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ORIGINAL INVESTIGATION

A DNA vaccine expressing CFP21 and MPT64 fusion protein enhances BCG-induced protective immunity against *Mycobacterium tuberculosis* infection in mice

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Abstract The efficacy of Bacillus Calmette–Guérin (BCG) vaccine in preventing adult tuberculosis (TB) is highly variable. Genetic differences between BCG vaccine substrains, which can be divided into early strains and late strains based on the loss of region of difference two (RD2), may result in the variability and BCG substrains. The effect of lack of RD2 on the protective efficacy of BCG substrains against TB remains unknown. In this study, we demonstrated that CFP21 and MPT64(rCM) fusion protein, encoded by RD2 of *Mycobacterium tuberculosis*, could stimulate higher level of interferon (IFN)-γ in tuberculin skin test (TST)-positive healthy population than in TST-negative healthy population. Compared with naive mice challenged with virulent *M. tuberculosis* H37Rv, C57BL/6 mice vaccinated with pcD2164 DNA expressing rCM protein resulted in a greater decrease in the bacterial load of lung. Moreover, pcD2164 could boost the protective immunity in mice primed by BCG than BCG alone or DNA vaccination alone, as evidenced by lower bacterial load in the lung tissue and reduced lung pathology. The protection induced by BCG prime-DNA vaccine boost strategy was associated with significant increases in rCM protein-specific IFN-γ. Therefore, our results clearly indicate that the loss of RD2 has an important influence on the protective efficacy of different BCG substrains. These findings will benefit the optimal choice of BCG substrain for neonatal immunization and rational design of new vaccines for the prevention of TB.

Keywords Tuberculosis · DNA vaccine · *Mycobacterium tuberculosis* · BCG · Prime-boost strategy

Introduction

Bacillus Calmette–Guérin (BCG) vaccine was developed between 1908 and 1921 by Albert Calmette and Camille Guérin after 230 passages of culturing *Mycobacterium bovis* on bile medium. It is the only available vaccine against tuberculosis (TB) and has been included in World Health Organization Expanded Program on Immunization since 1974. While BCG has demonstrated its proven efficacy at preventing tuberculosis (TB) in children for over 80 years [1], it provides variable efficacies against adult pulmonary TB, ranging from no protection in India to about 80% protection in England [2]. Strain variations in BCG preparations [3], environmental mycobacteria [3, 4], or host genetic and nutritional factors [3, 5] may account for the variability in BCG's protective efficacy.

During the past decade, comparative genomic analyses of BCG substrains distributed worldwide have uncovered extensive genotypic diversity, including deletions, insertions, duplications, and single-nucleotide polymorphisms (SNPs) [6–9]. A molecular genealogy of BCG substrains is established, which is consistent with the historical records of BCG dissemination [8, 10]. BCG substrains are divided into early

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substrains (BCG Japan, Russia, Moreau, Sweden, and Birkhaug) and late substrains (BCG Tice, Frappier, Phipps, Prague, Danish, Pasteur, Glaxo), characterized by the deletion of RD2 region during 1927 and 1931. The primary deletion of RD1 between 1908 and 1921, which is absent from all BCG substrains but present in virulent *M. tuberculosis* and *M. bovis*, is thought to be the most obvious reason for the attenuation of BCG [11, 12]. Three early substrains (BCG Japan, Russia, Moreau) and three late substrains (BCG Pasteur, Danish, and Glaxo) are main BCG vaccine strains currently manufactured and clinically used worldwide [3, 13]. The genetic difference between BCG vaccine substrains has attracted increasing interest in the influence of different BCG substrains on the protective efficacy against TB. Although the passage of BCG vaccine strains may induce progressive loss of efficacy [8, 14], epidemiological and experimental studies indicate that variation in the efficacy that can be attributed to difference between BCG early and late substrains remains controversial [15–20].

Recent studies including overexpression of RD1 in BCG [21] or BCG supplementation with subunit or DNA vaccine encoding RDs [22–24] suggested that RDs encode some important immunodominant antigens of *M. tuberculosis* and RD1 could enhance the protective efficacy of BCG in the forms of recombinant BCG or subunit vaccine. The direct impact of RD2 deletion on the unstable efficacy of different BCG substrains used for vaccination has not been determined. CFP10(Rv3874) and ESAT-6(Rv3875), CFP21(Rv1984c), and MPT64(Rv1980c) have been considered important immunodominant antigens encoded by RD1 and RD2 of *M. tuberculosis*, respectively. In order to elucidate the effect of loss of RD2 on the efficacy of BCG, the immune responses generated against DNA vaccines expressing CFP21-MPT64 and ESAT-6-CFP10 fusion proteins and the immunogenicity of both fusion proteins in tuberculin skin test (TST)-positive healthy population were compared in the present study. More importantly, we compared the use of a BCG prime-DNA vaccines boost strategy to induce protection against virulent *M. tuberculosis* challenge in mice.

Materials and methods

Bacterial strains and cultures

Escherichia coli DH5α and BL21 (DE3) strains were used for cloning and overexpression, respectively. Both bacteria were cultured in Luria–Bertani (LB) medium (Oxoid, England) with or without agar. When required, ampicillin was added to a final concentration of 100 μg/ml. Both *M. bovis* BCG China strain (derivative of BCG Danish strain) and *M. tuberculosis* H37Rv strain were cultivated on Middlebrook 7H9 medium or enumerated on 7H11 agar (Difco Laboratories), supplemented with 10% ADC, 0.5% glycerol, and 0.05% Tween 80.

Overexpression and purification of recombinant proteins

Recombinant CFP21/MPT64 fusion protein (rCM) was produced by induction of *E. coli* BL21 (DE3) harboring the plasmid pPro2164 and purified as previously described [25]. Recombinant ESAT-6/CFP10 fusion protein (rEC) was produced as the following procedures. The coding sequences of ESAT-6 and CFP10 proteins were amplified by PCR using the specific primers (Sangon Biotech, Shanghai, China), and cycle parameters are listed in Table 1, with the genomic DNA of *M. tuberculosis* H37Rv as a template. The PCR fragment encoding the fusion protein of ESAT-6 and CFP10 with a 45-bp linker was generated by a second PCR according to the method of gene splicing with overlap extension [26]. The fusion gene was digested with *Bam*HI and *Hin*dIII and then inserted into the expression vector pProEXHTb (Invitrogen, Carlsbad, CA), resulting in the recombinant plasmid pPro610 with the fusion gene under the control of the Trc promoter. The correctness of the cloned sequences was confirmed by enzyme digestion and DNA sequencing.

E. coli BL21 (DE3) harboring the plasmid pPro610 was cultured overnight. Overnight cultures (10 ml) were inoculated into 1 liter of LB medium containing 100 μg/ml ampicillin and incubated at 37°C with shaking until the optical density at 600 nm reached 0.6. The expression of the fusion protein rEC was induced with isopropyl-D-thiogalactopyranoside (IPTG) at a final concentration of 0.4 mM for 2 to 6 h. Expression of rEC fusion protein was confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE).

Protein purification was performed using a Ni–NTA purification system according to the manufacturer's instruction manual (Invitrogen). The purified rEC protein was lyophilized, aliquoted, and stored at −20°C. The preparation of rEC protein was diluted in normal saline, using pyrogen-free reagents, and tested to exclude endotoxin contamination as described previously [25].

To verify the specificity of purified rEC fusion protein, the protein samples were separated by SDS–PAGE, electrically transferred into nitrocellulose membranes, and detected with polyclonal rabbit anti-ESAT-6 antibody (Ab45073, Abcam, Cambridge, UK) or anti-CFP-10 antibody (Ab45074) as the primary antibody and alkaline phosphatase–conjugated goat anti-rabbit immunoglobulin G antibody (Santa Cruz Biotech, Santa Cruz, CA) as the secondary antibody, respectively.

Immunogenicity of rCM and rEC proteins

Eighteen household contacts (mean age, 37 ± 18 years; male/female ratio, 8/10) with recent sputum-positive TB patients

Table 1 Primers and thermal cycle parameters for cloning of *M. tuberculosis* antigens

Primer name	Primer sequence and orientation (5′–3′)	Cycle parameters	Amplicon size (bp)
ESAT-6F	GCG GAT CCA TGA CAG AGC AGC AGT G	94°C for 5 min, then 30 cycles of 94°C for 1 min, then 60°C for 1 min, 72°C for 45 s, then 72°C for 5 min	338
ESAT-6R	GCT GCC GCC ACC GCC GCT TCC GCC ACC GGC GCT TCC ACC GCC ACC TGC GAA CAT CCC AGT GAC GTT GCC TTC		
CFP10 F	GGT GGC GGT GGA AGC GCC GGT GGC GGA AGC GGC GGT GGA AGC GGC GGT GGC GGA ATG AAG ACC GAT G	94°C for 5 min, then 30 cycles of 94°C for 1 min, then 60°C for 1 min, 72°C for 45 s, then 72°C for 5 min	356
CFP10 R	CCA AGC TTT CAG AAG CCC ATT TGC GAG		
rECF	ESAT-6F	94°C for 5 min, then 30 cycles of 94°C for 1 min, then 60°C for 1 min, 72°C for 1 min, then 72°C for 10 min	654
rECR	CFP10 R		

were enrolled in the study. Active TB was excluded from all household contacts based on radiologic and clinical examinations, sputum microscopy, and bacteriologic culture. TST was performed as described previously [25]. Reactions of <5 mm and ≥5 mm were considered TST negative and positive, respectively. After heparinized, whole blood from each participant was collected; the whole-blood IFN-γ assay (WBIA) stimulated with the rEC or rCM fusion proteins was performed as described previously [25]. In brief, whole blood from each donor (1 ml) was seeded in 24-well plates and incubated with 20 μl rEC or rCM protein at final concentrations of 0 or 10 μg/ml for 24 h at 37°C. After stimulation, 200 μl of plasma was then taken from each well and stored at −20°C until use. The concentrations of IFN-γ in collected samples were determined in duplicate, using a commercial enzyme-linked immunosorbent assay kit according to the manufacturer's instructions (Dakewei Biotech, Shenzhen, China). The study protocol was approved by the Ethics Committee of Tongji Medical College. Written informed consent was obtained from all subjects involved in this study.

Construction of DNA vaccines

The recombinant plasmids pPro610 and pPro2164 were firstly digested with *Bam*HI and *Hin*dIII. The gene fragments encoding rEC and rCM were then subcloned separately into pcDNA3.1(−). The resulting plasmids were designated pcD610 and pcD2164, respectively. The insert DNA was confirmed by enzyme digestion and DNA sequencing. Plasmids for immunization were transformed into *E. coli* DH5α, and endotoxin-free plasmid DNA was purified using the Qiagen Plasmid Giga kit (Qiagen, Valencia, CA) according to the manufacturer's instructions.

Animals and immunization

Specific pathogen-free, 6- to 8-week-old, female C57BL/6 mice (Vital River Lab Animal, Beijing, China) were bred in cages on the animal feeding cabinet (VentiRack, Chester, CA, USA) in a BSL3 laboratory. The research protocol was reviewed and approved by Tongji Medical School Committees on Biosafety and Animal Care and Use Committee of China.

Mice were randomly assigned to the following experimental groups with twelve mice in each group: non-vaccinated control, vector control, pcD610, pcD2164, BCG, BCG prime plus vector booster, BCG prime plus pcD610 DNA booster, and BCG prime plus pcD2164 DNA booster. BCG (10^6 CFU) was applied once subcutaneously on the first day. Two weeks later, DNA vaccination was performed three times by injection of plasmid DNA (50 μg) into the quadricep muscle of each hind leg at 2-week intervals. Two weeks after the last DNA immunization, five mice from each group were killed for immunological analysis and seven mice were used for intravenous challenge with 10^6 CFU virulent *M. tuberculosis* H37Rv strain. The animal experiments were repeated twice.

Antigen-specific immunoglobulin G (IgG) antibody assay

Antigen-specific immunoglobulin G (IgG) antibody in the serum of each immunized mice was determined by enzyme-linked immunosorbent assay (ELISA). Microtiter plates were coated overnight at 4°C with 100 μl rCM, rEC,

or antigen control (5 μg/ml) in carbonate/bicarbonate buffer (pH9.6). A secondary HRP-conjugated rat anti-mouse IgG and orthophenyldiamine (OPD) were used for color development as an indicator. Sera from naive mice were used as negative control. The OD value from antigen control was subtracted as background to exclude antibody against non-specific *E. coli* protein. Data are presented as mean (±SEM) of optical density value at 490 nm.

IFN-γ production by splenocytes

Spleen cells from five mice per group were prepared individually as described previously [22] 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 microliters of rCM or rEC (5 μg/ml) in complete RPMI-1640 medium was used as the stimulator. RPMI-1640 medium and BCG-PPD (2 μg/ml, Chengdu Institute of Biological Products, Chengdu, China) were used as negative control and positive control, respectively. 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 use. 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 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 of five mice in each group.

Virulent *M. tuberculosis* H37Rv challenge

Two weeks after the last DNA immunization, seven mice of each group were infected with 10^6 CFU virulent *M. tuberculosis* H37Rv through the tail vein. Four weeks post-infection, mice were killed. Lungs and spleens were removed aseptically and homogenized in PBS for bacterial enumeration. Individual organs were plated in serial dilutions onto Middlebrook 7H11 agar supplemented with 10 μg of 2-thiophenecarboxylic acid hydrazide per ml to selectively inhibit the possible growth of residual BCG. The plates were incubated for 3 weeks at 37°C, and the number of CFU per organ was counted. Data were expressed as mean ± SEM of log 10 CFU for each group.

Histopathological staining

Partial lung lobes from different groups were harvested and fixed in 10% PBS-buffered formalin and then embedded in paraffin. Sections were prepared and stained with hematoxylin and eosin (HE). Representative slides were evaluated by a pathologist with no prior knowledge for the treatment group, recording the results under a light microscope and scoring as our described previously [27].

Statistical analysis

Student's *t* test was used to compare the mean of each group of mice, and a *P*-value less than 0.05 was considered statistically significant. Differences in data of hispathology were tested for significance using the rank-sum test.

Results

Construction of the recombinant plasmids

The DNA sequences of CFP10 and ESAT-6 were amplified by PCR from the genomic DNA of *M. tuberculosis* H37Rv strain (Fig. 1). The gene fragment encoding ESAT-6 and CFP10 fusion protein (rEC) with a 45-bp linker (encoding three copies of peptide G4S1) was first generated by the method of GeneSOEing and then subcloned into pProEXHTb, resulting in pPro610(Fig. 1). The fusion gene was also subcloned into pcDNA3.1(−), resulting in pcD610 (Fig. 1). The gene encoding CFP21 and MPT64 fusion protein (rCM) was also digested from the recombinant plasmid pPro2164 with *Bam*HI and *Hin*dIII and then subcloned into pcDNA3.1(−), resulting in pcD2164 (Fig. 1). DNA sequencing and enzyme digestion were used to confirm the desired constructs.

Purification of rEC and rCM fusion proteins

E. coli BL21 (DE3) strain harboring the plasmid pPro610 expressed a protein with a molecular mass of approximately 22 kDa (inclusion bodies) upon induction with IPTG, which was consistent with the expected molecular mass of the fusion protein rEC (Fig. 2). rEC protein was extracted by sonication, dissolved in urea, and then purified using a Ni–NTA purification system. The purified protein was examined by SDS–PAGE and verified by Western blotting with antibodies to ESAT-6 or CFP10. The results confirmed that the 22-kDa protein represented the expected protein rEC (Fig. 2). rCM was purified as previously described in detail [25]. The purified products of both rEC and rCM proteins were further confirmed by SDS–PAGE (Fig. 2).

Maximum level of IFN-γ detected in TST-positive contacts by rCM-WBIA

Whole-blood samples were collected from 18 household contacts with recent active TB patients and subjected to

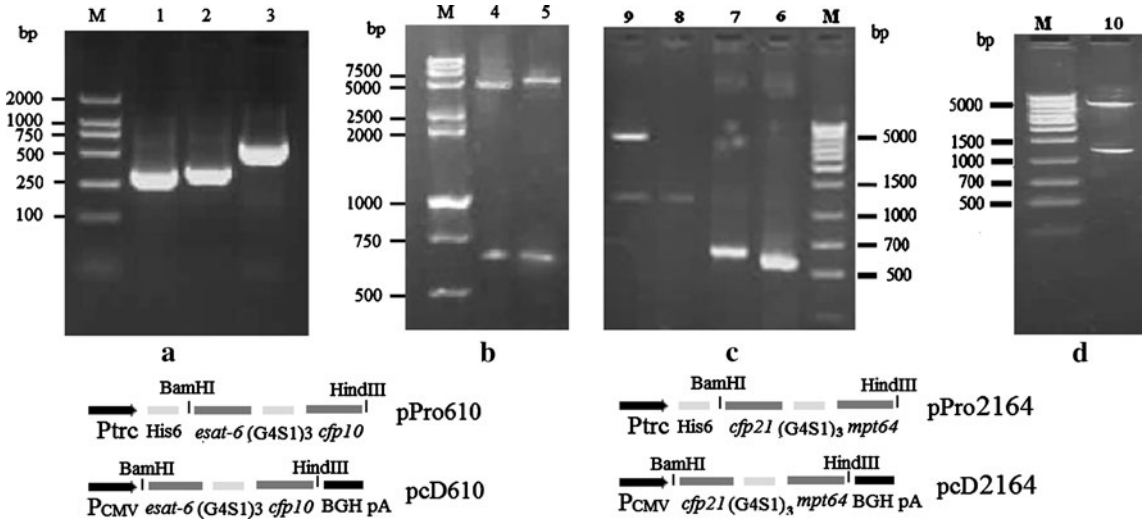

Fig. 1 Construction of recombinant plasmids. Genes encoding ESAT-6, CFP10, the fusion protein of ESAT-6 and CFP10 with a 45-bp linker, and CFP21, MPT64, the fusion protein of CFP21 and MPT64 with a 45-bp linker, were cloned from *M. tuberculosis* H37Rv genomic DNA, respectively (**a** and **c**). The PCR products of the fusion genes were inserted into the *Bam*HI and *Hin*dIII sites of pProExHTb or pcDNA3.1(−), resulting in the recombinant plasmids pPro610 and pcD610, pPro2164 and pcD2164, respectively. The recombinant plasmids were identified by enzyme digestion (**b**–**d**). *Lane M* DNA molecular marker; *lane A1* PCR product of *esat-6*; *lane A2* PCR product of *cfp10*; *lane A3* PCR product of *esat-6-cfp10*; *lane B4* products of pPro610 digested with enzymes; *lane B5* products of pcD610 digested with enzymes; *lane C6* PCR product of *cfp21*; *lane C7* PCR product of *mpt64*; *lane C8* PCR product of *cfp21-mpt64*; *lane C9* products of pPro2164 digested with enzymes; *lane D10* products of pcD2164 digested with enzymes

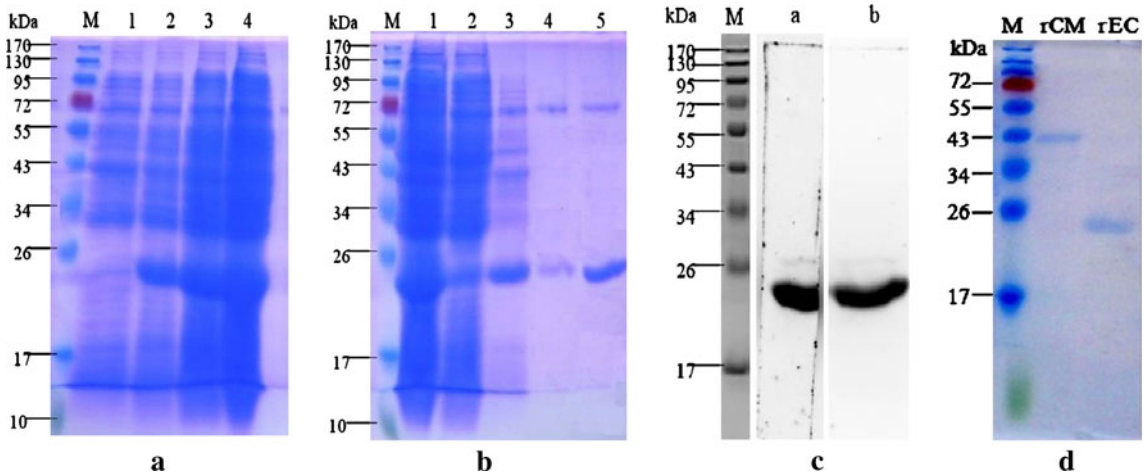

Fig. 2 Expression, purification, and identification of recombinant fusion proteins. *E. coli* BL21(DE3) harboring pPro610 was cultured with IPTG. The expression **a** and the purification procession of rEC fusion protein **b** were confirmed by SDS–PAGE and Western blotting (**c**). The expression and the purification procession of rCM fusion protein were described in detail in reference [25]. The final products of rEC and rCM were confirmed by SDS–PAGE (**d**). *Lane M* protein molecular size marker (kDa); *lane A1*, *E. coli* strain without IPTG; *lane A2*, *E. coli* strain 2 h after IPTG induction; *lane A3*, *E. coli* strain 4 h after IPTG induction; *lane A4*, *E. coli* strain 6 h after IPTG induction; *lane B1* cell lysis; *lane B2* fraction from Ni–NTA column after wash with denaturing binding buffer; *lanes B3* and *B4* fraction after wash with wash buffer; *lane B5* rEC protein eluted with native elution buffer; *lane Ca* anti-ESAT-6 antibody; *lane Cb* anti-CFP-10 antibody; *lane DrCM* the final products of rCM; *lane DrEC* the final products of rEC

both rEC-WBIA and rCM-WBIA (Fig. 3). The levels of IFN-γ in all samples without antigen stimuli were below 30 pg/ml (10.6 ± 5.9 pg/ml) but were significantly increased to $1{,}278.3 \pm 994.8$ pg/ml after rEC stimulation and $1{,}450.5 \pm 1{,}179$ pg/ml after rCM stimulation. Analysis of the distribution of IFN-γ levels in TST-positive and TST-negative samples showed a significant correlation between TST positivity and IFN-γ level, regardless of the proteins used (both $P < 0.05$). The mean IFN-γ levels in samples from both TST-positive group (2,012.4 pg/ml) and TST-negative group (567.5 pg/ml) detected by rCM-WBIA were higher than those by rEC-WBIA (1,797.4 and 462.7 pg/ml, respectively). However, the differences were not found to be statistically significant.

Antigen-specific IgG

Sera IgG antibodies against rEC and rCM proteins were determined by ELISA. As shown in Fig. 4, vaccinated mice only produced low level of IgG antibodies to both proteins. As expected, the most significant IgG-specific response to rEC protein was induced in both pcD610 and BCG plus pcD610 group, when rEC protein was used for detection. Both pcD2164- and BCG plus pcD2164-treated mice produced the strongest IgG response to rCM protein. No antigen-specific antibodies were detected in other groups, regardless of the proteins used.

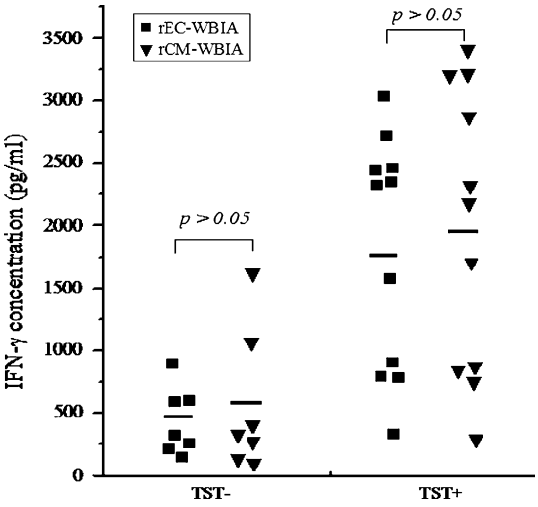

Fig. 3 Immunogenicity of rEC and rCM fusion protein in TB contacts. A total of 18 household contacts with active TB patients were tested by TST, rEC-WBIA, and rCM-WBIA. *Each square* represents the IFN-γ concentration in a sample, and median values for different groups are indicated by *horizontal lines*

Antigen-specific IFN-γ production by splenocytes

The level of IFN-γ production by splenocytes was determined by ELISA. As shown in Fig. 5, splenocytes from mice vaccinated with pcD610 and BCG prime plus pcD610 booster produced higher level of IFN-γ than with BCG ($P < 0.05$), when stimulated with rEC protein. Splenocytes from mice vaccinated with pcD2164 also showed a more significant IFN-γ response to rCM protein than that of BCG ($P < 0.05$). Moreover, BCG plus pcD2164 vaccination induced the strongest IFN-γ response to rCM protein. As expected, little IFN-γ response was observed for splenocytes from mice vaccinated with vector, BCG, and BCG plus vector whether stimulated with rEC or rCM proteins. When stimulated with BCG-PPD, all BCG-primed mice produced the similar IFN-γ response, regardless of DNA vaccines booster ($P > 0.05$).

pcD2164-vaccinated mice decreased comparable bacterial load in the lung with BCG vaccination and significantly enhanced BCG-primed protective efficacy

In order to determine the efficacies of different vaccination strategies, C57BL/6 mice were challenged with virulent *M. tuberculosis* strain. Four weeks later, mice were killed and bacterial load in the lung and spleen of each mouse was analyzed. The clear hierarchy of protective efficacy was seen in the different groups of vaccinated mice (Table 2). Highest bacterial load was seen in the lung and spleen in both naive and vector groups. pcD2164- or pcD610 DNA-vaccinated groups showed reductions in the bacterial load of lung and spleen to different degrees. pcD2164 DNA vaccination resulted in a comparable decrease in the bacterial load in the lung (−0.795 log) with BCG ($P > 0.05$). BCG

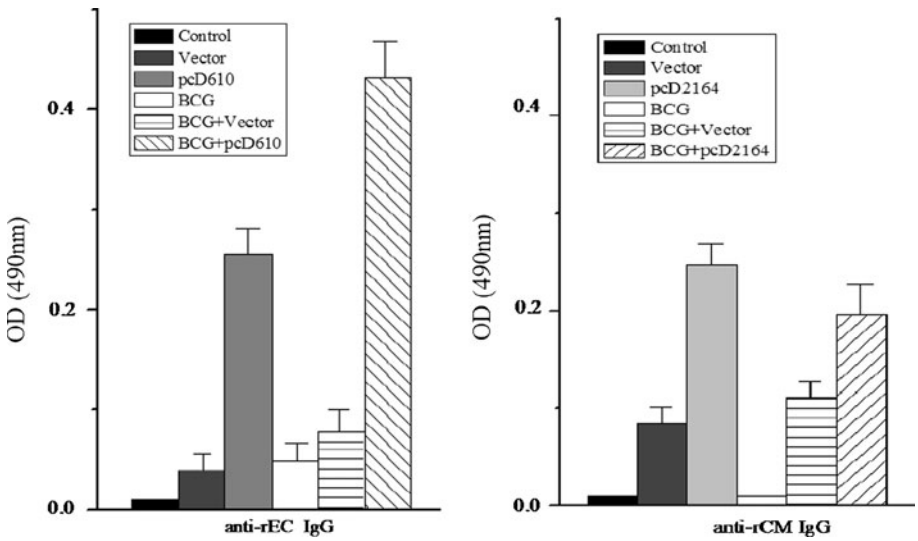

Fig. 4 Antigen-specific total IgG antibodies detected by ELISA. C57BL/6 mice ($n = 5$) were vaccinated with different vaccine candidates. Two weeks after the last immunization, the animals were bled and sera were obtained. rEC and rCM (5 μg/ml each) were separately used for the detection of IgG in different vaccine groups by ELISA. The results were expressed as mean (±SEM) of OD (490 nm) of each group

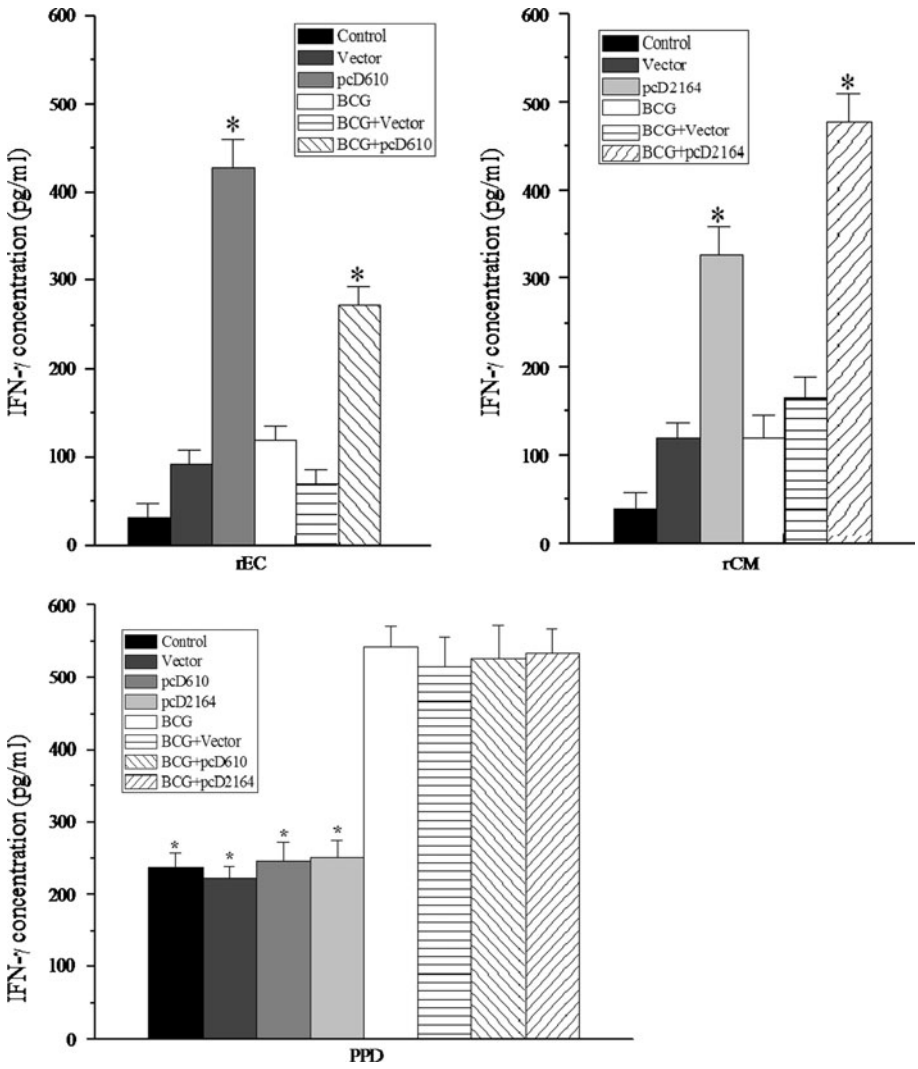

Fig. 5 Antigen-specific IFN-γ production of splenocytes from mice. C57BL/6 mice ($n = 5$) were vaccinated with different vaccines. Splenocytes were prepared 2 weeks after the last DNA immunization and incubated with rEC (5 μg/ml), rCM (5 μg/ml), or BCG-PPD (2 μg/ml). IFN-γ secreted by splenocytes was measured in triplicate by an ELISA. The results are expressed as mean (±SD, pg/ml) of five mice in each group. *$P < 0.05$ vs. BCG group

prime and pcD2164 or pcD610 booster inhibited the growth of *M. tuberculosis* more significantly in the lung and spleen of vaccinated mice than BCG or DNA vaccines alone, respectively ($P < 0.05$). The most significant decrease in bacterial load in the lung was seen in BCG prime and pcD2164 booster group.

Decreased lung pathology in mice vaccinated with BCG plus pcD2164 compared with BCG alone

Clear differences in lung pathology and pathological scores were found in different groups vaccinated with BCG and BCG plus pcD2164 challenged with virulent *M. tuberculosis* H37Rv (Figs. 6, 7). Both control and vector control mice showed severe interstitial pneumonia and intense inflammation throughout the lung. A large number of sites with significant granuloma formation appeared around alveolar tissues, bronchioles, and the small blood vessels.

Table 2 Bacterial load per lung and spleen in C57BL/6 mice at 4 weeks post-challenge i.v. with 10^6 CFU virulent *M. tuberculosis* H37Rv strain ($n = 7$)

Groups	(Mean ± SEM) log 10 CFU			
	Lung	Value*	Spleen	Value*
Control	6.251 ± 0.233^{b}		5.706 ± 0.234^{b}	
Vector	6.324 ± 0.11^{b}	−0.073	5.722 ± 0.132^{b}	−0.016
pcD610	$5.67 \pm 0.141^{a,b}$	0.581	$5.25 \pm 0.19^{a,b}$	0.456
pcD2164	5.456 ± 0.157^{a}	0.795	$5.248 \pm 0.126^{a,b}$	0.458
BCG	5.215 ± 0.222^{a}	1.036	4.826 ± 0.214^{a}	0.88
BCG + vector	4.911 ± 0.262^{a}	1.34	4.508 ± 0.23^{a}	1.198
BCG + pcD610	$4.78 \pm 0.129^{a,b}$	1.471	$4.23 \pm 0.25^{a,b}$	1.476
BCG + pcD2164	$4.565 \pm 0.2^{a,b}$	1.686	$4.231 \pm 0.162^{a,b}$	1.475

* The difference value between each groups and control

[a] $P < 0.05$ vs. control

[b] $P < 0.05$ vs. BCG. This experiment was repeated twice with similar results

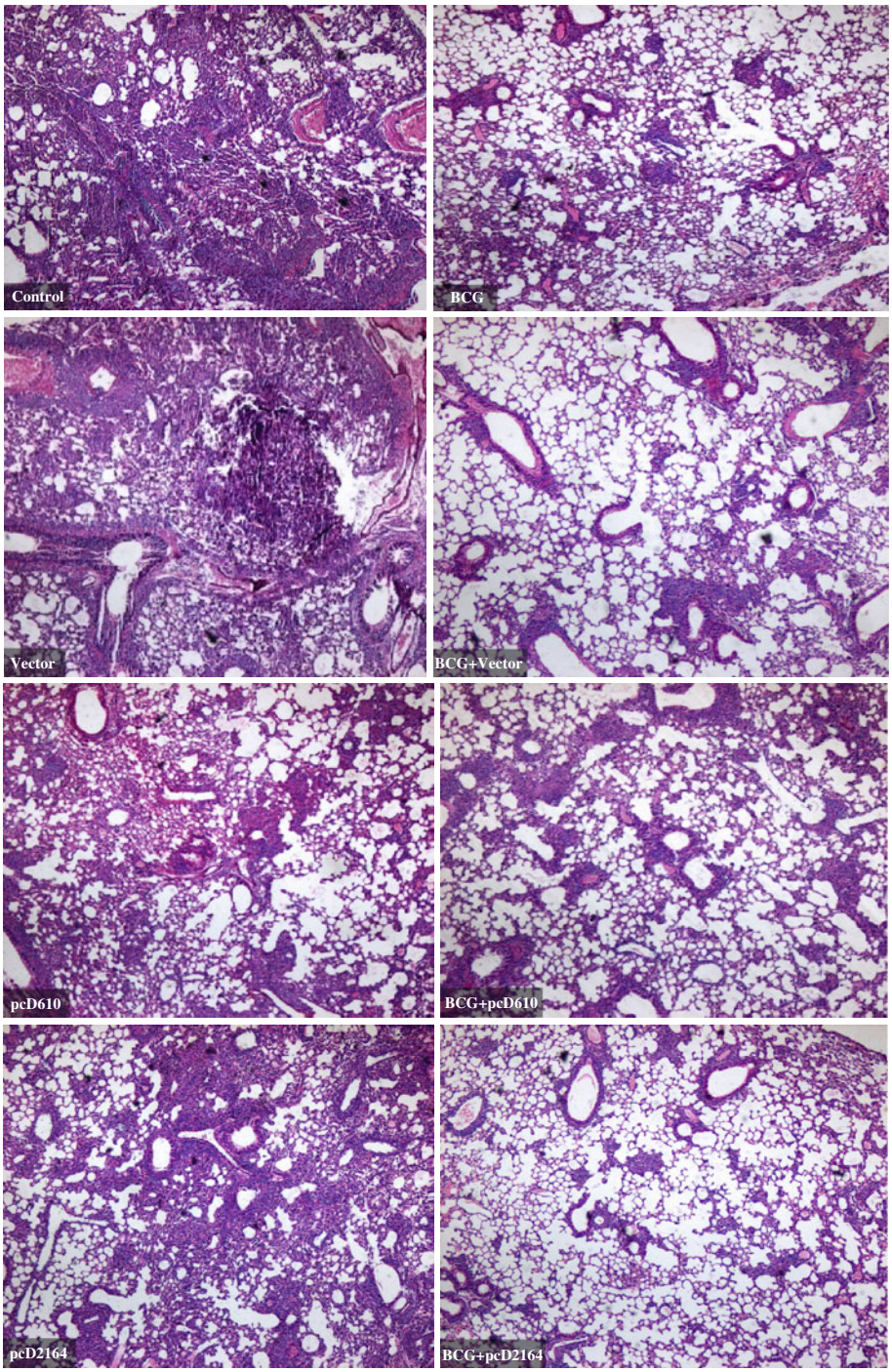

Fig. 6 Representative lung pathology of C57BL/6 mice after challenge. Vaccinated C57BL/6 mice were challenged i.v with 10^6 CFU virulent *M. tuberculosis* H37Rv strain. Four weeks after infection, lung tissue sections from different vaccine groups were prepared for HE staining (amplification 10×4)

There was relatively large number of small granuloma formation around alveolar tissues also observed from single pcD1264- or pcD610-vaccinated groups. Slight interstitial pneumonia appeared in BCG, BCG plus vector, and BCG plus pcD610 groups. Alveolar tissue from mice vaccination with BCG plus pcD2164 appeared to be intact with very limited lung inflammation and little granuloma formation around alveolar tissues (Fig. 6).

Discussion

TB prevention has a high priority in the area of global public health. However, the replacement of currently used BCG might not happen anytime soon. Although 11 new TB vaccines have been evaluated in clinical trials so far, most of these are administrated by BCG prime and a heterologous vaccine boost strategy [28]. Over 90% children are

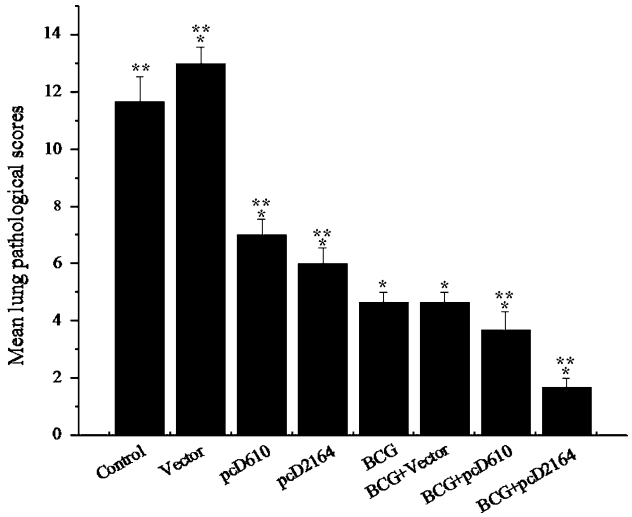

Fig. 7 Lung pathological scores of C57BL/6 mice after challenge. Vaccinated C57BL/6 mice were challenged i.v with 10^6 CFU virulent *M. tuberculosis* H37Rv strain. Four weeks after infection, pathology was evaluated from HE-stained lung tissue sections and scored. Data represent the mean ± SEM of 3 mice. $^*P < 0.05$ vs. control; $^{**}P < 0.05$ vs. BCG

immunized with BCG in most developing countries annually, making it the most widely used vaccine in human [29]. In addition, BCG has over 80% protective efficacy against severe forms of childhood TB [1, 3] and is generally considered the gold standard for evaluating new vaccine candidates. As a result, neonatal vaccination with BCG will continue to play a key role in the future TB vaccination strategy. The important problem needed to face is that the most protective BCG substrain for common use remains undetermined, although it is generally recognized that the currently used BCG vaccine substrains differ from each other and may induce different degrees of protective immunity. The sequential losses of RD1 and RD2 are the most iconic events during the original derivation of BCG and subculture, respectively. Previous studies have confirmed that the reintroduction of RD1 region of *M. tuberculosis* into BCG could enhance the protective efficacy of BCG [21]. In this study, pcD610 and pcD2164 DNA vaccines expressing rEC and rCM fusion proteins, based on RD1 and RD2 respectively, were constructed. The immunogenicity and protective efficacy of BCG prime and two DNA vaccines booster were assessed and compared in mice. Our results demonstrate that both DNA vaccines used as boosters could produce better protective immunity elicited by BCG prime than BCG or DNA vaccinations alone, as evidenced by lower bacterial load in the tissues and less lung pathology. Moreover, pcD2164 was, at the very least, comparable to pcD610 when used to boost BCG priming protective immunity in mice, because the least lung pathological changes and bacterial loads were observed in BCG prime and pcD2164 booster group. Therefore, our results clearly indicate that the deletion of RD2, through antigenic variation, has an important influence on the protective efficacy of different BCG substrains.

These results were consistent with our and other previous studies. A DNA vaccine expressing ESAT-6 protein could enhance the protective efficacy of BCG vaccination in mice [22]. A combined vaccine regimen of BCG and a DNA vaccine expressing the fusion protein of ESAT-6 and CFP10 might offer greater protection against TB in cattle [23]. BCG vaccination, supplementation with the mixtures of ESAT-6, CFP21, and MPT64 proteins, also resulted in significantly greater protection against experimental murine TB than BCG alone [24]. Both RD1 and RD2 are deleted in our BCG China substrain; pcD2164 and pcD610 may amplify overall anti-TB immunity in BCG immune animals by activating rCM- and rEC-specific T cells and play an important role in producing the booster effect on BCG prime, as evidenced by significantly increased expression of IFN-γ after splenocytes were restimulated with specific antigens.

Previously, the hypothesis that early substrains of BCG that contain fewer chromosomal deletions than late substrains might have more protective efficacy remains controversial. As early as 1953, a contrasting result was reported by Yakuwa that BCG Pasteur provided better protection of vaccinated guinea pigs against virulent *M. tuberculosis* challenge than BCG Japan did [15]. Wang compared three BCG China strains with BCG Denmark and one BCG strain derived from BCG Japan in a guinea pig model, and the results showed that animals immunized with BCG Denmark had the lowest *M. tuberculosis* CFU recovered from the spleen [16]. However, Yamamoto showed that BCG Tokyo 172 strain possessed a significant protective efficacy against *M. tuberculosis* as well as BCG Copenhagen 1331 strain in vaccinated guinea pigs [17], supported by the results of Horwitz in a more recent study [18]. In fact, we cannot exclude the possibility that other early substrains may be more potent than late substrains based on the discordant results about BCG Japan. BCG Russia could provide protection against mycobacterial challenge in a mouse model, but BCG Japan did not [19]. Recently, Castillo-Rodal reported that mice vaccinated with BCG Birkhaug also showed significantly lower CFU compared with the other nine substrains [20]. In addition, differential immunogenicity of BCG Japan was also reported. Vaccination with BCG Japan resulted in greater secretion of Th1 cytokines such as IFN-γ, TNF-α, and IL-2, when compared with BCG Danish-vaccinated infants [30], while Wu reported that BCG Japan-vaccinated infants had a significantly greater expression of proinflammatory cytokines in PBMC, such as IL-1, IL-1β, IL-6, and IL-24, than BCG Denmark or BCG Brazil did [31]. Taken together, we can conclude that the discrepancy of above results about immunogenicity and

protective efficacy induced by BCG substrains is very likely a reflection to the effects of genetic diversity among BCG substrains on the efficacy, because different BCG vaccine strains might have specific genetic differences besides RD1 and RD2 and these may have further influence on the efficacy [7, 32, 33]. Although Kozark reported that multiple genes within the RD2 region could contribute to the virulence of *M. tuberculosis* through regulating the host innate immune response [34], BCG early substrains under clinical use have been proved that BCG vaccine substrains with the RD2 region are safe for over 80 years. Protective effects of immunization with RD2-deficient BCG vaccine complemented with CFP21 and MPT64 would eventually prove the finding of the present work.

However, mice were not challenged by aerosol exposure but intravenous injection, which does not represent the natural infection route for TB. The infection by the traditional aerosol challenge may be different and will be primarily controlled by mucosal immunity at the first encounter. In addition, the results obtained by DNA vaccination in a mouse model may be different for humans, due to species differences. Therefore, direct comparison of protective efficacy induced by BCG early and late substrains in vaccinated human population remains necessary and will help to confirm the effect of genetic difference on their efficacy.

In conclusion, our present work demonstrates that DNA vaccine expressing RD2 could enhance the protective efficacy of BCG by a BCG prime and DNA vaccine boost strategy, which provided the direct evidence that RD2 deleted in BCG genome might have an important influence on the protective efficacy of different BCG substrains. These findings will benefit the optimal choice of BCG substrain for neonatal immunization and direct the rational design of new vaccines for improved prevention of TB.

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