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ORIGINAL ARTICLE

Evaluation of protective efficacy conferred by a recombinant *Mycobacterium bovis* BCG expressing a fusion protein of Ag85A-ESAT-6

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KEYWORDS
Ag85A;
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Background: We previously constructed a recombinant bacille Calmette-Guérin (rBCG-AE) strain that could express a fused Ag85A-ESAT-6 protein. That study suggested that the rBCG-AE strain was able to induce a higher titer of antibody and elicit a more long-lived and stronger Th1-type cellular immune responses than the parental BCG strain, the rBCG-A strain (i.e., expressing Ag85A), or the rBCG-E strain (i.e., expressing ESAT-6).
Methods: In the current study, we further investigated the strain's protective efficacy against *Mycobacterium tuberculosis* H37Rv infection in BALB/c mice through evaluating organ bacterial loads, lung histopathology, lung immunohistochemistry, and net weight gain or loss by using conventional BCG, rBCG-A, and rBCG-E as the controls.
Results: From the 3rd to 9th weeks after the challenge infection, the bacterial counts were significantly lower in tissues (e.g., spleen and lung tissues) in the mice immunized with rBCG-AE than in the control group, but were higher than the counts in the BCG group. The pathological damage in the lung tissues of the rBCG-AE group gradually improved from the 6th to 9th weeks after being infected with *M. tuberculosis* H37Rv, but the score of pathological changes in the rBCG-AE group was obviously higher than the score in the BCG group. There was no difference in the percentage of IFN-γ and iNOS positive cells in the lung tissues of the rBCG-AE and BCG groups.
Conclusion: The results suggest that rBCG-AE can not promote protective efficacy against *M. tuberculosis* H37Rv infection, compared to the BCG vaccine.

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Introduction

Tuberculosis (TB) is a serious disease afflicting humans; it results in millions of deaths and new cases each year.[1,2] The only available vaccine against TB is attenuated *Mycobacterium bovis* bacille Calmette-Guérin (BCG). However, the protective efficacy of BCG against TB—especially adult TB—remains controversial.[3] A more efficacious and satisfactory vaccine to fight against TB is therefore urgently required.

Studies have been performed in past decades to develop novel TB vaccines such as rBCG vaccines,[4,5] live attenuated vaccines,[6] subunit vaccines,[7] and DNA vaccines.[8] However, no vaccine has exceeded BCG in conferring protection from *Mycobacterium tuberculosis* infection in mankind. Recombinant BCG (rBCG) is a promising approach to promote the immune efficacy of the BCG vaccine by importing exogenous genes that encode immunodominant antigens of *M. tuberculosis* or immunoregulatory cytokines. By rBCG technology, the proteins secreted by BCG and recombinant proteins introduced to BCG can simultaneously stimulate the body to generate stronger immune responses to *M. tuberculosis* infection. The rBCG vaccine shares many advantages of the BCG vaccine such as safety and low adverse effects. It can be vaccinated in the form of a viable organism and it is capable of longterm self-production in the body,[9] allowing it to stimulate sustained immune responses against *M. tuberculosis* infection. In recent years, studies for developing a better TB vaccine than BCG have therefore been focused on the rBCG vaccine.

Early secretory antigen target 6 (ESAT-6) is only present in the virulent *M. tuberculosis*, but is lacking in BCG.[10] It can induce strong cell-mediated immunity.[11,12] It has been considered a protective antigen for developing TB vaccines.[13] Ag85A is a major fraction of the Ag85 complex (which comprises Ag85A, Ag85B, and Ag85C). These AG85 components are proteins that are secreted in *M. tuberculosis* culture filtrate.[14,15] Ag85A is the most essential component of *M. tuberculosis* for immunostimulation and has been used in numerous vaccine preparations that can induce outstanding protective efficacy.[16] Ag85A, moreover, is closely correlated with the longterm survival of *M. tuberculosis* within macrophages.[17] *M. tuberculosis* within macrophages may be effectively eliminated when the immune responses against Ag85A are induced. For these reasons, we previously chose Ag85A and ESAT-6 to construct rBCG (rBCG-AE) for stimulating more potent immune responses against *M. tuberculosis* infection; the study showed that in BALB/c mice the vaccine was able to induce a higher titer of antibody, elicit a greater $CD4^+$ T and $CD8^+$ T proliferation rate of splenocytes, and elevate the level of interferon-gamma (IFN-γ) production, compared to the parental BCG. The study suggested that the immunogenicity of the parental BCG was significantly improved.[18]

Eliciting prominent immunogenicity is insufficient for a satisfactory TB vaccine. Our ultimate goal is to develop a vaccine that effectively prevents *M. tuberculosis* infection. In the current study, we therefore further evaluated the protective efficacy conferred by rBCG-AE vaccine against *M. tuberculosis* H37Rv infection in BALB/c mice.

Materials and methods

Bacterial strains and media

M. bovis BCG-China was obtained from the Chengdu Biological Products Institute (Chengdu, China). *M. tuberculosis* H37Rv (strain ATCC 93009) was originally conserved in the Animal Bio-Safety Level 3 (ABSL-3) facility of Wuhan University (Wuhan, China). The rBCG vaccines were previously constructed and included rBCG-AE (which expresses the Ag85A-ESAT-6 fusion protein), rBCG-A (which only expresses the Ag85A protein of *M. tuberculosis*), rBCG-E (which only expresses the ESAT-6 protein of *M. tuberculosis)* and rBCG-361(BCG strains transformed with pMV361 plasmids).[18] They were cultivated in Sauton medium (i.e., 0.5 g $MgSO_4$, 0.5 g K_2HPO_4, 2 g citric acid, 8 g sodium glutamate, 60 mL glycerol, 0.01 g $ZnSO_4$, and 0.05 g ferrum-ammonium citrate in 1000 mL at a pH7 ranging approximately from 4 to 7.5).

Animals

Pathogen-free BALB/c female mice were obtained from the Laboratory Animal Research Center, Second Military Medical University (Shanghai, China). The mice were 4—5 weeks old at the time of vaccination. All of the animals were housed under controlled conditions at the ABSL-3 facility of Wuhan University (Wuhan, China). The animals were managed by using welfare animal practices.

Animal immunization

Fifty-four female BALB/c mice were randomly divided into 6 groups: (1) phosphate-buffered saline—Tween-80 (PBST) group; (2) BCG group; (3) rBCG-361 group; (4) rBCG-E group; (5) rBCG-A group; and (6) rBCG-AE group. The mice were immunized subcutaneously with either BCG or rBCG vaccine at a dose of 5×10^6 colony-forming units (CFUs), which was suspended in PBST (0.01M PBS containing 0.05% Tween-80, V/V) (Sigma, St. Louis, MO) in a volume of 0.1 mL. The control animals (i.e., PBST group) were sham-immunized with PBST only. All animals were immunized once. The protective experiment was performed at 10 weeks after vaccination.

Challenge infection

Ten weeks after immunization, the mice (nine mice per group) were infected intravenously (i.v.) via the lateral tail vein with 1×10^6 CFUs of virulent *M. tuberculosis* H37Rv. Three mice in each group were sacrificed at 3 weeks, 6 weeks, and 9 weeks after the challenge to evaluate the respective protective efficacy. The mice were weighed before being killed. The spleen and lung tissues from immunized mice were removed aseptically. One-half of the spleen (v/v) and the right lung lobe from each mouse were used for culture to determine the *M. tuberculosis* loads in the tissues. The left lung and the rest half spleen tissues were used for acid-fast staining, hematoxylin and eosin (H&E) staining, and evaluated by light microscopy.[19]