

Original Article

# The *Mycobacterium bovis* BCG prime-Rv0577 DNA boost vaccination induces a durable Th1 immune response in mice

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## Abstract

Tuberculosis remains a major global health problem and effective vaccines are urgently needed. In this study, we used the combined DNA- and protein-based vaccines of immunodominant antigen Rv0577 to boost BCG and evaluated their immunogenicity in BALB/c mice. Our data suggest that the booster vaccine may substantially enhance the immunogenicity of BCG and strengthen both CD4+ T cell-mediated Th1 and CD8+ T cell-mediated cytolytic responses. Compared with the protein-based vaccine, the DNA-based vaccine can induce more durable Th1 immune response, characterized by high levels of antibody response, proliferation response, percentages of CD4+/CD8+ and cytokine secretion in antigen-stimulated splenocyte cultures. In conclusion, we for the first time, developed a protein- and plasmid DNA-based booster vaccine based on Rv0577. Our findings suggest that antigen Rv0577-based DNA vaccine is immunogenic and can efficiently boost BCG, which could be helpful in the design of an efficient vaccination strategy against TB.

**Key words:** *Mycobacterium tuberculosis*, prime-boost strategy, Rv0577, BCG, DNA vaccine

## Introduction

Tuberculosis (TB) is one of the major chronic infectious diseases which is mainly caused by the *Mycobacterium tuberculosis* (Mtb). In 2013, it killed ~1.5 million people and ~9 million people developed into TB worldwide [1]. Moreover, the emergence of HIV/Mtb co-infection and increasing prevalence of multiple drug-resistant and extensively drug-resistant TB pose great challenge to the global TB control [2]. A vaccine that could protect people from Mtb infection is urgently needed. The only licensed TB vaccine, Bacillus Calmette–Guerin (BCG), is an attenuated *Mycobacterium bovis* strain which has been used since 1921. BCG is effective in protecting against TB meningitis and miliary TB in children. However, it is not so potent to prevent pulmonary TB in adult [3,4]. The efficacy of BCG is highly variable and revaccination with BCG does not increase protection [5]. Despite these drawbacks, BCG remains to be one of the first-line therapeutic options for vaccination of the newborns. In fact, as a vaccine, BCG is attractive in terms of its extensive safety, clinical record, heat stability, and low

production cost. Immunologically, BCG can induce long-lasting type 1 helper T cell (Th1) immunity and trigger CD8+ T-cell responses [6]. Therefore, it is a promising strategy to develop an effective booster vaccine following primary immunization of BCG to enhance the immunity induced by initial vaccination.

Previously, much effort has been made by our group and others in the development of safe and effective boost vaccine candidates after the BCG priming vaccination, such as giving a BCG prime followed by boosting with a viral vector, recombinant protein, or DNA vaccine [7–9]. These studies revealed that BCG prime-boost approach could activate T cell to evoke strong immune responses including both CD4+ T cell and CD8+ T cell immune responses [10]. We thus focused on the prime-boost strategy that combines BCG with a subsequent booster vaccine candidate.

Virulence-associated protein Rv0577 is a 32 kDa Mtb complex-restricted protein in the culture filtrate [11]. It plays a critical role in the maturation of dendritic cells and in triggering Th1 immune

responses, and it is important for protecting against challenge with Mtb in the mouse models [12–14]. These fascinating researches suggest that Rv0577 could be promising in designing a novel vaccine to boost BCG. In this study, we compared the ability of DNA vaccine with protein vaccine of antigen Rv0577 to boost BCG. Our aim is to evaluate the immune efficacy and strategy of using Rv0577 as a novel vaccine candidate.

## Materials and Methods

### Animals and immunization protocol

Female BALB/c mice (4–5 weeks old) were obtained from the Dashuo Animal Science and Technology Corporation (Chengdu, China). They were given water and food *ad libitum*. Animals were immunized subcutaneously or intramuscularly in five groups corresponding to one of the following treatment conditions: (i) PBST group: mice immunized subcutaneously with 0.1 ml PBST (0.1 M PBS containing 0.05% Tween 80) three times at Weeks 0, 3, and 6 as a blank control; (ii) BCG group: immunized subcutaneously with a dose of  $5 \times 10^6$  CFU BCG in a volume of 0.1 ml at Week 0, PBST was given twice with a dose of 0.1 ml at Weeks 3 and 6; (iii) BCG + P: prime immunized with 0.1 ml of  $5 \times 10^6$  CFU BCG at Week 0 and boosted subcutaneously with 50 µg purified Rv0577 protein each time at Weeks 3 and 6; (iv) BCG + D: prime immunized with BCG and boosted intramuscularly with Rv0577 DNA plasmid (50 µg) each time at Weeks 3 and 6; (v) BCG + DNA3.1: prime immunized with BCG and boosted with blank pcDNA3.1 plasmid (50 µg) intramuscularly at Weeks 3 and 6.

All immunization regimens are described in Table 1. Mice were sacrificed at 4, 8, 12, and 16 weeks (4 mice from each group at each time point) post-immunization. The serum and spleens were collected to evaluate the antigen-specific antibody response and cellular immune response. All animal experiments in this study were complied with laboratory animal care and used by designated members of Ethical Review Committee of Sichuan Laboratory Animal Society. All procedures were conducted in accordance with the national regulations and animal welfare requirements.

### Bacterial strains and plasmids

BCG-Pasteur was obtained from the Chengdu Biological Products Institute (Chengdu, China). It was cultivated in Sauton's medium (MgSO<sub>4</sub> 0.5 g, K<sub>2</sub>HPO<sub>4</sub> 0.5 g, citric acid 2.0 g, sodium glutamate 8.0 g, glycerol 60.0 ml, ZnSO<sub>4</sub> 0.01 g, and ferrum-ammonium citrate 0.05 g in 1000 ml, pH 7.4–7.5) and prepared at a concentration of  $5 \times 10^6$  CFU. Prokaryotic expression vector pET28a (+) and eukaryotic plasmid pcDNA3.1 preserved in our laboratory were used.

### Construction of plasmid DNA vaccine

Briefly, Rv0577 gene was amplified by PCR using the genome of H37Rv as template. The primers are as follows: F, 5'-GCGGATCCATGCCAAGAGAAGCGA-3'; R, 5-GAGAATTCCTATTGCTGCGGTGG-3'. The amplicons were cloned into pcDNA3.1(+) plasmid. The

recombinant pcDNA3.1-Rv0577 plasmid was confirmed by both restriction analysis and sequencing.

### Identification of recombinant Rv0577 protein by western blot analysis

The recombinant Rv0577 protein was obtained by the previously described methods [13]. Purified endotoxin-free recombinant protein was analyzed by 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis. Briefly, the protein samples were first separated by SDS-PAGE, and then transferred onto a PVDF membrane. The membrane was incubated with anti-His6 monoclonal antibody (TianGen Biotechnology, Beijing, China) and then with horseradish peroxidase-conjugated secondary antibody (TianGen Biotechnology) followed by DBA assay coloration. The concentration of purified protein was estimated by BCA Protein Assay Kit (Pierce, Rockford, USA) with bovine serum albumin as the standard. The protein was stored at  $-70^{\circ}\text{C}$  until used.

### Antibody measurement

Serum was separated from the eyeball blood of the immunized mice and stored at  $-30^{\circ}\text{C}$ . Indirect enzyme linked immunosorbent assay (ELISA) was used to determine the levels of antigen-specific antibodies in the serum. Briefly, 96-well high binding plates (Corning, Corning, USA) were coated with Rv0577 protein and blocked with 5% skim milk in PBS (pH 7.2). After incubation with 2-fold serially diluted sera, the plates were incubated with HRP-conjugated goat anti-mouse IgG, IgG1, and IgG2a (eBioscience, San Diego, USA) at a dilution of 1:2000, 1:5000, and 1:5000, respectively. Then, the ELISA plates were detected by TMB substrate and the reaction was stopped by addition of 0.1 M sulfuric acid. Finally, the absorbance was determined at 450 nm with an automatic microplate reader (Bio-Rad, Hercules, USA).

### Proliferation of splenocytes

At different time point, four mice from each group were splenectomized under aseptic conditions. The spleens were mechanically disrupted, and splenocytes were processed as single cell suspensions by cell strainer (BD, Franklin Lakes, USA). The cells were cultured in RPMI-1640 medium (Invitrogen, Carlsbad, USA) containing 10% fetal calf serum (FCS), 100 U/ml penicillin, and 100 µg/ml streptomycin. The proliferation of splenocytes was measured by Cell Counting Kit-8 (BOSTER, Wuhan, China). Briefly, the splenocytes were plated in 96-well flat-bottom plates ( $5 \times 10^5$  cells per well) with 10 µl protein Rv0577 (100 µg/ml) in each well, whereas the negative controls were treated with 10 µl PBS only. After 48 h of incubation at  $37^{\circ}\text{C}$ , 10 µl of CCK-8 reagent was added into each well, and incubated for another 1–4 h. Finally, the absorbance was measured at 450 nm by an automatic microplate reader. The results were expressed as the stimulation index (SI), which was calculated as the ratio of the mean absorbance of the stimulated/unstimulated wells.

### Analysis of the subsets of splenocytes

The splenocytes were cultured in 24-well flat-bottom plates ( $5 \times 10^6$  cells per well) to analyze CD4+ and CD8+ percentages of splenocytes. Briefly, the cells were stimulated with 100 µl Rv0577 protein (100 µg/ml) in each well. After 72 h of incubation at  $37^{\circ}\text{C}$  in 5% CO<sub>2</sub>, the cells were washed twice with PBS and were subsequently incubated with anti-mouse CD4+-FITC (eBioscience) and anti-mouse CD8+-PE (eBioscience). Flow cytometry was performed to analyze the percentages of CD4+ and CD8+ T cells.

**Table 1. Immunize regimens used in this study**

Group	Prime	First boost	Second boost
PBST	PBST	PBST	PBST
BCG	BCG	PBST	PBST
BCG + P	BCG	Protein Rv0577	Protein Rv0577
BCG + D	BCG	Plasmid Rv0577	Plasmid Rv0577
BCG + DNA3.1	BCG	Plasmid pcDNA3.1	Plasmid pcDNA3.1

### Analysis of the cytokines secreted by splenocytes

The supernatant from 72 h splenocytes culture was used to analyze the secretion of interferon- $\gamma$  (IFN- $\gamma$ ), interleukin-2 (IL-2), and interleukin-4 (IL-4). The concentrations of INF- $\gamma$ , IL-2, and IL-4 were determined by sandwich ELISA kits (eBioscience) following the manufacturer's instructions.

### Statistical analysis

Data were expressed as the mean  $\pm$  standard deviation (SD). The statistical analyses were performed using the SPSS21.0. One-way ANOVA test was carried out to calculate the significance of difference among groups. Post hoc test was applied to analyze the significance of difference between the two groups.  $P < 0.05$  was considered as statistically significant.  $P < 0.001$  was considered as highly significant.

## Results

### Rv0577 DNA boost resulted in long-lasting antibody responses

To evaluate the humoral immune response, the Rv0577-specific antibody responses including total IgG, IgG1, and IgG2a were detected using an indirect ELISA assay (Fig. 1). Both BCG + P and BCG + D groups elicited high levels of total IgG, IgG1, and IgG2a. Compared with PBST, BCG and BCG + DNA3.1 group, BCG + P group produced significantly higher levels of IgG2a at all detected time points, higher levels of IgG at the 4th, 12th, and 16th week, and higher levels of IgG1 at the 8th, 12th, and 16th week. Similarly, the BCG + D group produced significantly higher levels of total IgG at corresponding time points, higher levels of IgG1 at the 8th and 12th week, higher levels of IgG2a at the 8th, 12th, and 16th week. Importantly, compared

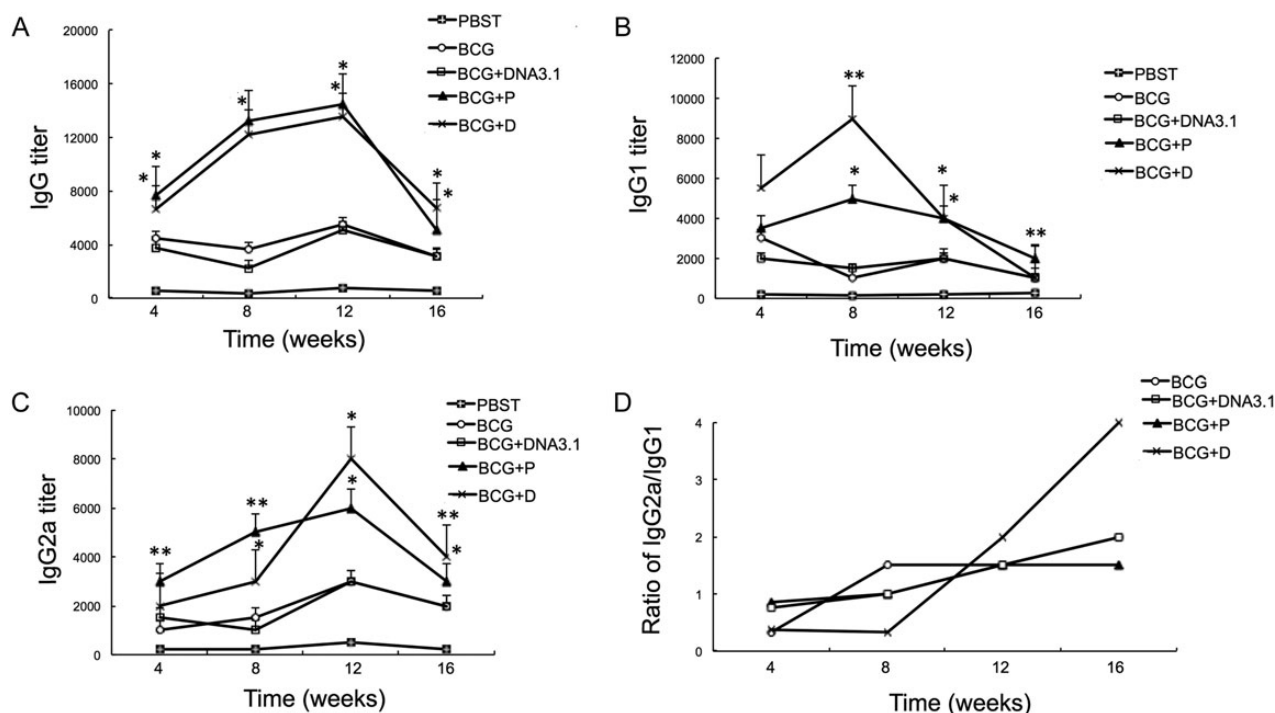
with BCG + P group, BCG + D group produced significantly higher levels of IgG1 at the 8th week and IgG2a at the 16th week. The ratio of IgG2a/IgG1 showed a shift toward a relatively Th1 immune response in BCG + D group at the 12th and 16th week.

### Rv0577 DNA boost induced a long-term splenocyte proliferation

The effect of Rv0577 on stimulating the proliferation of splenocytes was evaluated by CCK-8 (Fig. 2). The data showed that the proliferation of splenocytes in BCG + D and BCG + P groups was significantly higher than those in other groups at all corresponding time points. The proliferative response of BCG + D group was higher than that of the BCG + P group at the 12th and 16th week. These results indicate that BCG + P group may induce an immediate immune response, whereas BCG + D group may induce a long-term immune response.

### Rv0577 DNA boost induced high percentages of CD4+ and CD8+ T cells

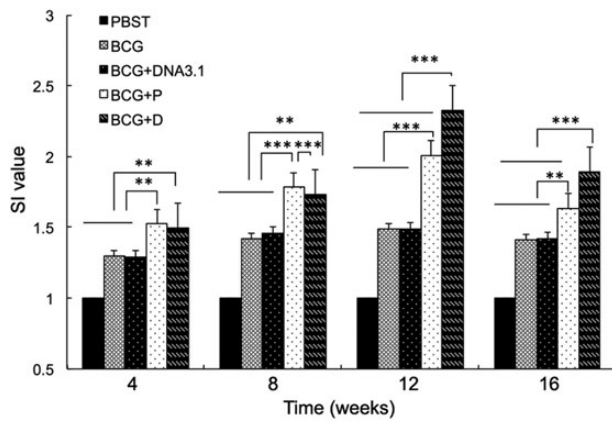
Both CD4+ and CD8+ T-cell immune responses are important to anti-TB; therefore, the percentages of responding CD4+/CD8+ T cells in splenocytes were determined by flow cytometry (Fig. 3). BCG + D group induced a significantly higher ratio of CD4+ T cells at the 4th and 8th week compared with BCG + P group. On the other hand, the proportions of CD8+ T cells in the BCG + D group were higher than that of the BCG + P group at the 4th and 12th week, whereas the proportions of CD8+ T cells in the BCG + P group reached its peak at the 8th week and it was higher than those of the other groups. Overall, these results indicate that BCG + D group may induce high percentages of CD4+ and CD8+ T cells.



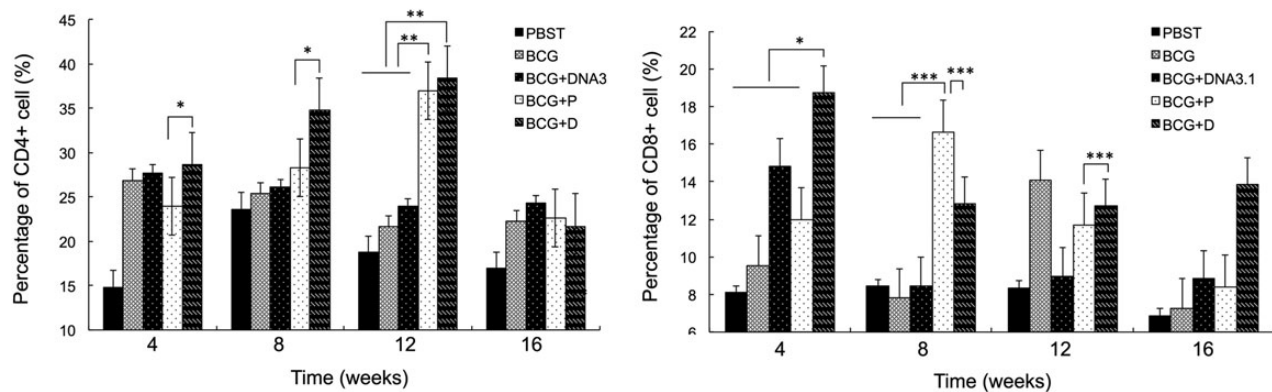
**Figure 1. Analysis of the Rv0577-specific total IgG and subtype IgG1 and IgG2a titer after immunization** Mice were anesthetized for blood sample collection at the indicated time points. The serum was obtained and screened by ELISA. The Rv0577-specific antibody levels including IgG (A), IgG1 (B), IgG2a (C), and IgG2a/IgG1 (D) are shown. \* $P < 0.05$  vs. PBST, BCG, BCG + DNA3.1 groups, \*\* $P < 0.05$  vs. PBST, BCG, BCG + DNA3.1, BCG + P, or BCG + D groups.

Rv0577 DNA boost induced high levels of IFN- $\gamma$  and IL-2. Th1 immune response is important in controlling TB since the infection is caused by intracellular pathogens. Since IFN- $\gamma$ , IL-2 is a characteristic

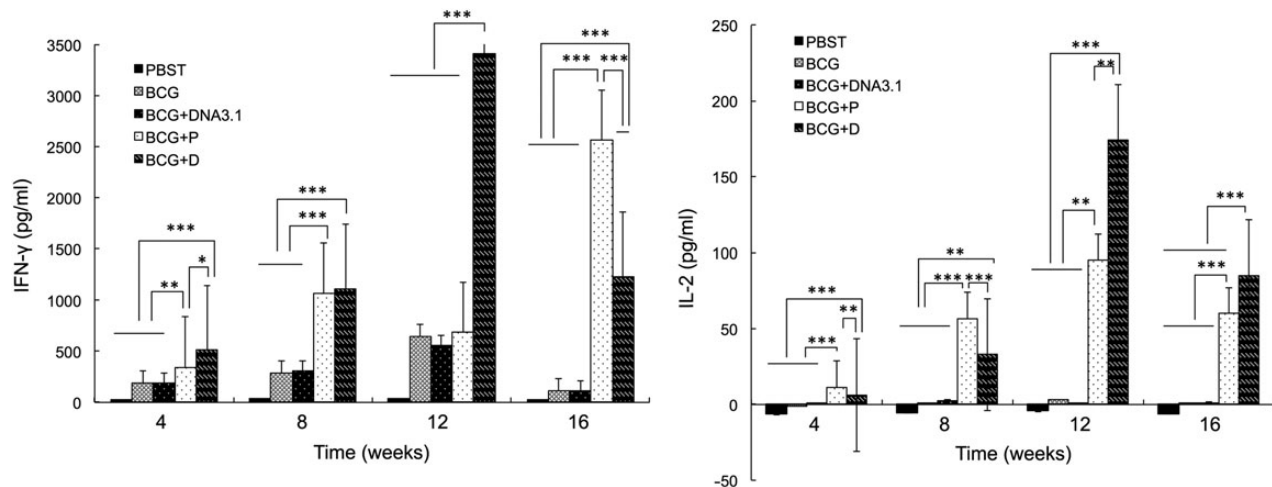
of Th1 response, and IL-4 is a characteristic of Th2 response, the concentrations of these cytokines were detected by ELISA (Fig. 4). The levels of IFN- $\gamma$  and IL-2 in BCG + D group are significantly higher than those in the PBST, BCG, and BCG + DNA3.1 groups at all tested time points. In addition, BCG + D group induced significantly higher levels of IFN- $\gamma$  than BCG + P group at the 4th and 12th week, and higher levels of IL-2 at the 12th and 16th week. The concentrations of IL-4 were too low to be characterized in all study groups ( $P > 0.05$ ) (data not shown). These results indicate that BCG + D group induces high levels of IFN- $\gamma$  and IL-2, which drives a Th1 immune response.



**Figure 2. Proliferative responses of splenocytes upon stimulation with Rv0577 protein *in vitro*** Splenocytes proliferation was analyzed by CCK-8 method. The proliferation was measured by SI. \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .



**Figure 3. The percentage of CD4+ and CD8+ T cells at different time point** The percentage of splenocyte subsets was determined by flow cytometry. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .



**Figure 4. The levels of IFN- $\gamma$  and IL-2 secreted by splenocytes** The cytokines secreted by splenocytes were detected by ELISA. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

are some of the key factors responsible for the limited efficiency of BCG in controlling TB [23,24]. In addition, the protection of subunit vaccines is not affected by environmental mycobacteria exposure. Therefore, improved subunit vaccines that could enhance the immunogenicity and protective efficacy of BCG both in children and in adults are needed.

There are three major types of heterologous boost vaccine candidates: protein-, plasmid DNA-, and viral-based vaccines which can express immunodominant protein [25]. Much attention has been paid to new and improved vaccines against TB focusing on mycobacterial secreted proteins, and these proteins have been shown to be protective in animals and recognized in infected humans [26–28]. Rv0577 protein is secreted by *M. tuberculosis* complex (MTBC) and participates in acetone aldehyde detoxification pathways [13]. It can be expressed in the lungs of TB patients and has been detected in the sputum samples of most Mtb-infected individuals. Mtb clinical isolates appear to express more Rv0577 protein than laboratory-adapted Mtb strains and other subspecies of MTBC, such as BCG [11]. Importantly, this protein can act as a specific recall antigen as well as a potent adjuvant triggering Th1-mediated immune responses [13]. These findings provide new insights into the potent activity of this protein as a vaccine against TB.

Cell-mediated immunity plays a key role in the defense against TB [29–31]. Most novel TB vaccine candidates currently under development are designed to induce high levels of cellular immunity. In this study, we analyzed proliferative responses of splenocytes under different immune strategies. The results suggested that BCG + D group induced a high level of proliferation reaction even at the 16th week, indicating that this regimen effectively enhanced the antigen-specific T-cell proliferation and induced durable T-cell responses. We also analyzed the percentage of CD4+/CD8+ among the splenocytes. Our data showed that BCG + P group induced an immediate immune response, whereas BCG + D group induced a long-term immune response. The CD8+ cytotoxic T cells (CTL) play a critical role in the immunity to certain intracellular bacterial infections and protection after reinfection by producing high amounts of IFN- $\gamma$  [32,33]. Infected human dendritic cells and macrophages are lysed by CD1- and MHC class I-restricted CD8+ T cells, and these CD8+ T cells can reduce intracellular bacterial numbers through the Fas/Fas L pathway [34]. By secreting granulysin, granzymes, and perforins, CD8+ T cells can kill mycobacteria-infected cells and protect against secondary mycobacterial challenge in the absence of CD4+ T cells [35,36]. Importantly, compared with vaccination with BCG/protein and BCG alone, BCG/DNA regimen can induce higher levels of IFN- $\gamma$  and IL-2 which are characteristics of Th1 response. The majority of researches indicated that Th1 immune response is essential for Mtb growth control [37], and the strong Th1 response induced by BCG is favorable to aid the maturation and maintenance of CTL [38], which strongly amplifies CTL responses. Furthermore, Th1 cells mainly develop after intracellular bacteria and some virus infection [39]. They are able to activate macrophages [40], natural killer cells, and CD8+ T cells. By secreting IFN- $\gamma$ , TNF- $\alpha$ , and IL-2, they are able to help B cells to produce immunoglobulins such as IgG2a in mice [41]. IFN- $\gamma$  is an essential component of protective immune response and a key effector cytokine in the control of Mtb infection [42]. In particular, IFN- $\gamma$  is widely used as an indicator for vaccine efficacy [43], whereas IL-2 is associated with memory T cells and plays an important role in the long-term survival [44,45]. Several studies have demonstrated that IL-2 can influence the course of Mtb infections, either alone or in combination with other cytokines [46,47]. In addition, IL-2 is also a growth factor of both CD4+ and CD8+ T cells. These cells that have a high potential of IL-2 secretion can play a functional role in the protection against TB [48,49].

Additionally, both BCG/DNA and BCG/protein immunization produced significant high levels of antibody responses, including increased total IgG, subtypes IgG1 and IgG2a. The IgG2a level induced by BCG/protein was high at early stage, whereas BCG/DNA group induced higher IgG2a levels compared with BCG/protein group at latter stages. These results demonstrated that BCG/DNA can induce long-lasting antibody responses. The ratio of IgG2a/IgG1 further suggested that the cellular responses in BCG/DNA are Th1-like predominant.

In conclusion, to develop novel BCG-based vaccines for controlling TB, we tried to utilize BCG as a primer with a heterologous prime-boost regimen. We evaluated the ability of Rv0577 DNA or purified Rv0577 protein as a booster combined with BCG to immune BALB/c mice. Our results suggested that the durability of BCG-induced specific T-cell response was significantly augmented by a heterologous BCG prime-DNA boost vaccination regimen, characterized by high levels of antibody response, proliferation response, percentages of CD4+/CD8+, and cytokine secretion. These results suggested that BCG/DNA vaccination might be a potential candidate since it was able to generate a much prolonged Th1 immune response when compared with BCG and BCG+P groups. As many previous studies demonstrated a positive correlation between Th1 immune response and protection [15,50,51], we infer that the improved Th1 immune response elicited by the BCG prime/DNA boost strategy may result in a stronger protection than other groups in this study; however, it still needs to be confirmed by further studies. For future studies, we are planning to develop a viral-based vaccine and rBCG expressing Rv0577 protein, then use subunit vaccines to boost rBCG and to evaluate the protective efficacy and safety of this promising vaccines.

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