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ORIGINAL ARTICLE

Recombinant tuberculosis vaccine AEC/BC02 induces antigen-specific cellular responses in mice and protects guinea pigs in a model of latent infection

Jin-biao Lu [a,c], Bao-wen Chen [a,c], Guo-zhi Wang [a], Li-li Fu [b], Xiao-bin Shen [a], Cheng Su [a], Wei-xin Du [a], Lei Yang [a], Miao Xu [a,*]

[a] *Division of Tuberculosis Vaccines, National Institutes for Food and Drug Control, Number 2, Tiantan Xili, Beijing 100050, PR China*
[b] *School of Public Health, Wenzhou Medical College, Wenzhou, Zhejiang 325000, PR China*

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Purpose: To preliminarily evaluate the immunogenicity and efficacy of the recombinant tuberculosis vaccine AEC/BC02 in which Ag85b and fusion protein ESAT6-CFP10 were combined with bacillus Calmette-Guérin CpG and an aluminum salt-based adjuvant system.
Methods: Groups of BALB/c mice were immunized intramuscularly three times at 10-day intervals with AEC/BC02 or the adjuvant alone and the vaccine-induced cell-mediated immune responses were evaluated. The efficacy of AEC/BC02 was evaluated in two guinea pig models, one a model of prevention and the other a model of latent infection.
Results: The AEC/BC02 vaccine induced strong cellular immune responses characterized by a high frequency of antigen-specific interferon-γ-secreting T cells in mice at different time points after the last vaccination. In the preventive model of guinea pig, AEC/BC02 did not protect against *Mycobacterium tuberculosis* as a pre-exposure vaccine. However, in a latent infection model of guinea pig, it effectively controlled the reactivation of *M. tuberculosis* and lowered the bacterial load in the lung and spleen.

* Corresponding author. Division of Tuberculosis Vaccines, National Institutes for Food and Drug Control, Number 2, Tiantan Xili, Beijing 100050, PR China.
E-mail address: xumiaobj@126.com (M. Xu).
[c] J.L. and B.C. contributed equally to this work.

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Conclusion: These results indicate AEC/BC02 can protect against reactivation of latent infection and may function as a therapeutic vaccine.

Introduction

China ranks second in the world tuberculosis (TB) epidemic, behind only India. The World Health Organization estimated that the number of incident cases of TB in China in 2012 was 0.9—1.1 million, accounting for 12% of global cases.[1] The bacillus Calmette-Guérin (BCG) vaccine is widely used as part of China's immunization planning, but it does not control the spread of TB in adults and cannot prevent latent TB infection in individuals. Therefore, novel TB vaccines are urgently needed in China.

At present, 16 TB vaccine candidates have entered clinical trials, with seven in clinical Phase II.[2] Several new candidates are waiting to enter clinical testing. Of the 16 vaccine candidates, six are recombinant protein vaccines of *Mycobacterium tuberculosis* (Mtb)-specific proteins and novel adjuvants such as AS01 or AS02 developed by GlaxoSmithKline,[3] IC31 and CAF01 from the Statens Serum Institut,[4,5] and GLA-SE from the Infectious Disease Research Institute.[6] The new adjuvants effectively promote cellular immunity induced by the vaccine, a step thought to be key in preventing and controlling TB infection. With the importance of adjuvants for subunit vaccines, we developed a new adjuvant system, BC02, based on BCG-derived CpG and aluminum salt. Some studies have shown that, due to a synergistic effect of CpG and aluminum, the combination of CpG and aluminum can induce a stronger Th1 and Th2 immune response.[7,8] In our previous study, we demonstrated that BC02 is safe and increased both antigen-specific interleukin-12 (IL-12) secretion by peritoneal macrophages and the number of antigen-specific T cells that release interferon-γ (IFN-γ).[9] Using the new adjuvant, we used Ag85b antigen and ESAT-6/CFP-10 (EC) fusion protein to construct a new TB vaccine candidate called AEC/BC02.

The T cell-mediated immune response is a critical defense against TB. Vaccine-induced antigen-specific polyfunctional T cells are thought to effectively control Mtb infection or postpone TB onset, and expression of Th1 cytokines such as IFN-γ, tumor necrosis factor-α, and IL-12 is important.[10] However, the contribution of humoral immunity to protection is controversial.[11] Therefore, most studies on the preclinical or clinical development of new TB vaccines focus on cellular immune responses induced by vaccination.[5,12,13]

At the preclinical stage, vaccine protection of animal models is an important indicator. The ability of the vaccine to mitigate organ lesions and reduce bacterial loads as well as extend survival time in Mtb-infected animals directly reflects its efficacy. Mice are the most widely used animal models. Guinea pigs are used less frequently for evaluating the efficacy of new TB vaccines, but they could be more appropriate and more stringent for vaccine testing than mice because TB pathogenesis in guinea pigs is similar to the process in humans.[14] However, because of a limited range of immunology reagents for evaluating the immune response in guinea pigs, they might not be as suitable as mice. Nonetheless, indicators relevant to vaccine protection such as organ lesions and bacterial loads can be verified in guinea pigs.

Therefore, in this study, we measured the frequency of antigen-specific IFN-γ secretion from T cells as a representative of cellular immune response induced by the AEC/BC02 vaccine in mice. We evaluated the efficacy of AEC/BC02 in two guinea pig models, one a model of prevention and the other a model of latent infection.

Materials and methods

Mice and guinea pigs

Specific-pathogen-free (SPF) BALB/c mice (female, 6—8 weeks) and Hartley guinea pigs (200—300 g) were obtained from the Institute for Laboratory Animal Resources, National Institutes for Food and Drug Control (NIFDC). Mice were maintained under SPF conditions and guinea pigs were held under barrier conditions in a Biosafety Level III animal laboratory. All animals used in this study were treated according to the standards of animal welfare and reviewed by the Animal Care and Welfare Committee of NIFDC.

Experimental vaccine

One dose of AEC/BC02 comprised 10 μg Ag85B + 10 μg EC in 0.2 mL (for mice) or 0.5 mL (for guinea pigs) adjuvant BC02. The adjuvant BC02 per animal contained 75 μg CpG and 0.2 mg aluminum hydroxide in phosphate-buffered saline.

Bacterial strains

BCG vaccine Shanghai line (D2 PB302 line) was obtained from the Chengdu Institute of Biological Products. The challenge strain Mtb (American Type Culture Collection 35810 strain) was grown on Löwenstein—Jensen medium at 37°C for approximately 4 weeks before washing with Sauton's medium and grinding. Bacterial suspensions were measured with a McNamara turbidimetric tube assay and diluted to 1 mg/mL. The samples were stored at −70°C in aliquots.

Cellular immune response in mice

Forty-eight mice were randomly divided into two groups. One group was immunized intramuscularly three times at 10-day intervals with AEC/BC02; the other was injected with the adjuvant BC02 for a control. At 1, 2, 4, and 8 weeks after the last vaccination, IFN-γ enzyme-linked immunosorbent spot (ELISPOT) was performed according to the manufacturer's instructions (Mabtech AB, Nacka Strand, Sweden). In brief, spleen lymphocytes (2.5×10^5/well) were stimulated with Ag85b peptide pools (15 amino acids, overlapping 10 amino acids, 2 μg/mL each), EC peptide pools (15 amino acids, overlapping 10 amino acids, 3.5 μg/mL each), media (negative control), or concanavalin A (positive control) for 48 hours. Spots were quantified using an ELISPOT reader (Cellular Technology Ltd, USA). Media background was subtracted from the antigen-specific response at each time point. Meanwhile, at 4 and 8 weeks after the last vaccination, lymphoproliferation responses were measured by cell counting kit-8 (CCK-8) assays (Dojindo, Kumamoto, Japan). In brief, spleen lymphocytes (2.5×10^5/well) were stimulated with Ag85b peptide pools (15 amino acids, overlapping 10 amino acids, 2 μg/mL each), EC peptide pools (15 amino acids, overlapping 10 amino acids, 3.5 μg/mL each) or media (negative control) for 72 hours and 10 μL of CCK-8 solution was added to each well for the final 6 hours of incubation. Results were shown as stimulation index, the fold-increase in $A_{450\ nm/650\ nm}$ over the negative control.

Evaluation in guinea pig preventive model

Guinea pigs were injected three times (Fig. 1A, weeks 9, 7, and 5) with one dose of AEC/BC02 vaccine intramuscularly 2 weeks apart. Negative-control guinea pigs received three equivalent doses of normal saline, and positive-control guinea pigs were subcutaneously given a single dose of 5×10^3 colony forming units (CFU) BCG 5 weeks before challenge. Five weeks after the last vaccination, guinea pigs were challenged subcutaneously with 0.5 mL of Mtb (10^2—10^3 CFU). Six weeks after infection, all guinea pigs were killed for necropsy.

Evaluation in latent infection model

Guinea pigs were challenged subcutaneously with 0.5 mL of Mtb (10^3—10^4 CFU). At Week 2 (Fig. 1B), animals had positive EC skin tests (Supplementary Fig. 1) and began to receive isoniazid treatment (5 mg/animal) by oral gavage. The animals were treated three times a week for 4 weeks. At the 6th week after infection, guinea pigs were intramuscularly given the single dose of AEC/BC02 vaccine or adjuvant BC02 six times at 10-day intervals; the negative-control group was vaccinated with normal saline. One week after final immunotherapy, all guinea pigs were killed for necropsy.

Determination of gross pathological scores and bacterial loads

After necropsy, pathological scores for the liver, spleen, and lung were evaluated separately, as described previously[9] and the sum was the gross pathological scores. Half of the harvested spleens or lungs were ground with 3 mL sterile normal saline. Homogenates were serially diluted and plated on a modified LJ medium base. CFU were determined after 4 weeks of incubation at 37°C.

Statistical analysis

Data analysis was performed using GraphPad Prism 6.0 (GraphPad Software, USA). The one-way analysis of variance

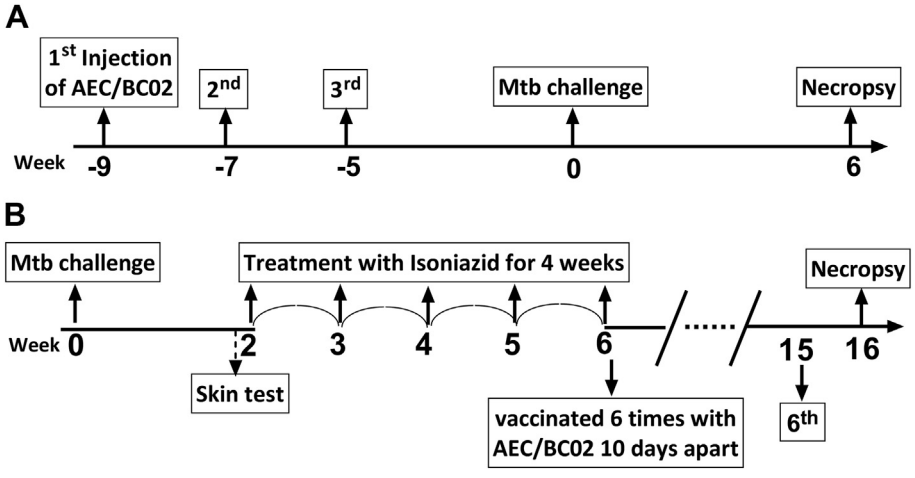

Figure 1. Timeline of evaluation in two guinea pig models. (A) Preventive model: 5 weeks after vaccination with AEC/BC02 (10 μg Ag85B and 10 μg EC in 0.5 mL BC02), animals were challenged with 10^2—10^3 colony forming units (CFU) *Mycobacterium tuberculosis* (Mtb) and killed 6 weeks later. The normal saline group was the negative-control and the bacillus Calmette-Guérin group (1 dose 5×10^3 CFU 5 weeks before challenge) was the positive control. (B) Latent infection model: 2 weeks after being challenged with 10^3—10^4 CFU Mtb, animals were treated with isoniazid (5 mg each) three times a week for 4 weeks and vaccinated with AEC/BC02 vaccine or adjuvant BC02 six times and killed 1 week after the last immunization. Negative-control guinea pigs were given normal saline. EC skin test was used to detect infection status.

followed by Tukey's multiple comparison test or *t* test was used for these studies. A p value < 0.05 was considered significant.

Results

Antigen-specific IFN-γ responses and lymphocyte proliferation

Antigen-specific IFN-γ ELISPOTs were analyzed after two or three doses of AEC/BC02. At 1 week after the last vaccination, antigen-specific IFN-γ responses were the same for both doses. After 1 week, responses in the two-dose group decreased with time; a slight increase in responses in the three-dose group was observed until the 8th week (Fig. 2A), suggesting a better response from three doses. Responses to Ag85b were stronger than responses to EC at each time point, indicating that Ag85b was more strongly immunogenic. We also performed lymphocyte proliferation assays to study antigen-specific responses in the three-dose vaccine group. At 4 and 8 weeks after the last vaccination, stimulation *in vitro* with either Ag85b or EC significantly enhanced lymphocyte proliferation in the three-dose vaccine group compared with the control group (Fig. 2B). Taken together, these data supported that AEC/BC02 vaccination induced long-term specific cellular immune responses in mice.

Evaluation in preventive model

Comparative evaluation of AEC/BC02 and current BCG, a test that would be conducted if AEC/BC02 was used as a preventive vaccine, was performed. As shown in Fig. 2, gross pathological scores for the liver, spleen, and lung in an AEC/BC02-vaccinated group were 35.8 ± 27.3, significantly lower than in a normal saline control group (62.5 ± 11.3, $p = 0.0441$) and significantly higher than a BCG-vaccinated group (5.0 ± 5.5, $p = 0.0196$). A significant difference between the BCG-vaccinated and normal saline groups was also observed ($p = 0.0001$). Bacterial loads in the spleen had a similar tendency. The AEC/BC02-vaccinated group had slightly lower bacterial counts (5.31 ± 0.91 $\log_{10}$ CFU) than the normal saline group (5.74 ± 0.47 $\log_{10}$ CFU), but the difference was not significant. No bacteria were detected in BCG-vaccinated guinea pigs. These results indicated that BCG provided strong protection against TB and AEC/BC02 had lower efficacy than BCG in this model.

Evaluation in latent infection model

Mtb-infected guinea pigs were treated with antibiotics to induce latent infection. After 4 weeks of treatment, bacterial loads were too low to be detected (Supplementary

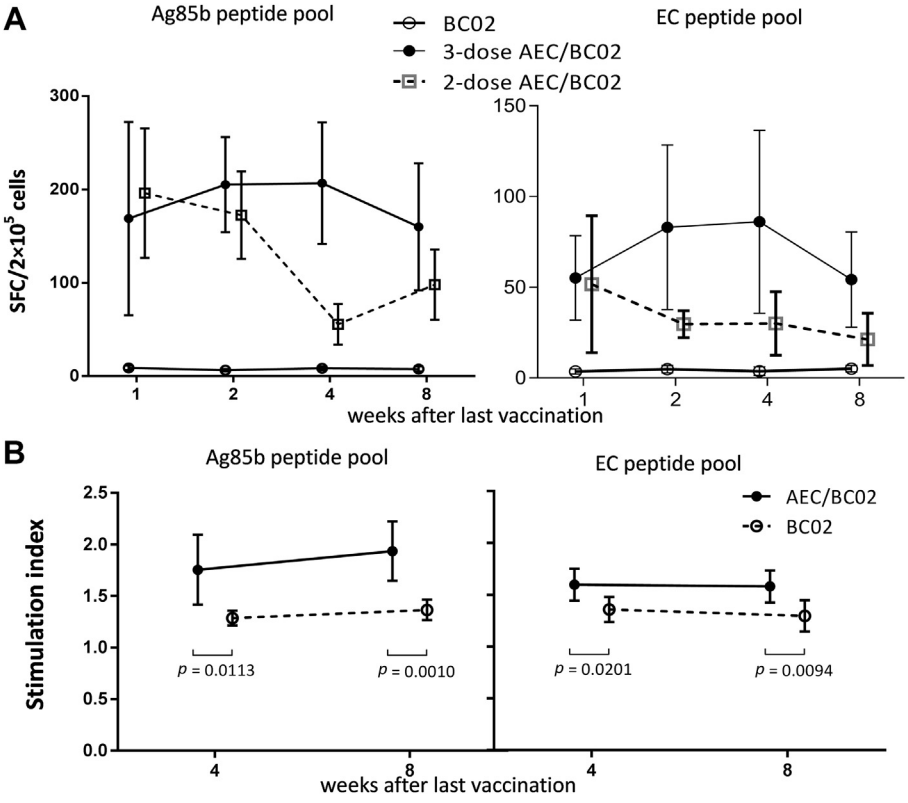

Figure 2. Antigen-specific T-cell responses after AEC/BC02 vaccination with 10-day intervals. (A) Interferon-γ enzyme-linked immunosorbent spot responses to Ag85b peptide pool and EC peptide pool [for spot-forming cells (SFCs)/2 × 10^5 cells] in mice vaccinated with two or three doses of AEC/BC02. Data are mean ± standard deviation (SD; $n = 6$/group). (B) Lymphoproliferative responses in BC02-vaccinated and AEC/BC02-vaccinated mice (3 doses) as stimulation index of lymphocytes in response to Ag85b peptide pool and EC peptide pool. The p values for BC02 and AEC/BC02 vaccination responses at each time point were evaluated with two-tailed *t* test. Data are mean ± SD ($n = 6$/group).

Fig. 2). Guinea pigs were vaccinated six times with AEC/BC02 or adjuvant BC02. In two independent experiments (Fig. 3), after AEC/BC02 immunotherapy, guinea pigs had lower gross pathological scores and fewer bacilli in both the spleen (1.78 $\log_{10}$ CFU and 2.04 $\log_{10}$ CFU reduction) and lung (1.70 $\log_{10}$ CFU and 2.29 $\log_{10}$ CFU reduction) compared with the normal saline group. Similar but lower reductions were observed in gross pathological scores and bacterial loads in the adjuvant BC02-treated group. Differences in bacterial loads between the normal saline and AEC/BC02-treated groups were significant in the first experiment ($n = 10$/group) but not in the second ($n = 8$/group), possibly because of a decrease in the number of animals and sampling error.

Discussion

In this study, we evaluated antigen-specific cell-mediated immunity in mice using IFN-γ ELISPOT assays and lymphocyte proliferation tests. Long-term cellular immune responses were induced in mice receiving either two or three vaccinations of AEC/BC02. The cellular responses were characterized by antigen-specific IFN-γ, and it was observed that three doses gave a better response than two. Other Th1 cytokine responses and the polyfunctional T-cell frequency induced by AEC/BC02 remain to be determined.

At present, BCG, the only available TB vaccine, does not protect against adult pulmonary TB. Although 10-week-old infants who receive the BCG vaccination induce mycobacterium-specific T-cell expression of Th1 cytokines such as IFN-γ and IL-2, protection by the BCG vaccine might decrease with time and not recover, even if vaccination is repeated.[11,12] Therefore, new recombinant BCG and attenuated or modified Mtb vaccine candidates have been developed and are expected to provide higher efficacy than BCG by enhancing antigen immunogenicity.[13] Thus, all new vaccine candidates should be compared with BCG as the gold standard. Much evidence indicates that BCG confers superior protective immunity against TB in mice, guinea pig, and monkey models.[5,15,16] For example, BCG maintains protection for up to 50–70 weeks in guinea pigs.[17] However, the new candidate vaccine AEC/BC02 did not have similar performance in a preventive model and did not effectively decrease either organ lesions or bacterial load in the spleen compared with BCG (Fig. 4). Thus, AEC/BC02 was not appropriate as a preventive, pre-exposure vaccine. The reason is probably that for the recombinant protein vaccine only containing several antigens it is difficult to induce sufficient protective immunity equal to that of BCG, a live bacterial vaccine containing a variety of antigens. In fact, in the clinical stage many recombinant protein vaccines against TB are not to be considered for pre-exposure prophylaxis but for other immunization strategies, such as booster after the BCG prime and immunotherapy of latent infection.[2]

An estimated one-third of the world's population, or approximately 2.2 billion people, have a latent Mtb infection and 10% of these will develop active disease.[18,19] The large population with latent infections means that vaccines will be an important preventive measure for controlling the spread of TB. Many new vaccines have been used for immunotherapy for latent infection. Evaluation of these vaccines is performed in latent-infection models, often established by infecting animals with low-dose Mtb or using Mtb-infected animals after anti-TB drug treatment. Mouse models have been the most frequently and earliest studied, with few applications of guinea pig models. Treatment of Mtb-infected guinea pigs with traditional drugs such as isoniazid and rifampin results in a rapid drop in the number of viable bacteria in the lung to nondetectable levels.[20,21] In our study, guinea pigs challenged with Mtb subcutaneously had positive EC skin tests after 12–14 days (Supplementary Fig. 1). At 4 weeks after treatment with isoniazid, EC skin tests were positive, but no bacteria were detected in the lung (Supplementary Fig. 2) and TB relapsed over time. This indicated that during the drug treatment period, a small number of Mtb were not killed and might remain latent. Therefore, after drug withdrawal, we vaccinated guinea pigs with AEC/BC02 to inhibit or delay Mtb recovery. Two independent experiments administering AEC/BC02 six times showed that Mtb in guinea pigs

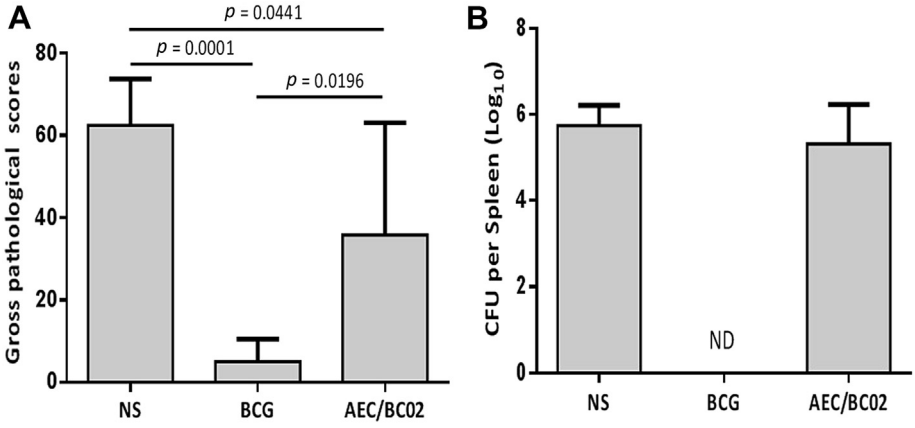

Figure 3. Efficacy of AEC/BC02 compared with bacillus Calmette-Guérin (BCG) in the preventive model ($n = 6$/group). (A) One-way analysis of variance followed by Tukey's multiple comparisons test. Data are means ± standard deviation. CFU = colony forming units; ND = no bacteria detected; NS = normal saline. A: Gross pathological scores; B: Bacterial loads in spleen.

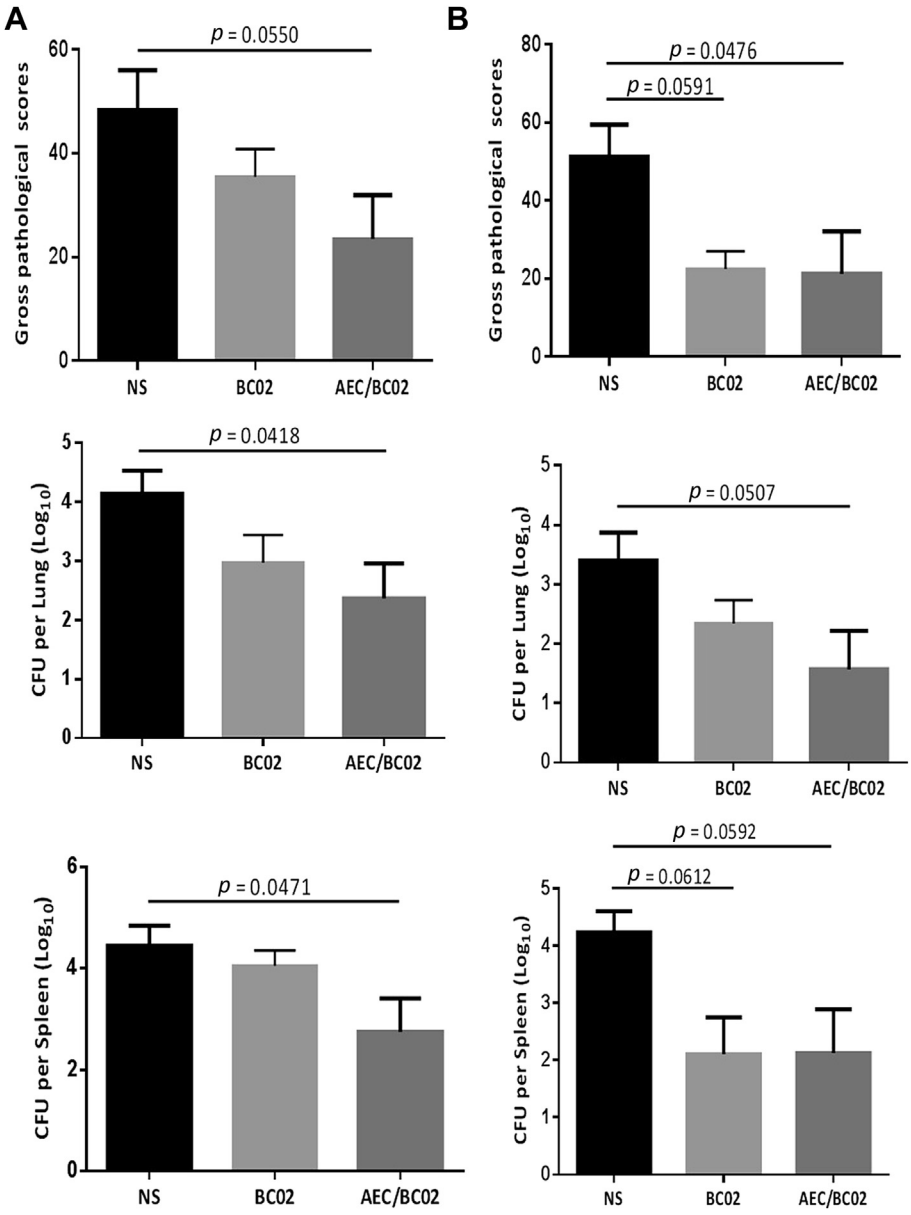

Figure 4. Evaluation of AEC/BC02 in a latent infection model. Gross pathological scores of the liver, spleen, and lung and bacterial loads in the lung and spleen were measured in two independent experiments [$n = 10$ (A) or 8 (B)/group, respectively]. Analysis was one-way analysis of variance followed by Tukey's multiple comparisons test for comparison among groups. Data are means $\pm$ standard deviation. CFU = colony forming units; NS = normal saline. A: Gross pathological scores; B: Bacterial loads in spleen.

was effectively controlled with fewer visible bacteria in the lung and spleen, and milder lesions on organs. By contrast, a saline-treated group showed increased numbers of bacteria and severe lesions on the organs. Guinea pigs treated with adjuvant BC02 also had fewer severe organ lesions and lower bacterial loads than guinea pigs treated with saline. This result might be because BCG-derived CpG is a component of BC02 and had a therapeutic effect on Mtb infection.[22,23]

It needs to be pointed out that the challenge route we performed in this study was a subcutaneous model. Although most researchers preferred the aerosol infection model, which was consistent with Mtb infection in human and more appropriate, our previous study suggested that protection of vaccines in guinea pigs could be judged indifferently in these two different challenge models[24] and subcutaneous route may be more convenient for challenging large quantities of animals. Nevertheless, in our future study, the challenge model will still require optimization to copy the natural infection.

In conclusion, we developed a subunit vaccine AEC/BC02, comprising Ag85b and EC with a new adjuvant system based on BCG-derived CpG and aluminum salt. This vaccine-induced long-term cellular immune response in mice, but is not suitable as a preventive vaccine for guinea pigs. However, it protected guinea pigs against progressive disease in a model of infection and might be used as a therapeutic vaccine.

Conflicts of interest

All authors declare no conflicts of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jmii.2014.03.005.

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