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DNA vaccine encoding ESAT-6 enhances the protective efficacy of BCG against *Mycobacterium tuberculosis* infection in mice

X. Fan, Q. Gao & R. Fu

Laboratory of Biosafety, Department of Pathogen Biology, Tongji Medical College, Huazhong University of Science & Technology, Wuhan, China

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Correspondence to: X. Fan, MD, PhD, Associate Prof., Laboratory of Biosafety, Department of Pathogen Biology, Tongji Medical College, Huazhong University of Science & Technology, Wuhan 430030, China. E-mail: xionglinfan@yahoo.com.cn

Abstract

ESAT-6 is a protein produced by virulent *Mycobacterium tuberculosis* but absent in *Mycobacterium bovis* bacillus Calmette–Guerin (BCG) and is considered as a promising vaccine subunit against tuberculosis. The protective efficacy of a new strategy based on the combination of DNA vaccine encoding ESAT-6 (DNA-E6) and BCG immunization was investigated in the present study. BALB/*c* mice were immunized with DNA-E6, BCG, DNA-E6 plus BCG, vector plus BCG or vector alone. Three weeks after the last immunization, antigen-specific interferon (IFN)-γ secreted upon stimulation with BCG-PPD or ESAT-6 protein by splenocytes of the different groups was determined by an ELISA. The immunized mice were challenged intravenously with low-dose virulent *M. tuberculosis* H37Rv and bacterial load of the infected mice was measured in the spleen and lung 6 weeks later. Pathological changes in the lung were observed by haematoxylin–eosin (HE) or acid-fast staining. Our study demonstrated that ESAT-6-specific IFN-γ was improved in mice vaccinated with DNA-E6 plus BCG, compared with those mice immunized with BCG alone. The combination of DNA-E6 and BCG resulted in the maximum reduction in bacterial load in both the lung and spleen, and only slight pathological changes were detected in the lung. These results suggested that the combination of DNA-E6 and BCG vaccination could be a better strategy against *M. tuberculosis* infections in human.

Introduction

Human tuberculosis (TB) is a major public health problem and causes about two million deaths annually. Bacillus Calmette–Guerin (BCG), an attenuated strain of *Mycobacterium bovis*, has been widely used against childhood meningeal TB and phthisis miliaris. However, BCG vaccination, in childhood, only provides protection for a period of 5–10 years and the efficacy of protection gradually wanes [1]. Furthermore, the protective efficacy of BCG against adult pulmonary disease, the major cause of TB mortality, is variable, ranging from 0% to 80% [2]. Therefore, a more effective vaccination strategy against TB is urgently needed.

The varying protective efficacy of BCG may have many reasons. The pre-exposure to environmental mycobacteria may prevent or mask BCG-induced protective immunity [3]. In addition, the protective efficacy of BCG could be weakened by continuing attenuation and loss of some genes encoding immunodominant antigens. By a comparative genomics analysis, Behr *et al.* reported that all BCG strains distributed worldwide have deleted RD1 regions [4]. *Rv3875*, belonging to one of nine open-reading frames in RD1 region, encodes an antigen ESAT-6 with human cytotoxic T lymphocyte (CTL) epitopes [5]. Antigen ESAT-6 has been shown to be a major target for memory T cells in mice infected with *M. tuberculosis* and therefore important for cell-mediated immunity [6, 7]. Therefore, the improvement of BCG may be achieved by reintroduction of deleted genes with immunological determinants. This idea has been proven to be correct in animal models in the recent investigations [8, 9]. For example, the immunogenicity and protective efficacy was improved by the reintroduction of *esat-6* into BCG. However, the safety of this strategy must be investigated thoroughly to

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avoid the potential risk of recovered virulence of BCG strain [10].

Recent studies have shown that DNA vaccine is effective to prime immune responses against TB [11–13]. Combination of DNA vaccines encoding the lost antigens of BCG with BCG may be useful for enhancing protection against *M. tuberculosis* infections. This approach takes advantage of BCG as neonatal vaccine and reduces the risk of virulence recovery. Previous studies showed that DNA vaccines expressing ESAT-6 provided a strong protection against TB [12, 14]. In this study, we investigated whether BCG-induced protection in mice could be improved by combination with DNA encoding ESAT-6.

Materials and methods

Plasmid construction. The DNA sequence encoding ESAT-6 was amplified by PCR from the genomic DNA of *M. tuberculosis* H37Rv strain using the forward primer and the reverse primer 5′-GGG ATC CAT GAC AGA GCA GCA GTG-3′ and 5′-GGA ATT CAT CGA TCT ATG CGA ACA TCC CAG TGA CG-3′ respectively. PCR was performed using the cycling parameters 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min over 30 cycles. PCR product was digested with *Bam*HI and *Eco*RI and cloned into pcDNA3.1 (Invitrogen, Shanghai, China) predigested with the same enzymes, resulting in the plasmid DNA-E6 [14]. The correctness of the cloned fragment was confirmed by sequencing. Following the recombinant plasmid transfection into P815 cells, the expression of ESAT-6 was verified by RT-PCR assay for specific mRNA or indirect immunofluorescence using rabbit anti-ESAT-6 antibodies, as previously described [9]. *E. coli* JM109 cells harbouring DNA-E6 were cultured and amplified in liquid LB medium containing 100 μg/ml ampicillin. The DNA plasmids for vaccination were purified using Qiagen endoFree plasmid maxi kit (Qiagen, Shanghai, China), adjusted to a final concentration of 1 mg/ml in phosphate-buffered saline (PBS), and stored at −20 °C.

Animal. Specific pathogen-free, 6- to 8-week-old, female BALB/*c* mice were provided by animal centre of Tongji Medical College (Wuhan, China) and were bred in cages on the animal feeding cabinet (VentiRack, Chester, CA, USA) in a biosafety laboratory. The research protocol was reviewed and approved by Tongji Medical School Committees on Biosaftey. Animal experiments were performed in accordance with the guidelines of Chinese Council on Animal Care.

DNA and BCG vaccination. Mice were vaccinated with control vector (pcDNA3.1), DNA-E6, BCG, DNA-E6 plus BCG and vector plus BCG. Ten mice were included in each group. DNA vaccination was performed three times by injection of plasmid DNA with 100 μg into the quadriceps muscle of each hind leg at 2-week intervals (at days 1, 15 and 29). CFU (10^6) of BCG (Chengdu Institute of Bioproducts, Chengdu, China) was applied once subcutaneously on the first day. Three weeks after the last DNA immunization, five mice from each group were killed for IFN-γ production by splenocytes, and five mice were used for intravenous challenge with a low dose of virulent *M. tuberculosis* strain.

IFN-γ production by splenocytes after antigenic stimulus. Three weeks after the last immunization, mice were killed and spleens were aseptically removed. Spleen cells from five mice per group were prepared individually and resuspended in complete RPMI-1640 medium (Invitrogen) with 10% fetal calf serum. The concentration of cell suspension was adjusted to 2×10^6 cells/ml. Cells (2×10^5) were added into each well in 96-well microtiter plates. One hundred microlitres of BCG-PPD (10 μg/ml, Chengdu Institute of Bioproducts, Chengdu, China) or recombinant ESAT-6 protein (5 μg/ml) [14] in complete RPMI-1640 medium were used as the stimulator. A medium control with RPMI-1640 medium was used as negative control. The plates were incubated at 37 °C in a humidified CO_2 incubator for 72 h. Supernatants from three separate wells were pooled and stored frozen at −20 °C until further processing. IFN-γ in the supernatants was measured in triplicate by an enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's recommendations (Jingmei Biotech, Shenzhen, China). The recombinant mouse IFN-γ was used as the standard at concentrations ranging from 3.9 to 1000 pg/ml. The amount of IFN-γ in the test samples was calculated from the standard curve. The detection limit of the ELISA is 4 pg/ml. The results were shown as mean ± SD (pg/ml) of five mice per group.

M. tuberculosis challenge. Mycobacterium tuberculosis H37Rv strain, passed through mice once to recover virulence, was cultured and enumerated, as previously described [9]. Mice were challenged 3 weeks after the third immunization by the injection of 5×10^3 CFU of *M. tuberculosis* H37Rv through a lateral tail vein. Animals were killed 6 weeks after challenge. The spleen and lung were aseptically removed to determine bacterial load. Organs were homogenized in 3 ml of 0.05% Tween 80-sterile distilled water solution. The serial 1:10 dilutions of homogenates were enumerated on three solid Middlebrook 7H10 agar plates. Samples from BCG-vaccinated mice were plated on Middlebrook 7H10 agar containing 2 μg/ml of 2-thiophenecarboxylic acid hydrazide (TCH, Beijing Luqiao Corp., Beijing, China). Colonies were counted after 4 weeks. CFU counts obtained from three dilutions were used to calculate the total number of CFU/organ/mouse. CFU data were expressed as mean ± SEM of $\log_{10}$CFU for each group.

Histopathological staining. Six weeks after challenge infection, left lung lobes from different vaccine groups were removed and fixed overnight in 10% phosphate-buffered formalin, embedded in paraffin, sectioned and stained by haematoxylin–eosin (HE) or acid-fast staining.

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Statistical analysis. Student's *t*-test was performed to analyse the mean organ burdens of each group of mice. A value of $P < 0.05$ was considered statistically significant.

Results

IFN-γ production

To determine cell-mediated immune responses to vaccines, splenocytes from immunized mice were prepared 3 weeks after the last DNA vaccination and stimulated with antigens. The level of IFN-γ production by splenocytes was determined by an ELISA. As shown in Fig. 1, splenocytes from mice vaccinated with BCG, DNA-E6 plus BCG or vector plus BCG produced similar levels of IFN-γ, while stimulated with BCG-PPD. Splenocytes from mice vaccinated with DNA-E6 did not show a significant response to BCG-PPD, when compared with the control. However, stimulation with ESAT-6 led to IFN-γ production of splenocytes from mice vaccinated with DNA-E6 and DNA-E6 plus BCG group. By contrast, no response was observed for splenocytes from mice of BCG and vector plus BCG group.

Protective efficacy of DNA vaccines

A challenge led to a high bacterial load over 4.5 log in the lung and spleen of control animals. Vaccination with DNA-E6 plus BCG reduced most strongly the bacterial loads in the lung (1.48 log) and spleen (1.53 log) of mice. Other vaccinated groups showed reductions in bacterial loads in the lung and spleen with different extents. DNA-E6 vaccination was not efficient in terms of reducing the bacterial loads in both organs (0.53 log in the lung and 0.66 log in the spleen). The statistical analysis revealed that only the DNA-E6 plus BCG combination resulted in a significant decrease in the bacterial load in the lung ($P < 0.05$, Fig. 2) and spleen ($P < 0.05$, Fig. 3) compared with vaccination with BCG.

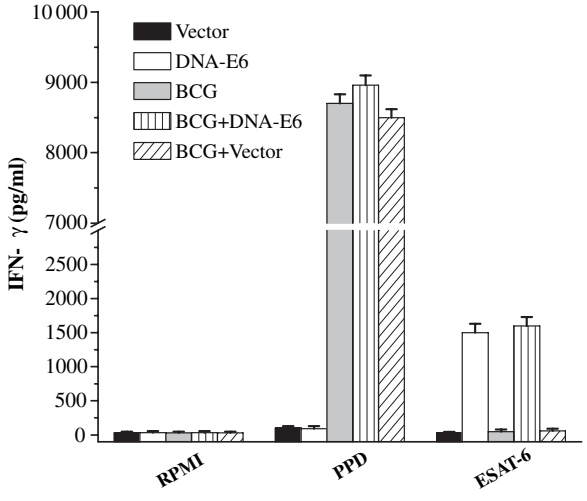

Figure 1 Level of antigen-specific IFN-γ production of splenocytes from mice. BALB/*c* mice ($n = 5$) were vaccinated with different vaccines. Splenocytes were prepared 3 weeks after the third DNA immunization and incubated with RPMI-1640 medium, 10 μg/ml of BCG-PPD or 5 μg/ml of recombinant ESAT-6. IFN-γ secreted by splenocytes was measured in triplicate by an ELISA. The results are expressed as mean ± SD of five mice in each group.

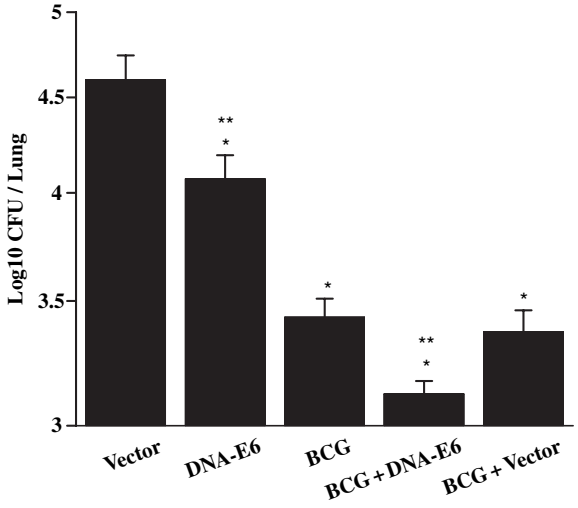

Figure 2 Bacterial load in the lungs of mice. Vaccinated BALB/*c* mice ($n = 5$) were challenged with 5×10^3 CFU virulent *Mycobacterium tuberculosis* H37Rv strain. The lungs were harvested and the numbers of CFU per organ in each group were enumerated 6 weeks post-challenge. Bars represent the mean ± SEM ($\log_{10}$CFU)/lung. *$P < 0.05$ versus vector; **$P < 0.05$ versus BCG.

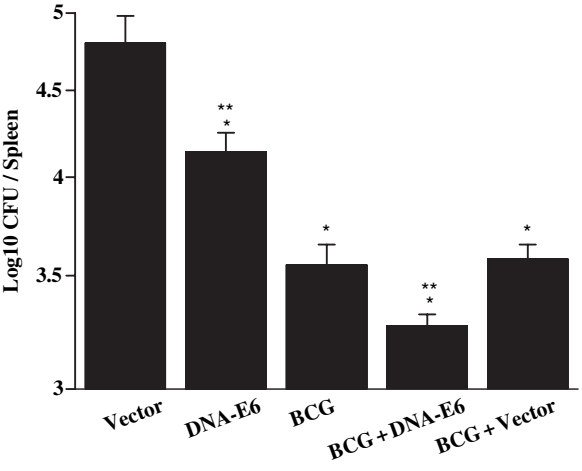

Figure 3 Bacterial load in the spleens of mice. Vaccinated BALB/*c* mice ($n = 5$) were challenged with 5×10^3 CFU virulent *Mycobacterium tuberculosis* H37Rv strain. Spleens were harvested and the numbers of CFU per organ in each group were enumerated 6 weeks post-challenge. Bars represent the mean ± SEM ($\log_{10}$CFU)/spleen. * $P < 0.05$ versus vector; **$P < 0.05$ versus BCG.

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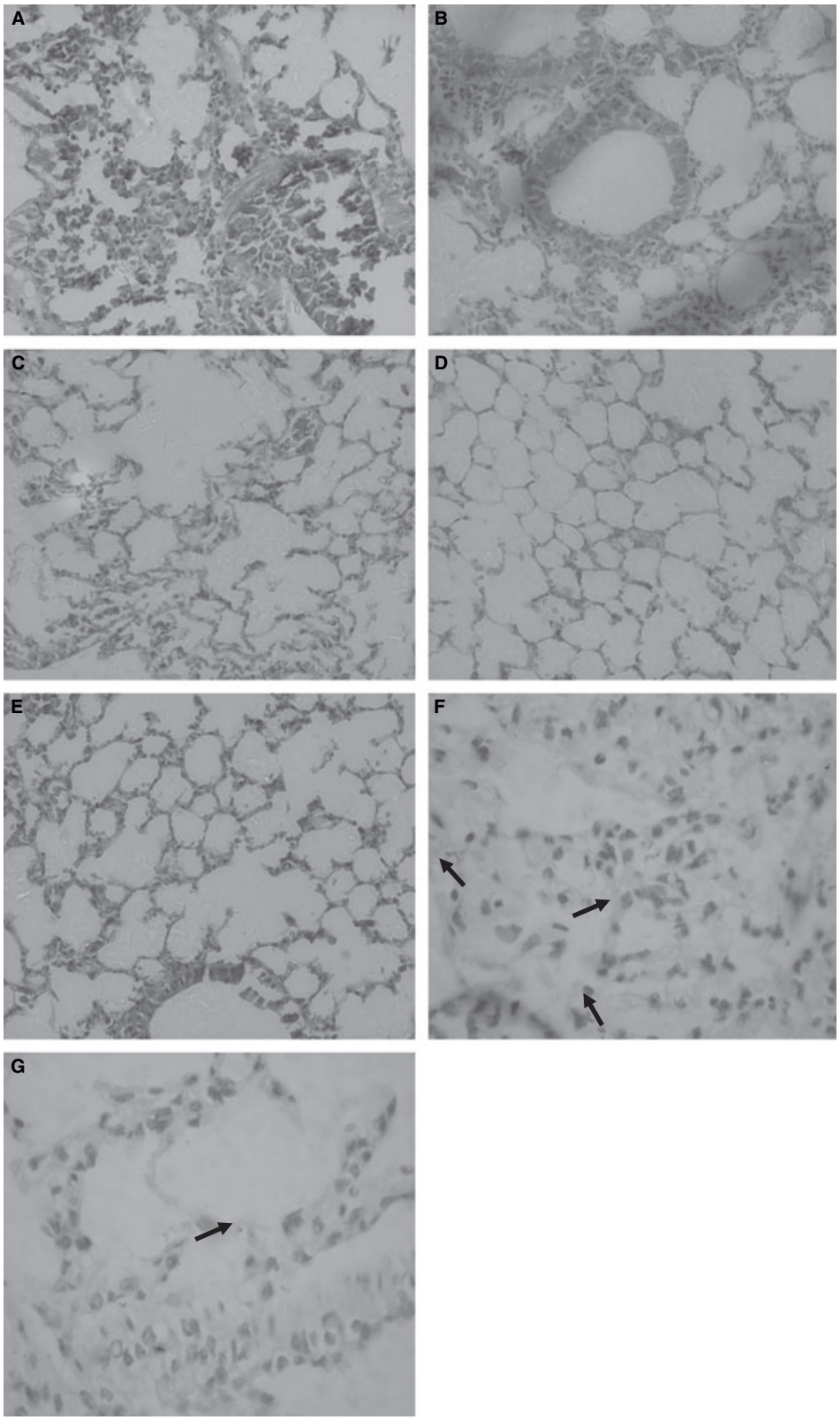

Figure 4 Lung pathology of mice after challenge. Vaccinated BALB/*c* mice (n = 5) were challenged with 5×10^3 CFU virulent *Mycobacterium tuberculosis* H37Rv strain. Six weeks after infection, lung tissue sections from different vaccine groups were prepared and stained. HE staining (×20): (A) vector DNA-vaccinated group; (B) DNA-E6 group; (C) BCG group; (D) DNA-E6 plus BCG group; (E) vector plus BCG group. Acid-fast staining (×1000): (F) vector DNA-vaccinated group; (G) DNA-E6 group; arrow indicates the acid-fast bacilli presented in the lung tissue sections.

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Pathological examinations

Consistent with varying bacterial loads in the lung, HE-stained sections from different groups showed clear differences (Fig. 4). The lung sections from control mice showed severe interstitial pneumonia and intense inflammation throughout the lung. Some inflammatory foci had the appearance of early granuloma formation (Fig. 4A). Slight damage in alveolar tissues with aggregated, relatively large number of lymphocytes was also observed in the lung from single DNA-E6-vaccinated group (Fig. 4B). BCG-vaccinated mice showed slight interstitial pneumonia (Fig. 4C). Alveolar tissue from mice vaccination with DNA-E6 plus BCG combination appeared to be intact with very limited lung inflammation (Fig. 4D). Acid-fast bacilli were found only in inflamed areas of lung tissues in control vector (Fig. 4F) and DNA-E6 (Fig. 4G) vaccinated groups.

Discussion

In this work, we demonstrated that the protective efficacy of the combination of BCG with DNA vaccine encoding ESAT-6 was significantly higher than that of BCG immunization alone. The results consistently showed that the vaccination with this combination led to a stronger T-cell immunity, reduced bacterial loads in the lung and spleen, and reduced lung histopathology. Thus, it is clear that ESAT-6 DNA vaccine may be suitable for enhancing immunogenicity and protective efficacy of BCG against virulent *M. tuberculosis* challenge in humans.

Previously, candidate vaccines including BCG with the reintroduction of RD1 [8] and BCG over-expressing antigen Ag85B [15], or priming/boost sequentially with DNA vaccine encoding Ag85B and BCG [16] were reported to show a better protection than BCG. Thus, there are different ways to enhance the protection efficacy of BCG. We could show that DNA vaccination was able to induce cell-mediated immune responses to ESAT-6. DNA vaccination is preferentially inducing Th1-type immune responses, which plays a key role in anti-mycobacteria infection. IFN-γ, a Th1 cytokine, is an essential mediator of the protective immune response to TB [17]. Here, splenocytes of mice vaccinated with DNA-E6 plus BCG group could produce the higher levels of IFN-γ after antigen stimulation, showing a direct correlation with the better protection against challenge. Recent investigations demonstrated the essential role of $CD8^+$ T cells in protection against TB [17]. ESAT-6 is one of several targets from *M. tuberculosis* for human CTL [5]. The effect of CTL against ESAT-6 epitope in the context of $H\text{-}2^d$ mice was demonstrated in our recent report [9]. Because the precise CTL epitope is not known yet, $CD8^+$ CTL responses induced by DNA-E6 that contributed to protective immunity was not analysed in detail in this study but should be done in future.

Consistent with the previously findings, the protective efficacy of DNA-E6 alone is much lower than that of BCG [12]. Because *esat-6* gene is absent in all BCG strains, DNA-E6 might induce immune responses that would be absent by vaccination with BCG alone. Our results support the idea to combine BCG with other components for a better protection against TB.

A limitation of the present study is that mice were challenged by intravenous injection, which does not represent the natural infection route for TB. Intravenous injection has the advantage that the number of CFU injected into the mice can be controlled and is therefore widely used in the preclinical investigations. However, the infection by aerosol may be different and will be primarily controlled by mucosal immunity at the first encounter. Thus, further studies using infections through the respiratory tract by intra-tracheal injection are need to be performed to evaluate the protection efficacy of our approach.

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