

Immunogenicity of a DNA Vaccine Encoding Ag85a-Tb10.4 Antigens from *Mycobacterium Tuberculosis*

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ABSTRACT

Background: Tuberculosis is a life threatening disease that is partially prevented by BCG vaccine. Development of more effective vaccines is an urgent priority in TB control. Ag85a and Tb10.4 are the members of culture filter protein (CFP) of *M. tuberculosis* that have high immunogenicity. **Objective:** To analyze the immunogenicity of Ag85a-Tb10.4 DNA vaccine by enzyme-linked immunosorbent assay (ELISA). **Methods:** In this study a previously described plasmid DNA vaccine encoding Ag85a-Tb10.4 was used to examine its capability in the stimulation of immune responses in an animal model. Female BALB/c mice were vaccinated with 100 µg of purified recombinant vector intramuscularly 3 times at two-week intervals and the levels of five cytokines including IFN-γ, IL-12, IL-4, IL-10 and TGF-β were measured. **Results:** The levels of IFN-γ and IL-12 for the mice following immunization with Ag85A-Tb10.4 was significantly greater than that of the BCG and control group (p<0.05). However there was no significant difference in the levels of IL-4, IL-10 and TGF-β between groups. **Conclusion:** IFN-γ and IL-12 Th1 cytokines increased significantly in mice vaccinated with Ag85a-TB10.4 DNA vaccine in comparison to the control and BCG groups. Our results may serve as groundwork for further research into the prevention and treatment of tuberculosis.

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Keywords: Antigen 85, BALB/c Mice, DNA Vaccine, Mycolyltransferase, Tb10.4

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INTRODUCTION

Tuberculosis (TB) is the second leading cause of death provoked by *M. tuberculosis*. While the incidence of reported tuberculosis disease had fallen to below 25 cases per 100 000 in Europe and North America, in other districts it has peaked and reached over 8 million new cases. Therefore, in 1993, World Health Organization (WHO) ranked TB as a Global Public Health Threat (1-3). In this regard, bacillus calmette Guerin (BCG) is the first and only available vaccine against tuberculosis. Despite extensive use of BCG vaccine around the world and the availability of effective treatment, tuberculosis still remains a serious disease worldwide. BCG is protective against childhood forms of the disease, but has not proved effective protection against adult pulmonary tuberculosis (4). currently many studies are underway to overcome this problem and find better solutions. Ag85A has mycolyltransferase activity which is required for biogenesis of trehalosedimycolate (TDM). This molecule is involved in cell wall integrity, bacterial growth in nutrient-poor conditions and intracellular infections (5). TB10.4 belongs to Esat-6 family that is present only in strains of the *M. tuberculosis* complex. TB10.4 is encoded by the Rv0288 gene and is essential for the virulence of *M. tuberculosis* (6). Cellular immune responses are essential for the control of *M. tuberculosis* infection. Ag85a and Tb10.4 are the most promising candidate antigens for vaccine development against TB. These two antigens are able to stimulate strong and broad T-cell response and CTL activity; hence, they are suitable candidates for new vaccine designs. DNA vaccination is a powerful technique for inducing strong cell mediated immune responses and protective immunity against tuberculosis infection in mice. Here we have described the potential of a DNA vaccine encoding the immunodominant Ag85A and Tb10.4 antigens for causing immune responses in BALB/c mice. Based on this hypothesis, the mice in this study were vaccinated with a DNA vaccine encoding Ag85a and Tb10.4 the efficacy of which was evaluated.

MATERIALS AND METHODS

Plasmid construction and DNA Vaccine Preparation. DNA vaccine encoding Ag85A-Tb10.4 was prepared as described previously (7). Following the culture of *E. coli JM109* cells containing the Ag85A-Tb10.4 vector, plasmid purification in large scale was performed by using phenol-chloroform protocol (8). To remove any endotoxin, the purified plasmid was passed through the QIAprep Spin filter (QIAGEN,USA).

Vaccination Schedule. At the start of vaccination, 6 to 8 weeks old female BALB/c mice were used. All of the mice were kept maintained under the ethical policies of Mashhad University of Medical Sciences.

Two groups containing 10 mice were vaccinated intramuscularly three times at 2-week intervals with 100 µg of Ag85A-Tb10.4 encoding DNA vaccine and empty vector in the vaccine group and control group, respectively. Subcutaneously, 5×10^5 cfu of BCG vaccine was injected to 10 mice in the BCG group (7,9,10).

Stimulation of Splenocytes. Splenocytes were extracted from the BCG vaccinated mice and then were plated with different concentrations (10^4 - 10^5 cfu) of live BCG bacterium (BCG antigen) and incubated at 37°C in 5% CO₂ for 72 h. After stimulation, the supernatants were drawn and IFN-γ was measured using ELISA kits (ebioscience,

SanDiego, CA) according to the manufacturer's recommendations. The maximum amount of IFN- γ was seen in 2×10^4 cfu of BCG antigen in PBS. Finally, all the splenocytes acquired from all of the groups encountered this concentration of BCG antigen (10-12).

Cytokine Assay. All of the vaccinated mice were sacrificed 4 weeks after the last immunization, and spleens were removed aseptically. 3×10^5 cells of the spleen cells were grown in flat-bottom microwell plates (Nunc, USA), in RPMI 1640 (Invitrogen, USA) medium supplemented with antibiotics, and 10% heat-inactivated fetal calf serum (Invitrogen, USA). 20 μ l (2×10^4 cfu) of B antigen was added to 180 μ l of cell suspension, respectively. The cells were incubated at 37°C in a humidified CO₂ incubator, and supernatants were harvested after 72 h. The supernatants from three separate wells were mixed and stored frozen at -20°C until assay (10-12).

ELISA. The amount of IFN- γ , IL-12, IL-4, IL-10 and TGF- β were quantified by ELISA using eBioscience kit according to the manufacturer's guidelines (eBioscience, USA). Cytokine concentrations were expressed as mean \pm SD. Briefly, Micro plates (96-well; Corning Costar 9018) were coated with specific monoclonal anti-murine IFN- γ , IL-12, IL-4, IL-10 and TGF- β antibody. Free binding sites were blocked with 2% (w/v) milk powder in PBS. Culture supernatants were harvested from lymphocyte cultures after 72 h of incubation. All of the cytokines were detected with biotin-labeled anti-murine antibody and HRP-conjugated streptavidin. The enzyme reaction was developed with tetramethylbenzidine (TMB), hydrogen peroxide and stopped with 1 M HCL. The plates were read at 450 nm using an ELISA reader (10-12).

Statistical Analysis. Normality was checked by using Kolmogorov-Smirnov test and one way ANOVA test was used to compare the difference between the groups. The difference was considered statistically significant when the p value was less than 0.05.

RESULTS

The mice were killed 4 weeks after the immunization with either recombinant vector, BCG or empty vector. Ag85A-Tb10.4 DNA and pCDNA3.1⁺ vector were administered three times at 2-week intervals to 10 BALB/c mice. Moreover, one group received one dose of BCG vaccine subcutaneously. The mice were killed 4 weeks after the last DNA inoculation. Cytokine productions in culture supernatants stimulated by B antigen were categorized. The detection limits were IFN- γ : 15 pg/ml, IL-12p70: 15 pg/ml, IL-4: 4 pg/ml, IL-10: 30 pg/ml and 8 pg/mL for TGF- β . The vaccinated and BCG groups produced significantly more IL-10, IL-12, IFN- γ , TGF- β and IL-4 than in mice vaccinated with empty vector and a significant difference was seen ($p < 0.05$). As shown in Figure 1 and Table 1, the level of IL-12 (39.35 ± 8.81 pg/ml) and IFN- γ (429.46 ± 195 pg/ml) increased more in the vaccinated group compared with the BCG (IL-12: 24.78 ± 6.60 pg/ml, IFN- γ : 85.24 ± 5.4 pg/ml) group. The elevation of IL-12 and IFN- γ was strongly indicative of Th1 activation. Productions of IL-4, IL-10 and TGF- β at 15.72 ± 5.72 , 1753.73 ± 881.49 and 9.50 ± 1.57 pg/ml respectively in the vaccine group, and 15.14 ± 5.08 , 1230.86 ± 247.86 and 8.71 ± 0.50 pg/ml in the BCG group were seen without any significant differences between the two groups.

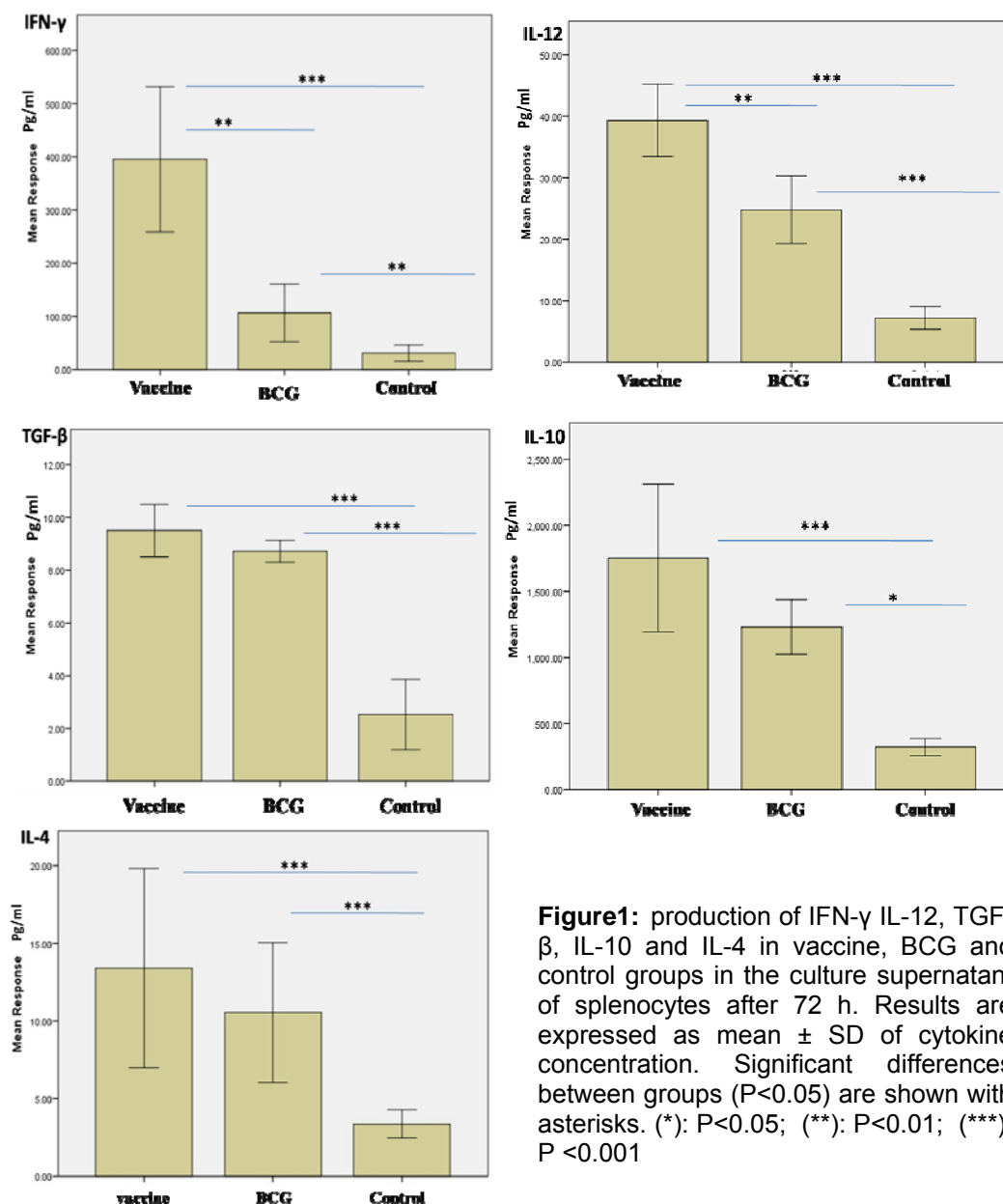


Figure1: production of IFN-γ IL-12, TGF-β, IL-10 and IL-4 in vaccine, BCG and control groups in the culture supernatant of splenocytes after 72 h. Results are expressed as mean ± SD of cytokine concentration. Significant differences between groups (P<0.05) are shown with asterisks. (*): P<0.05; (**): P<0.01; (***): P <0.001

Table 1. Spleen cytokine profile in different groups of BALB/c mice

Vaccination groups	IL-12p70 pg/ml	IL-4 pg/ml	IL-10 pg/ml	IFN-γ pg/ml	TGF-β pg/ml
control	7.16 ± 1.7	3.5 ± 1.80	320.32 ± 62.36	15.40 ± 3.60	2.52 ± 1.26
vaccine	39.35 ± 8.81	15.72 ± 5.72	1753.73 ± 881.49	429.46 ± 195.00	9.50 ± 1.57
BCG	24.78 ± 6.60	15.14 ± 5.08	1230.86 ± 247.86	135.06 ± 39.00	8.71 ± 0.50

4 weeks after the last Ag85a-Tb10.4 injection, ten mice from each group were bled and the obtained spleen cells were cultured in the presence of B antigen. The concentration of cytokines was tested in a 72 h culture supernatant.

DISCUSSION

According to the WHO fact sheet in 2014, one third of the world population were infected with the TB bacillus. Major challenges to TB control include co-infection with HIV, emergence of multidrug-resistant strains and poor efficacy of the current vaccines (13). Clearly, development of a better vaccine could be an impressive remedy to the global threat of tuberculosis. Previous studies confirm that BCG vaccine is not an ideal one against TB; thus, new generation vaccines are urgently needed. DNA vaccine is an interesting area of research that was first invented in the early of 1990s. Simplicity and versatility of this vaccine confer several advantages over the conventional vaccines.

DNA vaccine is safe and well tolerated but it has low immunogenicity in humans (14). Use of molecular adjuvants and advanced delivery methods can greatly enhance the immunogenicity of the DNA vaccines (14). Ag85a is a secreted protein with enzyme activity that is essential for the generation of envelope lipid and survival in macrophages. Recent studies showed that Ag85 complex protein induces strong T-cell proliferation and IFN- γ production in mice and humans and can be considered as a promising candidate for vaccine development (15-17). Although Tb10.4 function is unknown, this antigen along with Ag85a play an essential role in *M. tuberculosis* pathogenesis. Rindiet *al.* showed that Tb10.4 expression is notably down-regulated in the attenuated H37Ra strain in comparison with virulent H37Rv strain (18). Furthermore, TCD8⁺ and TCD4⁺ play a vital role in generating impressive immune responses against Tuberculosis. TCD8⁺ cells are able to produce type 1 cytokines IFN- γ and TNF- α that activate macrophage bactericidal mechanisms and CTL function. Ag85a has a specific epitope that induces strong cytolytic responses of TCD8⁺ (5,19,20).

A review of the previous studies reveals that Tb10.4 is more antigenic than Esat-6 (21,22). In this study, we report that the vaccination of mice with DNA vaccine encoding Ag85a. Tb10.4 can be viewed as a powerful means for inducing strong cell-mediated immune responses. Immunogenicity and protective efficacy of a DNA vaccine encoding Ag85A-Tb10.4 from *M. tuberculosis* was examined in BALB/c mice. The amount of IFN- γ and IL-12 was elevated in the culture supernatant of spleen lymphocyte. These cytokines have a central role in control of TB, since the disruption in IFN- γ gene and IL-12 gene leads to susceptibility to mycobacterial infections (23-25). IL-4 and IL-10 are indicative of Th2 activation following the injection and this study showed that there was no significant difference between the BCG and vaccine groups. These results indicate that Ag85a-Tb10.4 DNA vaccine capable to induce protective Th1 type immune responses in mouse immunization. Mouse immunization studies have shown that plasmid DNA vaccine encoding Ag85a and Tb10.4 causes a high level production of IL-12 and IFN- γ . Based on these findings, it can be concluded that this vaccine has potential to shift the immune system toward cellular immune responses. Jingyuet *al.* reported recombinant adenovirus expressing g85a and Tb10.4 (AdAg85a-Tb10.4) as a fusion protein that has protective efficacy on pulmonary TB in animals. they concluded that fusion of Ag85a and Tb10.4 provides superior protection relative to its monovalent counterparts (26).

In a study, Dietrich *et al* substituted Tb10.4 for Esat-6 in Ag85B-ESAT-6 vaccine which induced a significant protection against tuberculosis (20). Fusing Ag85B to TB10.4 provides more effective protection against tuberculosis compared to the individual Ag components (21). Other studies showed that rBCG over expressing

Ag85a and Ag85b makes stronger protective immunity than conventional BCG vaccines (26, 27).

In the present study, the production of IL-4 and IL-10 as Th2-type cytokines in spleen cells from the vaccine group was not significant like the BCG group. The aim of this study was to analyze immunogenicity of the recombinant vector based on Ag85A-Tb10.4 by ELISA assay in BALB/c mice. The amount of IFN- γ and IL-12 in the mice receiving DNA vaccine was significantly greater than the BCG and control groups. In comparison with the control group, all of the cytokines increased. Compared to the BCG group, the amount of IL-12 and IFN- γ raised which indicates that this recombinant vaccine had strong capability to shift immune responses toward cell mediated immunity. These data demonstrate that the Ag85A-Tb10.4 DNA vaccine is able to produce a strong and broad T-cell response. Also, Fusing Ag85A to TB10.4 produces a more effective vaccine compared to BCG vaccination. Moreover, Ag85a-Tb10.4 DNA vaccine is a new candidate in the field of tuberculosis.

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