

Recombinant *Bacille Calmette-Guérin* coexpressing Ag85B-IFN- γ enhances the cell-mediated immunity in C57BL/6 mice

WEI LIU, YING XU, HONGBO SHEN, JINGRAN YAN, ENZHUO YANG and HONGHAI WANG

State Key Laboratory of Genetic Engineering, Institute of Genetics,
School of Life Sciences, Fudan University, Shanghai 200433, P.R. China

Received June 2, 2016; Accepted January 31, 2017

DOI: 10.3892/etm.2017.4273

Abstract. The only available vaccine against pulmonary tuberculosis is *Bacille Calmette-Guérin* (BCG). As the efficacy reported of the vaccine is not up to the mark, there is an urgent need to develop improved anti-tuberculosis vaccines. Antigen 85B (Ag85B) is a very promising vaccine candidate molecule of *Mycobacterium tuberculosis* and interferon (IFN)- γ and has been considered the most attractive correlate of protective immunity. The aim of this study was to construct a novel recombinant BCG (rBCG) to secrete Ag85B and mouse IFN- γ under control of the Mycobacterial heat shock protein 60 (*hsp60*) promoter and the antigen signal sequence. Second aim of the present study is to evaluate the immune response in C57BL/6 elicited by the new rBCG. Expression of the fusion protein was readily detectable by western blotting and IFN- γ bioactivity was detected indirectly by enzyme-linked immunosorbent assay (ELISA). Compared with BCG, rBCG::Ag85B-IFN- γ was substantially more active in inducing the production of IFN- γ and tumor necrosis factor (TNF)- α from mouse splenocytes. ELISA analysis for IgG, IgG1 and IgG2c showed that rBCG::Ag85B-IFN- γ induced higher titer of Ag85B and facilitated Th1 type immune response. rBCG::Ag85B-IFN- γ also improved nitric oxide production levels and enhanced antigen-specific splenocyte proliferation. Moreover, rBCG::Ag85B-IFN- γ induced human monocytes such as THP-1 cells to enhance expression of CD80, CD86, CD40 and HLA-DR. Flow cytometry analysis confirmed that rBCG::Ag85B-IFN- γ significantly activated CD4⁺ T cells. Assessing combinations of IFN- γ , TNF- α and interleukin-2 at the single-cell level by multiparameter flow cytometry, we found that rBCG::Ag85B-IFN- γ improved the multifunctional T cells level in comparison to BCG. In conclusion, the present study indicates that rBCG::Ag85B-IFN- γ

increases cell mediated immune response and is a potential candidate vaccine for immunotherapeutic protocols against pulmonary tuberculosis.

Introduction

Tuberculosis (TB) is one of the leading infectious diseases worldwide. The latest surveillance data by the World Health Organization (WHO) reveals that in 2006, there were 9.2 million new cases and 1.7 million deaths from TB (1). The TB pandemic has continued despite widespread use of the only available licensed TB vaccine *Bacille Calmette-Guérin* (BCG) under the WHO expanded program on immunization and despite the use of directly observed chemotherapy programs for those diagnosed with active disease. Although BCG seems to provide protection against disseminated disease in newborns and children, its efficacy against pulmonary TB is poor especially in adults, which highlights the need for a better vaccine regimen (2).

During the past decades, progress has been made in explanation of BCG failure. The main potential vaccination strategies against TB included recombinant BCG (rBCG), attenuated strains of *Mycobacterium tuberculosis* (*M. tb*), subunit vaccine approaches and live, and non-replicating viral vector-based delivery systems. The most important strategy is to stimulate more potent immune responses against *M. tb*. In production of rBCG, three strategies have been mainly used. The first is based on the restoration of deleted BCG genes (deleted in the process of *M. bovis* attenuation) that encode immunogenic antigens. Another approach is based on rBCG that produces large amounts of strong immunogenic protective antigens, and the third is to construct rBCG that secrete cytokines relevant in anti-TB immune response such as interleukin (IL)-2, interferon (IFN)- γ , or IL-18 (3-5). These cytokines have been shown to activate macrophages and kill or control the growth of the *M. tb*. Although imperfect, INF- γ remains the best available correlate of protective immunity (6,7). Vaccines which failed to induce sufficient levels of IFN- γ are unable to protect effectively against tuberculosis in animal models (8).

Antigen 85B (Ag85B) is a secretory and immunogenic protein of *M. tb* and BCG with mycolyl-transferase activity, which is required for mycobacterial cell wall synthesis (9), and a study has observed rBCG30 overexpressing Ag85B resulted in better protection as compared to its parental BCG

Correspondence to: Dr Honghai Wang, State Key Laboratory of Genetic Engineering, Institute of Genetics, School of Life Sciences, Fudan University, 220 Handan Road, Shanghai 200433, P.R. China
E-mail: hhwangfudan@163.com

Key words: *Bacille Calmette-Guérin*, IFN- γ , tuberculosis, *Mycobacterium tuberculosis*

strain (10,11). Moreover, there was no significant safety issues in a phase I clinical trial of rBCG30 in more than 30 healthy adult volunteers (12,13). In the present study, the *ag85b* and *ifn- γ* genes were inserted into Mycobacterial-*E. coli* shuttle vector pMV361 in order to form novel rBCG viz rBCG::Ag85B-IFN- γ and its efficacy was tested in C57BL/6 mice. The Ag85B-IFN- γ fusion protein was expressed under control of the Mycobacterial heat shock protein 60 (*hsp60*) promoter and the *ag85b* signal sequence was utilized to secret it into the supernatant of the culture continuously. Expression of the fusion protein was readily detectable and IFN- γ bioactivity maintained unaffected.

Compared with BCG, rBCG::Ag85B-IFN- γ was substantially more active in inducing the production of IFN- γ and tumor necrosis factor (TNF)- α from mouse splenocytes by ELISPOT and enzyme-linked immunosorbent assay (ELISA). ELISA analysis for Ag85B special IgG, IgG1, and IgG2c displayed that rBCG::Ag85B-IFN- γ can induce high level antibody titer and facilitate Th1 type immune response. Also, rBCG::Ag85B-IFN- γ improved nitric oxide (NO) production level and enhanced antigen-specific splenocyte proliferation.

Expression of various surface molecules on activated macrophages is required for optimal development of protective T cell response in tuberculosis. We characterized the expression of surface markers in human monocytes such as THP-1 cell line infection with rBCG::Ag85B-IFN- γ . It increased expression of CD80, CD86 and CD40 significantly compared with BCG.

Flow cytometry analysis showed that rBCG::Ag85B-IFN- γ activated CD4⁺ T cells remarkably and improved CD8⁺ T cells gradually with time. Although IFN- γ is clearly necessary, using it as a single immune parameter may not always sufficient to bring protection (14). Multifunctional T cells secreting IFN- γ , TNF- α , and IL-2 have been shown to correlate with protection in *Leishmania major* infection in mice (15). We assess all combinations of IFN- γ , TNF- α and IL-2 at the single-cell level by intracellular cytokine staining and parameter flow cytometry analysis.

The immunostimulatory properties of rBCG::Ag85B-IFN- γ increased cell mediated immune response compared to the parent BCG strain. Considering the action of IFN- γ in clearing the infection pathogen, rBCG::Ag85B-IFN- γ may be a potential candidate vaccine and a superior agent for immunotherapeutic protocols in the future.

Materials and methods

Bacterial strains and cultures. *M. bovis* BCG (obtained from Shanghai Institute of Biological Products Co., Ltd., Shanghai, China), *M. tb* H37Rv and rBCG::Ag85B-IFN- γ (constructed as below) were grown in Middlebrook 7H9 medium (BD Biosciences, Sparks, MD, USA) supplemented with 0.5% glycerol, 0.05% Tween-80 and 10% ADC (BD Biosciences) or on solid Middlebrook 7H10 Agar medium (BD Biosciences) supplemented with 0.5% glycerol and 10% ADC. When required, the antibiotic kanamycin was added at a concentration of 25 μ g/ml. *E. coli* DH5- α was grown in Luria-Bertani medium (Oxoid Ltd., Basingstoke, Hampshire, UK) and used for cloning and extracting plasmid.

Construction of a rBCG expression Ag85B and IFN- γ . Coding sequences for *ag85b* (containing signal sequence) and mouse *ifn- γ* were amplified from the *M. tb* H37Rv genomic DNA and mouse spleen tissue cDNA respectively by PCR using the primers shown in Table I. The *ag85B* and murine *ifn- γ* coding regions were cloned into the Mycobacterial-*E. coli* shuttle vector pMV361, in which gene expression is under the control of the strong *M. bovis* hsp60 promoter. Inserted genes were sequencing confirmed and the rBCG substrain was produced by electroporation transfecting BCG-Danish strain cells with the recombinant plasmid pMV361-Ag85B-IFN- γ . The transformed BCG was plated on 7H10 solid medium supplemented with 25 μ g/ml kanamycin and grown at 37°C for 3 weeks. The resulting recombinant clones containing pMV361-Ag85B-IFN- γ were designated rBCG::Ag85B-IFN- γ .

Western blotting detection of expressed Ag85B and INF- γ . Expression of the recombinant fusion protein by rBCG::Ag85B-IFN- γ was determined by western blotting. The individual rBCG clone was selected and grown in Sauton medium (0.25 g MgSO₄·7H₂O, 0.25 g K₂HPO₄, 1.0 g citric acid, 4.0 g sodium glutamate, 30 ml glycerol, 5 mg ZnSO₄ and 25 mg ferrum-ammonium citrate in 500 ml ddH₂O) containing 25 μ g/ml kanamycin. After 2 weeks of growth, protein expression was induced by heating at 45°C for 60 min. The bacterial cultures were centrifuged at 8,000 x g for 20 min. The culture supernatants were concentrated as previously described (16) for western blot detection. A total of 25 μ l of the concentrated culture filtrates from rBCG::Ag85B-IFN- γ were analyzed for expression of the recombinant fusion protein by western blotting using anti-Ag85B rabbit polyclonal sera.

Determination of IFN- γ levels and bioactivity in the bacterial culture supernatants. The ability of the bacilli to secrete murine recombinant IFN- γ was verified by measuring the concentration of the cytokine by ELISA in the bacterial culture supernatants (17). The mouse INF- γ Quantikine ELISA kit (R&D Systems, Inc., Minneapolis, MN, USA) was used to determine the IFN- γ activity of rBCG::Ag85B-IFN- γ following the manufacturer's instructions. The bioactivity of IFN- γ produced by the rBCG::Ag85B-IFN- γ strain was calculated according to the standard curve. The experiment was performed twice.

Animal vaccination. Five-week-old female C57BL/6 mice (Slaccas Laboratory Inc., Shanghai, China) were used in the ABSL-2 animal facility at Second Military Medical University (Shanghai, China). Mice received free access to food and water throughout this study. All experiments were performed in accordance with the local Ethics Committee guidelines. C57BL/6 mice (12 per group) were immunized subcutaneously at the dosage 5x10⁶ CFU of BCG or rBCG in 200 μ l phosphate-buffered saline (PBS). Mice were sacrificed to analyze the immune responses at 6 and 12 weeks after the immunization. As an additional control, mice of the other group were injected with PBS only. The experiment was repeated twice.

ELISPOT assay for IFN- γ from splenocyte cultures. At 6 and 12 weeks after the vaccination, mice were sacrificed, and

Table I. Primers used to engineer the recombinant *Bacille Calmette-Guérin*.

Primers	Sequence
Ag85B	Primer F: 5'-TGTGAATTCATGACAGACGTGAGCCGAAAG-3' (<i>EcoRI</i>)
	Primer R: 5'-TACGGATCCGCCGCGCCTAACGAACTCTG-3' (<i>BamHI</i>)
IFN- γ	Primer F: 5'-TCTGGATCCATGAACGCTACACACTGC-3' (<i>BamHI</i>)
	Primer R: 5'-ACTAAGCTTTCAGCAGCGACTCCTTTTCC-3' (<i>HindIII</i>)

For construction of the pMV361-Ag85B-IFN- γ fusion molecule, the coding region (with the secretory signal sequence) of Ag85B was amplified from *M. tb* H37Rv genome DNA using the Ag85B primers. The polymerase chain reaction (PCR) product was digested by *EcoRI* and *BamHI*. Coding sequences for IFN- γ were amplified from mouse IFN- γ cDNA using the IFN- γ primers. The PCR product was digested by *BamHI* and *HindIII*. The two digested gene fragments were cloned into the predigested pMV361 plasmid with *EcoRI* and *HindIII*. Underlined text, restriction endonuclease sequence. *M. tb*, *Mycobacterium tuberculosis*; Ag85B, antigen 85B; INF, interferon; F, forward; R, reverse.

their spleens removed aseptically into RPMI-1640 medium containing 10% fetal calf serum (FCS), 2 mM glutamine, 50 μ M β -mercaptoethanol, 100 μ g/ml streptomycin and 100 U/ml penicillin. Spleens were gently ground through a 70 μ m cell strainer, and the single-cell suspensions were prepared with Lympholyte-M (Cedarlane Laboratories, Burlington, NC, USA) by density-gradient centrifugation procedure. We used the mouse IFN- γ ELISPOT kit (U-CyTech Biosciences, Utrecht, The Netherlands). Analyses were conducted on the splenocytes from five mice each group. Diluted cells were added to the wells of the ELISPOT plate at 5×10^5 cells per well in culture medium as described above containing purified protein derivatives (PPD, 5 μ g/ml), Ag85B (5 μ g/ml) or phytohemagglutinin (2 μ g/ml, as positive control) as stimulus. The plates were incubated at 37°C, 5% CO₂, 100% humidity for 36 h and the IFN- γ secreting T cells were evaluated. Spots were counted by use of an immunospot image analyzer. Wells with <5 spots were not used for calculations.

ELISA analysis for IFN- γ , TNF- α and IL-4. Single cell suspensions were acquired and diluted in 2 ml culture medium containing the same concentration of stimulus as described above to the 12-well plates at 1×10^6 cells per well. The plates were incubated at 37°C, 5% CO₂, 100% humidity for 36 h. The suspensions were collected of the splenocyte cultures for sandwich ELISA to detect the level of the cytokines. The cell deposits were harvested to prepare for flow cytometry analysis. We used the mouse IFN- γ ELISA kit, TNF- α ELISA kit and IL-4 ELISA kit (eBioscience, Inc., San Diego, CA, USA). The concentration of the cytokines was calculated in the suspension according to the standards curve.

NO production determination. This procedure for NO determination was based on the Griess reaction. Culture supernatants were acquired as described above. A total of 100 μ l of the splenocyte culture supernatants or sodium nitrite standard 100 μ l were mixed with an equal volume of Griess reagent (Sigma-Aldrich, St. Louis, MO, USA). In addition anaerobic conditions were achieved by purging the reaction

mixture with high purity argon gas for ≥ 5 min. After 5 min at room temperature, the absorbance at 550 nm (A550) was measured. The NO concentration was calculated in the culture supernatants according to the sodium nitrite standard.

ELISA analysis for IgG, IgG1, and IgG2c. Sera were collected from the immunized mice to monitor the antibody response by ELISA. Corning Costar 9018 ELISA plates (Corning Costar, Inc., Corning, NY, USA) were coated with Ag85B (5 μ g/ml) overnight at 4°C. The plates were blocked with PBS containing 1% bovine serum albumin (BSA; Bovogen Biologicals Pty Ltd., East Keilor, VIC, Australia). Sera were added at serial 2-fold dilution (beginning at a 1/50 dilution). After washing, and addition of horseradish peroxidase conjugated goat anti-mouse IgG, IgG1 and IgG2c (SouthernBiotech, Birmingham, AL, USA) diluted at 1/5,000, 1/1,000 and 1/1,000, respectively in blocking buffer (PBS containing 1% BSA) for 1 h, the plates displayed the staining by o-phenylenediamine substrate. Antibody titers were expressed as reciprocal end point titers. The mean antibody titers were the antilog of the average logarithm for antibody titer of 5 mice.

Antigen-specific splenocyte proliferation. Spleens of the immunized mice were removed aseptically to acquire the lymphocytes as described above. The isolated lymphocytes were washed in medium twice and cultured in RPMI-1640 + 10% FCS containing 100 μ g/ml streptomycin and 100 U/ml penicillin. Cells were adjusted to 1×10^5 cells/ml and 0.1 ml was plated with either 20 μ l PBS or 20 μ l Ag85B (300 μ g/ml) in 96-well plates. Cells were cultured for 2 days in 5% CO₂, 100% humidity and 37°C for MTS assay (CellTiter 96[®] Aqueous Non-Radioactive Cell Proliferation Assay kit; Promega Corp., Madison, WI, USA). The proliferation was assessed with splenocyte stimulation index (SI) (18).

Flow cytometry analysis for CD4⁺ and CD8⁺ subsets. The spleen tissue and single cell suspensions were prepared as described above in cell staining buffer (BioLegend, Inc., San Diego, CA, USA). The debris was removed by filtration of the

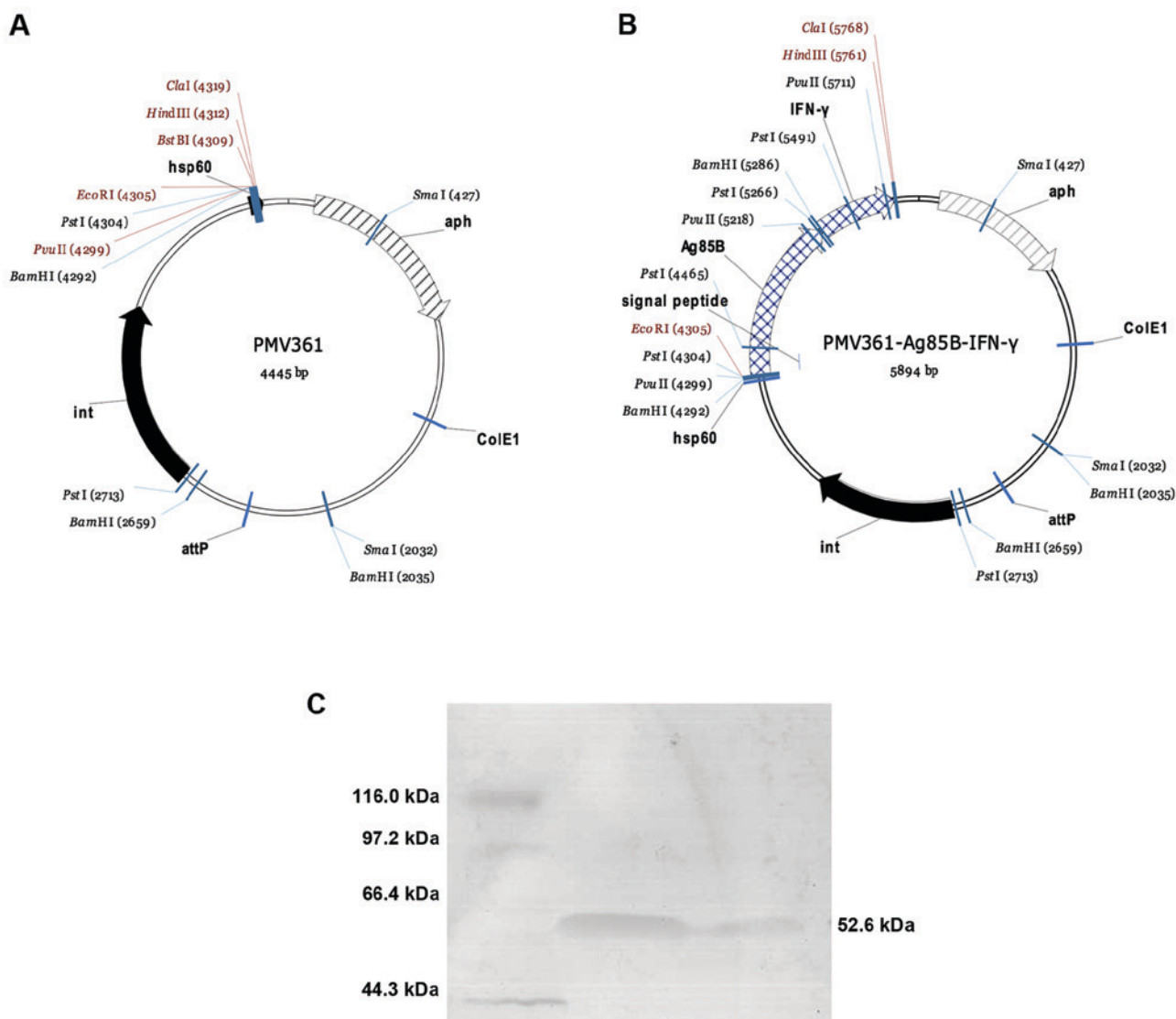


Figure 1. Construction of the recombinant *Bacille Calmette-Guérin* (BCG) expression antigen 85B (Ag85B) and interferon (IFN)- γ . (A) Plasmid pMV361 and (B) recombinant plasmid pMV361-Ag85B-IFN- γ . Recombinant plasmid pMV361-Ag85B-IFN- γ contains a full-length copy of the *Mycobacterium tuberculosis* 30 kDa major secretory protein (Ag85B) gene and mouse IFN- γ gene regions including the promoter heat shock protein 60 (hsp60) region. (C) Western blot analysis. Culture filtrates concentrated were analyzed for expression of recombinant protein by western blot using antibodies recognizing Ag85B. Introduction of the *ag85B* and *ifn- γ* coding regions into BCG resulted in a 52.6 kDa product in culture filtrate.

cell suspension through 70 μ m nylon mesh strainer. Viable cells were counted and suspended in cell staining buffer at 1×10^7 cells/ml. Cell suspension (100 μ l) was distributed into aseptic Eppendorf plastic tubes. Cells were blocked with PBS containing 1% BSA. Isotype controls of fluorescein isothiocyanate (FITC) and phycoerythrin (PE) conjugated anti-mouse IgG2b were used. FITC 0.25 μ g anti-mouse CD4 and 0.25 μ g PE anti-mouse CD8 (eBioscience, Inc.) per million cells in a 100 μ l total staining volume were added, then incubated in the dark at 4°C for 20 min. Cells were washed twice and the cell pellets were resuspend in 0.5 ml cell staining buffer for analyzes by flow cytometry (FACSCalibur; BD Biosciences). A total of 10,000 events per test were collected.

THP-1 cell culture and rBCG::Ag85B-IFN- γ treatment. THP-1 cells were cultured in complete medium RPMI-1640 supplemented with 10% FBS. The cells were seeded in complete medium containing Phorbol-12-myristate 13-acetate

(20 ng/ml) in 12-well plates at 1×10^6 cells/well for 18 h and induced to macrophages. Then the monocyte derived macrophage were treated with PBS (control group), BCG (1×10^7 CFU) or rBCG::Ag85B-IFN- γ (1×10^7 CFU) for 36 h. Supernatants were collected for cytokine assays, and cells were collected for surface marker flow cytometry analysis.

Cells surface marker flow cytometry analysis. For flow cytometry analysis, cells were collected, washed and resuspended in PBS supplemented with 1% FBS, thereafter cells were stained with fluorochrome-conjugated mAb. We divided samples into two parts at 1×10^6 cells/tube. One was stained with FITC-conjugate anti-human CD40 and PE conjugate HLA-DR and the other was stained with FITC conjugate CD86 and PE conjugate CD80 (Caltag Laboratories; Invitrogen Life Technologies, Carlsbad, CA, USA). Cells were incubated in the dark at 4°C for 20 min. Following two washes, cell pellets were resuspend in 0.5 ml cell staining buffer for analyses by flow

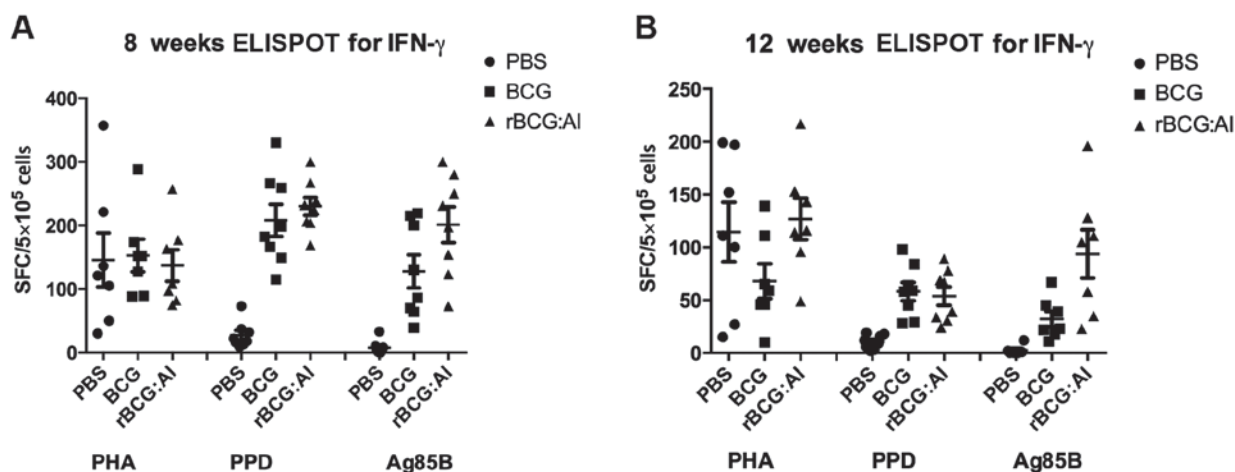


Figure 2. The cellular immune response was measured in an ELISPOT assay with splenocytes isolated from C57BL/6 mice immunized with different vaccines at (A) 6 weeks and (B) 12 weeks. Splenocytes were plated at 5×10^5 cells per well in duplicate and incubated with phytohemagglutinin (PHA) ($2 \mu\text{g/ml}$), purified protein derivatives (PPD) ($5 \mu\text{g/ml}$) or antigen 85B (Ag85B) ($5 \mu\text{g/ml}$) at 37°C , 5% CO_2 , 100% humidity for 36 h. INF, interferon; PBS, phosphate-buffered saline, BCG, *Bacille Calmette-Guérin*; rBCG, recombinant BCG.

cytometry. A total of 10,000 events per test were collected. Data were analyzed using CellQuest software (version 7.5.3.; BD Biosciences) or FACS Express 3 software (DeNovo Software, Ontario, ON, Canada).

Intracellular cytokine staining analysis. The peripheral blood from the vaccinated mice were used to acquire peripheral blood mononuclear cell (PBMC) single-cell suspensions using Histopaque-1077 kit (Sigma-Aldrich) based on density centrifugation. The cells were diluted in 2 ml culture medium containing the same concentration of stimulus as described above to the 12-well plate at 1×10^6 cells per well. The plate was incubated at 37°C , 5% CO_2 , 100% humidity for 36 h. Brefeldin A solution (eBioscience, Inc.) of $1 \mu\text{l}$ was added into the cell culture for 4 h to inhibit the cytokine transport. Cells were harvested and $100 \mu\text{l}$ of Reagent A was added (fixation medium, fix and perm cell permeabilization reagent; Caltag Laboratories; Invitrogen Life Technologies) and incubated for 15 min at room temperature. Cells were washed, and the cell pellets resuspended, then adding $100 \mu\text{l}$ of Reagent B (permeabilization medium) and the $1 \mu\text{l}$ FITC conjugate anti-mouse IL-2, $1.25 \mu\text{l}$ PE conjugate IFN- γ and $20 \mu\text{l}$ PerCP-Cy5.5 conjugate TNF- α (eBioscience, Inc.) per million cells. Vortexed and incubated for 20 min in the dark at 4°C . Cells were wash and the cell pellets resuspend in 0.5 ml of cell staining buffer for analyzes by flow cytometry.

Data analysis. Statistical significance was determined using one-way ANOVA with Kruskal-Wallis tests, Tukey and Dunnett tests of GraphPad Prism 5.0 for Windows (GraphPad Software, Inc., San Diego, USA). PBS group was regard as negative control. All the other groups were compared with BCG group. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Construction of rBCG secreting Ag85B and IFN- γ . Novel rBCG capable of expressing Ag85B and IFN- γ , on

a shuttle vector pMV361 is shown in (Fig. 1A). Further, rBCG::Ag85B-IFN- γ expressed and secreted a relatively high level of fusion protein as shown in Fig. 1B. The bioactivity of IFN- γ produced by the rBCG::Ag85B-IFN- γ strain was $21.85 \pm 7.06 \mu\text{g/ml}$ (equal to $2.2 \times 10^6 \text{ U/mg}$).

Cytokine response. Cell-mediated immune responses were evaluated by IFN- γ ELISPOT assay and sandwich ELISA analysis for IFN- γ , TNF- α and IL-4. The amount of IFN- γ secreted is shown in Fig. 2. It was noted that in response to purified Ag85B, IFN- γ secretion at 6 weeks was higher than that at 12 weeks. The IFN- γ level of rBCG::Ag85B-IFN- γ was 1.6- and 3-fold that of BCG at 6 and 12 weeks, respectively.

Moreover, the amount of IFN- γ secreted exhibited a decline at 12 weeks in all the groups. The effect of rBCG::Ag85B-IFN- γ on the Th1 or Th2 immune response is shown in Fig. 3. Mice immunized with rBCG::Ag85B-IFN- γ showed a significant increase in release of IFN- γ in response to stimulation with Ag85B compared with BCG at 6 and 12 weeks. Mice immunized with rBCG::Ag85B-IFN- γ showed no difference in releasing TNF- α in response to Ag85B compared with BCG at 6 weeks, but at 12 weeks they showed significantly higher level than those immunized with BCG.

NO production levels. The NO levels of the culture supernatant of the mice vaccinated with rBCG::Ag85B-IFN- γ or BCG ($>450 \mu\text{mol/l}$) showed higher levels than the mice vaccinated with PBS ($<100 \mu\text{mol/l}$) at 6 and 12 weeks. The NO level of the rBCG::Ag85B-IFN- γ group was significant higher than BCG group at 6 and 12 weeks (Fig. 4).

Humoral immune responses. Fig. 5A and B illustrate the levels of antibody response in the sera of mice against purified Ag85B. Compared with the PBS control group, mice vaccinated either with BCG or rBCG::Ag85B-IFN- γ strains induced higher levels of antibody against Ag85B. However, the mean IgG titers were as high as 1:33,900 and 1:2,460 in mice immunized with rBCG::Ag85B-IFN- γ at 6 and 12 weeks, which were significant higher than those immunized with

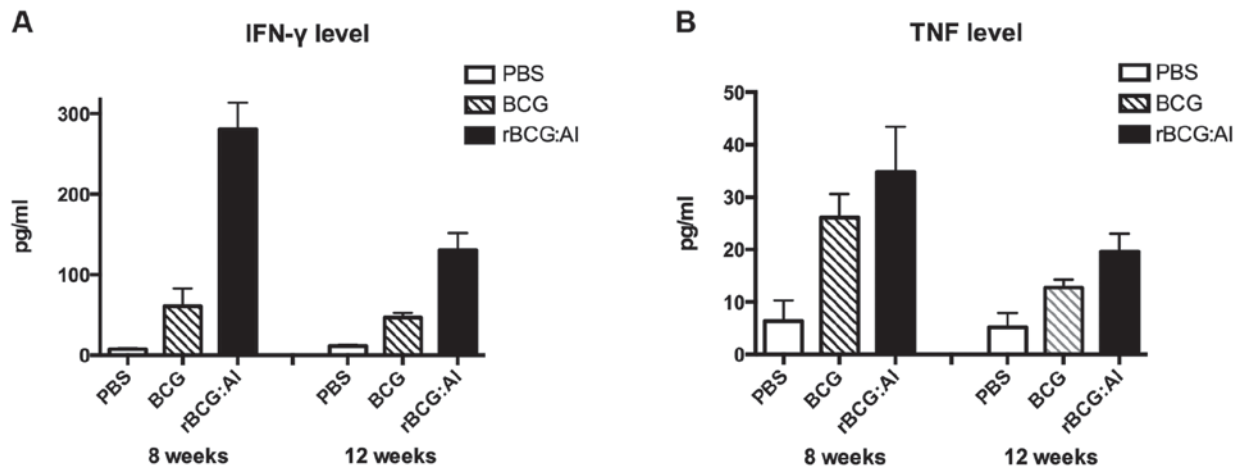


Figure 3. The (A) interferon (IFN)- γ and (B) tumor necrosis factor (TNF)- α level in the splenocytes culture supernatants by sandwich ELISA assay. Harvest the supernatants from the splenocytes culture restimulated with antigen 85B (5 μ g/ml) at 37°C, 5% CO₂, 100% humidity for 36 h. Shown are the means \pm standard deviations. PBS, phosphate-buffered saline; BCG, *Bacille Calmette-Guérin*; rBCG, recombinant BCG.

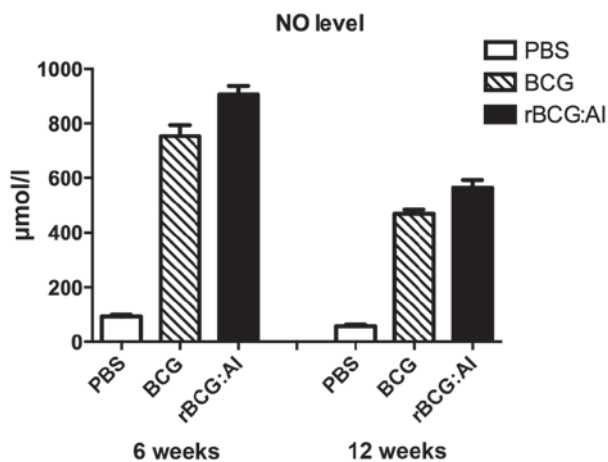


Figure 4. The nitric oxide (NO) level in the MDM culture supernatants detected by Griess reagent. Harvest the supernatants from the MDM culture treated with phosphate-buffered saline (PBS), *Bacille Calmette-Guérin* (BCG) or rBCG::Ag85B-IFN- γ at 37°C, 5% CO₂, 100% humidity for 36 and 72 h. Shown are the means \pm standard deviations *0.01<P<0.05. rBCG, recombinant BCG; IFN, interferon.

BCG (1:10,600 and 1:300, $p < 0.01$). The antibody titers of the PBS control group were only $\sim 1:50$.

Fig. 5C illustrates the antibody levels of IgG1 and IgG2c isotype against Ag85B. The ratios of IgG2c/IgG1 were calculated to determine the induction of Th1 or Th2 responses in animals (19). As a result in response to Ag85B, the ratio of mice immunized with rBCG::Ag85B-IFN- γ was much higher than that of BCG at 6 weeks and the ratio was higher than BCG at 12 weeks also. The results collectively revealed that the capability of the induction of Th1 protection immune response of rBCG::Ag85B-IFN- γ was higher than BCG in the experiments.

Antigen-specific splenocyte proliferation. The antigen-specific proliferation of the splenocytes isolated from the mice immunized with rBCG::Ag85B-IFN- γ was significantly higher than that of BCG or PBS as determined by SI also at 6 or 12 weeks (Fig. 6).

CD4⁺ and CD8⁺ T cell subset analysis. After vaccination with different vaccines, the lymphocyte subsets were examined for differences in their percentages by flow cytometry. As shown in Fig. 7, the levels of the CD4⁺ and CD8⁺ T cell populations were significantly higher in the rBCG::Ag85B-IFN- γ or BCG group than that in the PBS group. The percentage of CD4⁺ T cells of rBCG::Ag85B-IFN- γ was significant higher than that of BCG at 6 and 12 weeks whether stimulated with Ag85B or PPD.

The percentage of CD8⁺ T cells of rBCG::Ag85B-IFN- γ was significant lower than that of BCG at 6 weeks when stimulated with Ag85B, but at 12 weeks, they showed no difference. The percentage of CD8⁺ T cells in the mice vaccinated with rBCG::Ag85B-IFN- γ showed no difference with BCG at 6 weeks when stimulated with PPD, but at 12 weeks they were higher than that of BCG. Moreover, the CD4⁺/CD8⁺ ratio in mice immunized with rBCG::Ag85B-IFN- γ was significant higher than that of BCG. Therefore, the rBCG::Ag85B-IFN- γ enhanced CD4⁺ T cells were significantly higher and hence showed increased CD4⁺/CD8⁺ ratio.

Cells surface marker expression level. rBCG::Ag85B-IFN- γ enhanced expression of CD80, CD86, CD40 and HLA-DR. Changes in expression were found to be more significant for CD80, CD86 and CD40. The cells infected by rBCG::Ag85B-IFN- γ produced a 12-38% increase in the percentage of positive cells of these markers compared to infected with BCG and demonstrated small increases in expression of HLA-DR. ALs especially showed a trend of increasing CD80 expression when compared with BCG.

Multifunctional T cell analysis. Using multiparameter flow cytometry, we compared the quality of the immune response in different vaccination groups to Ag85B. PBMC were analyzed for their intracellular expression of IFN- γ , TNF- α and IL-2, allowing us to follow the frequency of cells that produced various combinations of IFN- γ , TNF- α and IL-2 positive for either a single or up to three cytokines simultaneously. The results showed half of the responses in the rBCG::Ag85B-IFN- γ group was 3⁺ and 2⁺ T cells, whereas,

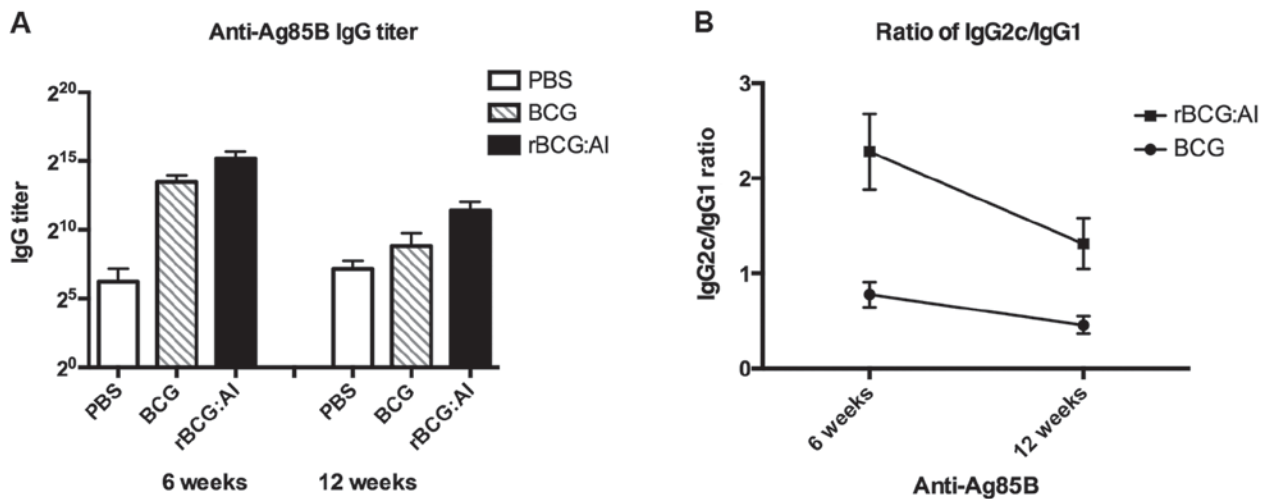


Figure 5. Antibody response against antigen 85B (Ag85B) of the mice immunized after (A) 6 and 12 weeks. The ratio of (B) IgG2c/IgG1. Sera were collected from mice immunized at 6 and 12 weeks, then examined with horseradish peroxidase conjugated goat anti-mouse IgG, IgG1 and IgG2c for IgG titer and the ratio of IgG2c/IgG1. Shown are the means \pm standard deviations. PBS, phosphate-buffered saline, BCG, *Bacille Calmette-Guérin*; rBCG, recombinant BCG.

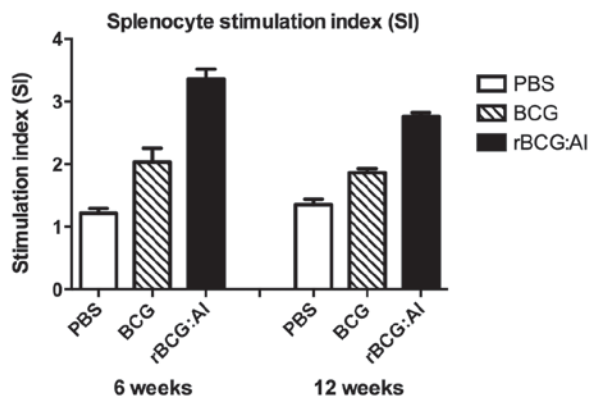


Figure 6. Antigen-specific splenocyte proliferation analysis. Splenocytes isolated from mice at 6 and 12 weeks after vaccination cultured at 37°C, 5% CO₂, 100% humidity for 2 days and analyzed with MTS assay. Splenocytes stimulation index (SI) represents the proliferation efficiency. The results are expressed as the mean \pm standard deviations. PBS, phosphate-buffered saline, BCG, *Bacille Calmette-Guérin*; rBCG, recombinant BCG.

the response after BCG vaccination was dominated by 1⁺ and 2⁺ T cells.

Discussion

rBCG has several advantages over other novel vaccines by reasons of low cost, easy production and convenient storage. However, BCG could not be easily replaced by novel vaccine candidates despite the controversies over efficiency. rBCG expressing protective antigens represents a very promising possibility for use as an efficient vaccine against TB. It could be envisaged that continuous overexpression of selected candidate epitopes as the case for a live vaccine like BCG may be pivotal for vaccine efficacy (7). The results of phase 1 trial of rBCG30 provide proof of principal that rBCG30 could safely enhance human TB immunity and support further development of rBCG overexpressing Ag85B for TB vaccination (13). Moreover, cytokines are considered as potent immune

responses and the potent IFN- γ producers are crucial for protection against *M. tb* (17,20). IFN- γ , which synergizes with TNF- α , is central to the activation of macrophages and the isolation of *M. tb* inside the granuloma (2). A previous study indicated that BCG strains secreting cytokines, in particular GM-CSF, IL-2 and IFN- γ , could modify and potentiate the immune response to BCG antigens (3). The local expression of IFN- γ by the rBCG results in more efficient bacterial clearance which is accompanied by a reduction in tissue pathology (21). Similarly, mouse IFN- γ was coexpressed with Ag85B in the present study. Further it has been proved that the IFN- γ produced by the rBCG::Ag85B-IFN- γ strain was bioactive and was probably negatively affected by fusion Ag85B sustained as an efficient antigen. It is believed that the stronger cell mediated immune response was induced by rBCG::Ag85B-IFN- γ and may be due to either IFN- γ in the fusion construct promoting the development of immunity or that Ag85B may act as an efficient antigen.

The dominant immunological consequence of infection with *M. tb* or vaccination with rBCG is priming of Th1-oriented CD4⁺ T cells which in turn, potentiates the antimicrobial function of macrophages through release of IFN- γ . Interestingly, CD4⁺ T cells prominently produce IFN- γ during the first few weeks of infection, while CD8⁺ T cell production of IFN- γ occurs later. Thus, CD4⁺ T cells play a greater role in early defense against *M. tb* infection, and both CD4⁺ and CD8⁺ T cells control latent mycobacteria (22). Both the lymphocyte proliferation and IFN- γ secretion in response to Ag85B, including the percentage CD4⁺ T cells of rBCG::Ag85B-IFN- γ were significant higher than those of BCG.

IFN- γ production may act in part by inducing the production of inducible NO synthase. Inhibitors of the NO production aggravate tuberculosis infection (23). Activated macrophages could act directly as effector cells by producing microbicidal molecules such as reactive oxygen intermediates, NO and lysosomal enzymes (24). rBCG::Ag85B-IFN- γ induced higher NO levels than BCG in the splenocyte culture and suggested that it activated macrophages efficiently. The levels of IgG1 and IgG2c reflect the stimulation of

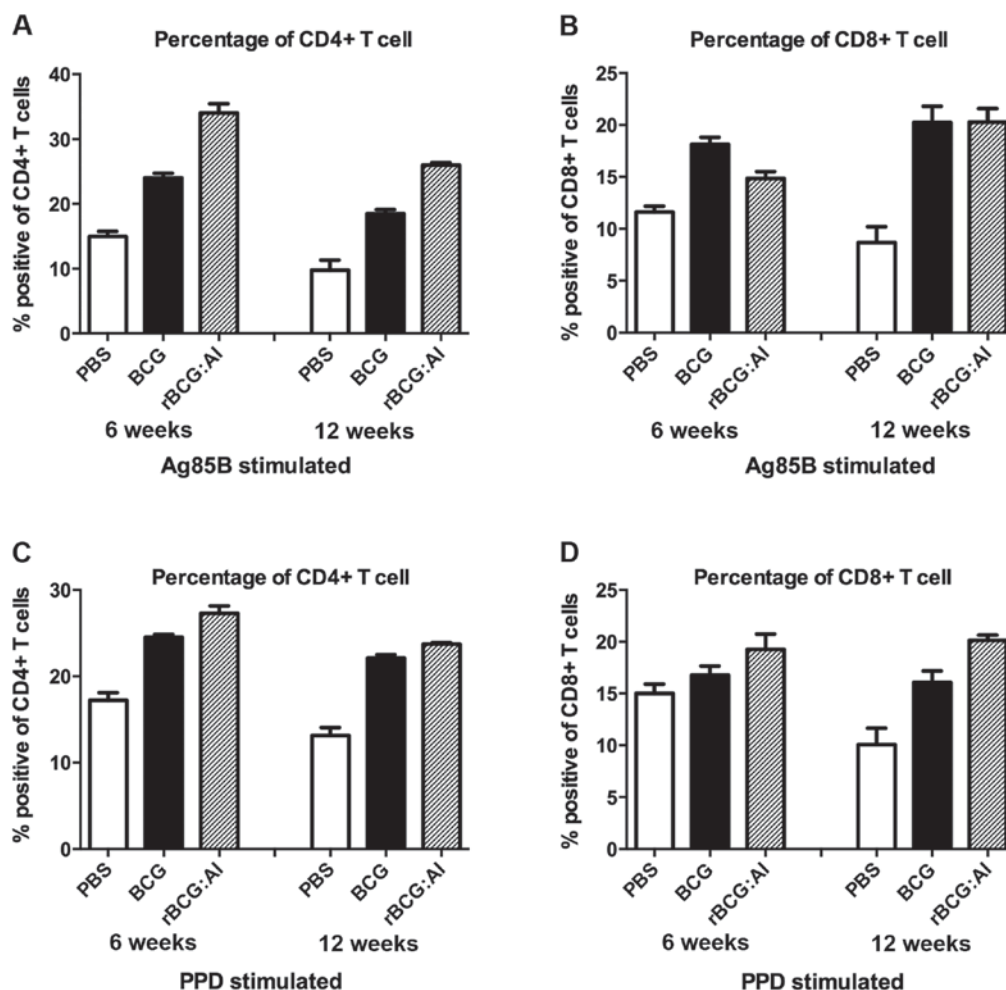


Figure 7. Analysis of T cell subset percentages stimulated by (A and B) antigen 85B (Ag85B) and (C and D) purified protein derivatives (PPD). Splenocytes were isolated from mice vaccinated after 6 and 12 weeks and incubated with Ag85B (5 μ g/ml) or PPD (5 μ g/ml) at 37°C, 5% CO₂, 100% humidity for 36 h. Then splenocytes deposits were collected and incubated with fluorescein isothiocyanate-conjugated anti-CD4 and phycoerythrin-conjugated anti-CD8. The percentages of CD4⁺ T and CD8⁺ T cells were determined by flow cytometry. A total of 10,000 events per test were collected. The results are expressed as the mean \pm standard deviations. PBS, phosphate-buffered saline; BCG, *Bacille Calmette-Guérin*; rBCG, recombinant BCG.

Th2 and Th1 cells, respectively. Also in the present study, rBCG::Ag85B-IFN- γ increased the Th1 responses, and weakened the Th2 responses when memory was suppressed by regulatory mechanisms.

In addition to production of cytokines, macrophages also influence T cell response. Expression of various surface molecules on activated macrophages, including MHC-II (HLA-DR), CD80, CD86 and CD40, is required for optimal development of protective T cell response in tuberculosis (25). The use of costimulation-based immunomodulators may have significant repercussions on the induction of host protective immunity against tuberculosis (26). In light of previous work, we investigated how rBCG::Ag85B-IFN- γ modulated the activities of macrophages by characterizing the expression of surface markers during infection. THP-1 cell derived macrophages increased the four surface marker expression significantly when infected with rBCG::Ag85B-IFN- γ . HLA-DR is a major antigen presenting molecule and its expression is impaired upon infection by *M. tb* (27). High levels of expression of MHC-II along with CD40, CD80, and CD86 contribute to the efficiency of antigen presenting cells for stimulating T cells (28). rBCG::Ag85B-IFN- γ improved the capabilities of

multifunctional T cell population compared with BCG and enhanced IL-2 level (IL-2⁺, IL-2⁺ TNF- α ⁺ and IL-2⁺ TNF- α ⁺ IFN- γ) significantly. IL-2-expressing cells were significantly more likely to have a central memory phenotype. Furthermore, the induction of memory T cells with multifunctional properties appears to provide a good correlate for protection against tuberculosis (29).

In conclusion, the present study indicated that rBCG::Ag85B-IFN- γ strain could enhance the cell mediated immune response. Moreover, it could be a good candidate vaccine against TB and may be a superior agent for immunotherapeutic vaccine in the future. However, the safety as well as stability concerns of the rBCG::Ag85B-IFN- γ should be considered.

Acknowledgements

This study was supported by the Science Research Project of Eleventh Five-Year Plan grant (nos. 2008ZX-103-013 and 2008ZX-103-011) and the National High Technology Research and Development Program of China (863 Program, grant no. 2006AA02Z445).

References

- World Health Organization (WHO): Global tuberculosis control: Surveillance, planning, financing: WHO report 2008. WHO, Geneva, 2008.
- Skeiky YA and Sadoff JC: Advances in tuberculosis vaccine strategies. *Nat Rev Microbiol* 4: 469-476, 2006.
- Murray PJ, Aldovini A and Young RA: Manipulation and potentiation of antimycobacterial immunity using recombinant bacille Calmette-Guérin strains that secrete cytokines. *Proc Natl Acad Sci USA* 93: 934-939, 1996.
- O'Donnell MA, Aldovini A, Duda RB, Yang H, Szilvasi A, Young RA and DeWolf WC: Recombinant *Mycobacterium bovis* BCG secreting functional interleukin-2 enhances gamma interferon production by splenocytes. *Infect Immun* 62: 2508-2514, 1994.
- Luo Y, Yamada H, Chen X, Ryan AA, Evanoff DP, Triccas JA and O'Donnell MA: Recombinant *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) expressing mouse IL-18 augments Th1 immunity and macrophage cytotoxicity. *Clin Exp Immunol* 137: 24-34, 2004.
- Flynn JL and Chan J: Immunology of tuberculosis. *Annu Rev Immunol* 19: 93-129, 2001.
- Nasser Eddine A and Kaufmann SH: Improved protection by recombinant BCG. *Microbes Infect* 7: 939-946, 2005.
- Agger EM and Andersen P: Tuberculosis subunit vaccine development: On the role of interferon- γ . *Vaccine* 19: 2298-2302, 2001.
- D'Souza S, Rosseels V, Romano M, Tanghe A, Denis O, Jurion F, Castiglione N, Vanonckelen A, Palfliet K and Huygen K: Mapping of murine Th1 helper T-Cell epitopes of mycolyl transferases Ag85A, Ag85B, and Ag85C from *Mycobacterium tuberculosis*. *Infect Immun* 71: 483-493, 2003.
- Horwitz MA and Harth G: A new vaccine against tuberculosis affords greater survival after challenge than the current vaccine in the guinea pig model of pulmonary tuberculosis. *Infect Immun* 71: 1672-1679, 2003.
- Horwitz MA, Harth G, Dillon BJ and Maslesa-Galic' S: Recombinant bacillus calmette-guerin (BCG) vaccines expressing the *Mycobacterium tuberculosis* 30-kDa major secretory protein induce greater protective immunity against tuberculosis than conventional BCG vaccines in a highly susceptible animal model. *Proc Natl Acad Sci USA* 97: 13853-13858, 2000.
- Horwitz MA: Recombinant BCG expressing *Mycobacterium tuberculosis* major extracellular proteins. *Microbes Infect* 7: 947-954, 2005.
- Hoft DF, Blazevic A, Abate G, Hanekom WA, Kaplan G, Soler JH, Weichold F, Geiter L, Sadoff JC and Horwitz MA: A new recombinant bacille Calmette-Guérin vaccine safely induces significantly enhanced tuberculosis-specific immunity in human volunteers. *J Infect Dis* 198: 1491-1501, 2008.
- Xu Y, Liu W, Shen H, Yan J, Qu D and Wang H: Recombinant *Mycobacterium bovis* BCG expressing the chimeric protein of antigen 85B and ESAT-6 enhances the Th1 cell-mediated response. *Clin Vaccine Immunol* 16: 1121-1126, 2009.
- Forbes EK, Sander C, Ronan EO, McShane H, Hill AV, Beverley PC and Tchilian EZ: Multifunctional, high-level cytokine-producing Th1 cells in the lung, but not spleen, correlate with protection against *Mycobacterium tuberculosis* aerosol challenge in mice. *J Immunol* 181: 4955-4964, 2008.
- Xu Y, Zhu B, Wang Q, Chen J, Qie Y, Wang J, Wang H, Wang B and Wang H: Recombinant BCG coexpressing Ag85B, ESAT-6 and mouse-IFN- γ confers effective protection against *Mycobacterium tuberculosis* in C57BL/6 mice. *FEMS Immunol Med Microbiol* 51: 480-487, 2007.
- Moreira AL, Tsenova L, Murray PJ, Freeman S, Bergtold A, Chiriboga L and Kaplan G: Aerosol infection of mice with recombinant BCG secreting murine IFN- γ partially reconstitutes local protective immunity. *Microb Pathog* 29: 175-185, 2000.
- Yi Z, Fu Y, Yang C, Li J, Luo X, Chen Q, Zeng W, Jiang S, Jiang Y, He Y, *et al*: Recombinant *M. smegmatis* vaccine targeted delivering IL-12/GLS into macrophages can induce specific cellular immunity against *M. tuberculosis* in BALB/c mice. *Vaccine* 25: 638-648, 2007.
- Martin RM and Lew AM: Is IgG2a a good Th1 marker in mice? *Immunol Today* 19: 49, 1998.
- Luo Y, Chen X, Han R and O'Donnell MA: Recombinant bacille Calmette-Guérin (BCG) expressing human interferon-alpha 2B demonstrates enhanced immunogenicity. *Clin Exp Immunol* 123: 264-270, 2001.
- Wangoo A, Brown IN, Marshall BG, Cook HT, Young DB and Shaw RJ: Bacille Calmette-Guérin (BCG)-associated inflammation and fibrosis: Modulation by recombinant BCG expressing interferon- γ (IFN- γ). *Clin Exp Immunol* 119: 92-98, 2000.
- Wong P and Pamer EG: CD8 T cell responses to infectious pathogens. *Annu Rev Immunol* 21: 29-70, 2003.
- Cooper AM, Adams LB, Dalton DK, Appelberg R and Ehlers S: IFN- γ and NO in mycobacterial disease: New jobs for old hands. *Trends Microbiol* 10: 221-226, 2002.
- Rook GA, Seah G and Ustianowski A: *M. tuberculosis*: Immunology and vaccination. *Eur Respir J* 17: 537-557, 2001.
- Feng Y, Yang X, Liu Z, Liu Y, Su B, Ding Y, Qin L, Yang H, Zheng R and Hu Z: Continuous treatment with recombinant *Mycobacterium tuberculosis* CFP-10-ESAT-6 protein activated human monocyte while deactivated LPS-stimulated macrophage. *Biochem Biophys Res Commun* 365: 534-540, 2008.
- Bhatt K, Uzelac A, Mathur S, McBride A, Potian J and Salgame P: B7 costimulation is critical for host control of chronic *Mycobacterium tuberculosis* infection. *J Immunol* 182: 3793-3800, 2009.
- Kan-Sutton C, Jagannath C and Hunter RL Jr: Trehalose 6,6'-dimycolate on the surface of *Mycobacterium tuberculosis* modulates surface marker expression for antigen presentation and costimulation in murine macrophages. *Microbes Infect* 11: 40-48, 2009.
- Seder RA, Darrah PA and Roederer M: T-cell quality in memory and protection: Implications for vaccine design. *Nat Rev Immunol* 8: 247-258, 2008.
- Lindenstrøm T, Agger EM, Korsholm KS, Darrah PA, Aagaard C, Seder RA, Rosenkrands I and Andersen P: Tuberculosis subunit vaccination provides long-term protective immunity characterized by multifunctional CD4 memory T cells. *J Immunol* 182: 8047-8055, 2009.