Journal of Microbiology, Immunology and Infection (2017) **50**, 90–96

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ORIGINAL ARTICLE

Recombinant bacille Calmette–Guerin coexpressing Ag85b, CFP10, and interleukin-12 elicits effective protection against *Mycobacterium tuberculosis*

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Received 4 September 2014; received in revised form 11 November 2014; accepted 29 November 2014
Available online 11 December 2014

KEYWORDS
Mycobacterium tuberculosis;
recombinant bacille Calmette–Guerin;
vaccine

Background: The tuberculosis (TB) pandemic remains a leading cause of human morbidity and mortality, despite widespread use of the only licensed anti-TB vaccine, bacille Calmette-Guerin (BCG). The protective efficacy of BCG in preventing pulmonary TB is highly variable; therefore, an effective new vaccine is urgently required.
Methods: In the present study, we assessed the ability of novel recombinant BCG vaccine (rBCG) against *Mycobacterium tuberculosis* by using modern immunological methods.
Results: Enzyme-linked immunospot assays demonstrated that the rBCG vaccine, which coexpresses two mycobacterial antigens (Ag85B and CFP10) and human interleukin (IL)-12 (rBCG2) elicits greater interferon-γ (IFN-γ) release in the mouse lung and spleen, compared to the parental BCG. In addition, rBCG2 triggers a Th1-polarized response. Our results also showed that rBCG2 vaccination significantly limits *M. tuberculosis* H37Rv multiplication in macrophages. The rBCG2 vaccine surprisingly induces significantly higher tumor necrosis factor-α (TNF-α) production by peripheral blood mononuclear cells that were exposed to a nonmycobacterial stimulus, compared to the parental BCG.

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http://dx.doi.org/10.1016/j.jmii.2014.11.019
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Conclusion: In this study, we demonstrated that the novel rBCG2 vaccine may be a promising candidate vaccine against *M. tuberculosis* infection.
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Introduction

Tuberculosis (TB) is one of the most prevalent and deadly infectious diseases worldwide. In 2012, 12,845 new cases of TB were reported in Taiwan with an estimated annual incidence of 54 cases per 100,000 people (Center for Disease Control, Taiwan). The current vaccine against *Mycobacterium tuberculosis* is attenuated *Mycobacterium bovis* bacille Calmette-Guerin (BCG), which is widely used in preventing TB diseases such as meningitis, especially during early childhood.[1] However, the efficacy of BCG against adult pulmonary TB remains controversial.[2,3] Taiwan has undertaken comprehensive BCG vaccination since the 1950s and has witnessed an effective reduction in TB incidence and mortality rates. However, the disease has continued to spread. Numerous clinical trials have investigated the protective ability of BCG and have reported estimates of 0%—80%.[2,3] Thus, a more effective TB vaccine is urgently required. In previous studies, we found that modern Beijing lineages are the predominant *M. tuberculosis* strains in Taiwan.[4,5] For example, as many as 64% of young patients with TB are infected by a Beijing strain.[6] These results suggest that BCG vaccination may favor the positive selection of modern Beijing strains.

Efforts to improve the protective efficacy of anti-TB vaccines include enhancing the current BCG vaccine by boosting it with new vaccines and developing more effective vaccines to replace the traditional BCG vaccine. Recombinant BCG (rBCG) vaccines now undergoing clinical assessment employ two strategies to enhance antigenicity and protective efficacy: (1) overexpression of immunodominant antigens and (2) overexpression of identified antigens that potentially aid in the phagosomal escape of bacilli to the cytosol. Good examples of such proteins are culture filtrate protein-10 (CFP10) and 6 kDa early secretory antigenic target (ESAT-6).[6—8]

The BCG vaccines used in most countries are derived from four major strains: Glaxo strain 1077, Danish strain 1331, French Pasteur strain 1173 P2, and Tokyo strain 172.[9] The rBCG vaccines in the present study were created by using BCG Tokyo strain 172 as the host bacterium and expressing the cloned *Mycobacterium*-specific genes *Ag85B* and *CFP10* in combination with human interleukin (IL)-12 in an attempt to increase the immune response.[10] We previously constructed two novel rBCG strains: rBCG1(pMVAg85b-CFP10) and rBCG2(pMVAg85b-CFP10; pVVhIL-12).[10] We also evaluated the immune responses elicited by these strains by the characterization of peptide-specific $CD4^+$ and $CD8^+$ T cell responses in the lung and spleen. We found that both rBCG vaccines enhanced Th1 immunity, compared with the traditional BCG vaccine[10]; however, the protective effect of the rBCG vaccines toward *M. tuberculosis* was not explicitly determined. In the present study, we therefore first assessed the ability of the two rBCG strains and BCG to elicit interferon-γ (IFNγ)-releasing cells after immunization. In this paper, we compared the protective ability elicited by immunization with the parental BCG and with the two rBCG strains.

Materials and methods

Bacterial strains and culture

Mycobacterium bovis BCG (Tokyo 172) and the recombinant BCG strains (i.e., rBCG1 and rBCG2) and *M. tuberculosis* H37Rv were grown on Middlebrook 7H9 medium (Difco Laboratories, Detroit, MI, USA), which was supplemented with 0.5% glycerol, 0.05% Tween-80, and 10% albumin-dextrose-catalase, or grown on solid 7H11 medium (Difco Laboratories), which was supplemented with oleic-acid-albumin-dextrose-catalase. Oleic-acid-albumin-dextrose-catalase is added to 7H10 and 7H11 basic media to enhance the growth of the mycobacteria. When required, the antibiotic kanamycin was added at a concentration of 25 μg/mL.

Mice and immunization

Female C57BL/6 and C3H/HeJ mice (6—8 weeks old) used in this study were purchased from the National Laboratory Animal Center in Taiwan (Taipei, Taiwan). All animals were maintained under specific pathogen-free conditions. The mice (5 mice per group) were immunized subcutaneously with 1×10^7 colony-forming units (CFU) of the BCG strain or rBCG strain in 100 μL of phosphate-buffered saline (PBS). The control group received 100 μL PBS. The mice were sacrificed to obtain their sera and splenocytes. All animal work was approved by the Animal Experimentation Committee of the National Health Research Institutes (Zhunan, Taiwan), and performed in accordance with the guidelines of the committee.

Growth inhibition assay

The assays were performed by coculturing splenocytes from vaccinated C57BL/6 mice with *M. tuberculosis*-infected macrophages. For the assay, murine bone marrow macrophages (BMM) were the target cells for *M. tuberculosis* H37Rv infection. The BMMϕ were prepared by flushing the femurs of the C57BL/6 female mice with Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Grand Island, NY, USA), which was supplemented with 10% heat-inactivated fetal bovine serum (FBS), 10% L-929a conditioned medium, 1 mM L-glutamine, 1 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer, 1 mM nonessential amino acids, and 1 mM sodium pyruvate [complete DMEM (cDMEM)]. On Days 1

and 3, the BMMϕ were plated at 1×10^6 cells per well on a 24-well plate (Costar, Corning, NY, USA) in cDMEM supplemented with 100 units/mL of M-CSF (**ProSpec**-Tany TechnoGene, Ltd., East Brunswick, NJ, USA). They were incubated for 7 days at 37°C in 5% carbon dioxide (CO_2). The BMMϕ were infected with *M. tuberculosis* H37Rv at a multiplicity of infection of 5:1 (i.e., the bacterium:BMMϕ ratio) for 4 hours at 37°C in 5% CO_2. The wells were then washed three times with PBS to eliminate extracellular and nonadhering bacteria. After the last wash, the PBS was replaced with fresh cDMEM medium at 37°C in 5% CO_2. Splenocytes from mice immunized with the BCG, rBCG1, or rBCG2 vaccines were used to evaluate the protective ability of the vaccines against *M. tuberculosis* infection in BMMϕ. At specific times after vaccination (4 weeks, 8 weeks, and 12 weeks), the spleens from immunized mice were aseptically removed and temporarily placed in sterile PBS. Based on the description by Parra et al,[11] the spleens were passed through a 100-μm cell strainer (BD Biosciences, Mountain View, CA, USA) to generate a single-cell suspension and were incubated in ammonium-potassium-chloride lysis buffer [0.15M ammonium chloride (NH_4Cl) and 1mM potassium bicarbonate ($KHCO_3$)] for 4 minutes. To remove adherent cells, the cells were then resuspended in cold DMEM and added to culture flasks for 2 hours of incubation at 37°C. Nonadherent splenocytes were harvested by gently pipetting the suspensions. Cell viability was assessed by trypan blue exclusion. Nonadherent splenocytes (5×10^6 cells) were overlaid on *M. tuberculosis*-infected BMMϕ. At selected time points, the presence of intracellular bacteria in BMMϕ was determined by lysing a fraction of the cells with 0.1% saponin and counting the diluted cell lysates by plating.

Interferon-γ enzyme-linked immunospot assay

As in our previous study, we used the mycobacterial shared peptides $Ag85B_{241\text{-}255aa}$ QDAYNAAGGHNAVFN, $Ag85B_{261\text{-}280aa}$ THSWEYWGAQLNAMKGDLQS, $Ag85B_{1\text{-}19aa}$ FSRPGLPVEYLQVPSMG, $CFP10_{11\text{-}25aa}$ LAQEAGNFERISGDL, and $CFP10_{32\text{-}39}$ VESTAGSL as antigenic stimuli in an IFN-γ enzyme-linked immunospot (ELISPOT) assay.[10] The C3H/HeJ mice were euthanized and their spleens and lungs were removed aseptically. Cells from the spleens and lungs were diluted in a culture medium containing an appropriate stimulus [i.e., 10 μg/mL tuberculin purified protein derivative (PPD; Statens Serum Institute, Copenhagen, Denmark) and 5 μg/mL Ag85B/CFP10]. They were then placed in the wells of the ELISPOT plate at a density of 5×10^5 cells per

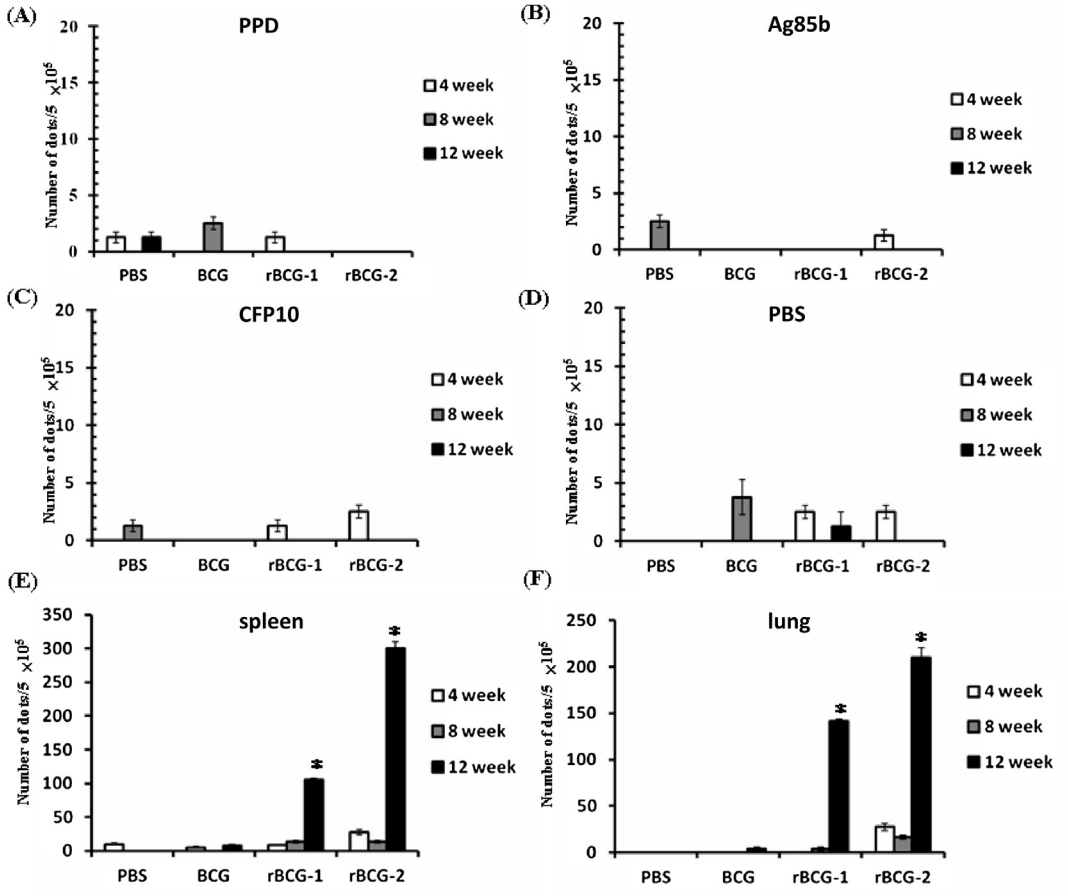

Figure 1. Analysis of antigen-specific interferon-γ production. The immune response was measured by enzyme-linked immunospot assay. Cells from (A, C, E) the spleen and (B, D, and F) the lung were isolated from mice immunized with PBS, BCG, rBCG1, or rBCG2. The cells were stimulated with (A and B) 5 μg/mL Ag85 B and 5 μg/mL CFP10, (C and D) PBS, or (E and F) 10 μg/mL tuberculin purified protein derivative (PPD). The number of cells secreting interferon-γ in single-cell suspensions was counted. * Indicates that the number of dots is significantly increased, compared to the number in the group immunized with BCG ($p < 0.05$). For all figures, BCG = bacille Calmette-Guerin; PBS = phosphate-buffered saline; rBCG1 = recombinant BCG1; rBCG2 = recombinant BCG2.

well. A mouse IFN-γ ELISPOT kit (U-Cytech Biosciences, Utrecht, Netherlands) was used in accordance with the manufacturer's instructions to determine the relative number of IFN-γ-expressing cells in the suspension of single cells. The spots were visualized and counted. Wells with fewer than 10 spots were not used for the calculations.

Antibody isotype analysis

For the evaluation of the Th1 and Th2 immune response, sera from the immunized C3H/HeJ mice were collected and analyzed by indirect enzyme-linked immunosorbent assay (ELISA). The ELISA plates were coated overnight at 4°C with recombinant protein Ag85B or CFP10 (DIATHEVA, Fano, Italy) at a final concentration of 5 μg/mL. The coated plates were blocked with 200 μL PBS containing 3% bovine serum albumin at 37°C for 1 hour. The plates were washed three times with PBS containing 0.05% Tween-20. Serum was added for 2 hours of incubation, and then washed out, followed by addition of horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin G1 (IgG1) or immunoglobulin G2a (IgG2a) antibody (Jackson ImmunoResearch Laboratories Inc, West Grove, PA, USA). The plates were incubated at 37°C for 1 hour, washed, and developed with o-phenylenediamine and hydrogen peroxide substrate. Reactions were stopped by adding to each well 50 μL of 1N sulfuric acid (H_2SO_4). The product was measured on an ELISA plate reader at 492 nm.

Peripheral blood mononuclear cell stimulation assays

Female C57BL/6 mice were immunized with BCG or rBCG and sacrificed after 7 days. The peripheral blood mononuclear cell (PBMC) fraction was isolated from the blood of the immunized mice by density centrifugation using a Ficoll-Paque centrifuge (Pharmacia Biotech, Piscataway, NJ). Cells were washed three times in saline and resuspended in a culture medium [Roswell Park Memorial Institute (RPMI) 1640 medium; Invitrogen], which was supplemented with 2mM L-glutamine, 10 mM HEPES and 10% FBS. A total of 2×10^5 mononuclear cells in a 100-μL volume was added to 96-well plates containing RPMI or sonicated *M. tuberculosis* H37Rv (optical density = 1) or *Pseudomonas aeruginosa* ATCC27853 (optical density = 1). After 48 hours, the supernatants were collected and stored at −20°C. The level of tumor necrosis factor (TNF)-α was assessed by ELISA.

Results

Immunization by rBCG elicits IFN-γ spot-forming cells in the spleen and lung

An ELISPOT assay was used to examine the relative numbers of IFN-γ-expressing cells in single-cell suspensions of spleen and lung tissue from mice immunized with BCG Tokyo 172, rBCG1, or rBCG2. The antigens PPD and Ag85B/CFP10 were used to determine the cellular immunity after vaccination with BCG, rBCG1, or rBCG2. Fig. 1 shows the results at 4 weeks, 8 weeks, and 12 weeks after the mice were immunized with the vaccines. In response to stimulation by the Ag85B and CFP10 peptides, mice immunized with rBCG1 or rBCG2 produced high numbers of IFN-γ-releasing cells, compared to mice immunized with PBS (i.e., the control) or BCG (Fig. 1E and F). Furthermore, the number of IFN-γ-expressing cells in the mice immunized with rBCG1 or

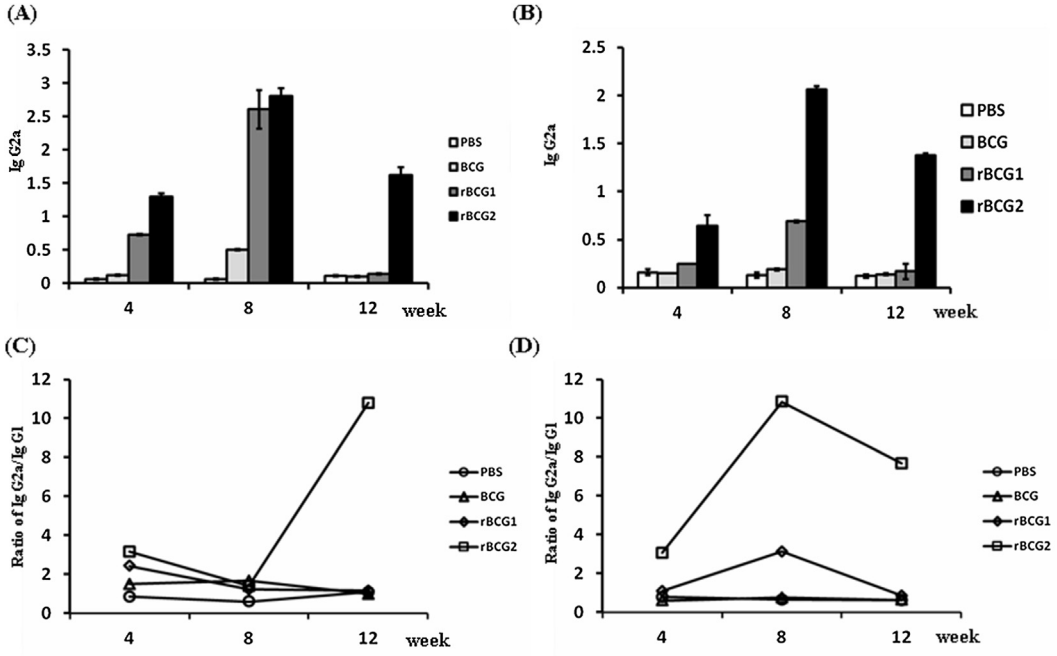

Figure 2. Antibody response against Ag85b or CFP10 in mice immunized with PBS, BCG, rBCG1 or rBCG2. Four groups of mice were immunized with PBS, BCG, rBCG1, or rBCG2, respectively, and sacrificed after 4 weeks, 8 weeks, and 12 weeks to prepare their serum for examining (A, B) the antibody response and (C, D) the ratio of IgG2a/IgG1. The antigens used were (A, C) Ag85B or (B, D) CFP10.

rBCG2 reached its highest level at 12 weeks (Fig. 1E and F). There were no significant differences among the cells stimulated by PBS or PPD (Fig. 1A—D).

Immunization by rBCG promotes the Th1-type immune response

Fig. 2 illustrates the expression levels of antibody responses in the sera of mice immunized with BCG, rBCG1, rBCG2, or PBS (i.e., the control) on using recombinant purified Ag85B (Fig. 2, left panels) protein or CFP10 (Fig. 2, right panels) protein as the antigen. Mice immunized with the rBCG1 or rBCG2 strains produced higher levels of IgG2a antibody against Ag85B or CFP10, compared to mice immunized with BCG (Fig. 2A and B). The highest titer of IgG2a antibody against Ag85B or CFP10 was reached at 8 weeks, and declined somewhat by 12 weeks (Fig. 2A and B).

Because the Th1 polarized arm of the cellular immune response is important for protective immunity against *Mycobacterium* infection, we also calculated the ratio of IgG2a to IgG1 to gauge the Th1/Th2 nature of the immune response in these mice. The IgG2a/IgG1 ratio was highest in mice immunized with rBCG2 for Ag85B (Fig. 2C) and for CFP10 (Fig. 2D). The IgG2a/IgG1 ratio in the rBCG2 group reached its highest point at 8 weeks for CFP10, which was similar to the IgG2a antibody response (Fig. 2B and D). However, for Ag85B, the IgG2a/IgG1 ratio in the rBCG2 group reached its highest point at 12 weeks (Fig. 2C). These results reveal that the ability of the vaccines to induce Th1/Th2 immunity increased in the following order: PBS, BCG, rBCG1, and rBCG2. A Th1-polarized response overall was initiated by both rBCG vaccines.

Vaccination with rBCG limits *M. tuberculosis* H37Rv multiplication in macrophages

To further evaluate the protective efficacy of the rBCG1 and rBCG2 vaccines, the ability of splenocytes isolated from vaccinated mice to inhibit *Mycobacterium* growth in macrophages was examined. Animals immunized with rBCG1 or rBCG2 exhibited significantly reduced *M. tuberculosis* growth in their macrophages, compared to the PBS or BCG treatment groups (Fig. 3A—C). The results in Fig. 3B show that the level of *Mycobacterium* growth in mice immunized with rBCG2 for 8 weeks was reduced to approximately 30% at day 4 and 10% at day 7 in mice immunized with BCG. After 12 weeks of immunization, the levels of *Mycobacterium* growth were reduced to approximately 48% and 33% at days 4 and 7, respectively (Fig. 3C). These results indicate that rBCG2 vaccination elicited a strong protective immune response against *M. tuberculosis* infection.

Vaccination with rBCG promotes nonspecific protection against *P. aeruginosa*

To evaluate the innate immunity induced by rBCG vaccination, the ability of PBMCs collected from rBCG-vaccinated mice to produce TNF-α in response to an unrelated pathogen was tested. The PBMCs collected from rBCG2-vaccinated mice and treated with *M. tuberculosis* H37Rv induced 10-fold higher TNF-α production, compared

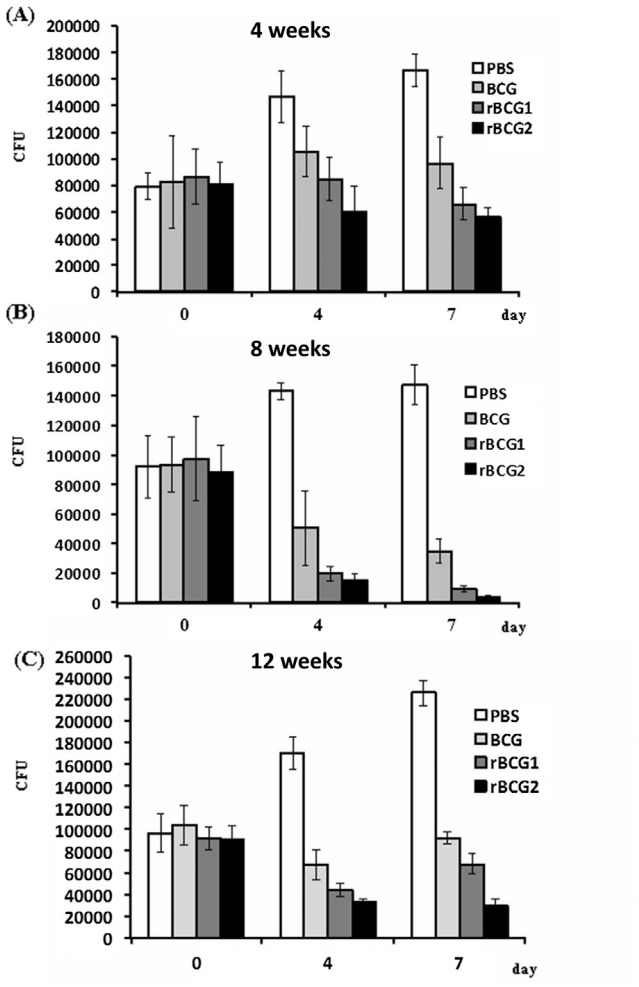

Figure 3. Vaccine inhibition of *Mycobacterium tuberculosis* H37Rv growth at days 0, 4, and 7. After (A) 4 weeks, (B) 8 weeks, and (C) 12 weeks, four groups of mice vaccinated with PBS, BCG, rBCG1 and rBCG2 were sacrificed. Their spleens were aseptically removed. *Mycobacterium tuberculosis* H37Rv-infected macrophages were cocultured with splenocytes from mice immunized with the vaccine. At the specified time points, bacterial uptake by murine bone marrow-derived macrophages was determined. * Indicates that the number of dots is significantly decreased, compared to the number in mice immunized with BCG ($p < 0.05$).

to cells from the BCG group (Table 1). The production of TNF-α interestingly was also enhanced when the cells were exposed to a nonmycobacterial stimulus: PBMCs collected from the rBCG2 group and treated with *P. aeruginosa* induced 9-fold to 10-fold higher TNF-α production, compared with cells from the BCG group (Table 1). It was surprising that the rBCG2 cells treated with *P. aeruginosa* induced 1.4-fold higher TNF-α production, compared to rBCG2 cells treated with *M. tuberculosis* H37Rv. These data demonstrated that rBCG vaccination increased the functional state of circulating mononuclear cells.

Discussion

Bacillus Calmette—Guerin is widely used as a human vaccine against TB. In past decades, numerous laboratories

Table 1 Bacille Calmette-Geurin and recombinant bacille Calmette-Geurin vaccination increases the nonspecific production of tumor necrosis factor-α by peripheral blood mononuclear cells

	PBS	BCG	rBCG1	rBCG2
No stimulus	0	0	0	0
M. tuberculosis H37Rv	0	16.78 ± 10.66	66.82 ± 25.28	168.09 ± 15.13
P. aeruginosa	0	23.53 ± 4.94	73.18 ± 8.63	237.83 ± 13.61

All data (pg/mL) are presented by the mean ± the standard deviation.
BCG = bacille Calmette-Geurin; *M. tuberculosis* = *Mycobacterium tuberculosis*; *P. aeruginosa* = *Pseudomonas aeruginosa*; PBS = phosphate-buffered saline; rBCG = recombinant bacille Calmette-Geurin.

have cultured and passaged BCG, which has resulted in various genetically different BCG sub-strains.[12] Bacillus Calmette—Guerin has also demonstrated efficacy against severe forms of TB in children, especially tuberculosis meningitis; however, it is not effective against pulmonary TB, which is the predominant form in all age groups.[13] Bacillus Calmette—Guerin is widely used in many countries for routine vaccination pre-exposure, but it is not used for individuals with latent TB infection (LTBI).

Various strategies are used to enhance the antitubercular immunity of the BCG vaccine. In the early 1990s, BCG was used as a platform for the heterologous expression of foreign proteins driven by mycobacterial expression vectors.[14] More recent studies indicate that secreted proteins can be strong candidates for improving the effectiveness of TB vaccines.[15–17] For example, using Ag85B and CFP10 as the secreted antigens enhances the immune response of mice against tuberculosis infection.[18,19] The expression of cytokines IL-2, IL-18, and IFN-γ by rBCG similarly upregulates the Th-1 immune response *in vitro*.[20,21] *Mycobacterium tuberculosis* and *M. bovis* secrete Ag85B, and Ag85B generally behaves as an immunodominant antigen.[22] In guinea pig models, vaccination by rBCG that overexpresses Ag85B can induce better protection against *M. tuberculosis* infection *via* aerosol challenge.[23,24] Culture filtrate protein-10 is also an immunodominant antigen secreted by *M. tuberculosis*, but it is missing in *M. bovis* BCG.[25] In humans, $CD8^+$ T cells that recognize CFP10 are elicited after *M. tuberculosis* infection.[26,27]

Interferon-γ is critical for innate immunity and adaptive immunity against intracellular infection by pathogens such as *M. tuberculosis*. Dendritic cells engulfing mycobacteria triggers the production of IFN-γ and TNF-α, which stimulate the first antimycobacterial responses that help restrict *M. tuberculosis* growth.[28] Furthermore, IL-12 produced by dendritic cells induces a type-1 T cell response.[29] In the present study, the ELISPOT assay demonstrated that rBCG coexpressing human IL-12, Ag85B, and CFP10 can elicit strong IFN-γ production in the lung and spleen (Fig. 1). This may help establish a barrier against *M. tuberculosis* infection. In addition, rBCG2 can induce a Th-1-polarized immune response, as demonstrated by the IgG2/IgG1 ratio (Fig. 2).

To verify the protective efficacy, we first isolated splenocytes from vaccine-immunized mice and cocultured them with macrophages to measure the proliferation rates of *M. tuberculosis* in the macrophages. The results demonstrated that rBCG2 induced strong protection against *M. tuberculosis* H37Rv infection, compared to the protection by the parental BCG strain. However, it will be necessary to test this effect further in a mouse model using aerosol infection. It has been reported that mice immunized with a rBCG vaccine exhibit stronger cellular and humoral immune responses, compared to mice immunized with the parental BCG vaccine; however, the two groups do not differ with respect to overall protective efficacy.[30] In the near future, we plan to test the protective efficacy of the rBCG vaccine by infecting mice with epidemic *M. tuberculosis* reference stains such as *M. tuberculosis* H37Rv *via* the respiratory route using a nose-only exposure device.

The effectiveness of BCG vaccination in preventing adult pulmonary TB is highly variable[2,3]; however, epidemiological studies and scientific reports have suggested that BCG may provide some special health benefits to vaccines, including reducing the impact of asthma and possibly malaria.[11] Kleinnijenhuis et al indicated that BCG could induce trained immunity-nonspecific protection from infections through epigenetic reprogramming.[31] In addition, cytokine production (e.g., IFN-γ and TNF-α) by PBMCs isolated from naïve volunteers after vaccination with BCG increases significantly when the subjects are exposed to unrelated pathogens such as *Candida albicans* and *Staphylococcus aureus*.[31] Kato et al report that BCG-vaccinated Japanese flounder is able to mount nonspecific immune responses against *Nocardia seriolae* infection—most likely through producing bacteriolytic lysozymes.[27] Our results indicated that when rBCG2 cells were exposed to a nonmycobacterial stimulus, they were able to induce significantly higher TNF-α production by PBMCs, compared to the parental BCG strain (Table 1). To further explore this finding, animal models incorporating protective assays should be investigated. Our results showed that rBCG2 may be a good vaccine candidate against *M. tuberculosis* infection.

Conflicts of interest

The authors have no conflicts of interest to declare.

Acknowledgements

We thank Dr Daryl Henderson for his kind help in improving the English of this manuscript. We acknowledge all participants of this consortium for valuable discussions. This project was supported by grants from the National Health Research Institutes (IV-103-SP-06 and PH103-PP-05) (Zhunan, Taiwan) and the National Science Council (NSC102-2320-B-400-006) (Taipei, Taiwan).

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