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Original Article

Commonly administered bacille Calmette-Guerin strains induce comparable immune response

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Abstract: Bacille Calmette-Guerin (BCG) is currently the only available vaccine against tuberculosis (TB), but its protective efficacy in adults is highly variable. This study aimed to compare the immune response induced by two widely used BCG strains: BCG China strain (derivative of BCG Danish strain) in DU2-III group and BCG Pasteur in DU2 -IV group. Healthy BALB/c mice were immunized with BCG China strain or BCG Pasteur strain. Specific IgG, IgG1, and IgG2a antibodies titers, the proliferation of splenocytes, the percentages of splenocyte subsets and the concentrations of induced IFN-γ and IL-4 at 6^{th}, 8^{th}, 10^{th}, and 12^{th} weeks after the immunization were detected. We found that BCG Pasteur strain induced higher specific IgG and IgG1 titers, higher proliferation of splenocytes, higher percentages of $CD4^+$ or $CD8^+$ T cells, and higher concentration of secreted IFN-γ than BCG China strain. However, there were no significant differences in IgG2a titer and IL-4 concentration between both strains. In conclusion, our study shows that immune responses to BCG vaccine differ by strain, which may account for variable outcomes of BCG immunization.

Keywords: BCG China, BCG Pasteur, immunogenicity

Introduction

Mycobacterium bovis bacillus Calmette-Guérin (BCG) is part of the WHO's Expanded Program on Immunization against tuberculosis and has reached 90% of global coverage [1]. Vaccination with BCG provides efficient protection against childhood tuberculosis, especially the severe extrapulmonary tuberculosis [2, 3]. Immunization campaigns are easy to be implemented in neonates and infants [4]. However, impaired protective efficacy of BCG was revealed in the establishment of latent TB and the recurrence of tuberculosis in adults, or for multi-drug resistant M. tuberculosis (MDR-TB) infection [5].

A number of presumptions for the failure of BCG as an effective vaccine have been proposed: a) geographic latitude and climate [6]; b) prior exposure or cross-reactivity of the immune response to BCG to environmental *mycobacteria bovis* [7]; c) host genetic and nutritional factor; d) the direction of immune response toward the Th2 or Th1 type accompanied with parasite infection [8]; e) BCG strains with similar genomic content, but different phenotype [9]; f) sex divisions [10]; g) strain variations in BCG preparations. After numerous passages, biological characteristics, immunological profiles, protection effect and the residual virulence differ significantly in sub-strains differentiated from each other [11, 12]. At present, more than 20 genetically distinct daughter strains exist in the world with heterogeneous phenotypic and genotypic that may affect their ability to provide protection against TB.

Among BCG strains, BCG China strain (or Shanghai strain, derivative of BCG Danish strain) and BCG Pasteur (1173P2) are currently the most commonly used in large field trials. To reveal a potential factor in the variable outcomes of BCG immunization, in this study we compared the immunogenicity profiles of BCG China strain and BCG Pasteur strain in mice model. Our results help provide the basis for testing novel vaccine strategies or developing better protective vaccines to control TB.

Materials and methods

Bacterial strains

M. bovis BCG China strain (or BCG Shanghai strain, derivative of BCG Danish strain) and *M.*

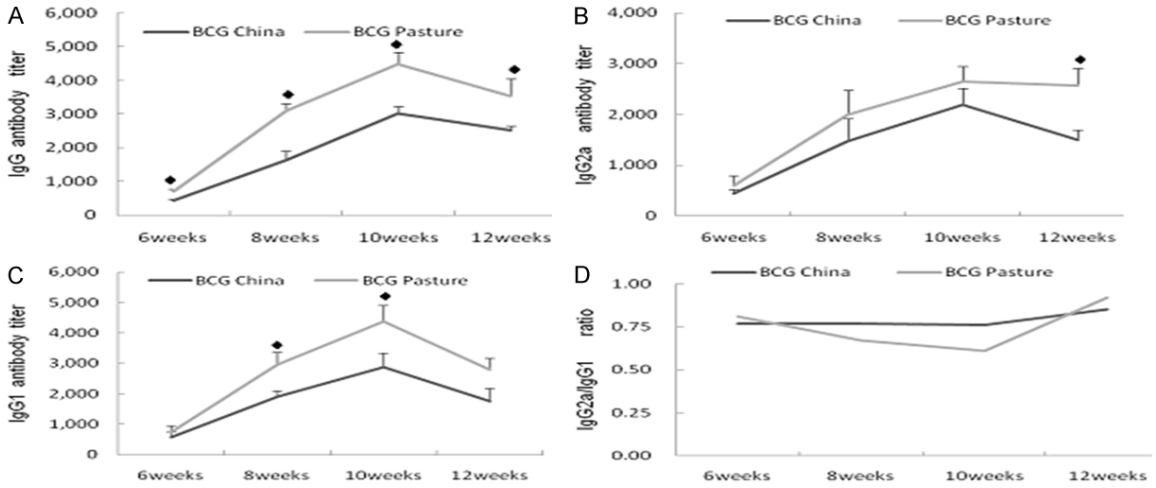

Figure 1. Mice sera were collected at the 6th, 8th, 10th, 12th weeks after immunization and specific IgG (A), IgG2a (B), and IgG1 (C) antibodies response to TB-PPD were measured by ELISA. The ratio of IgG2a/IgG1 (D).

bovis BCG Pasteur strain (BCG Pasteur 1173P) were obtained from Chengdu Institute of Biological Products (Chengdu, China). Both strains were cultured at the same time to mid-log phase as surface pellicles, unshaken cultures for 5 weeks at 37°C, in 50 ml of Sauton vaccine production medium ($MgSO_4$ 0.5 g, K_2HPO_4 0.5 g, citric acid 2 g, sodium glutamate 8 g, glycerol 60 mL, $ZnSO_4$ 0.01 g, and ferrum-ammonium citrate 0.05 g in 1000 mL, pH7.4).

Animals

Healthy BALB/c mice, equal amount of male and female, were obtained from Laboratory Animal Center, West China Center of Medical sciences, Sichuan University (Chengdu, China). The animals were individually housed in stainless steel cages with free access to standard laboratory food and water. The animals were weighed every two weeks. The mice were 4-5 weeks old at the time of vaccination. All animal experiments were performed according to Animal Welfare guidelines of Institute of Biomedical Sciences.

Vaccine preparation

BCG China and BCG Pasteur vaccines were prepared from midlog-phase liquid cultures. The liquid cultures were centrifuged at 4,000 g, resuspended in 0.01 M phosphate buffered saline (PBS), sonicated to separate clumps and washed three times. Single cell suspensions were resuspended in PBST (0.01 M PBS containing 0.05% tween-80, V/V, Sigma, St. Louis, MO, USA), counted in a Petroff-Hauser counting chamber, and then adjusted to 5×10^6 CFU.

BCG immunization

4-5 weeks old BALB/c mice (48 animals) were randomly divided into three groups: PBST group, BCG China group, BCG Pasteur group, which received a subdermal injection with PBST, BCG China strain or BCG Pasteur strain at a dose of 5×10^6 CFU in a volume of 0.1 ml, respectively. The animals were immunized once. Four mice in each group were killed for analysis at the 6th, 8th, 10th, and 12th weeks after the immunization, respectively. Sera samples were collected and maintained frozen at -20°C until used.

Measurement of specific IgG, IgG2a, and IgG1 antibodies

Specific antibodies were assayed by sandwich ELISA as described previously [13]. ELISA plates were coated overnight at 4°C with 0.1 mL of TB-PPD (1 μg/ml, XiangRui Biotech, Beijing, China). After washing with PBST buffer (0.01 M PBS, 0.05% Tween-20, V/V, pH 7.2) three times, the plated were blocked with 5% skim milk in PBS (pH 7.2) for 2 h at 37°C. Subsequently, serum samples were added to the wells at serial twofold dilutions, and incubated for 2 h at 37°C. The plates were washed and then incubated with HRP conjugated goat anti-mouse IgG (ZSGB Biotech, Ltd, Beijing,

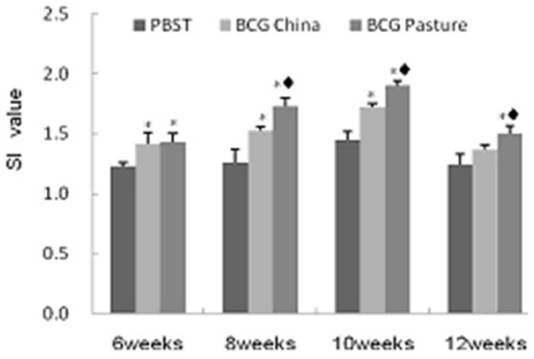

Figure 2. The splenocytes were co-cultured with TB-PPD at the 6th, 8th, 10th, 12th weeks after immunization and proliferation activity was determinate by stimulation index (SI).

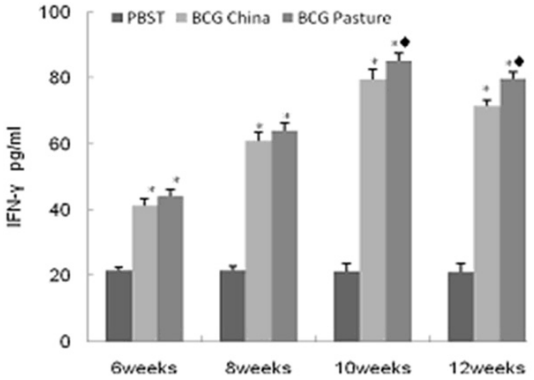

Figure 3. IFN-γ concentration secreted by splenocytes stimulated with TB-PPD (1 μg/ml) or un-stimulated was quantified by ELISA.

China), IgG1 and IgG2a (Southern Biotech, USA), respectively, for 1 h at 37°C. Next, the plates were washed and developed with 100 μl fresh TMB solution (NeoBioscience, China) in the dark for 30 min. Finally, 50 μl of 2 M sulfuric acid was added into each well to stop the reaction, and the plates were detected at 450 nm by the automated plate reader (Bio-Rad, Hercules, CA, USA). The concentrations of each specific isotype were tested three times and recorded as P. Sera from PBST group were tested as the negative control (N). Sera with P/N ≥2 were considered positive. Th e results were expressed as mean ± SE.

Proliferation of splenocytes

At 6th, 8th, 10th, and 12th weeks after immunization, four mice were killed at each time point in each group. Spleen was isolated aseptically and grinded with PBS, then filtered by a cell strainer to prepare single cell suspension. The cells were washed twice with PBS and resuspended in cRPMI (RPMI-1640 medium containing 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin), and counted after staining with 0.4% trypan blue for viability. Subsequently, splenocytes were seeded into 96-well plates at a concentration of 5×105 cells/well in 100 μl of cRPMI in triplicate and stimulated with 100 μl of TB-PPD (1 μg/ml) or cRPMI as negative control (nonstimulated) as described previously [14]. The plates were incubated in 5% CO_2 humidified incubator at 37°C for 72 h. After incubation with 50 μl XTT working fluid (4 ml 1 g/L XTT containing 0.1 ml 0.15 g/L PMS, Sigma) for 4 h, the absorbance was measured at 450 nm (Bio-Rad). The average was calculated from triplicate wells stimulated or nonstimulated. The results were presented as the stimulation index (SI): OD of stimulated cultures/OD of nonstimulated cultures.

Flow cytometry analysis of splenocyte subsets

The splenocytes were seeded in six-well flat bottomed plates (5×10^6 cells in 1 ml of cRPMI per well) in triplicate, then treated with 1 mL TB-PPD (1 μg/ml) at 37°C for 72 h with 5% CO_2. The plates were washed with 0.01 M PBS (pH 7.2), then incubated with rabbit anti-mouse $CD4^+$-PE (eBioscience, USA) or rabbit anti-mouse $CD8^+$-FITC (eBioscience, USA) for 1 h at 4°C. The plates were washed twice and the proportions of $CD4^+$ T and $CD8^+$ T cells were analyzed by flow cytometry (FACSCalibur, BD, USA).

Measurement of cytokines

The splenocytes were treated with 1 ml TB-PPD (1 μg/ml) at 37°C for 72 h with 5% CO_2, and the culture supernatants were collected and frozen at -20°C. The concentrations of IFN-γ and IL-4 secreted into the medium were quantified by ELISA kits (eBioscience, USA) according to the manufacturer's protocol. Values were expressed as pg/ml deduced from standards run in parallel.

Statistical analysis

One-way ANOVA was used to compare with the levels of difference among all groups. Differences were considered significant for a *P*-value < 0.05. The statistical analyses were performed by using SPSS17.0.

Table 1. The percentages of splenocyte subsets

		6 weeks	8 weeks	10 weeks	12 weeks
PBST	$CD4^+$	23.4±0.8	22.5±0.7	23.2±1	23.5±0.6
	$CD8^+$	18.6±0.9	17.4±0.6	18.3±0.6	17.2±0.8
BCG China	$CD4^+$	28.7±1*	30.6±1.3*	32.7±0.8*	30.5±1.1*
	$CD8^+$	22.5±1.2*	25.6±1.4*	28.3±0.9*	26.5±1.1*
BCG Pasture	$CD4^+$	30.5±1.7*	33.4±0.9*,♦	35.7±1.8*,♦	35.1±2.3*,♦
	$CD8^+$	24.7±1.4*	28.5±1.5*,♦	30.4±0.9*,♦	29.5±2.2*

The splenocyte subsets were determined at the 6th, 8th, 10th, 12th weeks after immunization by flow cytometry. Results were shown as mean ± SE. *P < 0.01 vs. the PBST group; ♦P < 0.01 vs. BCG China group at the same time point.

Results

Antibody responses after immunization with BCG strains

Serum was isolated from the blood obtained at 6th, 8th, 10th, and 12th weeks after immunization, and specific IgG, IgG2a, and IgG1 antibody titers in serum specimens were evaluated by ELISA. The results showed that IgG titers from 6th to 12th weeks (**Figure 1A**), IgG2a titers at 12th weeks (**Figure 1B**), and IgG1 titers from 8th to 10th weeks (**Figure 1C**) after immunization with BCG Pasture were higher than those from BCG China (P < 0.01). In addition, the ratio between the IgG2a and IgG1 titers (**Figure 1D**) in both BCG groups showed constant upward trends that indicated a shift towards a Th1 immune response.

Proliferation of splenocytes after immunization with BCG strains

The splenocytes were co-cultured with TB-PPD at 6th, 8th, 10th, 12th weeks after immunization and proliferation activity was determined by stimulation index (SI). The results showed that the proliferation of splenocytes induced by BCG China was higher than that by PBST group except for 12th weeks (P < 0.01). Moreover, SI was higher in BCG Pasture than PBST group at all time point after immunization (P < 0.01). The significant difference between BCG Pasture and BCG China was sustained from the 8th weeks to the 12th weeks (P < 0.01) (**Figure 2**).

Percentages of splenocyte subsets after immunization with BCG strains

The proportions of splenocyte subsets stimulated with TB-PPD were determined by flow cytometry. The percentage of $CD4^+$ T cells was higher in both BCG groups than in PBST group (P < 0.01) from the 6th to 12th weeks after immunization, and it was higher in BCG Pasture group than in BCG China group from the 8th weeks to the 12th weeks (P < 0.01). Similarly, the percentage of $CD8^+$ T cells in both vaccine groups was higher than PBST group (P < 0.01), but it was lower in BCG China group than in BCG Pasture group at the 8th and the 10th weeks (P < 0.01) (**Table 1**).

Secretion of cytokines after immunization with BCG strains

The induction of both Th1 (IFN-γ) and Th2 (IL-4) type cytokines secreted by lymphocytes was evaluated by ELISA. The results showed that from the 6th weeks to the 12th weeks after the immunization, the concentrations of IFN-γ in both BCG vaccine groups were higher than in PBST group (P < 0.01). In addition, significant difference in IFN-γ level was observed between BCG Pasture group and BCG China group at the 10th and 12th weeks (P < 0.01). Meanwhile, the concentration of IL-4 secreted was at low level in both BCG vaccine groups (**Figure 3**).

Discussion

BCG China strain (or BCG Shanghai strain) was generated from the BCG- Danish- 823 maintained at the Statens Serum Institute, Copenhagen, Denmark. BCG China strain was sub-cultured for a total of 10 continuous passages on non-bile Sauton medium at Shanghai Institute of Biological Products of China to ensure the stability of strain and then freeze-drying preserved, thereafter named Shanghai D2PB302 strain. BCG China strain was widely utilized in China as vaccine production in 1985 and then conferred to be unique licensed BCG strain of vaccine production till now.

BCG Pasteur (1173P2) was one of the main BCG strains currently commonly manufactured and clinically used worldwide. The name comes from the 1,173 passages when the archive seed lots were established in the 1960 s. In 2007, the complete genomic sequence of *Mycobacterium bovis* BCG Pasteur 1173P2 was determined [15]. Based on comparative genomics studies, all BCG strains were divided into the early and the late strains, the major difference being that the latter strains have lost

deletion region 2. BCG Pasteur strain and BCG Danish strain all belonged to the late strains [16].

Mycobacterium tuberculosis (Mtb) is a facultative intracellular pathogen that mainly infects alveolar macrophages to replicate and disseminate within the host. $CD4^+$ and $CD8^+$ T cells play a pivotal role in providing effective protection against *Mycobacterium bovis* infection [17, 18]. For example, HIV patients with impaired $CD4^+$ system were susceptible to TB. Helper T-cell type 1 (Th1) cytokine IFN-γ, primarily secreted by T lymphocytes, NK and NKT cells, and are crucial to defending against intracellular pathogens. IFN-γ can promote macrophage activation; produce superoxide and nitric oxide (NO), decrease intra-lysosomal pH, and accelerate the fusion of the phagosome and lysosome [19, 20]. Nevertheless, Th2 type cytokines such as IL-4 may inhibit Th1 response and provide poor protection against *M. tuberculosis* [21].

In this study, to evaluate the immunogenicity of BCG strains, TB-PPD was chosen as the stimulator or coating antigen, which is a kind of purified protein derivative (PPD) of *M. tuberculosis* H37Rv [22]. We found that both groups immunized with BCG produced higher and prolonged titer of antibody against TB-PPD. Especially in BCG Pasture, we observed a shift towards a Th1 immune response. Sustained antibody production is an essential feature of immunological memory that indicates vaccine efficiency. Flow cytometry analysis showed that the ratios of both $CD4^+$ and $CD8^+$ subsets increased obviously after the immunization, especially the $CD4^+$ subset. $CD4^+$ immune response in BCG Pasture group persisted longer than in BCG China group. Consistently, the concentration of IFN-γ in BCG Pasture group peaked at 10^{th} weeks and remained at a high level at the 12^{th} weeks post-vaccination. These data suggest that BCG Pasture vaccine could provide long-term immunity, and are in agreement with previous findings that the variability in T cell proliferation and IFN-γ production depends on the strains and the route of administration [23, 24].

In this study we found that specific IgG, IgG2a and IgG1antibodies titers, SI value, the percentages of $CD4^+$ or $CD8^+$ T cells, and IFN-γ concentration were all higher in BCG Pasteur group than in BCG China group, which may be related to the surface antigen of BCG Pasteur strain. Comparative genome analyses of BCG strains have shown that the genome of BCG Pasteur contains two independent tandem duplications, DU1 and DU2. BCG strains can be divided into four categories DU2-I to DU2-IV, according to DU2 [15]. BCG Pasteur belongs to DU2-IV group. Among four DU2 groups, DU2-III group is the most attenuated, and the mother of BCG China -BCG Danish is one of them. Notably, desA3 gene encoding desaturase was expressed obviously in *M. bovis* and patients with active tuberculosis, and also was expressed in DU2-IV group but not in DU2-III group [15, 25]. In addition, some secreted proteins were detected in BCG Pasteur strain including MPB83 and MPB70 antigens [15]. These cell antigens or diverse secretion of proteins probably contribute to the better immune responses induced by BCG Pasteur strain. Our study shows that immune responses to BCG vaccine differ by strain, which may account for variable outcomes of BCG immunization. These data help provide the basis for testing novel vaccine strategies or developing better protective vaccines to control TB.

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Disclosure of conflict of interest

None.

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