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Original Article

Improved immunogenicity of *mycobacterium tuberculosis* Rv0577 by a heterologous prime-boost vaccination strategy in mice

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Abstract: Tuberculosis (TB) remains a major cause of global mortality and morbidity. However, the only available vaccine, *Mycobacterium bovis* bacillus Calmette-Guerin (BCG), is insufficiently effective against this disease. Therefore, the development of a potent vaccine against TB is urgently needed. Virulence-associated protein Rv0577 from *Mycobacterium tuberculosis* was demonstrated to induce the maturation of dendritic cells and enhance cellular immunity, and it could be helpful in designing efficacious TB vaccines. In this research, we constructed both protein vaccine and DNA vaccine of Rv0577. We have generated, characterized and studied the immunogenicity induced by different immunization strategies. Antigen-specific antibody and cellular immune responses were evaluated in mice. The best vaccination strategy was achieved with a heterologous DNA prime and protein boost. This strategy proliferated splenocytes, induced high levels of specific antibody responses, a high proportion of $CD4^+$ and $CD8^+$ T cells and Th1 type cytokines. The results in this study showed that the improved immunogenicity can be elicited by Rv0577 with an appropriate vaccination strategy, and it could be considered for developing a novel vaccine against TB.

Keywords: *Mycobacterium tuberculosis*, prime-boost, Rv0577, cell-mediated immunity, vaccine

Introduction

Mycobacterium tuberculosis (Mtb), the causative agent of TB, is a facultative intracellular pathogen which could establish lifelong infection within human [1]. Globally, Mtb caused 9.0 million new cases and 1.5 million deaths in 2013 [2]. In recent years, the increase of Mtb/HIV co-infection and the prevalence of multidrug-resistant and extensively drug-resistant strains, posed great challenges to global TB control [3, 4]. The only available vaccine BCG is effective in infants but has a limited and variable effect (0%-89%) in adults [5-7]. Therefore, the development of a new vaccine which would provide an effective protection against Mtb among general populations is critically needed.

Previous research demonstrated that virulence-associated Mtb antigens as novel vaccine candidates were important for inducing host protective immunity [8-10]. The Mtb complex-restricted gene Rv0577 encodes a 32 kDa protein (also known as CFP32 and TB27.3) which is involved in the methylglyoxal detoxification pathway [11, 12]. Neutral red staining suggested an association of this gene with virulence [13]. The diagnostic potential of this protein and its molecular basis of immunological function have also been explored [14, 15]. These works indicated that Rv0577 could be a promising target for designing a new TB vaccine. Importantly, we have demonstrated that durable Th1 immune response was induced in mice by M. bovis BCG prime-Rv0577 DNA boost vaccination [16].

An effective vaccine requires an optimized immunization regimen. Previous studies suggested that prime-boost vaccination strategy could improve the immunogenicity of the vaccine and enhance the immune response [17, 18]. This strategy has been proved to be attractive in the development of new TB vaccines. In this study, we used Rv0577 as an immunodom-

inant antigen and evaluated its immune efficiency using different prime-boost vaccination strategies in BALB/c mice including DNA/DNA, DNA/Protein, Protein/Protein. We tried to explore the best strategy for using this protein as a potential vaccine of TB.

Materials and methods

Bacterial strains, media and plasmids

M. bovis BCG-Pasteur was obtained from the Chengdu Biological Products Institute. BCG was cultivated in Sautons medium ($MgSO_4$ 0.5 g, K_2HPO_4 0.5 g, citric acid 2.0 g, sodium glutamate 8.0 g, glycerol 60.0 ml, $ZnSO_4$ 0.01 g and ferrum-ammonium citrate 0.05 g in 1000 ml, pH 7.4-7.5). The plasmids pcDNA3.1 (+) and pET28a (+) were conserved in our laboratory.

Recombinant Rv0577 protein

The Rv0577 gene was amplified by PCR using genomic DNA of Mtb H37Rv (ATCC27294) with Taq platinum DNA polymerase (Thermo, France). The gene-specific primers were described elsewhere [15]. The PCR products of Rv0577 were double-digested by *Nde*I and *Hin*dIII (Thermo, France). Then the products were inserted into pET28a (+) vector and the recombinant plasmids containing Rv0577 were transformed into *E. coli* BL21 cells. After being induced with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG, Amresco, USA), the *E. coli* cells were harvested and lysed by sonication. Rv0577 protein was purified by Ni-NTA affinity columns (GE Healthcare, UK). Each purification step was analyzed by 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western-blotting. The purified protein was concentrated with Millipore, and the protein was incubated with polymyxin B (PmB)-agarose (Sigma) for 6 h at 4°C to remove endotoxin contamination. Lastly, purified endotoxin-free recombinant protein was frozen at -70°C. The protein concentration was estimated by the BCA Protein Assay Kit (PIERCE, China).

Rv0577 DNA vaccine

The Rv0577 gene was amplified by PCR, the primers are as following: F, 5'-GCGGATCCATGCCCAAGAGAAGCGA-3', and R, 5'-GAGAATTCCTATTGCTGCGGTGCGG-3'. The PCR products were cloned into pcDNA3.1 (+). Positive clones were verified by restriction analysis and DNA sequencing (Invitrogen, Shanghai, China). Plasmid DNA was isolated using Endofree Plasmid Maxikit (OMEGA, USA) and was diluted in PBS at a concentration of 1 mg/ml.

Animals and immunization regimens

4-5 week-old pathogen-free BALB/c female mice were purchased from the Dashuo Laboratory Animal Technology Corporation (Chengdu, China). BALB/c mice were vaccinated with either Rv0577 protein or Rv0577 DNA plasmid with different prime-boost strategies. Each group was immunized and treated with one of the following strategies:

(a) PBST group: animals were immunized 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.

(b) BCG group: initial immunization was given with a dose of 5×10^6 CFU in a volume of 0.1 ml at week 0. PBST was given with a dose of 0.1 ml at weeks 3 and 6.

(c) P+P group: immunized with 50 μg Rv0577 proteins at weeks 0, 3 and 6.

(d) D+D group: immunized with 50 μg Rv0577 DNA plasmids each time at weeks 0, 3 and 6.

(e) DNA3.1 plasmid group: immunized with 50 μg pcDNA3.1 plasmids each time at weeks 0, 3 and 6.

(f) P+D group: prime immunized with 50 μg Rv0577 DNA plasmids at week 0 and then boosted with 50 μg proteins at weeks 3 and 6.

(g) DNA3.1 plasmid protein group: prime immunized with 50 μg pcDNA3.1 plasmids at week 0 and boosted with 50 μg proteins at weeks 3 and 6.

PBST, BCG and protein were immunized subcutaneously, while the plasmids were injected intramuscularly. Four mice in each group were sacrificed at weeks 10, 14, 18 and 22. Blood was collected by removing the eyeballs, the spleens were removed aseptically as well. The blood was static at 4°C till sera was separated, then sera was collected by centrifugation and stored at -20°C to detect the antibody response. The splenocytes were used to analyze

Table 1. Immunize regimens used in this study

Group	Prime	1st boost	2nd boost
PBST	PBST	PBST	PBST
BCG	BCG	PBST	PBST
P+P	Protein Rv0577	Protein Rv0577	Protein Rv0577
D+D	Plasmid Rv0577	Plasmid Rv0577	Plasmid Rv0577
DNA3.1	Plasmid pcDNA3.1	Plasmid pcDNA3.1	Plasmid pcDNA3.1
D+P	Plasmid Rv0577	Protein Rv0577	Protein Rv0577
DNA3.1+P	Plasmid pcDNA3.1	Protein Rv0577	Protein Rv0577

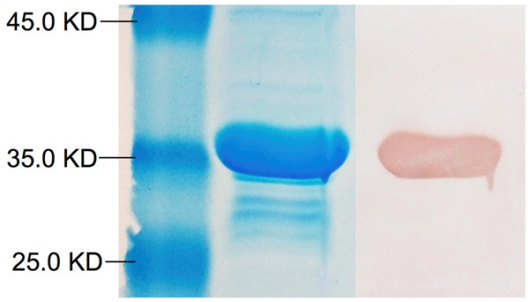

Figure 1. SDS-PAGE and western blotting of the expressed products of pET-Rv0577. Lane 1, Unstained Protein MV Maker (Ferments, China). Lane 2, Rv0577 protein was analyzed by 15% SDS-PAGE. Lane 3, Rv0577 protein was analyzed by western blotting using anti-His antibodies.

proliferation, cytokine secretion and subtypes. All immunize regimens are described in **Table 1**. All procedures were conducted in accordance with national regulations and animal welfare requirements. All activities with animals in this study were complied with laboratory animal care and used by designated members of Ethical Review Committee of Schuan Laboratory Animal Society.

Antibody responses

The antigen-specific IgG and subtype IgG1 and IgG2a in the sera were detected by an indirect enzyme-linked immunosorbent assay (ELISA). Firstly, 96-well high binding plates (Corning Costar3590, USA) were coated overnight at 4°C with 0.1 ml Rv0577 protein (10 μg/ml). After being washed three times with PBST (PBS plus 0.05% Tween 20, pH = 7.2), the plates were blocked with 5% skim milk in PBS (pH 7.2) for 2 h at 37°C and then washed as previously mentioned. The plates were incubated with twofold serially diluted sera for 2 h at 37°C. After the plates were washed another three times, HRP-conjugated goat anti-mouse IgG, IgG1 and IgG2a (eBioscience, USA) were added at a dilution of 1:2000, 1:5000 and 1:5000, respectively, and then incubated for 1 h at 37°C. The plates were scoured with PBST six more times and then detected by TMB substrate. After 20 minutes of incubation at room temperature in the dark, the reaction was stopped with 50 μl solution (0.1 N sulfuric acid). Finally, the absorbance was read at 450 nm in an automatic microplate reader (Bio-Rad, USA). Serum titers were determined as the highest dilution of immune serum at which the absorbance at OD450 greater than or equal to twice the absorbance of negative control wells.

Proliferation of splenocytes

The proliferation of splenocytes was tested by CCK-8 assay (Cell Counting Kit-8, BOSTER). The mice were sacrificed and the spleens were homogenized manually. Firstly, the spleens were prepared as single cell suspensions using cell strainer (BD falcon, USA) and the red blood cells were lysed with RBC Lysis Buffer (Sigma). Then, splenocytes were suspended in cRPMI (RPMI-1640 medium (Invitrogen, USA) plus 10% fetal calf serum (FCS), 100 U/ml of Penicillin, 100 μg/ml of Streptomycin) and plated in 96-well flat-bottom plates at a concentration of 5×10^5 cells per well in 100 μl of cRPMI. Each well was stimulated with 10 μl Rv0577 protein (100 μg/ml), while the negative controls were treated with 10 μl PBS. After being incubated for 48 h at 37°C, the plates were added 10 μl CCK-8 each well, and then the incubation continued for 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.

Percentages of splenocyte subsets

Freshly isolated splenocytes were harvested and cultured in 24 well flat-bottom plates (5×10^6 cells in 1 ml of cRPMI per well). Then the splenocytes were stimulated with 100 μl Rv0577 protein (100 μg/ml) in each well. The supernatants were harvested after 72 h of

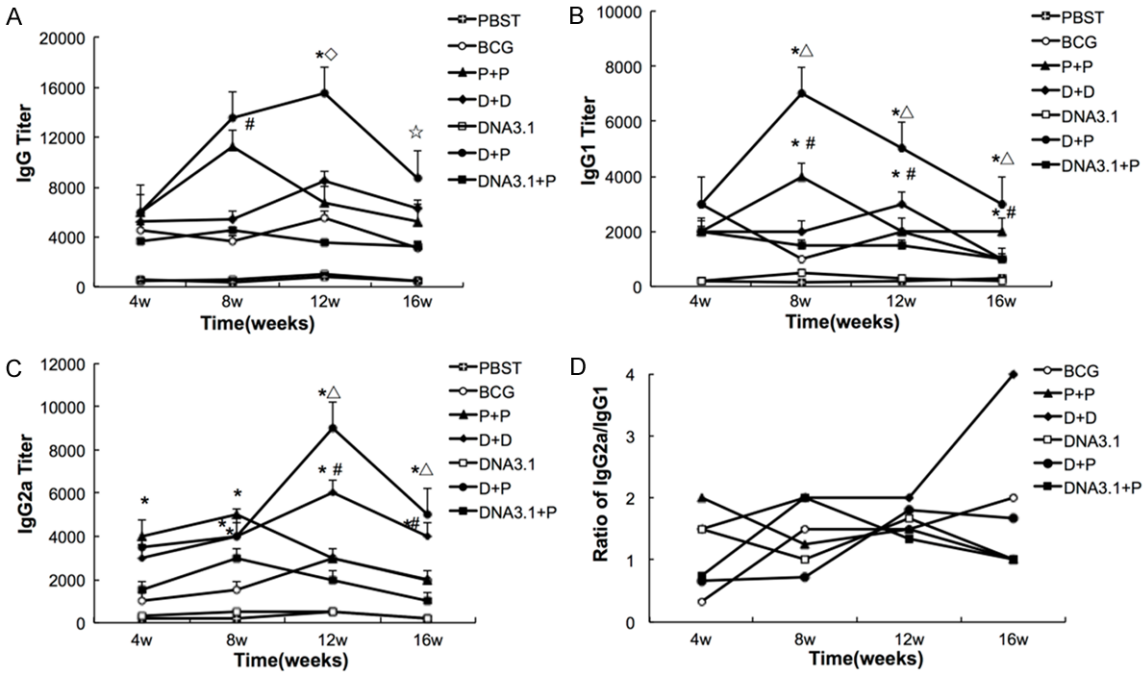

Figure 2. The titers of total IgG and subtype IgG1 and IgG2a. The Rv0577-specific antibody responses were assessed by indirect ELISA. The antibody levels including IgG (A), IgG1 (B), IgG2a (C) and IgG2a/IgG1 (D) were shown. **P*<0.001 versus PBST, BCG, DNA3.1 or DNA3.1+P group, Δ*P*<0.001 versus D+P, P+P or D+D group, ◊*P*<0.05 versus D+P or D+D group, ☆*P*<0.05 versus D+P or P+P group, #*P*<0.05 versus P+P or D+D group.

incubation (37°C, 5% CO_2) and stored at -80°C until cytokine detection. Firstly, the cells were washed with 0.1 M PBS three times. Then the cells were suspended in 200 μl PBS and were incubated with rabbit anti-Mouse $CD4^+$ PE and $CD8^+$ FITC (eBioscience, USA) in ice bath for 30 min. At last, the cells were washed twice with PBS, and the proportions of $CD4^+$ and $CD8^+$ T cells were measured by flow cytometry (BD Accuri™, USA).

Cytokine analyses

Concentrations of IFN-γ, IL-2 and IL-4 secreted by the lymphocyte were measured by ELISA according to the manufacturer's instructions (eBioscience, USA). The levels of cytokines released into culture were measured by an automatic microplate reader.

Statistical analysis

Data were analyzed by one-way ANOVA Test. Differences between the groups were assessed for statistical significance using Tukey's post hoc test using SPSS21.0 software. P<0.05 were considered as statistically significant. P<0.001 were considered as highly significant. The results are presented as the mean ± standard deviation (SD).

Result

Construction of the protein and DNA vaccine of Rv0577

To evaluate the immunological potent of Rv0577, we constructed eukaryotic recombinant plasmid pcDNA3.1-Rv0577 and the prokaryotic recombinant plasmid pET28a-Rv0577. The protein of Rv0577 was expressed and purified with a molecular mass of 32 kDa (**Figure 1**).

Serological immune response

To evaluate the immunogenicity of the immunized animals, the antibody responses in the sera of the immunized mice at different time points were evaluated by ELISA (**Figure 2**). Group D+P induced the highest levels of IgG1 and IgG2a than other groups at 12th and 16th week. Otherwise, the total IgG levels of D+P group were higher than D+D group at 12th week and P+P group at 16th week. IgG2a and IgG1 in sera are associated with Th1 (IgG2a) and Th2

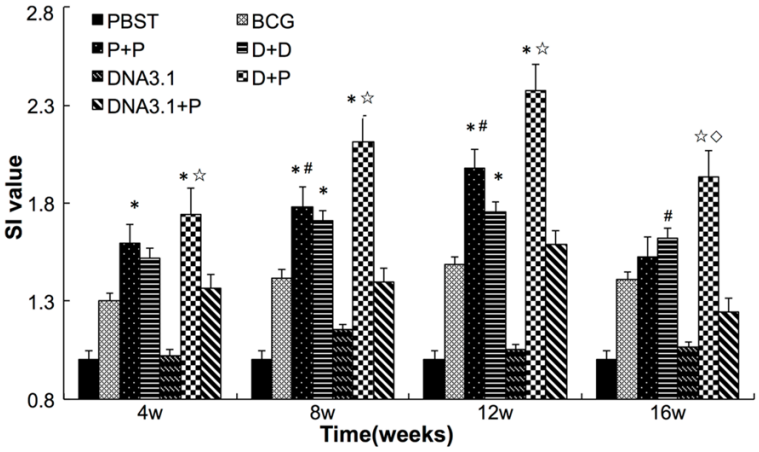

Figure 3. The proliferative responses of splenocytes *in vitro*. The proliferation of splenocytes was analyzed by CCK-8 method and the results were measured by SI. **P*<0.001 versus PBST, BCG, DNA3.1 or DNA3.1+P group, ☆*P*<0.05 versus D+P or P+P group, ◊*P*<0.05 versus D+P or D+D group, #*P*<0.05 versus P+P or D+D group.

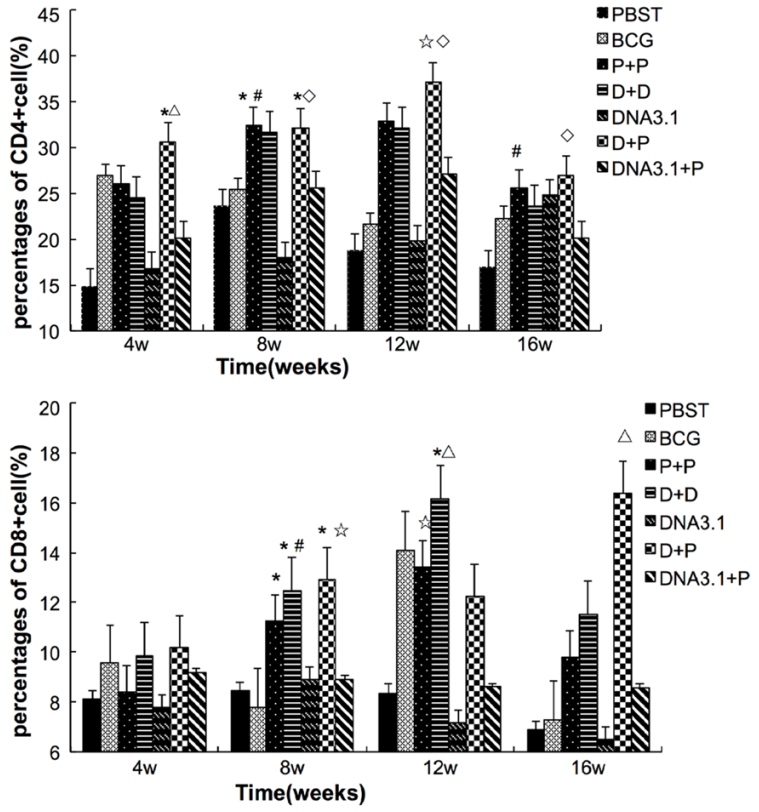

Figure 4. The percentages of $CD4^+$ and $CD8^+$ T cell at different time point (%). The proportions of splenocyte subsets were determined by flow cytometry. **P*<0.001 versus PBST, BCG, DNA3.1 or DNA3.1+P group, Δ*P*<0.001 versus D+P, P+P or D+D group, ☆*P*<0.05 versus D+P or P+P group, ◊*P*<0.05 versus D+P or D+D group, #*P*<0.05 versus P+P or D+D group.

(IgG1) response respectively. D+P group showed relatively mixed Th1/Th2 response at 4-8th weeks, and the higher IgG2a/IgG1 ratio at 12-16th weeks suggested its strong ability shift towards relatively Th1-based response.

Proliferation of splenocytes

To evaluate cell-mediated immune response, Rv0577 protein stimulated spleen lymphocyte proliferation was assessed by CCK-8 assay (**Figure 3**). We found that the proliferation of splenocytes in the D+P group was significantly higher than the other six groups at 8th and 12th week. Importantly, the proliferation reaction of D+P group still maintained at a high level even at the 16th week.

Percentages of $CD4^+$ and $CD8^+$ T cells in immunized mice

Both $CD4^+$ and $CD8^+$ T cell immune responses are important to anti-TB. The percentages of splenocyte subsets were measured by flow cytometry (**Figure 4**). We found that D+P group induced a significantly higher ratio of $CD4^+$ T cells than D+D group at any time point. In addition, D+P group generated markedly higher $CD4^+$ T cell responses compared with P+P group at 4th and 12th week. On the other hand, the proportions of $CD8^+$ T cells in the D+P group reached the peak at 16th week and were remarkably higher than P+P and D+D groups.

Cytokine profiles

The cellular immune responses are important for controlling infection caused by intracellular pathogens. Therefore, we assessed the functional profile of the cellular immune responses elicited by Rv0577 with different immunization

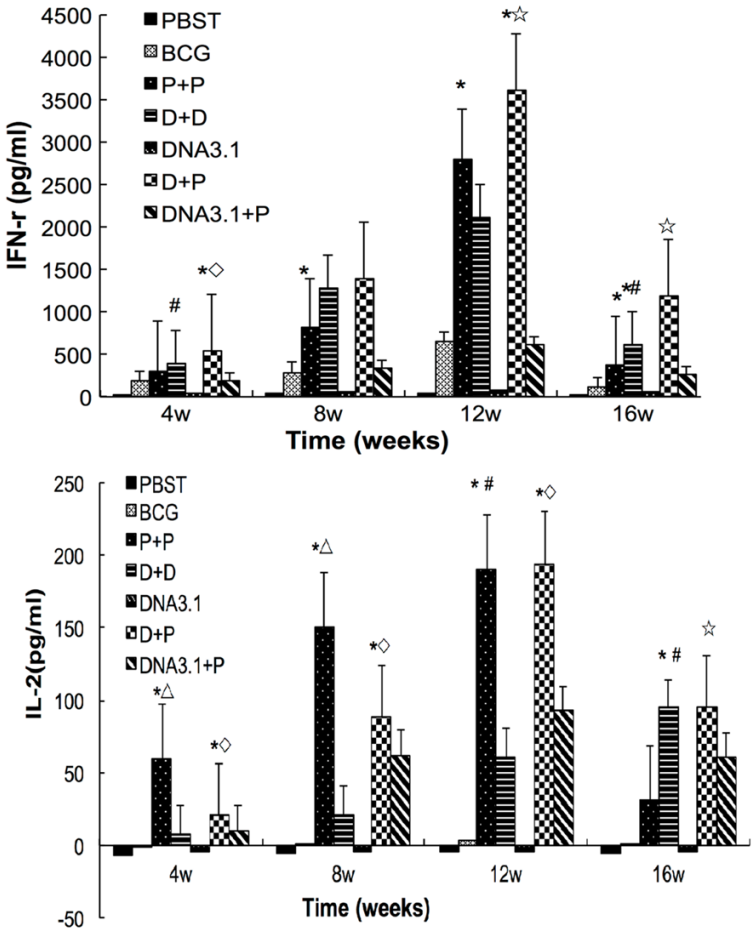

Figure 5. The concentrations of IFN-γ, IL-2 and IL-4. The cytokines secreted by splenocytes were detected by ELISA. **P*<0.001 versus PBST, BCG, DNA3.1 or DNA3.1+P group, Δ*P*<0.001 versus D+P, P+P or D+D group, ☆*P*<0.05 versus D+P or P+P group, ◊*P*<0.05 versus D+P or D+D group, #*P*<0.05 versus P+P or D+D group.

strategies. The levels of IFN-γ, IL-2 and IL-4 were measured by ELISA (**Figure 5**). The concentrations of IFN-γ and IL-2 in D+P group went up from 4th week and reached the peak at 12th week, and the levels of IFN-γ in D+P group were significantly higher than P+P group at 4th, 12th and 16th week. In addition, the concentrations of IL-2 in D+P group were significantly higher than other groups except for P+P group at 4th, 8th and 12th week. The secretion of IL-4 maintained low, and the changes were not statistically significant in any groups (*P*>0.05) (data not shown).

Discussion

Both an immunodominant protein and a novel vaccine strategy which could elicit strong and long-lasting immune responses especially the cell-mediated immunity (CMI) are usually needed to develop an effective vaccine against TB since Mtb is an intracellular bacterium [19, 20]. Two to six weeks after infection, T cells differentiate into class II MHC-restricted $CD4^+$ and class I MHC-restricted $CD8^+$ T cells. Then the activated $CD4^+$ T cells further differentiate into Th1 cells which by producing IFN-γ, IL-2 and TNF-α to control infection [21].

Culture filtrate proteins are secreted proteins in Mtb culture supernatants. In the Mtb infection animal models, live vaccine of Mtb could efficiently stimulate immune cells to secrete cytokines such as IFN-γ and promote better protective immunity than inactivated vaccine. It is thought to be related to the secreted proteins. Previous studies have suggested the importance of secreted proteins in the immune protections such as CFP10, ESAT-6, CFP21 and so on [7, 22-24]. As a highly antigenic secreted protein, Rv0577 has been proved to provide a protection against challenge with Mtb in the mouse model and contribute to the maturation of dendritic cells and drive Th1 immune responses [15, 20, 25]. It was expressed in the lungs of TB patients and could also stimulate a humoral response. Therefore, we speculate that this protein may be a potent antigen in serodiagnostic test and vaccine development for TB. In our previous study, we used a post-BCG vaccination boost with protein or DNA vaccine of Rv0577 to immune BALB/c mice and the results suggested that the BCG prime-Rv0577 DNA boost vaccination could induce a durable Th1 immune response [16]. Therefore, we further evaluated the immunogenicity of Rv0577 and tested the potential of this gene as an immunological strategic tool in this study.

The role of antibody response in protection against Mtb is considered less important than

the CMI. However, recent research has demonstrated antibodies may play a role in protection against TB [26, 27]. The results in our study suggested that D+P group can stimulate antigen-specific antibody responses, and compared to other groups, the antibody response in this group lasted longer.

The spleen is an immune organ. As an important immune reaction center of anti-TB infection, the proliferation of splenocytes is important in controlling Mtb infection. When the body suffers from bacterial, the splenocytes will produce specific proliferative responses to control the infection. Therefore, the proliferation of splenocytes can reflect the protective effect of vaccine indirectly. D+P group showed significantly higher proliferation of splenocytes than D+D, P+P or other control groups. The proliferation responses of D+P group were even efficiently maintained at 16th week, indicating that HPB immunization greatly enhanced the antigen-specific T cell proliferation and this proliferative activity can last longer.

In the early stage of infection, antigen presenting cells will deal with Mtb and MHC- class II molecules to form an antigen complex for presentation to $CD4^+$ T cells which by secreting IFN-γ, IL-2 and other cytokines to control infection [28]. In the later stage of infection, the anti-TB response mainly depends on $CD8^+$ T cells. By secreting perforin, granzyme and Fas/FasL, $CD8^+$ T cells can play the cytotoxic effect on pathogens and infected macrophages, thereby inducing the apoptosis of target cells [29, 30]. After Mtb infection, $CD4^+$ and $CD8^+$ T cells expand into effector cells and then differentiate into long-lived memory cells [31]. These cells potently and specifically inhibit the growth of the intracellular Mtb [32]. As the targets for vaccine design, $CD4^+$ and $CD8^+$ T cells are the essential components of cell-mediated immune responses. Our results suggested that HPB regimen may induce significantly stronger and longer-lasting $CD4^+$ and $CD8^+$ T cell immune responses than protein- or DNA-based vaccines alone.

To further understand the immune response, cytokine profiling was carried out. Cytokines secreted by D+P group significantly toward polarized Th1-type responses as the high levels of IFN-γ, IL-2 and down regulation of IL-4. The strong Th1 cellular response mainly develops following infections of intracellular bacteria and some viruses. Th1 cells could induce macrophages mediated phagocyte-dependent killing via the production of reactive nitrogen and oxygen intermediates [33-35]. Moreover, IFN-γ is necessary for resistance to *mycobacterial* infections [36, 37]. IL-2 plays an important role in the long-term survival of primed $CD4^+$ T cells *in vivo* and is predominantly associated with central memory T cells (TCM) [38, 39]. However, IL-4, the maker cytokine of Th2 response, was hardly detected by ELISA in any groups, which further demonstrated the anti-TB responses in our job are Th1 predominant.

To our knowledge, DNA vaccines have been proved to provoke a potent cytotoxic T cell response, exhibit a good safety profile and feature a low production cost. However, they are poorly immunogenic and could not elicit effective antibody responses [40, 41]. On the other hand, protein vaccines probably induce $CD4^+$ T cells and enhance humoral responses but fail to elicit strong and durable immune responses when administered alone [42]. Previous studies have suggested the heterologous prime-boost (HPB) immunization regimen which combined DNA and protein vaccines could take the advantages of both DNA and protein vaccines to induce robust and long-lasting cellular immunity as well as antibody responses against infection. Furthermore, this strategy has been demonstrated to provide significant protection against bacterial, viral, influenza and parasitic infections [43-46].

HPB immunization strategies could induce significantly stronger antigen specific T cell responses than other homologous prime-boost strategies. We speculate that different vectors expressing the same antigen could minimize the development of antivector immunity [17, 47]. Importantly, DNA vaccines are particularly suited for priming immune responses since they have the strong potential to induce memory responses and the poor immunogenicity of DNA vaccines could be overcome by the corresponding protein to boost [48]. Furthermore, compared to our previous study [16], we found that BCG prime-DNA boost vaccination prefers to induce robust cellular immune responses at early stage, while DNA prime-protein boost strategy could induce more robust and durable immune responses.

Conclusions

In summary, compared to the simple homologous prime-boost vaccination and BCG vaccination alone, our results demonstrated that Rv0577 with heterologous DNA-protein/prime-boost immunization strategy can induce robust antibody and cell-mediated immune response in mice. These immune responses became more and more prominent as time went on and can last till 16th week. As an important index of vaccine evaluation, immunogenicity has been demonstrated to have a positive correlation with protection in many previous studies [7, 49]. Therefore, we infer that the improved immunogenicity elicited by the heterologous DNA-protein/prime-boost strategy may result in a potent protection although it still needs to be confirmed by further studies. Moreover, since this vaccination strategy doesn't rely on BCG, it may bring gospel to the prevention of Mtb infection in immunodeficiency patients such as HIV infected individuals. On this basis, we believe that Rv0577 with HPB strategy could be a promising candidate vaccine for TB. In addition, proper adjuvant and protective efficacy of this candidate vaccine will be needed in our further studies.

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Disclosure of conflict of interest

None.

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