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Immunogenicity and protective efficacy against tuberculosis with DNA expressing ESAT6 protein

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The greatest challenges in tuberculosis (TB) vaccine development include optimization of vaccines for use in humans, creation of effective single-dose vaccines, development of delivery systems that do not involve live viruses, and the identification of effective new adjuvants. In this study, we examined the immunogenicity and protective efficacy of DNA vaccine (pVAX1-ESAT6) expressing ESAT6 protein and its booster effects in mice for control tuberculosis. The results showed that ESAT6 DNA vaccine led to a dramatic augmentation of humoral and cellular responses. The novel immunogenic ESAT6 DNA vaccine revealed in this study provided a new candidate target for tuberculosis vaccine development.

Key words: Tuberculosis, Immunogenicity, ESAT6, humoral immune, cellular immune.

INTRODUCTION

It is estimated that, in China, there are 550 million people latently infected with *Mycobacterium tuberculosis* (MTB), 5 million patients with pulmonary tuberculosis (TB) and nearly 2 million patients with smear-positive pulmonary TB (Sala et al., 2011). Most latently infected individuals as well as many patients with active TB are smear-negative and/or culturenegative, complicating the diagnosis of TB (Rouanet et al., 2010; Kernodle et al., 2010). *Mycobacterium bovis*, bacilli Calmette-Guérin (BCG) has been widely administered to newborns throughout the world showing to be effective in the prevention of childhood tuberculosis (TB) but not in the reactivation of pulmonary disease or human immunodeficiency virusassociated TB (Amaral et al., 2011). Development of a more effective, standardized, affordable vaccine with durable activity and fewer side effects has been considered a major international public health priority (Hanif et al., 2010).

The RD1 genetic region is present in the genomes of MTB and *M. bovis*, but is absent from all strains of *M. bovis* BCG, as well as most non-tuberculous *Mycobacteria* (NTM) (Pym et al., 2003; Fan et al., 2007).

The early secreted antigen target6 (ESAT6) gene that is 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 humans(Maue et al., 2007; Kalra et al., 2007). DNA vaccine expressing ESAT-6 protein could enhance the protective efficacy of BCG vaccination in mice vaccinated (Maue et al., 2007). However, DNA vaccine expressing ESAT-6 could induce strong humoral and cell-mediated immunity in vaccinated mice, which has little report. In the present study, we evaluated the immune responses generated against DNA vaccine expressing the fusion protein of ESAT-6.

MATERIALS AND METHODS

Bacterial strain and culture media

Escherichia coli DH5α strains were used for cloning. Both bacteria were cultured in Luria-Bertani (LB) medium with or without agar. When required, ampicillin was added to a final concentration of 100 µg/ml. *M. tuberculosis* H37Rv and *M. bovis* BCG China were cultivated in Middlebrook 7H9 medium or enumerated on 7H11

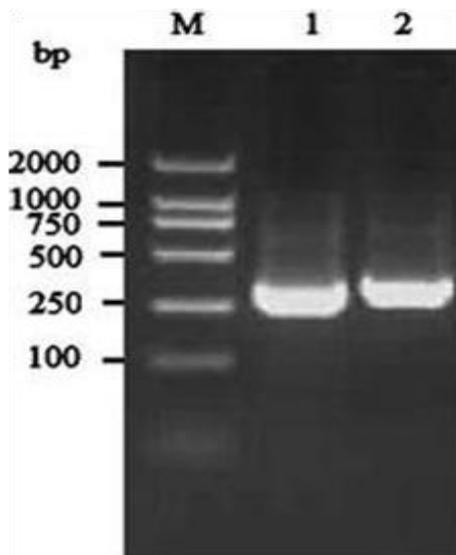*Corresponding authors: E-mail: sanglin518@yahoo.cn.

Figure 1. Construction of recombinant plasmids. Genes encoding ESAT-6 was cloned from M. tuberculosis H37Rv genomic DNA. M: Mark DL2000, line1 and 2: The PCR product of ESAT6.

agar (BD, Sparks, USA), supplemented with 10% ADC, 0.5% glycerol, and 0.05% Tween 80.

Construction of recombinant plasmids (pVAX1-ESAT6)

Genes coding ESAT-6 (*esxA, Rv3875*) was amplified by PCR with primers and the genomic DNA of *M. tuberculosis* H37Rv as the template. The PCR products were first digested with BamHI and EcoRI and then cloned into the corresponding sites prokaryotic expression vector eukaryotic expression vector pVAX1, resulting in recombinant plasmids named pVAX1-ESAT6, respectively. The correctness of recombinant plasmids was confirmed by DNA sequencing and enzyme digestion. Plasmids pVAX1-ESAT6 for DNA immunization were transformed into competent *E. coli* DH5α, and endotoxin-free plasmid DNA was purified using the Qiagen Plasmid Giga kit (Qiagen, Valencia,CA) according to the manufacturer's instructions.

Transfection of BHK-21 Cells

BHK-21(ATCC) cells were transfected with pVAX1-ESAT6 and pVAX1 by Lipofectamine 2000 (Invitrogen, USA). Cells at 90 to 95% confluence were transfected with 5 μg vectors. After 6 h exposure, the normal culture medium, RPMI 1640 supplanted with 15% horse serum (Gibco, USA) was added into cells for another 24h-48h.Tuberculosis polyclonal antibody (1:100) was used as primal antibody while FITC marked Rabbit anti bovine IgG (1:100) was used as 2nd antibody, each for 2 h at 37°C. Pictures were taken under fluorescence microscope. Transfected cells were collected and total RNA was isolated and then translated into cDNA as template. RT- PCR was performed for gene detection.

Animal immunization

Specific pathogen-free (SPF) 6 to 8 week-old, female C57BL/6 mice (Lab Animal, Changchun, 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 Changchun Medical School Committees on Biosafety and Animal Care and Use Committee of China. Mice were randomly divided into (10 mice in each group): nonvaccinated control, vector control, pVAX1-ESAT6, BCG. Pasmid DNA (100 μg) was injected intramuscularly in the same area, and immunization was repeated thrice with 2 weeks intervals.

Antibody response

Sera were collected from each mouse two weeks after immunization. Antigen-specific antibody responses were measured in an ELISA by microtiter plates, precoated overnight at 4°C with 100 μl ESAT6 protein (5 μg/ml) in carbonate/bicarbonate buffer (pH 9.6). After blocking with 1% BSA in PBS, serum samples were diluted to appropriate concentrations and were incubated for 2 h at 37°C. After washing, the plates were incubated for 2 h at 37°C with HRP-conjugated goat antimouse IgG antibody. Orthophenylenediamine (OPD) was used for color development as an indicator. Sera from naive mice were used as negative controls. Data are presented as mean of optical density value at 490 nm per group.

ELISA analysis IFN-γ

To detect IFN-γ in serum of rats after immune vaccine, ELISA was conducted by using assay kit. The rats IFN-γ Enzyme-linked Immunosorbent Assay (ELISA) kit (Leikai, China) is an in vitro ELISA for the quantitative measurement of rats IFN-γ in serum, plasma, cell culture supernatants and urine. This assay employs an antibody specific for rats IFN-γ coated on a 96-well plate. Standards and samples are pipetted into the wells and rats IFN-γ present in a sample is bound to the wells by the immobilized antibody. The wells are washed, and then biotinylated anti-rats IFN-γ 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 IFN-γ 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.

Statistics analysis

The statistical package SPSS13.0 (SPSS Incorporated, Chicago) was used for all analysis. One-way ANOVA followed by Bonferroni's post hoc test were utilized to determine the significant difference among multiple groups. Student's *t* test was used to determine the significance of differences between the groups. All values were expressed as mean ± SD. In general, p values less than 0.05 were considered statistically significant.

RESULTS

Construction and expression of pVAX1-ESAT6 in BHK-21 cells

The genes of ESAT-6 were first amplified by PCR and *M. tuberculosis* H37Rv genomic DNA as the template (Figure 1). The purified PCR productions were digested
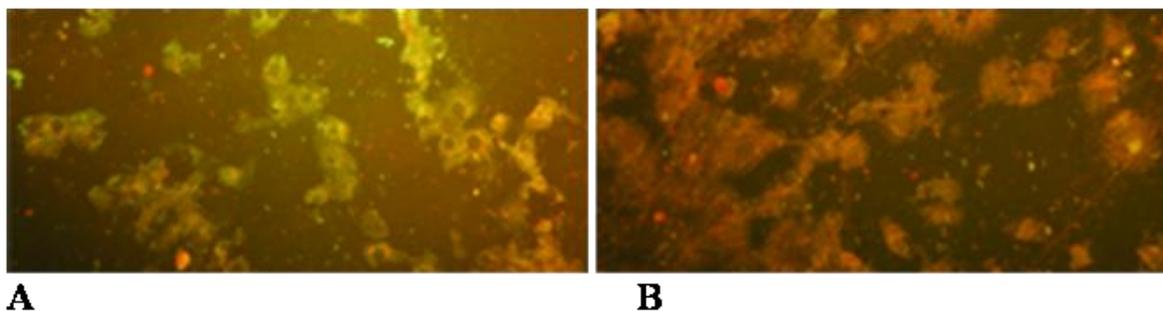
Figure 2. Recombinant plasmid pVAX1-ESAT-6 transfected into BHK-21 cell line(x 200) A: pVAX1-ESAT-6; B: pVAX1.

with BamHI and EcoRI and separately cloned into the plasmid and pVAX1 predigested with the same restriction enzymes, in order to construct eukaryotic expression plasmid pVAX1. DNA sequencing and enzyme digestion confirmed the successful constructions.

The genes of ESAT-6 transfected in BHK cells were detected by fluorescence antibody test. Specific kelly fluorescence could be detected in BHK-21 translated with recombination expressing plasmid pVAX1-ESAT-6, while no specific kelly fluorescence could be detected in BHK-21 translated with pVAX1 control vector (Figure 2). This means the ESAT6 was expressed in BHK-21 cell, which showed that recombination expressing plasmid pVAX1-ESAT-6 was successfully constructed.

Antibody response in serum

The ESAT6 expressed and purified by the recombination expressing plasmid pVAX1-ESAT6, was used as antigen. ELISA was used to detect the antibody titer in the mice serum. The result of titer was shown in Figure 3. The antibody titer in the serum was obviously increased significantly in comparing with control group, and ESAT6 group and BCG group had no significantly difference, which showed that ESAT6 vaccine could induce strong humoral immunity in vaccinated mice.

Expression of IFN-γ 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 IFN-γin serum of vaccinated mice were determined by ELISA. As shown in Figure 4, IFN-γ response increased in all groups except the vector control group. Mice vaccinated with BCG induced the highest levels of IFN-γ responses in the serum. Moreover, ESAT6 group were significantly higher than control group ($P<0.05$), and ESAT6 group and BCG group had no significantly difference. These results showed that ESAT6 could cause cell-mediated immune responses.

DISCUSSION

BCG is the only available vaccine against tuberculosis (TB) and has been included in World Health Organization Expanded Program on Immunization since 1974. While BCG has demonstrated its prove efficacy at preventing tuberculosis (TB) in children for over 80 years (Rodrigues et al., 1993), it provides variable efficacy against adult pulmonary TB, ranging from no protection in china to about 80% protection in England (Colditz et al., 1994). Environmental mycobacteria (Fine et al., 1999; Brandt et al., 2002), strain variations in BCG preparations (Fine et al., 1999), or host genetic and nutritional factors (Fine et al., 1999; Fernando et al., 2006) may explain the variability in BCG's protective efficacy. Development of recombinant DNA Vaccine expressing promising immune-dominant antigens of *M. tuberculosis* represents one of the potential approaches for the development of vaccines against TB.

Previous studies including over expression of RD1 in BCG (Pym et al., 2003) or BCG supplementation with subunit or DNA vaccine encoding RDs (Fan et al., 2007; Maue et al., 2007; Kalra et al., 2007) suggested that RDs encode some important immune-dominant antigens of *M. tuberculosis* and RD1 could enhance the protective efficacy of BCG in the forms of recombinant BCG or subunit vaccine. ESAT-6(Rv3875), had been considered important immune-dominant antigens encoded by RD1and RD2 of *M. tuberculosis*, respectively (Maue et al., 2007).ESAT-6 is considered a dominant antigen for cell-mediated immunity (Horwitz et al., 2009) and is a major target for memory T cells in mice infected with *M. tuberculosis*. Recently study demonstrated that DNA vaccine expressing the gene of ESAT-6 could strength protection efficacy (Kaufmann et al., 2010), which comply with our results, however, we used pVAX1 as vector in this study, which is safety and efficacy compared to other vector.

In conclusion, our results clearly demonstrated the vaccination of C57BL/6 mice with DNA vaccine (pVAX1-ESAT-6) expressing the protein of ESAT-6 resulted in significant protection against challenge with virulent *M. tuberculosis* H37Rv when compared with the control
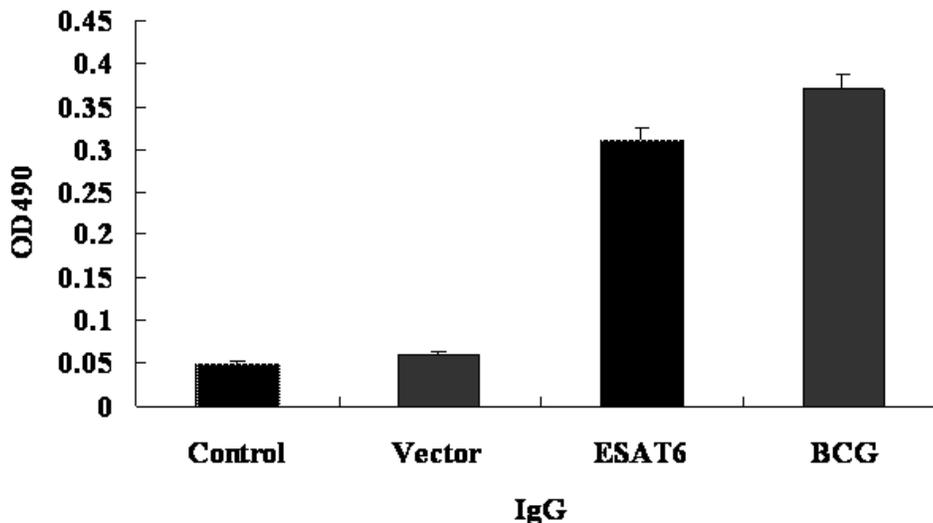
Figure 3. Antigen-specifc total IgG antibodies detected by ELISA. C57BL/6 mice (*n* = 10) were vaccinated with different vaccine candidates. Two weeks after the last immunization, the animals were bled and sera were obtained. The results were expressed as mean of OD (490 nm) of each group.
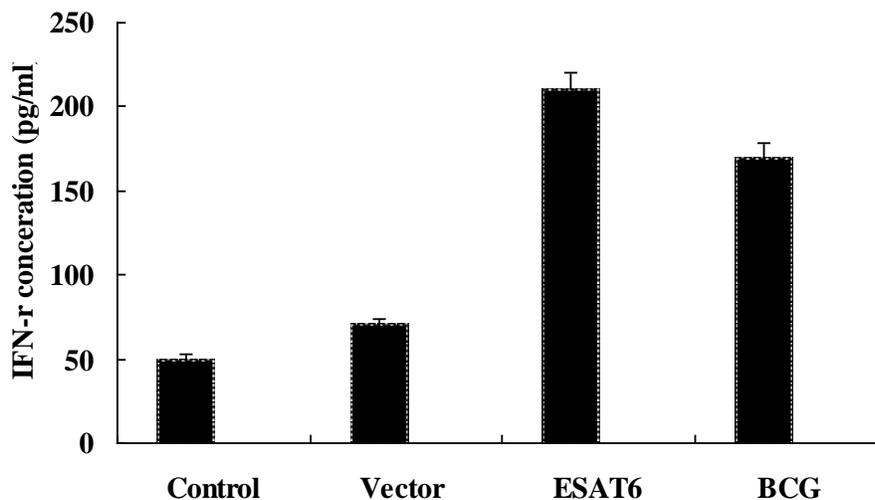

Figure 4. Antigen-specific IFN-γ production of serum from mice. C57BL/6 mice (*n* = 10) were vaccinated with different vaccines. Serum was prepared 2 weeks after the last DNA immunization. IFN-γ was measured in triplicate by an ELISA. The results are expressed as mean (pg/ml) of ten mice in each group, different letter represent the significant difference at p<0.05.

group. Thus, our study indicates that ESAT6 may be an efficient booster vaccine against TB with a strong ability to enhance prior BCG immunity, which will benefit for direct the rational design of new vaccines for improved prevention of TB.

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