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Activity in mice of recombinant BCG-EgG1Y162 vaccine for Echinococcus granulosus infection

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Cystic hydatid disease is a zoonotic parasitic disease caused by *Echinococcus granulosus* which is distributed worldwide. The disease is difficult to treat with surgery removal is the only cure treatment. In the high endemic areas, vaccination of humans is believed a way to protect communities from the disease. In this study we vaccinated BALB/c mice with rBCG-EgG1Y162, and then detected the level of IgG and IgE specifically against the recombinant protein by ELISA, rBCG-EgG1Y162 induced strong and specific cellular and humoral immune responses. In vitro study showed that rBCG-EgG1Y162 vaccine not only promote splenocytes proliferation but also active T cell. In addition, the rBCG-EgG1Y162 induced a protection in the mice against secondary infection of *Echinococcus granulosus*.

Introduction

Cystic hydatid disease or cystic echinococcosis is caused by the cystic stage of *Echinococcus granulosus* (*Eg*), which is distributed almost around the world with a strong link to sheep farmers. This disease is serious and potentially fatal. The disease often starts without symptoms for many years. In addition, currently there are no human vaccines against echinococcosis. Vaccination of humans in high endemic areas is believed a effective way to protect the communities from the disease.[1-2]

Studies have shown that the recombinant EG95 vaccine can protect cattle, sheep and other intermediate host species from primary infection of cystic echinococcosis (CE).[4-9] Our previous studies showed that a gene EgG1Y162 isolated from *E. granulosus* encodes a protein homologous to EG95.[10] Thus, EgG1Y162 is likely a vaccine candidate for intermediated host against larval stage infection.

Bacille Calmette-Guerin (BCG) is an ideal vector for carrying exogenous antigen because it is considered to be an alive recombinant vaccine.[11-12] Studies showed that the recombinant BCG (rBCG) has dual-function, which can be as a vaccine or an adjuvant. It has been shown thatBCG provides a strong and persistent immune response against infection diseases, malignant tumor, and parasitic diseases.[13-14] In the present study, we cloned *EgG1Y162* from *E. granulosus* and inserted the gene into *Mycobacterium* expressed plasmid vector pMV361. The recombinant plasmid was then transformed into the BCG to construct rBCG-EgG1Y162 vaccine. We showed that the rBCG-EgG1Y162 vaccine induced strong and specific cellular and humoral immune responses, indicating that rBCG-EgG1Y162 can be a new vaccine candidate for reducing the risk of human infected by *E.granulosus*.

Results

Construction of the rBCG-EgG1Y162 Plasmid

In order to probe whether the rBCG-EgG1Y162 vaccine has the antigenicity, we constructed and induced the expression of rBCG-EgG1Y162 Plasmid, then probed the antigenicity of rBCG-EgG1Y162 vaccine. Recombinant plasmid pMV361-EgG1Y162 contains EgG1Y162 in size of 369 bp after digested with *EcoRI and Hind*Ⅲ. After sequence confirmation of the recombinant pMV361-EgG1Y162 through sequencing, the plasmid pMV361-EgG1Y162 was transformed into BCG by electroporation. The recombinant BCG-EgG1Y162 gene was confirmed by restriction endonuclease digestion and identified by PCR. And then was induced protein expression at 45°C. The molecular weight of rBCG-EgG1Y162 protein is 71 k_D protein (**Fig. 1**). We collected sera from 11 patients infected with cystic echinococcosis. All the sera samples (lanes 1 to 11) recognized rBCG-EgG1Y162 (**Fig. 2**) by Western Blot analysis. There were no reaction observed using sera collected from health persons. The results indicate that EgG1Y162 can be recognized by the serum of CE patients and can be vaccine candidates.

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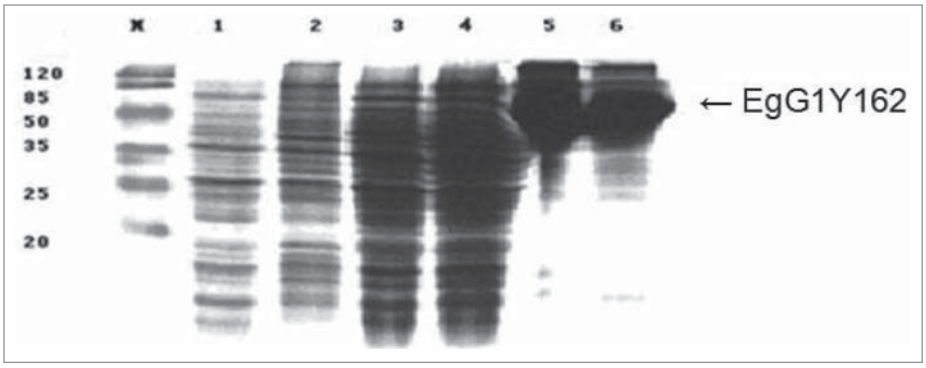

Figure 1. The expression of EgG1Y162 in BCG. M: Protein Marker; lanes 1,2: the expression of BCG and rBCG-EgG1Y162 were induced at room temperature for 1 h, respectively; lanes 3, 4: the expression of rBCG-EgG1Y162 were induced for 3 h, 6 h at room temperature, respectively; lanes 5 and 6: the high expression of rBCG-EgG1Y162 could be induced for 3 h at 45°C.

rBCG-EgG1Y162 vaccine promoted splenocytes proliferation and active T cell in vitro

In order to detect whether the rBCG-EgG1Y162 vaccine has the immunogenicity, in vitro cell proliferations were analyzed tested using the CCK-8 assay. Each culture plate containing cells was incubated at 37°C for 24 h, 48 h and 72 h. Dynamic cell growth showed that 48 hours of *in vitro* stimulation was the best time for cell growth (**Fig. 3**). Both the rBCG and EgG1Y162 groups showed a significant increase in cell proliferation compared to BCG and normal saline control groups.Meantime, splenocytes from mice vaccinated with the rBCG-EgG1Y162 produced significantly higher levels of IFN-γ, IL-2, IL-4, and IL-10 after antigen-specific stimulation compared to control mice. In contrast, there were no significant differences in the production of TNF-α induced by rBCG-EgG1Y162 (**Fig. 4**, **Table 1**). Obviously, rBCG-EgG1Y162 vaccine not only promote splenocytes proliferation but also active T cell in vitro.

rBCG-EgG1Y162 vaccine stimulated both cellular and humoral immune responses in vivo simultaneously

In order to understand the impact of rBCG-EgG1Y162 vaccine on both cellular and humoral immune responses in vivo, we vaccinated BALB/c mice with alive BCG containing rBCG-EgG1Y162. We collected sera from the mice and measured the levels of IgG and IgE, and then analyzed the subtypes of IgG in the sera. The results showed IgG1, IgG2a and IgG2b were significantly increased in the mice ($P > 0.05$) compared to the control mice. Levels of IgE, however, were not significantly different ($P > 0.05$). Given that the levels of IgG1 and IgG2b are indicators of Th2 response; and IgG2a reflects Th1 cellular immune responses. The vaccination results reveal that rBCG-EgG1Y162 vaccine is able to stimulate both Th1 and Th2 responses. (**Fig. 5**, **Table 2**).

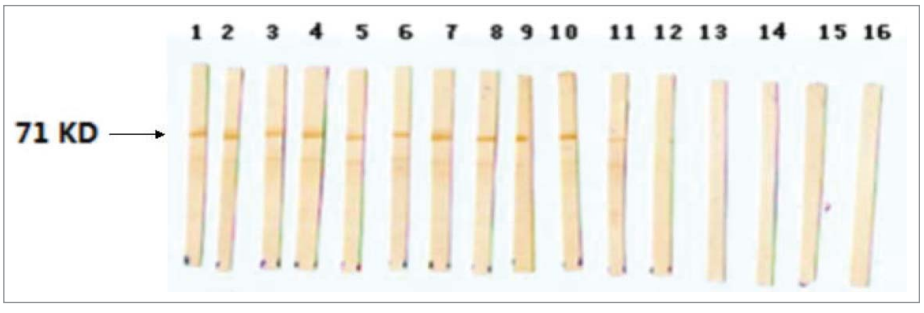

Figure 2. Detected the antigenicity of rBCG-EgG1Y162 by Western blots. M: Protein Marker, lanes 1 to 16: 1–6 serums from rabbits infected by *Eg*, 7–11 from patients infected by *Eg* and 12–16 from 5 normal serum samples as negative controls. All of serum samples were diluted 200-fold with PBS.

The rBCG-EgG1Y162 vaccine can arouse immune protection against *Eg* effectively in vivo

In order to observe protective effect of the rBCG-EgG1Y162 vaccine, the number of visible cysts in the sacrificed mice were counted. The results showed that rBCG-EgG1Y162 induced 75.52% of cyst reduction compared to the cyst nuer in the control mice. In addition, we also measured the size of the cysts isolated from the mice. The average size of cysts (0.39 mm) in vaccinated mice was significantly smaller than these cysts in an average of 3.65 mm from the control mice. These results indicated that the rBCG-EgG1Y162 inoculation could reduce protection response against CE infection in terms of reduction of cyst number and inhibition of cyst growth (**Fig. 6**).

Discussion

Control of the widespread distribution of hydatid disease in the world, particularly in developing countries needs special tools for protection of communities from the infection. BCG has been widely used as a vaccine for children against tuberculosis infection. In addition, BCG has also been studied as an adjuvant for making vaccines against different infectious diseases. The early stage of CE is normally asymptomatic, and the situation is maintained for many years until the cyst growing big enough to physically produce symptoms. The peak age group of CE patients with surgical treatment is at age 20–45. These patients are believed to have their infection at child ages. In fact, the epidemiological studies showed that children are most susceptible for hydatid disease infection. Therefore, making a vaccine for children against *E. granulosus* infection is important for protection of communities from CE infection. We believed it is easy to accept that children are vaccinated with recombinant BCG containing *E. granulosus* protective antigen(s) given that BCG has been accepted for vaccination of children.

In this study, we demonstrate rBCG-EgG1Y162 vaccine have both immunogenicity and antigenicity, this allowed us to further characterize its vaccination ability. Compared with other vaccine against echinococcosis, rBCG-EgG1Y162 vaccine could induce simultaneously significant cellular and humoral immune response to protecte against E. granulosus infection in terms of reduction of the number and inhibition of cyst growth. However, the immune protective effect of rBCG-EgG1Y162 vaccine is limited Therefore, It's necessary to find the immunodominant epitope of EgG1Y162 and reconstructe the rBCG-EgG1Y162 vaccine.

In order to confirm the authenticity of the immune protective effect, we challenge-infected the BALB/c mice with protoscoleces and detected the concentration of IgE after sixth weeks. The result showed all of mice infected by protoscoleces and there were no significant different between groups. So the immune protective effect of rBCG-EgG1Y162 vaccine is reliable. However the result still need be confirm in other animal such as dog, sheep, and even humans.

Although, the results showed that rBCG-EgG1Y162 could be a vaccine candidate for an effective human vaccine against echinococcosis. It's a long way to apply rBCG-EgG1Y162 vaccine to humans. In order to obtain higher titer of protective antibody, we will reconstruct rBCG-EgG1Y162 vaccine by analyzing the dominant epitope to improve its antigenicity, and then to observe it's immune protective effect in mice, dog, and sheep before apply to humans.

Materials and Methods

Bacteria and culture method

The DH5α strain of *Escherichia coli* (*E. coli*) was grown in LB medium. The *M. bovis* BCG strain (kindly provided by the Medical School, Shihezi University, China) was cultured in 7H9 media (Difco, USA) (30 μg/ml kanamycin).

The construction of the recombinant plasmid pMV361-EgG1Y162

The plasmid of pET41a/egG1Y162 was digested with *EcoR*I and *Hind*III. The purified fragment with the expected length of EgG1Y162 (GenBank: AB462014, 360 bp) was then subcloned into the *E.coli-Mycobacterium* shuttle plasmid pMV361.

The construction of the rBCG-EgG1Y162 plasmid

The recombinant plasmid pMV361- EgG1Y162 was transformed into the BCG by electroporation (Electroporator, Eppendorf). The transformed bacteria were cultured for 3 to 4 weeks at 37°C on Middlebrook 7H10 broth agar (BD), containing 10% oleic acid albumin-dextrose-catalase (OADC) and 0.5% Tween 80 in kanamycin selective medium (kanamycin: 30 μg/ml). The transformed bacteria were selected and amplified in the Middlebrook 7H9 liquid medium for 2 to 3 weeks. The number of cells used for vaccination was determined by the colony forming unit (cfu). The method for calculating BCG was based on previous study.[16] When A600 is 0.1, the cfu of the BCG or rBCG is approximately 2.5×10^6 cfu. The cells were centrifuged at 3000 rpm for 10 minutes, and diluted to 1×10^6 cfu in 100 μl PBS.

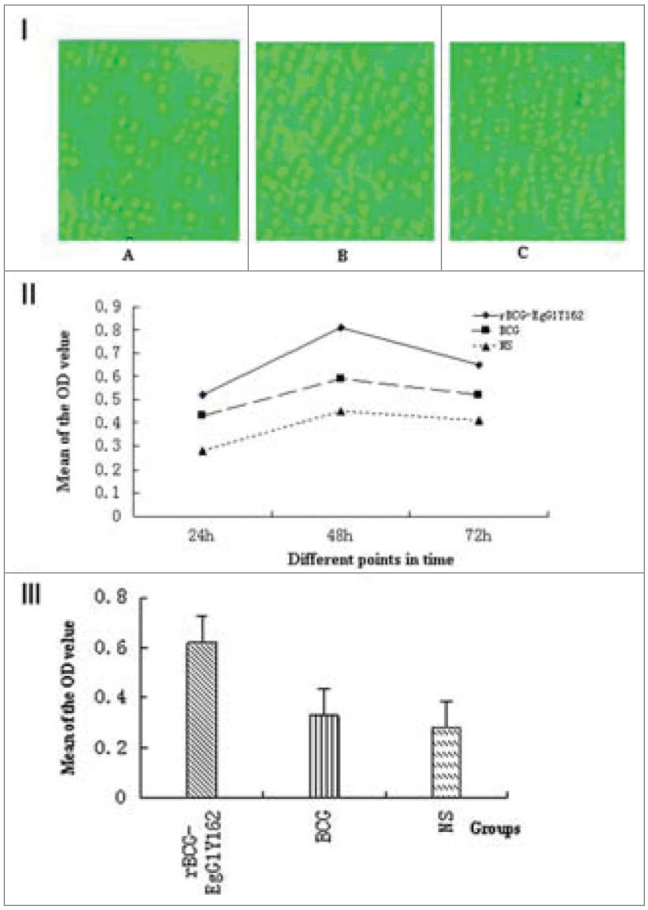

Figure 3. The proliferation of mouse splencytes cultured for 24 h, 48 h, and 72 h *in vitro*. Microscope images of mouse splencytes stimulated by normal saline, BCG and rBCG-EgG1Y162 for 48 h in vitro. A: normal saline B: BCG C: rBCG-EgG1Y162(I). Compared to normal saline, splenocytes stimulated by rBCG-EgG1Y162 proliferated significantly, while splenocytes stimulated by BCG proliferate slightly. The mean stimulation index for the immunized mice had higher than the index of the negative control mice ($P < 0.05$). The splenocytes from both groups showed equal responses after stimulated with ConA, suggested that the splencytes were functional and responsive to nonspecific mitogens.

Table 1. The concentrations of different cytokines in culture supernatants from splenocytes stimulated by rBCG-EgG1Y162 and BCG, respectively (μg/ml)

Group	n	IFN-γ	TNF-α	IL-2	IL-4	IL-10
rBCG-EgG1Y162	8	41.85* ± 4.80	15.37 ± 5.23	35.65* ± 4.98	39.57* ± 4.70	37.59* ± 5.10
BCG-pMV361	8	24.8 ± 1.62	16.08 ± 1.57	21.81 ± 2.05	22.95 ± 1.35	20.15 ± 1.24
NS	8	20.7 ± 1.16	14.32 ± 1.04	18.41 ± 1.48	20.36 ± 1.38	16.59 ± 1.00

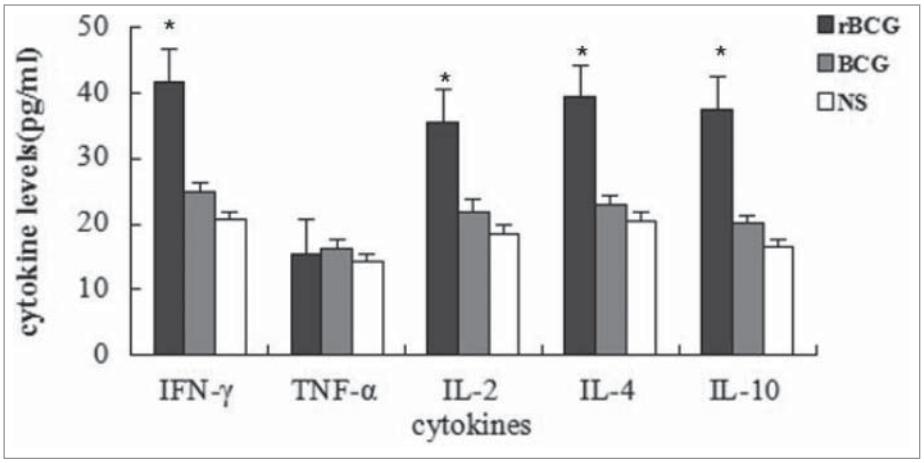

Figure 4. The level of different cytokine in culture supernatants from splenocytes stimulated by rBCG-EgG1Y162 and BCG, respectively. There were no significant differences in TNF-α among each group. Levelsof IL-4, IFN-γ, IL-10, and IL-2 increased significantly in culture supernatants from splenocytes stimulated by rBCG-EgG1Y162. (* means significant difference, $P < 0.05$).

The expression analysis

The transformed BCG were grown for about 2 weeks (mid-logarithmic phase) in the liquid medium 7H9 containing 10% oleic acid albumin-dextrose-catalase (OADC), 0.05% Tween 80 and 30 μg/ml kanamycin. After 14 d of culture at 37°C, the rBCG culture was induced at 45°C for 30 minutes and the culture was centrifuged at 1680 g for 10 minutes at 4°C. The pellets were washed twice in phosphate buffered saline (PBS), and proteins were separated on 12% SDS-PAGE gel.

The antigenicity analysis of rBCG-EgG1Y162 by using anti-serum collected from 6 rabbits infected Eg, 5 CE patients, 5 normal peoples. Western blot analysis was performed with 16 serum samples, all of the patients had never been vaccinated with BCG. The Western blotting was performed by first blocking the membrane with 5% skimmed milk in 0.05% Tween 20 in PBS for 2 hours at 37°C. Nitrocellulose membranes were first probed with a 1: 200 dilution of Eg patient serum as the primary antibody incubated overnight at 4°C, then washed with PBS 3 times. Then, they were incubated with 1:1000 dilution of the appropriate secondary antibodies for 2 hours at 37°C, followed by 3 washing steps. The positive protein bands were detected with diaminobenzoic acid using DAB (3,3′-diaminobenzidine) developer (SIGMA, Saint Louis, Missouri, USA).

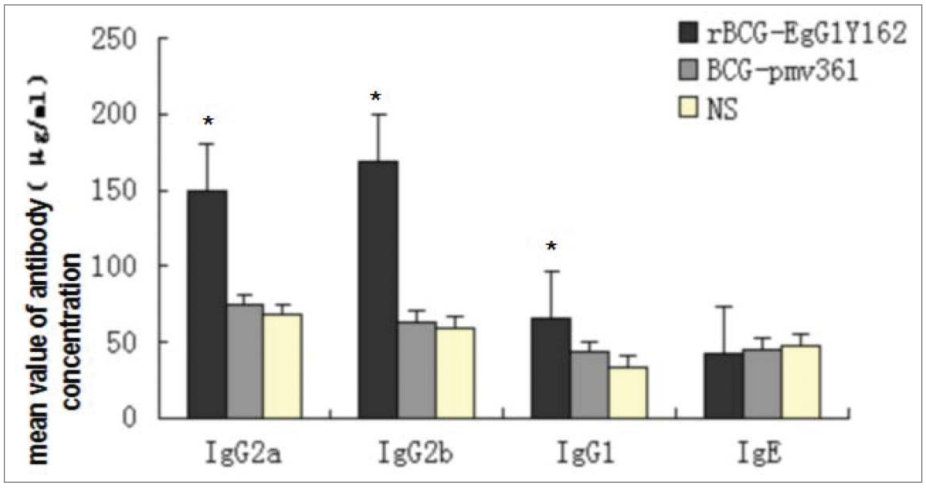

Figure 5. Concentration of the IgG in different groups. IgG sub-classes and the IgE antibodies response against rBCG-EgG1Y162 were detected by ELISA. Serum samples come from BALB/c mice 6 weeks after first immunization. Antibody levels were significant differences between the immunized group and the control group (*$P < 0.05$, **$P < 0.01$). Mice immunized by rBCG-EgG1Y162 generated specific high levels of IgG1, IgG2b, and IgG2a.

Animal vaccine

Animal experiments were strictly performed in accordance with the guidelines of the Chinese Council on Animal Care. Fifty female BALB/c mice at 6 weeks of age (sourced from the experimental animal center of The First Affiliated Hospital of Xinjiang Medical University, Urumqi, China) were used. Fifty BALB/c mice were divided into 3 groups; 20 mice were the rBCG-EgG1Y162 vaccine group, 20 mice were the BCG treated group, and the remaining 10 mice were the normal saline group. Mice were vaccinated in abdominal subcutaneous at 1×10^6 cfu with rBCG-EgG1Y162, and with BCG in a final volume of 100 μL of PBS. The control groups were injected with 100 μL saline. Six mice were sacrificed for the immunological assay after 6 weeks post immunization.

ELISA

Two weeks after the last immunization, 10 mice from the rBCG-EgG1Y162 and BCG groups, and 5 mice from normal saline group, were sacrificed and their serum were collected. The anti-serum including IgE, IgG, and its subclasses were quantified by ELISA following Kalantari's method.[17] Briefly, ELISA-plates (SIGMA) were coated with rBCG-EgG1Y162 μg/100 μL per well. Each serum collected from each of the mice vaccine with either rBCG-EgG1Y162, BCG or saline was diluted at 1:100. The serum from BCG and normal saline groups were used as a negative controls. Peroxidase-conjugated goat anti-mouse IgE, IgG, IgG1, IgG2a, and IgG2b

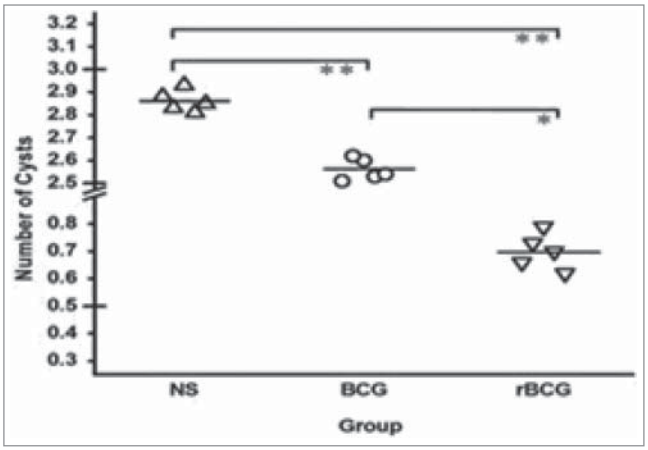

Figure 6. Immune protection of rBCG-EgG1Y162 vaccine against Eg in vivo. Each mouse was immunized with the rBCG-EgG1Y162 or BCG once every 2 weeks, a total of 3 times, respectively. Then challenged with 3000 viable protoscoleces intraperitoneally after the last immunization. The controls groups were immunized with normal saline. The average number of visible cysts decreased in the experimental group (0.7 per mouse), compared to the number in the BCG and normal saline group (control group) (2.86 per mouse).Values are the mean number of cysts ± SEM from 10 mice. *$P < 0.05$, **$P < 0.01$ compared to the mean value of each group.

(Sino-USA) were used in 1: 1000 dilution. Optical densities were measured at 490 nm.

Splenocytes preparation and culture

Two weeks after the last immunization, 10 mice from the rBCG-EgG1Y162 and BCG groups, and 5 mice from normal saline group, were sacrificed and their spleens were dissected under aseptic conditions. The suspension of splenocytes was prepared after removing erythrocytes resuspended in RPMI 1640 (GIBCOBRL) by vigorous pipetting. The cell suspension was added into the 96-well flat-bottomed tissue culture plates (Extra-Gene) at 100 μL/well, and cultured for 48 h, 48 h and 72 h, respectively, at 37°C and 5% CO_2.

The splenocyte proliferation assays

Splenocytes from the immunized and control mice were prepared in RPMI 1640 supplemented with 10% fetal calf serum. Viable cells (5×10^6 cells/mL) were cultured in a medium comprising 10 μg/mL of rBCG-EgG1Y162 and 5 μg/mL of concanavlin A (ConA; Sigma), and incubated for 72 h. Proliferation was assessed using a CCK-8 (Dojindo) assay follow the manufacturer's instructions. The absorption at 450 nm for each well was measured by using a microtiter plate reader (Bio-Rad, New Jersey, USA).

Table 2. The concentration (μg/ml) of the mouse serum antibodies in Balb/C mice sixth weeks after vaccination

Group	n	IgE	IgG1	IgG2a	IgG2b
rBCG-EgG1Y162	8	42.336	66.263	149.012	169.406
BCG-pMV361	8	45.726	43.328	74.223	63.565
normal saline	8	48.271	34.169	68.035	59.750

Note: *rBCG – with BCG – pMV361 EgG1Y162 group, saline group $P < 0.05$.

The cytokine assay

Culture supernatants from the proliferation assay were collected at 72 h for the assessment of both Th1 and Th2 cytokines, including IFN-γ, IL-2, IL-4, and IL-10. Cytokine profile was analyzed by sandwich ELISA, using a Quantikine M kit (SIGMA) following the manufacturer's instructions.

The protection test

Six months after challenge with protoscoleces of *Eg*, the mice (vaccinated with rBCG-EgG1Y16, BCG, and normal saline, respectively) were sacrificed and the visible hydatid cysts were counted and measured. At six weeks after the final immunization, the remaining 10 mice from each group were challenged with 3000 viable protoscoleces intraperitoneally as reported previously.[17-18] Six months post-challenge, the carcasses were dressed and examined for visible hydatid cysts. Protective immunity was calculated following the protocol outlined by vaccinated mice (%) = (1−average of cysts in test group/average of cysts in control group) × 100%.

Statistic analysis

All data comparisons were tested for significant differences by conducting one-way analysis of variance (ANOVA) using the software SPSS 17.0.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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