Original Article
Polyethylenimine modified nanoparticle adjuvant increases therapeutic efficacy of DNA vaccine Ag85A-ESAT-6-IL-21 in mice infected with Mycobacterium tuberculosis

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Abstract: Despite considerable research efforts, the treatment of tuberculosis (TB) remains to be a great challenge in the requirement of prolonged therapy with multiple high-dose drugs and associated side effects. As such, the development of an effective vaccine to treat TB may be feasible strategy. The aim of this study was to investigate the possibility of polyethylenimine (PEI) modified nanoparticle adjuvant increases therapeutic efficacy of DNA vaccine Ag85A-ESAT-6-IL-21 in mice that were aerosol infection with the standard virulent M. tuberculosis strain H37Rv. The therapeutic effect was observed in the mice after M. tuberculosis infectious mouse model was established. The results indicated that the PEI modified DNA vaccine significantly induced a powerful therapeutic efficacy compared with that of Bacille Calmette-Guérin (BCG) or other immunotherapeutic vaccinations, which was reflected in decreasing the M. tuberculosis numbers and pathological damage in lungs, and increasing the mouse weight gain. The effective mechanism was closely related with a strong immune response induced by the DNA vaccine in the mice. The findings provide further approaches for PEI modified DNA vaccine Ag85A-ESAT-6-IL-21 as a potential therapeutic vaccine to fight against TB.

Keywords: Mycobacterium tuberculosis, therapeutic vaccine, nanoparticles, interleukin IL-21, polyethylenimine

Introduction

Tuberculosis (TB), caused by the causative agent of Mycobacterium tuberculosis (M. tuberculosis), now ranks alongside human immunodeficiency virus (HIV) as a significant cause of morbidity and mortality worldwide, especially in developing countries. The latest estimate of the global in 2014, TB mortality among HIV-negative people was around 1.5 million and 0.4 million HIV-positive despite the availability of a live attenuated Bacille Calmette-Guérin (BCG) vaccine and anti-TB antibiotics [1-3]. Meanwhile, a rapid spread of multi-drug resistant (MDR) bacteria causing refractory TB cases per year has worsened the problem during recent years, which has pushed our available repertoire of anti-TB therapies to the limit of effectiveness. Therefore, there is an urgent need for improved vaccines as well as developed novel treatment modalities including investigation of therapeutic vaccines if we are to control the TB [4-6].

Interleukin-(IL-) 21 is a pleiotropic cytokine that regulates the wide range of relevant biological activity including innate and specific immunity. It is also suitable for combinational therapeutic regimens with other agents [7-9]. The 6 kDa early secretory antigenic target (ESAT-6) and antigen 85A (Ag85A) are an important protective antigens encoded by M. tuberculosis, which have been demonstrated by others [10-13] and by us [14-16]. Polyethylenimine (PEI), a cationic polymer widely used in non-viral gene delivery, is a potential adjuvant in DNA-based vaccination for induction of protective and therapeutic immunity [17, 18].

In our previous study [19], we had engineered Fe3O4 nanoparticle-based DNA vaccine express-
ing Ag85A and ESAT-6 of *M. tuberculosis* as well as mouse IL-21, and the vaccine could elicit mice to generate an anti-*M. tuberculosis* immune responses that showed that the increase of the protective efficacy against *M. tuberculosis* infection in the immunized mice. However, the therapeutic efficacy against TB in *M. tuberculosis* postexposure mice remains to be evaluated. In our current study, we used the engineered DNA vaccine expressing Ag85A-ESAT-6-IL-21 modified by PEI-nanoparticle to evaluate the therapeutic efficacy in mice infected with *M. tuberculosis*. Indeed, the PEI modified DNA vaccine markedly decreased the *M. tuberculosis* load and pathological change in lungs of mice infected with the standard virulent *M. tuberculosis* strain H37Rv compared with that of mice treated with bacille Calmette-Guérin (BCG) and other vaccines. Overall, this work was significantly contributed to support that the PEI-nanoparticle modified DNA vaccine Ag85A-ESAT-6-IL-21 may be a potential vaccine candidate based TB therapeutic strategy.

**Materials and methods**

**Mice**

Specific-pathogen-free male C57BL/6 mice between 5 and 6 weeks of age were obtained from Animal Center of Yang Zhou University of China and were raised at the Experimental Animal Center, Southeast University. All animal experiments were conducted following the guidelines of the Animal Research Ethics Board of Southeast University. Full details of the study approval can be found in the approval ID: 20080925.

*M. tuberculosis*, BCG, and DNA vaccine

H37Rv was used as the virulent *M. tuberculosis* strain for challenge study, and was grown in Sauton medium enriched with 0.5% sodium pyruvate and 0.5% glucose. BCG (Connaught strain; product ID: 2012080606) was a gift from the Centers for Disease Control and Prevention in Jiangsu Province of China. The DNA vaccine pIRES-IL21-Ag85A-ESAT-6 was developed in our previous work [14-16].

**Preparing of PEI-nanoparticle modified DNA vaccine Ag85A-ESAT-6-IL-21**

Nanoparticles (NPs) of Fe3O4 coated with glutamic acid (nano-Fe3O4-Glu) were a gift from Dr. Chen Zhongping of Southeast University of China; these NPs have no cytotoxicity to cell lines in vitro [20]. PEI was ordered from Sigma (Steinheim, Germany). The PEI modified DNA vaccine was performed as described previously [19, 20]. Briefly, PEI-NP coated DNA vaccine pIRES-IL21-Ag85A-ESAT-6 or pIRES mock plasmid was prepared by shaking the mixtures of 5 mL of the nano-Fe3O4-Glu to 1 mL PEI (5:1 ratio) for 60 minutes, and then shaking the mixtures with 2 g/L of DNA vaccine pIRES-IL21-Ag85A-ESAT-6 or pIRES mock plasmid (2:1 ratio) for another 60 minutes. The PEI modified DNA vaccine was identified by a scanning electron microscope, the zeta potential determination, RT-PCR, and Western blot, respectively, as described in our previous report [21].

Mice infected with *M. tuberculosis* and treated with PEI modified DNA vaccine

In mouse infectious model experiment, 6 mice infected with *M. tuberculosis* H37Rv aerosol with a low dose of bacteria. Briefly, the nebulizer compartment of an airborne infection apparatus (Glas-Col, Terre Haute, Ind.) was filled with 5 ml of a suspension containing 2×10^7 (colony-forming units, CFU) bacteria to allow uptake of 50 to 100 viable H37Rv bacteria per lung during a 30-minute exposure. The numbers of viable bacteria in the lungs were determined at 24 hours by plating serial dilutions of whole-lung homogenates onto nutrient medium and counting bacterial colonies after 21 days of incubation at 37°C in humidified air [22]. The infected mouse model was successfully established after the bacteria clone was identified in each mouse lungs.

In treated experiments, 162 mice were infected with *M. tuberculosis* H37Rv by aerosol as above-mentioned, and then were randomly divided into six groups (27 mice/each group) after 24 hours: (1) phosphate buffered saline (PBS) group, mice intramuscular injection (i.m.) treated with 100 μl PBS; (2) mock plasmid group (pIRES), mice i.m. treated with 100 μg mock plasmid; (3) nano-Fe3O4-Glu mock plasmid (Na-P) group, mice i.m. treated with 100 μl mixture suspensions containing 50 μg nano-Fe3O4-Glu and 50 μg mock plasmid; (4) DNA vaccine pIRES-Ag85A-ESAT-6 (PAEI) group, mice i.m. treated with 100 μg DNA vaccine; (5) PEI modified DNA vaccine Ag85A-ESAT-6-IL21 (NaPAEI) group, mice i.m. treated with 100
μl mixture suspensions containing 50 μg DNA vaccine and 50 μg nano-Fe$_3$O$_4$-Glu particles; (6) BCG group, mice received subcutaneous injection (s.c.) with 1x10$^6$ CFU of BCG [21, 22]; another 9 mice were not infected with M. tuberculosis as normal control group. Each mouse was therapeutically vaccinated three times (1, 3, and 5 weeks after infection) in the right hind legs with 2-week intervals between the immunotherapeutic vaccinations. Two weeks after every time therapeutic vaccination, 6 mice were sacrificed for detecting serum interferon-γ (IFN-γ), and the remnant mice were continuously observed for therapeutic effect until ten weeks after finishing three time therapeutic vaccinations, and all treated mice were killed for detecting the IFN-γ secreted by splenocytes, and bacterial burden, as well as the obtaining murine weight.

Measuring cytokine IFN-γ

Sera were separated from the M. tuberculosis infected mice treated with a various immunotherapeutic vaccinations for detecting IFN-γ (1:1000-fold dilution). A commercially available double antibody sandwich enzyme linked immunosorbent assay (ELISA) kit for detecting serum IFN-γ was performed by following the Kit’s protocol (eBioscience, USA) [23]. The IFN-γ secreted by splenocytes was measured by using enzyme-linked immunospot (ELISPOT) according to the manufacturer’s protocol (eBioscience company, USA). Briefly, 96-well plates (Millipore) were coated with 15 μg/ml anti-IFN-γ mAb 1-DIK (Mabtech) at 4°C overnight and blocked with 10% fetal calf serum for 1 hour. Then 1x10$^5$ splenocytes obtained from the mice treated with the different immunotherapeutic vaccinations were added to wells in 100 μl and incubated with the Ag85A. After incubation for 24 hours at 37°C in 5% CO$_2$, the cells were discarded, and the wells were washed three times with PBS containing 0.05% Tween 20 (Sigma-Aldrich). This was followed by incubation with 1 μg/ml biotinylated anti-IFN-γ mAb 7-B6-1-biotin (Mabtech) for 2 hours at room temperature. Wells were washed three times again and incubated with streptavidin-conjugated alkaline phosphatase (Mabtech) for other 1 hour. Individual cytokine-producing cells were identified as dark spots after a 30-min reaction with 5-bromo-4-chloro-3-indolyl phosphate and NBT by means of an alkaline phosphatase conjugate substrate kit (Bio-Rad). Spots were counted using an automated reader, (AID-Diagnostika), and results displayed as number of per 10$^5$ splenocytes secreting IFN-γ [24, 25]. The IFN-γ concentration secreted by splenocytes was measured by using ELISA.

Detecting the M. tuberculosis colony and analysis of histopathological change

Ten weeks after the M. tuberculosis infected mice finally treated with the various immunotherapeutic vaccinations, all treated mice were killed and all the right lungs were removed, and were placed in 4 mL of PBS with 0.05% Tween 80, and homogenized with a tissue homogenizer. The homogenates onto nutrient medium and counting bacterial colonies were performed as above-mentioned. The CFUs were counted under a dissection microscope and the bacterial colony counts are expressed as log$_{10}$ values of the mean numbers of bacteria recovered per organ [24, 26]. All the left lungs were fixed in 10% (v/v) neutral buffered formalin. The sections of lung lobes were processed with paraffin wax, and stained with hematoxylin and eosin (H.E.) for routine evaluation with Alizarin red to detect calcified lesions by a clinical pathologist who lacked prior knowledge of the treatment groups. The histopathological parameters including peribronchiolitis, perivasculitis, alveolitis, and granuloma formation were each semi-quantitative scored as absent, minimal, slight, moderate, marked, or strong, noted as 0, 1, 2, 3, 4, and 5, respectively. In this score the frequency and severity of the lesions were incorporated [19, 27, 28].

Statistical analysis

Statistical comparisons were performed using the Student’s t-test method or single factor analysis of variance to test for any statistically significant differences in the results between the experimental groups and control group. Bonferroni correction was used where multiple comparisons were made. The differences were considered as statistically significant when a values of $P<0.05$.

Results

PEI modified DNA vaccine Ag85A-ESAT-6-IL-21 augments immunotherapeutic efficacy in M. tuberculosis infected mice

The mice infected with M. tuberculosis by aerosol were treated with the various immunotherapeutic...
PEI modified Ag85A-ESAT-6-IL-21 vaccine therapy effect

Table 1. Immunotherapeutic efficacy in *M. tuberculosis* infected mice treated with the different vaccines

<table>
<thead>
<tr>
<th>Groups</th>
<th>Log_{10} CFU</th>
<th>Weight</th>
<th></th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-therapy</td>
<td>Post-therapy</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>PBS</td>
<td>6.21±0.65</td>
<td>20.50±0.70</td>
<td>20.76±0.88</td>
<td>0.26±0.67</td>
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<tr>
<td>PIRES</td>
<td>6.17±0.46</td>
<td>20.46±0.68</td>
<td>20.72±0.94</td>
<td>0.27±0.63</td>
<td></td>
</tr>
<tr>
<td>Na-P</td>
<td>6.18±0.28</td>
<td>20.40±1.06</td>
<td>20.66±1.02</td>
<td>0.26±0.81</td>
<td></td>
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<tr>
<td>PAEI</td>
<td>5.83±0.41</td>
<td>20.49±0.89</td>
<td>21.87±1.12</td>
<td>1.38±0.80</td>
<td></td>
</tr>
<tr>
<td>Na-PAEI</td>
<td>5.22±0.50</td>
<td>20.42±0.73</td>
<td>23.00±0.83</td>
<td>2.58±0.62</td>
<td></td>
</tr>
<tr>
<td>BCG</td>
<td>5.72±0.55</td>
<td>20.48±0.50</td>
<td>22.38±0.68</td>
<td>1.90±0.75</td>
<td></td>
</tr>
</tbody>
</table>

The bacterial CFU data and the different weight data do not come from the same mice, and the data from multiple experiments were pooled for calculations and statistics. The marks *show statistically significant differences between two groups as judged by Student’s t test and Bonferroni correction followed where multiple comparison tests were done. CFU: colony-forming unit; PBS, PIRES, Na-P, PAEI, Na-PAEI and BCG represent the PBS, the mock plasmid, the mock plasmid + nanoparticles, the DNA vaccine Ag85A-ESAT-6-IL-21, PEI modified DNA vaccine Ag85A-ESAT-6-IL-21, and the BCG vaccine groups, respectively. Bacterial CFUs were calculated from 9 mice per group, and weights were calculated from 9 mice per group. The experiments were repeated twice. *P<0.05, **P<0.03, and ***P<0.01.

Figure 1. Histology evaluation of lung tissue pathological change in *M. tuberculosis* infected mice treated with the different vaccines. Profile of the pathological change of lung tissues in mice that were infected with *M. tuberculosis* H37Rv by aerosol route, and respectively treated, as described in section of methods. Twenty-four hours after infection, the mice were respectively treated by the different vaccines three times with 2-week intervals between the immunotherapeutic vaccinations. Seventy days after finishing final treatment, the lungs were removed and the histological analysis was performed in H.E. staining. The main figures show all infected, treated mice as observed in the individual description per figure. Original magnification: 100×. A-F. The figures represent the pathological change in the treated mice in the PBS, the mock plasmid, the mock plasmid + nanoparticles, the DNA vaccine Ag85A-ESAT-6-IL-21, PEI modified DNA vaccine Ag85A-ESAT-6-IL-21, and the BCG vaccine groups in turn. G. It exhibits the normal alveolar architecture in lungs without any infection. H. It indicates the results from the semiquantitative analysis of histopathological change. Each histogram represents a set of data for 9 mice. *P<0.05, and ***P<0.01.

Immunotherapeutic vaccinations, and the immunotherapeutic efficacy against *M. tuberculosis* was found in all treated mice as is shown in Table 1. It is found that the *M. tuberculosis* growth was significantly inhibited in the total number of CFU (5.22±0.50 vs. 5.83±0.41, *P*<0.03) in the PEI modified DNA vaccine Ag85A-ESAT-6-IL-21 group compared with the DNA vaccine Ag85A-ESAT-6-IL-21 group or the BCG group (5.22±0.50 vs. 5.72±0.55, *P*<0.05) or the PBS group (5.22±0.50 vs. 6.21±0.65, *P*<0.005). In addition, the mouse weight in the PEI modified
DNA vaccine Ag85A-ESAT-6-IL-21 treated mice was significantly increased in the total net weight (2.58±0.62 vs. 1.38±0.80, P<0.03) in contrast to the DNA vaccine Ag85A-ESAT-6-IL-21 treated mice or the BCG treated mice (2.58±0.62 vs. 1.90±0.75, P<0.05) or the PBS treated mice (2.58±0.62 vs. 0.26±0.67, P<0.005). There was no significant difference in the total numbers of CFU between the PBS, the pIRES and the Na-P groups. The inhibiting bacterial growth and the obtaining murine weight were all true of the other vaccine immunotherapeutic groups. The results strongly suggested that the PEI modified DNA vaccine Ag85A-ESAT-6-IL-21 was an efficient fight against M. tuberculosis by the vaccine’s powerful therapeutic efficacy in decreasing the bacterial burdens in the mouse lungs, and in developing the murine net weight after the treated mice infected with M. tuberculosis.

Tuberculosis was significantly lessened in the infected mice treated with PEI modified DNA vaccine Ag85A-ESAT-6-IL-21

To directly examine the anti-TB immunotherapeutic efficacy in M. tuberculosis infected mice, we harvested the lung tissues ten weeks after final immunotherapeutic vaccination, fixed, sectioned, and then stained with H.E. Figure 1 indicates the images of the histopathological change of lung tissues, which represented clinical pictures of different groups on week 10 after final treatments. It is found that the lung lesions was effectively decreased in the M. tuberculosis infected mice treated with the PEI modified DNA vaccine Ag85A-ESAT-6-IL-21, and the coalescing small granulomas (the arrows) displayed extensively infiltrated numerous lymphocytes and neutrophils as is shown in Figure 1E. Whereas more lung tissues with extensive caseating and miliary lesions or caseation tuberculosis as well as less lymphocytes with necrosis were seen in the images as pointed out by arrows in lungs from the mice treated with PBS group (Figure 1A). Figure 1B, 1C represent the images of lungs from the mice treated with mock plasmid and nanoparticle-based mock plasmid, and the pathological change was serious, which is similar to that of Figure 1A. Less-contained granulomas and many more infiltrated inflammation cells, and tuberculosis lesions were seen in Figure 1A-C, in which the lesions grew progressively. As shown in Figure 1F, the pathological change was similar to that of Figure 1E, in which less alveolar architecture was observed in the BCG- immunotherapeutic mice but lung damage was not apparent. Figure 1D displays a slight inflammation response, peribronchiolitis, perivasculitis, alveolitis, and granuloma formation as well as some caseation necrosis with moderate lymphocytic infiltration in the mice treated with the DNA vaccine Ag85A-ESAT-6-IL-21 alone, displaying a slightly greater injury than that of mice treated with the PEI modified DNA vaccine. The normal alveolar architecture was found in mice without infection of M. tuberculosis (Figure 1G). Figure 1H presents the different score of the lung pathological changes.

Dynamic detection of serum cytokine IFN-γ

To understand the anti-TB mechanisms in the infected mice treated by PEI modified DNA vaccine Ag85A-ESAT-6-IL-21, we detected the level of the serum IFN-γ, which was closely related to immune response induced by immunotherapeutic vaccinations. As shown in Figure 2, the kinetic level of serum IFN-γ was gradually increased, especially in mice treated with PEI modified DNA vaccine, which was statistically significant compared with the groups of PBS, pIRES, and Na-P (2 weeks (P<0.05), 4 weeks (P<0.03), and 6 weeks (P<0.01)). Additionally, the serum IFN-γ level also was markedly enhanced in DNA vaccine Ag85A-ESAT-6-IL-21 and BCG groups compared with the groups of PBS, pIRES, and Na-P (6 weeks (P<0.03). The results from Figure 2 indicate that the PEI modified DNA vaccine Ag85A-ESAT-6-IL-21 elicited
a high level of cytokine IFN-γ 6 weeks after the mice treated with the vaccine three times.

Analysis of IFN-γ secreted by splenocytes in the infected mice treated with the various immunotherapeutic vaccinations

To know whether the spleen cells in mice were sensitized by immunotherapeutic vaccinations, we further assayed the IFN-γ level secreted by splenocytes after Ag85A stimulating, which closely typified the cell immune response induced by the various immunotherapeutic vaccinations. As shown in Figure 3A, the number of per $10^5$ splenocytes secreting IFN-γ was remarkably elevated in PEI modified DNA vaccine group in contrast with the BCG ($P<0.05$) or the DNA vaccine Ag85A-ESAT-6-IL-21 ($P<0.03$) or the PBS, pIRES, and Na-P groups ($P<0.01$). Coincidentally, the concentration of per $10^5$ splenocytes secreting IFN-γ (Figure 3B) was also significantly increased in PEI modified DNA vaccine group compared with the BCG ($P<0.03$) or the DNA vaccine Ag85A-ESAT-6-IL-21 ($P<0.03$) or the PBS, pIRES, and Na-P groups ($P<0.01$). The data suggested that the PEI modified DNA vaccine Ag85A-ESAT-6-IL-21 induced a vigorous splenocyte activity for generating a high level of IFN-γ in treated mice infected with M. tuberculosis.

Discussion

In previous study, we investigated the effect of nanoparticle-based adjuvant for enhanced protective efficacy of DNA vaccine Ag85A-ESAT-6-IL-21 against M. tuberculosis challenge. Latest study approaches based on immunotherapeutic vaccine has received much attention to potentially immunotherapeutic efficacy in tumor diseases and infectious diseases [29, 30]. Therefore, an alternative approach suggested in which the vaccine is given as a postexposure or immunotherapeutic activity after the individual has been infected with M. tuberculosis [4, 12, 23]. As such, in this study, we extended our previous study, and sought to investigate the possibility of PEI modified nanoparticle adjuvant enhance the therapeutic efficacy of DNA vaccine Ag85A-ESAT-6-IL-21 in mice infected with M. tuberculosis.

From the infected experiment results, we demonstrated that mice infected by aerosol route with a low dose of bacteria were feasible since all 6 mice were completely infected after the bacteria clone from the lung homogenate’s culture was identified in each infected mouse. Based on the establishment of mouse TB model, we carried out the different immunotherapeutic vaccinations in the infected mice with a protocol of immunotherapeutic inoculation of three times, two week intervals between the inoculations. From the immunotherapeutic results, we found that the mice treated with the PEI modified DNA vaccine Ag85A-ESAT-6-IL-21 during the M. tuberculosis infection exhibited marked reduction in the bacterial load seen in the lungs compared with the mice treated with other immunotherapeutic inoculations, and this certainly suggests an immunotherapeutic effect, that means hundreds or thousands fold reduction in lung bacterial counts. The spleen and brain were also true of reduced bacterial counts (data not shown here). Although the BCG immunotherapeutic inoculation elicited effective inhibition of TB growth effect as well, the efficacy was more efficient found in the PEI modified DNA vaccine treated mice. The results from the lung pathological change analysis were consistent with the bacterial colony counts in lungs. These data are in accordance
with our previous report that the nanoparticle-based DNA vaccine was given as prophylactic vaccine against *M. tuberculosis* challenge.

To understand the possible mechanisms involved in the increase of the immunotherapeutic efficacy [31], we sought and found a correlation between IFN-γ level and CFU numbers. Detecting IFN-γ production by Ag85A-specific splenocytes provides the available immunotherapeutic correlate of therapeutic vaccine to fight against TB. The increased IFN-γ production by splenocytes or in sera was obviously found in the mice treated with PEI modified DNA vaccine, whereas the significant reduction of CFU numbers was true of the PEI modified DNA vaccine treated mice. Accordingly, the strong inhibitory TB effect of PEI modified DNA vaccine Ag85A-ESAT-6-IL-21 was apparently associated with increased IFN-γ level.

It was known that IL-21 is an immune regulator that augments the immunogenicity of DNA vaccine expressing Ag85A-ESAT-6 and enhances the IFN-γ level, resulting in increase of the protection against *M. tuberculosis* infection in mice [16]. It was also known that IFN-γ professionally induces immune responses against viral and *M. tuberculosis* infection by inducing cell apoptosis [32-34]. One explanation for the efficiency of immunotherapeutic vaccination against TB could be that IL-21 enhanced the expression of Ag85A and ESAT-6 and presentation to lymphocytes by antigen presenting cells, which elicited immune responses to Ag85A and ESAT-6, and generated high level of IFN-γ to preferentially mediate cellular immunity against *M. tuberculosis*. In addition, PEI mixed Na-P served as delivery system as well as immune adjuvant for slow delivery of DNA vaccine and enhanced immune response against TB [35-37]. These data support manipulating PEI modified DNA vaccine Ag85A-ESAT-6-IL-21 as an immunotherapeutic vaccine in addition to serving as an immunoprophylaxis vaccine.

In conclusion, our data demonstrated that the PEI modified DNA vaccine Ag85A-ESAT-6-IL-21, administered in *M. tuberculosis*-infected mice with the protocol was safe, immunotherapeutic action, and decreased the *M. tuberculosis*-associated lung injury. Our study add to a growing body of evidence that supports the therapeutic vaccine may be tested further in the clinical trials to explore the therapeutic vaccine against TB.

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Disclosure of conflict of interest

None.

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