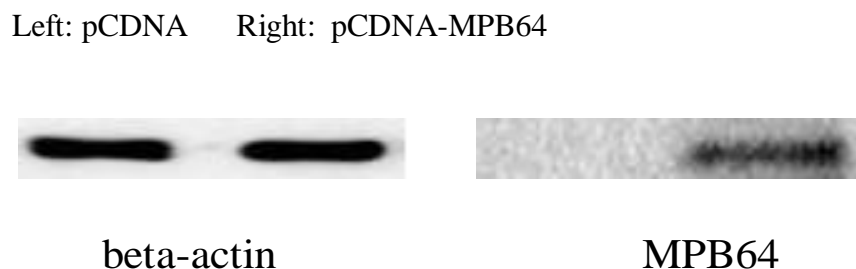


Figure 3. Protein assay for BHK-21 cell transfected with pCDNA and pCDNA-MPB64.

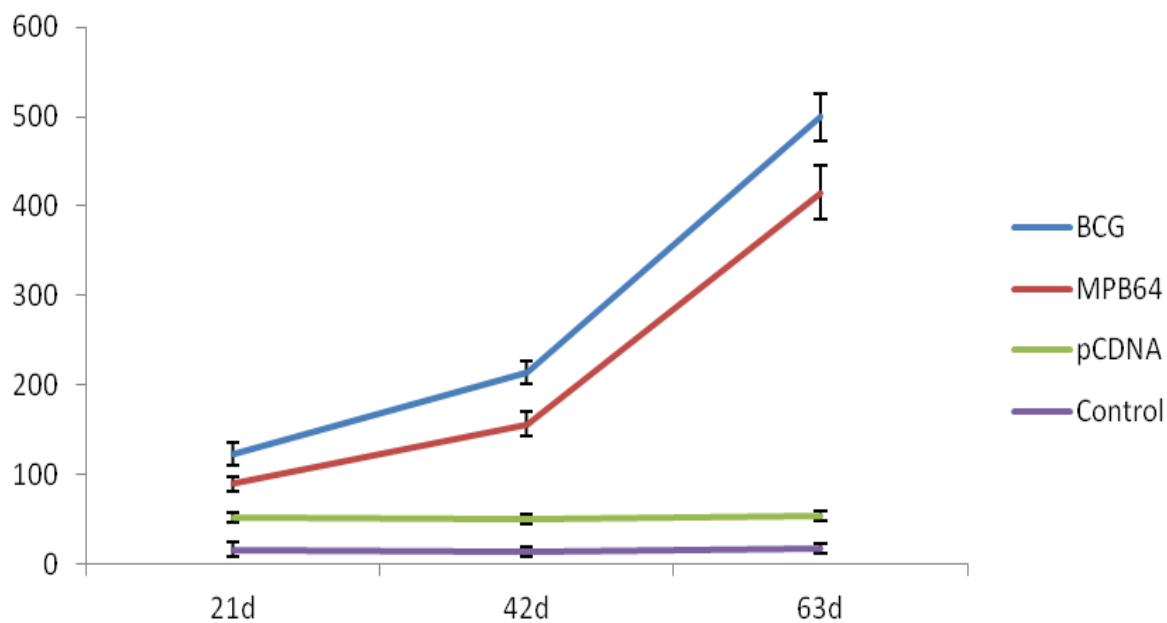


Figure 4. Antigen-specific total IgG antibodies detected by ELISA. C57BL/6 mice ($n = 20$) were vaccinated with different vaccine candidates.

response (Dunham et al., 2006). Vector design for maximal protein expression is essential and important. This is the reason we focus on the new DNA vaccination discovery.

Antibody titer measurement is a common way to test

The quantity of antibody in human body, which has produced that recognizes a particular epitope, expressed as the greatest dilution that still gives a positive result (Decker et al., 2006). ELISA is a common used method of determining antibody titers. Our research found that

IL-2

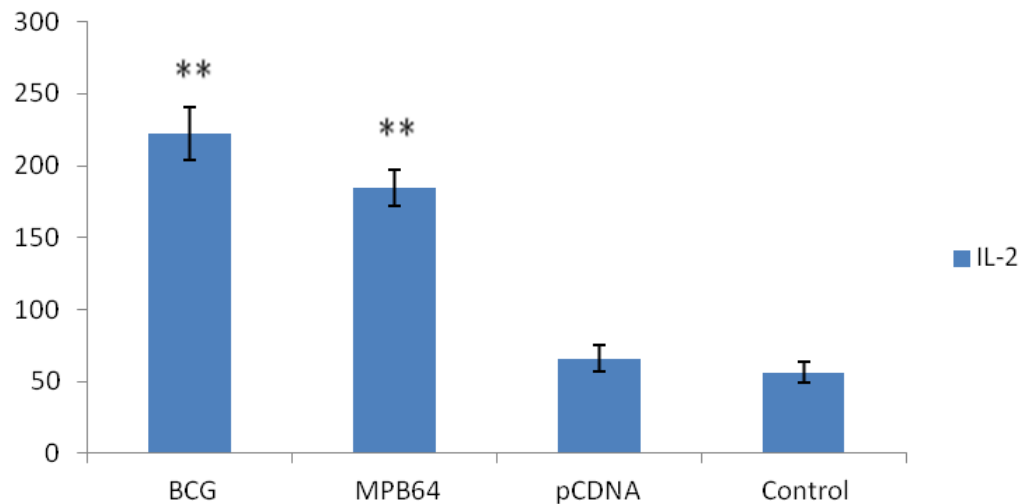


Figure 5. Antigen-specific IL-2 production of serum from mice. C57BL/6 mice ($n = 20$) were vaccinated with different vaccines. Serum was prepared 2 weeks after the last immunization. IL-2 was measured in triplicate using ELISA. The results are expressed as Mean (pg/ml) \pm SD of ten mice in each group. Data with significant difference when $p < 0.01$.

the MPB expressed and purified by the recombination expressing plasmid pCDNA-MPB64 could be enough to produce an effective immunological response. Immunogens could be targeted to various cellular compartments in order to improve antibodies or cytotoxic T-cell responses. In response to an antigen, cell-mediated immunity involves the activation of natural killer cells (NK), macrophages, antigen-specific cytotoxic T-lymphocytes, and cytokines, instead of antibodies. The immune system was separated into humoral immunity and cellular immunity, for which the protective function of immunization was associated with cells (Harris et al., 2009; Lutz et al., 2009). Cell-mediated immunity is directed primarily at microbes survive in phagocytes and microbes that infect non-phagocytic cells. It is most effective in removing virus-infected cells, but also participates in defending against fungi, protozoans, intracellular bacteria, and cancers. It also plays a major role in transplant rejection in human body (Cohen et al., 2008).

The RD1 genetic region is present in the genomes of MTB and *Mycobacterium bovis*, but absent from all strains of *M. bovis* BCG, as well as most non-tuberculous *Mycobacteria* (NTM) (Pym et al., 2003). The MPB64 gene, encoded by RD1, has been investigated extensively, and has been shown to have great potential in the specific *in vitro* diagnosis of MTB infection in human beings (Maue et al., 2007; Kaufmann et al., 2011). Previous studies showed that RDs encode some important immuno-dominant antigens of *M. tuberculosis* and RD1 could enhance the protective efficacy of BCG in the forms of recombinant BCG or subunit vaccine.

MPB64 had been considered important immuno-dominant antigens encoded by RD1 and RD2 of *M. tuberculosis*, respectively (Maue et al., 2007). Recent study demonstrated that DNA vaccine expressing the gene of MPB64 could strength protection efficacy, which was complied with our results (Kaufmann et al., 2011).

In conclusion, our results clearly demonstrated the vaccination of C57BL/6 mice with new DNA vaccine pCDNA-MPB64 resulted in significant protection against challenge with virulent *M. tuberculosis* H37Rv when compared with the control group, which indicates that MPB64 may be an efficient booster vaccine against TB with a strong ability to enhance prior BCG immunity. These results suggest that MPB64 is safe and effective vaccine against TB.

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