Ag85b/ESAT6-CFP10 adjuvanted with aluminum/poly-IC effectively protects guinea pigs from latent *mycobacterium tuberculosis* infection

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ABSTRACT

The high global burden of tuberculosis (TB) underscores the urgent need for an effective TB vaccine since the only licensed Bacillus Calmette-Guérin (BCG) vaccine is ineffective in preventing adult pulmonary TB and affords no protection against latent TB infection (LTBI). Herein we investigated the potential of *Mycobacterium tuberculosis* (*Mtb*) antigen proteins AEC comprised of Ag85b and ESAT6-CFP10 proteins in conjunction with aluminum (Al) and polyriboinosinic-polyribocytidylic acid (poly-IC) as a novel sub-unit vaccine against TB. The immunogenicity and protection induced by the adjuvanted vaccine were evaluated in two animal models. Mice vaccinated with AEC/Al/poly-IC exhibited significant antigen-specific humoral immune responses and cell-mediated immunity as determined by immunoassay and multicolor flow cytometric assay, and the protective effect of the vaccine was demonstrated in a guinea pig model of latent *Mtb* infection. Compared to the control group, the mean pathological scores and bacterial loads in lungs and spleens of AEC/Al/poly-IC-immunized guinea pigs were significantly reduced. These data indicate that the AEC/Al/poly-IC is highly immunogenic in mice and can effectively protect guinea pigs against latent *Mtb* infection; it may represent a promising candidate vaccine for the control of latent TB.

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1. Introduction

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (*Mtb*) complex, is one of the leading cause of death by communicable diseases worldwide [1]. There are approximately 10.0 million people infected with *Mtb* and 1.6 million TB patients died in 2017 [2]. TB affects all age groups, with most cases found in adults (aged ≥15 years), and almost two-third originated from eight countries: India, China, Indonesia, the Philippines, Pakistan, Nigeria, Bangladesh and South Africa. The global burden of TB remains high. Thus, the powerful strategies for TB epidemic control and prevention represents a global emergency.

Vaccination is the most effective means to prevent or contain infectious diseases. Bacillus Calmette-Guérin (BCG), an attenuated strain of *Mycobacterium bovis*, is currently the only licensed vaccine against TB which has been used for nearly 100 years [3–5].

However, the efficacy of BCG varies as demonstrated by different clinical trials [5–7]. Although BCG can efficiently protect children against TB, it is much less effective in protecting adolescents and adults against pulmonary TB. Moreover, BCG is ineffective in containing reactivation of latent TB infection (LTBI) [8–12]. Therefore, new vaccines should be explored to protect susceptible human populations against both naïve and latent pulmonary TB.

Currently, there are 12 TB vaccines under investigation in clinical trials, with two-thirds of them being protein-based subunit vaccines [2,13–15]. Great efforts are being made to determine which components in these subunit vaccines can induce protective immunity. Ag85b, early secretory antigen target 6 kDa protein (ESAT-6) and culture filtrate protein-10 (CFP-10) have been extensively studied, given that they are targets of T cell-mediated immunity (CMI) [16]. Ag85b is a major secreted *Mtb* protein in the Ag85 complex capable of eliciting T cell responses both in humans and mice, particularly increasing the levels of Th1 cytokines (IFN-γ) [17,18]. ESAT6 and CFP-10 are co-transcribed and secreted as heterodimers [19,20]. The two proteins are encoded by genes of the RD1 region, which is missing in the BCG strain but remains in all

pathogenic *Mtb* strains [21]. ESAT-6 induces a strong cellular immune response, with the potential to provide protection to individuals with prior TB exposure [18,22]. CFP10 can prime cytolytic CD8^{+} T cells *in vivo*, with cytolytic activity of CFP10-specific CD8^{+} T cells shown to be long-lasting following *Mtb* infection [23]. Previous studies have also shown that TB vaccines based on a mixture of culture filtrate proteins or recombinant fusion proteins could induce high levels of protective immunity [23–25]. However, these antigens only induce moderate immune responses when used alone, necessitating the use of adjuvants to enhance antigen-specific immune responses.

Aluminum and polyriboinosinic-polyribocytidylic acid (poly-IC) are two commonly used adjuvants [26]. Aluminum (Al) adjuvant is thought to facilitate interactions between vaccine components and immune cells [27], while Poly-IC, a synthetic double-stranded RNAs, could enhance immune responses through TLR3-mediated cellular pathway [28–30].

In this study, we investigated the potential of *Mtb* antigens comprised of Ag85b and ESAT6-CFP10 fusion protein (AEC) in conjunction with Al/poly-IC as a novel subunit vaccine against TB. The immunogenicity of the vaccine was studied in a mouse model, and the protection against latent *Mtb* infection was evaluated in the guinea pigs.

2. Materials and methods

2.1. Ethics statement

The animal experiments were conducted by strictly complying with the guidelines for the Care and Use of Laboratory Animals of the People's Republic of China. This study was approved by the Animal Care & Welfare Committee of National Institutes for Food and Drug Control (NIFDC). All procedures were performed under ethyl ether anesthesia; all efforts were made to minimize animal suffering.

2.2. Animals and bacteria

Female specific pathogen-free (SPF) BALB/c mice (6–8 weeks old) and Hartley guinea pigs (half male, half female) weighed 250–300 g were purchased from the Institute for Laboratory Animal Resources of NIFDC. Mice were maintained under SPF condition and guinea pigs were housed under barrier condition at Animal Biosafety Level (ABSL)-III laboratory. Animals were allowed free access to water and food.

The *Mtb* strain (ATCC 35810) used in this study was grown at 37 °C on Lowenstein Jenson medium for approximately 4 weeks. Bacterial suspension (1 mg/mL) was prepared and stored at −70 °C in aliquots.

2.3. Vaccine preparation and mouse immunization

The *Mtb* antigen proteins were provided by Anhui Zhifei Longcom Biologic Pharmacy Company (Hefei, Anhui). The mixed antigens (AEC) consisting of Ag85b and ESAT6-CFP10 proteins formulated with Al (Invivogen) or poly-IC (Sigma) or Al/poly-IC combinations were made in a biosafety levels 2 (BSL-2) laboratory of NIFDC and shipped to the study site as sterile suspensions for injection.

Thirty mice were randomly allocated into AEC/Al/poly-IC, AEC/Al, AEC/poly-IC, AEC and Al/poly-IC groups (n = 6). Of which, the latter four groups were designed as controls. Mice in AEC/Al/poly-IC group were immunized intramuscularly (i.m.) with a dose of AEC/Al/poly-IC vaccine comprised of 10 µg of Ag85b, 10 µg of ESAT6-CFP10 (EC), 0.2 mg of Al and 50 µg of poly-IC

adjuvants. Mice in AEC/Al group were immunized i.m. with a dose of 10 µg of each antigen and 0.2 mg of Al adjuvant. Mice in AEC/poly-IC group were immunized i.m. with a dose of 10 µg of each antigen and 50 µg of poly-IC adjuvant. Mice in AEC group were just inoculated i.m. with a dose of 10 µg of each antigen and mice in Al/poly-IC group were only injected i.m. with a dose of 0.2 mg of Al and 50 µg of poly-IC adjuvants. All mice were inoculated three times at 10-day intervals with a dosage volume of 0.2 mL and euthanized 7 days after the last immunization for the assessment of humoral and cellular immunogenicity of the investigated vaccine.

2.4. Enzyme-linked immunosorbent assay (ELISA)

Serum samples were collected from all immunized mice and the levels of antigen-specific total IgG and isotypes were subsequently measured by ELISA as previously described [31]. Briefly, Ag85b or EC proteins were coated on 96-well ELISA plates at 100 ng per well and incubated overnight at 4 °C. After blocking with 1% bovine serum albumin (BSA) at room temperature for 2 h and washing with PBST, serially 2-fold diluted (beginning at a 1/100 dilution) sera were added and incubated at 37 °C for 1 h. Then the plates were washed and the bound antibodies were reacted with horseradish peroxidase (HRP)-conjugated goat-anti-mouse IgG (1:10,000) (Abcam), IgG1 (1:5000) (Alpha Diagnostic International) and IgG2a (1:10,000) (Alpha Diagnostic International) at 37 °C for 1 h, followed by the addition of 3,3′,5,5′-tetramethylbenzidine. Thereafter, the reaction was stopped by adding 2 M H_2SO_4 and the absorbance was read at 450 nm with 570 nm as the reference wavelength using an ELISA plate reader (Thermo). The cut-off value was set to 2.1-fold above that of the negative control and antibody titer was expressed as reciprocal end point titer.

2.5. Isolation of splenocytes

Spleens of mice were aseptically harvested one week after the final immunization in each group. The splenic tissues were subsequently mashed using syringe plungers and passed the 300-mesh screens before the splenic lymphocytes separated from the cell suspensions using mouse 1× lymphocyte separation medium (Dakewe Biotech). Lymphocytes were enumerated and then diluted to the density of 2.5×10^6 or 5×10^6 viable cells/mL using RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS, Gibco) and 100 µg/mL of each Penicillin G and Streptomycin (Sigma).

2.6. IFN-γ Enzyme-linked immunospot (ELISPOT) assay

To quantify the number of antigen-specific IFN-γ secreting cells, an ELISPOT assay was performed using Mouse IFN-γ ELISpot[PLUS] kit (Mabtech) per the manufacturer's instruction. In brief, freshly harvested splenocytes were plated in duplicated at density of 2.5×10^6/mL in 100 µL per well and incubated in medium containing 2 µg/mL of the synthesized Ag85b or ESAT6 or CFP10 or EC peptide pools (15 amino acids, overlapping 10 amino acids) or 1 µg/mL of Con A (Sigma) or medium alone for 40 h in a 37 °C humidified incubator with 5% CO_2. Next, the biotinylated detection antibody and streptavidin-horseradish peroxidase were added and spots were developed by the addition of substrate solution at room temperature incubation in the dark. The reaction was stopped by washing the plates with deionized water, and plates were dried in the dark at room temperature. Finally, spots were quantified using an ELISPOT reader (Cellular Technology Ltd). Antigen-specific responses were background subtracted and reported as numbers of spot-forming cells (SFC) per 2.5×10^5 splenocytes.

2.7. Intracellular cytokine staining (ICS)

Antigen-specific IFN-γ/TNF-α/IL-2 responses in CD4^{+} and CD8^{+} T cells were measured using the 6-color ICS assay by flow cytometry. Briefly, freshly harvested splenocytes were seeded at density of 5×10^6/mL in 200 μL per well, which either left unstimulated (as a negative control) or stimulated with peptide pools of Ag85b, ESAT-6, CFP10 and EC, or stimulated with $1 \times$ PMA/Ionomycin mixture (as a positive control, MultiSciences). The stimulations were performed for 6 h at 37 °C in a humidified chamber with 5% CO_2 in the presence of brefeldin A (BioLegend). Cells were washed and subjected to staining surface markers with PerCP-Cy™5.5-labeled CD3e, FITC-labeled CD4 and APC-Cy7-labeled CD8a antibodies (BD Pharmingen). Then cells were further processed to stain for intracellular cytokines with PE-Cy™7-labeled IFN-γ, PE-labeled TNF-α and APC-labeled IL-2 antibodies (BD Pharmingen) after fixation and permeabilization using the Cytofix/Cytoperm™ plus Fixation/Permeabilization Kit (BD Pharmingen) according to the manufacturer's specification. Finally, samples were acquired on a BD FACSCanto™ II flow cytometer and data was subsequently analysed using FlowJo (Treestar) software (Fig. S1).

2.8. Protection of guinea pigs in the latent Mtb infection model

To determine the protective effect of the adjuvanted vaccine, we employed the guinea pigs latently infected by *Mtb*. Briefly, twenty-four guinea pigs were randomly divided into three groups (n = 8): AEC/Al/poly-IC, Al/poly-IC and PBS. Then the LTBI animal model was developed as previously described [32,33]. Each LTBI animal in above-mentioned three groups was inoculated with one dose of AEC/Al/poly-IC (referenced as mice immunization), Al/poly-IC (referenced as mice immunization) and PBS six times at 10-day intervals, respectively (Fig. 1). Finally, all animals were sacrificed for lung and spleen lesion scores and bacterial enumeration at 4 weeks after the last vaccination.

2.9. Lesion scoring and bacterial enumeration

The lesions of lung and spleen organs obtained from guinea pigs were evaluated by a previously reported method [34]. Meanwhile, half of the harvested lungs and spleens were ground with 3 mL sterile normal saline, followed by serial dilution and plating on Lowenstein Jenson medium at approximately 4 weeks at 37 °C for CFU determination.

2.10. Statistical analysis

Statistical analyses were conducted by the GraphPad Prism software (version 7.0), and the statistical significance of the difference between two groups was performed using the *t*-test, with one-way ANOVA for the analysis of differences between multiple groups. Differences were considered to be significant at *$p < 0.05$ or **$p < 0.01$.

3. Results

3.1. Antibodies induced by AEC/Al/poly-IC in mice

TB antigen-specific antibodies were measured in mice injected with AEC/Al/poly-IC, AEC/Al, AEC/poly-IC, AEC or Al/poly-IC one week after the last immunization. As shown in Fig. 2A, the
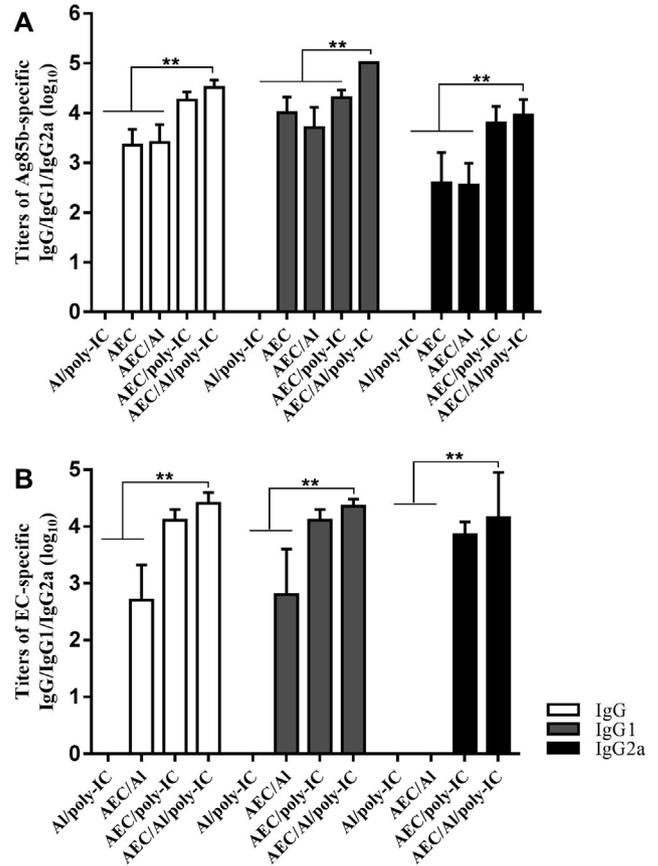

Fig. 2. Total IgG, IgG1 and IgG2a antibody titers induced in each group. (A), Ag85b-specific IgG, IgG1 and IgG2a in Al/poly-IC-, AEC-, AEC/Al-, AEC/poly-IC- and AEC/Al/poly-IC-immunized mice were determined by ELISA 7 days after the last immunization. (B), EC-specific IgG, IgG1 and IgG2a in Al/poly-IC-, AEC/Al-, AEC/poly-IC- and AEC/Al/poly-IC-immunized mice were determined by ELISA 7 days after the last immunization. All animals in the Al/poly-IC group were seronegative for Ag85b and EC. Statistical significance was set at **$p < 0.01$.
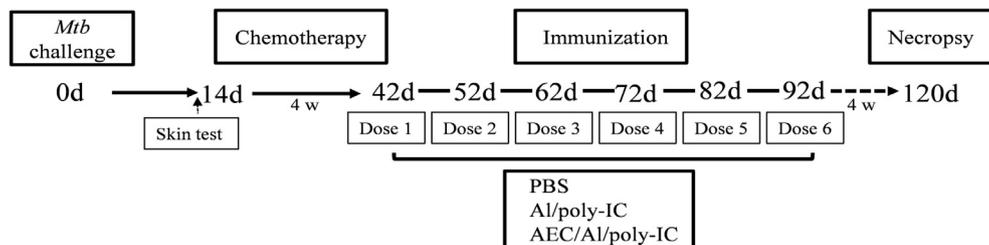

Fig. 1. Vaccination schedule for the potential candidate evaluation in *Mtb* latently infected guinea pig model. Guinea pigs were randomly divided into three groups and subcutaneously challenged with 5×10^3 CFU of *Mtb*, followed by antibiotic (isoniazid, 10 mg/animal) treatment at three times a week for 4 weeks after EC skin testing positive (2 weeks after challenge). Animals in the three groups were separately inoculated with one dose of AEC/Al/poly-IC, Al/poly-IC and PBS six times at 10-day intervals and euthanized 4 weeks post the last immunization for organs lesion scores and bacterial enumeration.

Ag85b-specific IgG, IgG1 and IgG2a were detectable in the sera of all the vaccinated groups, and the highest titers of IgG (4.51 ± 0.16) and IgG2a (3.99 ± 0.34) were induced in the AEC/Al/poly-IC group, with statistically significant difference between the AEC/Al/poly-IC group and AEC ($p < 0.01$) or AEC/Al ($p < 0.01$) control groups. Furthermore, there is significant difference between the AEC/Al/poly-IC group and other vaccinated control groups ($p < 0.01$), with AEC/Al/poly-IC elicited the highest titer of IgG1 (5.01 ± 0.00) in the mice.

As shown in Fig. 2B, AEC/Al/poly-IC immunization also induced the highest levels of the EC-specific IgG (4.41 ± 0.19), IgG1 (4.36 ± 0.12) and IgG2a (4.11 ± 0.33), with statistical difference between the AEC/Al/poly-IC group and AEC/Al control group ($p < 0.01$). It is of note that the titers of IgG, IgG1 and IgG2a in the sera of AEC-immunized mice and the titer of IgG2a in the sera of AEC/Al-immunized mice were undetectable 7 days after the last vaccination (Fig. 2B). Meanwhile, as expected, no Ag85b- and EC-specific antibody titers were detected in the adjuvant control group (Al/poly-IC). Collectively, these data indicate that the combination of the two adjuvants (Al/poly-IC) is more effective in inducing antigen-specific humoral immune responses than the two individual adjuvants when used alone.

3.2. IFN-γ-secreting cell response elicited by AEC/Al/poly-IC in mice

We next investigated cellular immune responses induced by AEC/Al/poly-IC. To this end, splenocytes of immunized mice were harvested one week after the last vaccination, with the numbers of antigen-specific IFN-γ-secreting cells quantified by ELISPOT. As shown in Fig. 3, the highest numbers of antigen-specific IFN-γ-secreting cells were found in AEC/Al/poly-IC group, with statistical significance observed between AEC/Al/poly-IC and other groups in the numbers of Ag85b-, ESAT6- and CFP10-specific IFN-γ-secreting cells (Fig. 3A–C); the same observation was made with regards to the numbers of EC-specific IFN-γ-secreting cells (Fig. 3D). Taken together, these results demonstrate that the AEC/Al/poly-IC vaccine has the capacity of eliciting strong antigen-specific CMI in mice.

3.3. Functional CD4+ and CD8+ T-cell responses induced by AEC/Al/poly-IC in mice

Experiments were then conducted to determine the types of cells-expressing Th1 cytokines including IFN-γ, TNF-α and IL-2 using ICS. Fig. 4 presents percentages of CD4+ (panel A) and CD8+
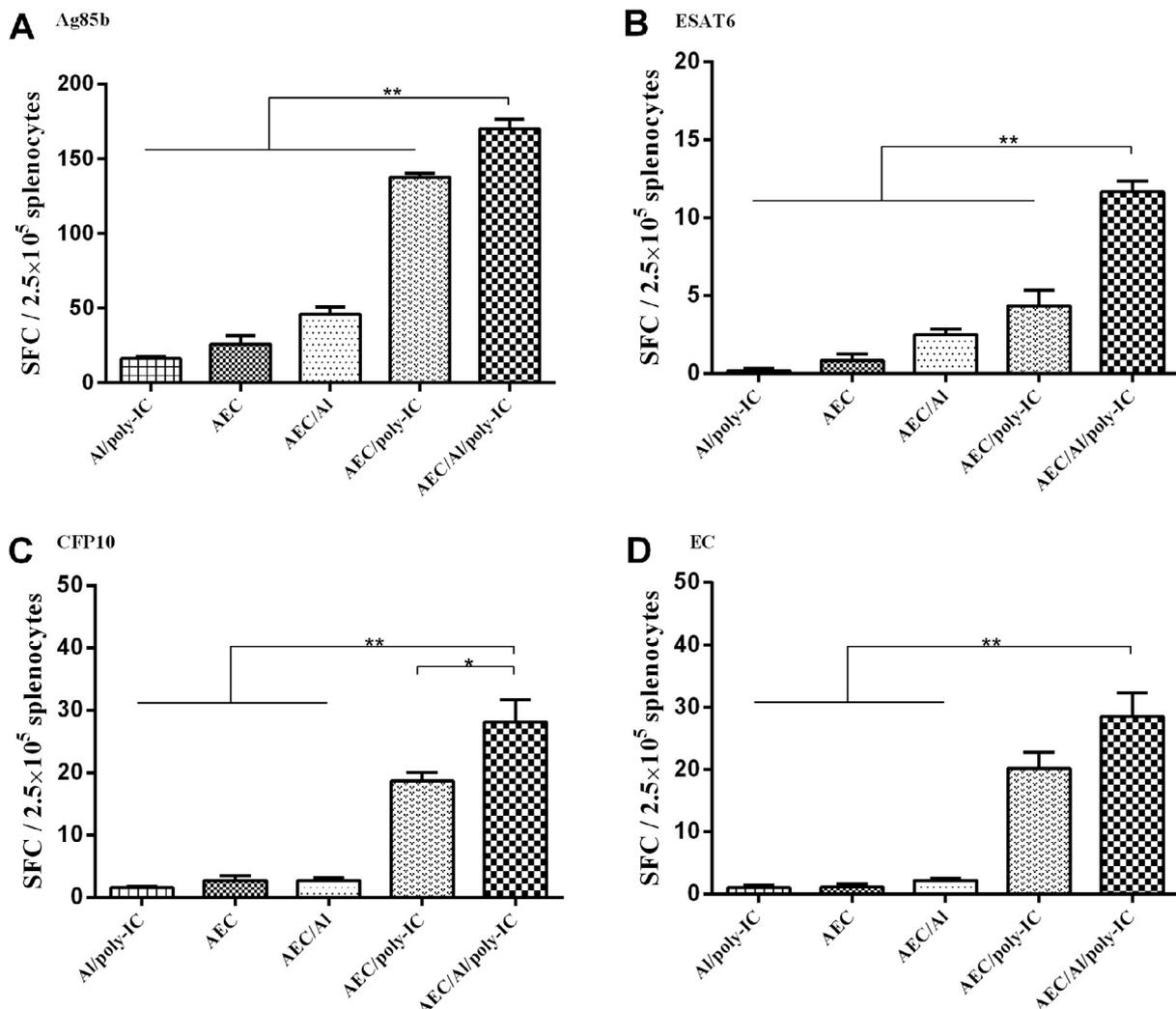

Fig. 3. The magnitude of IFN-γ-secreting lymphocyte responses in mice was determined by ELISPOT. Splenocytes isolated from Al/poly-IC-, AEC-, AEC/Al-, AEC/poly-IC- and AEC/Al/poly-IC-immunized mice were evaluated for antigen-specific IFN-γ secretion one week after the last vaccination. Data are expressed as spot-forming cells (SFC) per 2.5×10^5 splenocytes with antigen specificity to Ag85b (A), ESAT6 (B), CFP10 (C) and EC (D) peptides and presented as mean ± SEM. Statistical significance was set at *$p < 0.05$ or **$p < 0.01$.
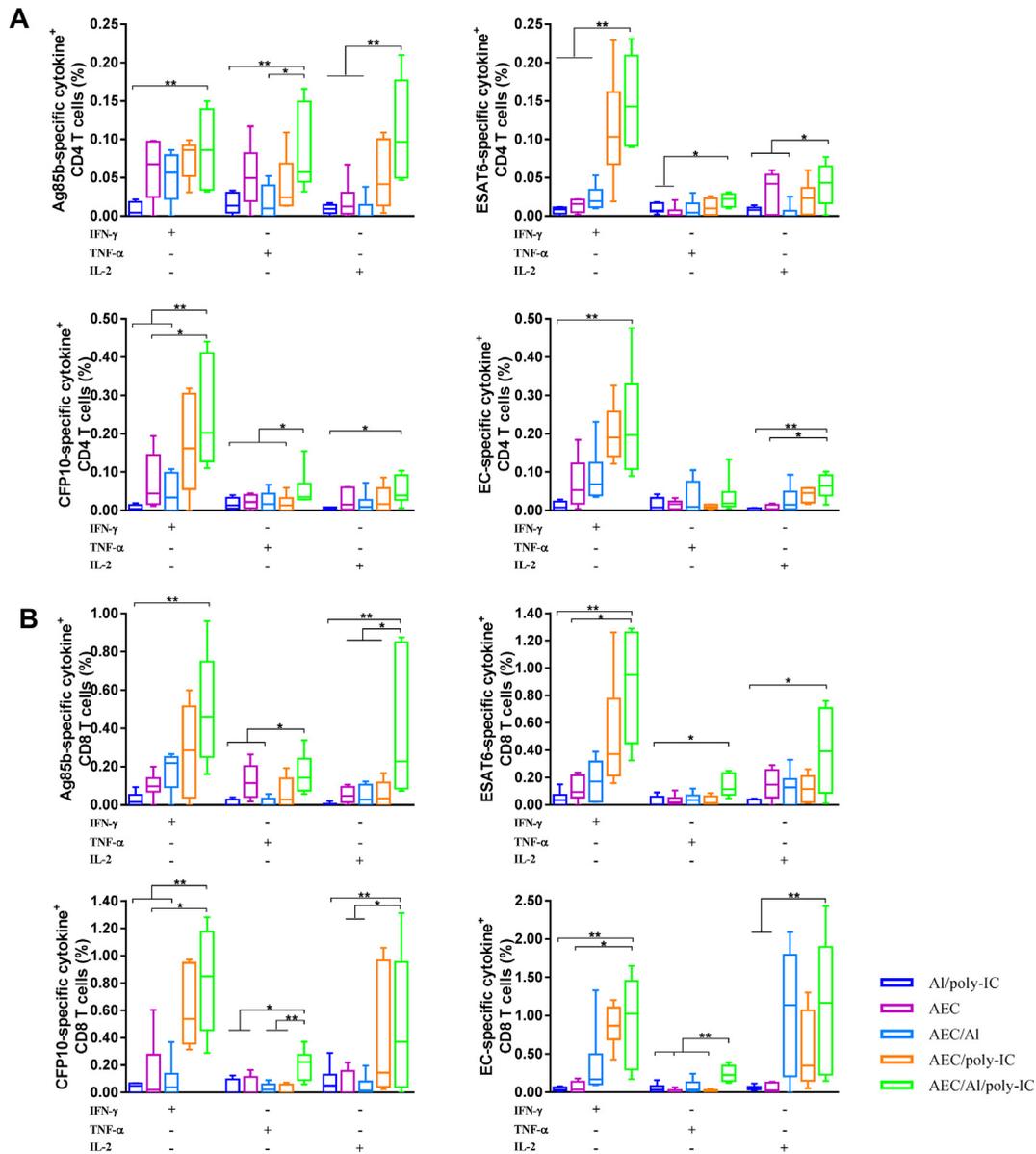
Fig. 4. The magnitude of antigen-specific T lymphocyte responses in mice was determined by ICS. Specific CD4$^+$ T cells (A) and CD8$^+$ T cells (B) of Al/poly-IC-, AEC-, AEC/Al-, AEC/poly-IC- and AEC/Al/poly-IC-immunized mice were determined for cytokine expression, including IFN-γ, TNF-α or IL-2 following stimulation with Ag85b, ESAT6, CFP10 or EC peptides *in vitro*. The percentages of IFN-γ$^+$, TNF-α$^+$, or IL-2$^+$ CD4 and CD8 T cells in the unstimulated control were subtracted. Medians and interquartile ranges are shown with the boxplots. The differences within groups were assessed by Kruskal-Wallis test. Statistical significance was set at *$p < 0.05$ or **$p < 0.01$.

(panel B) T cells producing intracellular cytokines after stimulation with Ag85b, ESAT6, CFP10 or EC peptides. Compared with the baseline level of CD4$^+$and CD8$^+$ T cell responses in adjuvant control group (Al/poly-IC), vaccination resulted in increased CD4$^+$ and CD8$^+$ responses, with the AEC/Al/poly-IC immunization elicited the strongest antigen-specific CD4$^+$and CD8$^+$ T cell responses. With respect to the total individual cytokine expression, CD4$^+$ T cells were mainly expressing IFN-γ, but little TNF-α or IL-2 unless Ag85b used for stimulation, while CD8$^+$ T cells were found to producing both total IFN-γ and IL-2 but little TNF-α. Overall, the induction of antigen-specific total IFN-γ or TNF-α or IL-2-producing CD8$^+$ T cells was greater than that with CD4$^+$ T cells. Importantly, statistical analysis revealed that AEC/Al/poly-IC immunization resulted in the largest numbers of antigen-specific CD4$^+$ or CD8$^+$ T cells producing Th1 cytokines compared to other control groups (Fig. 4).

Given the T cells capable of producing multiple cytokines (bifunctional or polyfunctional T cells) are a better indicator of cell-mediated immune response than single cytokine-secreting T cell, we further investigated the levels of polyfunctional T cells in mice injected with the vaccine. As shown in Fig. S2A, AEC/Al/poly-IC-induced antigen-specific cellular responses were predominantly comprised of bifunctional CD4$^+$ (TNF-α$^+$IL-2$^+$, IFN-γ$^+$TNF-α$^+$, IFN-γ$^+$IL-2$^+$) (Fig. S2A) and CD8$^+$ (IFN-γ$^+$TNF-α$^+$, IFN-γ$^+$IL-2$^+$) (Fig. S2B) T cell subsets. Furthermore, the AEC/Al/poly-IC immunization was also capable of eliciting antigen-specific polyfunctional CD4$^+$ and CD8$^+$ (IFN-γ$^+$TNF-α$^+$IL-2$^+$) T cell responses. Taken together, these data demonstrate that the AEC/Al/poly-IC immunization induces strong cell mediated immune responses in mice.

3.4. Protection of latently infected guinea pigs immunized with AEC/Al/poly-IC

Having established that AEC/Al/poly-IC was most effective in inducing humoral and cellular immune responses in mice, we next
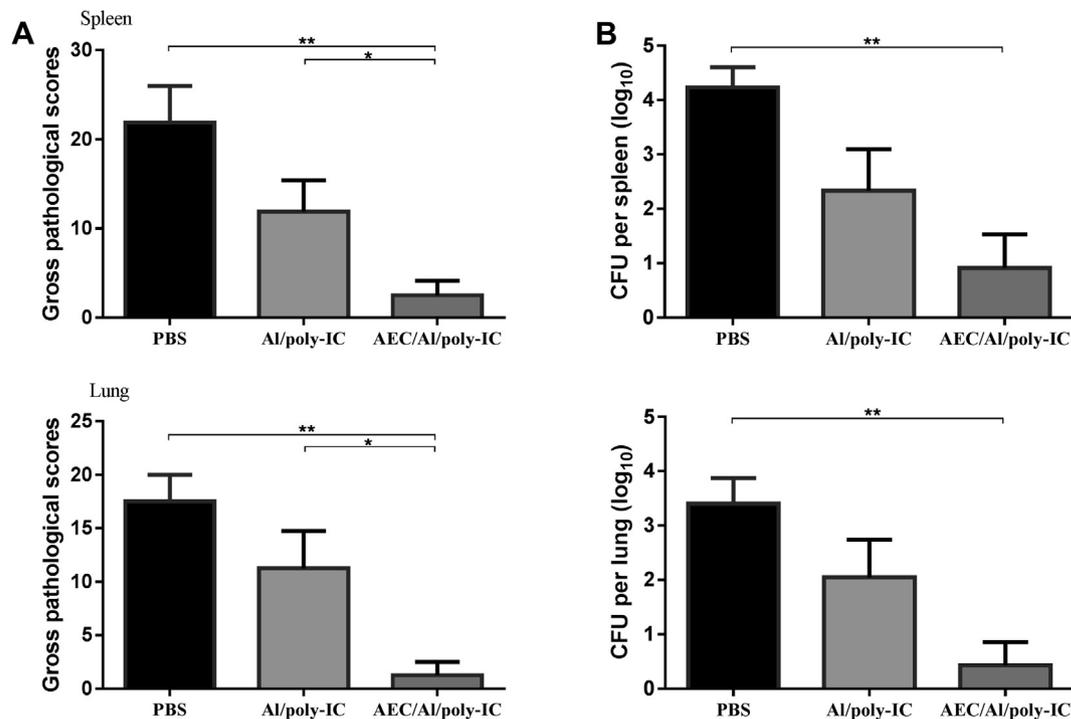
Fig. 5. Protection of the latently infected guinea pigs immunized with AEC/Al/poly-IC. The latently infected guinea pigs received PBS, Al/poly IC or AEC/Al/poly-IC immunizations were euthanized at 4 weeks post the last immunization. The gross pathological scores (A) and the bacterial loads (B) in the spleens and lungs of each guinea pig were determined. With regard to lesion scoring, gross pathological scores of spleen were graded as 35 (heavy), 20 (moderate), 10 (mild) and 0 (no lesions) and those of lung were graded as 30 (heavy), 20 (moderate), 10 (mild) and 0 (no lesions) based on the number and size of tubercles and areas of necrosis [34]. Data were shown as mean ± SEM. Statistical significance was set at $^*p < 0.05$ or $^{**}p < 0.01$.

investigate if AEC/Al/poly-IC vaccination could protect guinea pigs in latent *Mtb* infection model. To this end, guinea pigs latently infected with *Mtb* were injected with AEC/Al/poly-IC or controls with no antigens (Al/poly-IC and PBS). Protection was evaluated based on lesion scoring and bacterial enumeration 4 weeks after the last vaccination. As shown in Fig. 5, compared with the controls, gross pathological scores in spleens and lungs were the lowest in AEC/Al/poly-IC-immunized guinea pigs (Fig. 5A). Statistically significant differences were found in the pathological scores for both of spleen and lung between AEC/Al/poly-IC and PBS ($p < 0.001$; $p = 0.001$) or Al/poly-IC groups ($p = 0.032$; $p = 0.029$). Moreover, AEC/Al/poly-IC-induced protection is further supported by enumeration of the mycobacteria in the affected tissues. Specifically, the bacterial burdens of lungs and spleens were drastically reduced in AEC/Al/poly-IC-immunized guinea pigs, with fewer bacterial loads in both the spleen ($0.92 ± 1.74$) and lung ($0.43 ± 1.21$), compared with that either in PBS ($4.23 ± 1.06$; $3.40 ± 1.33$) or Al/poly IC ($2.33 ± 2.16$; $2.05 ± 1.97$) treated animals (Fig. 5B). The differences in bacterial enumeration between the AEC/Al/poly-IC and PBS groups are statistically significant, with p values being 0.004 (spleen) and 0.006 (lung). Taken together, our findings indicate that AEC/Al/poly-IC vaccination affords effective protection of guinea pigs against latent *Mtb* infection.

4. Discussion

Tuberculosis remains a severe threat to public health throughout the world. Millions of people suffer from this serious infectious disease every year [2,35]. Given the questionable effectiveness of BCG in protecting health adults and those with latent TB reactivation, new forms of vaccines should be explored. Here, AEC adjuvanted with Al/poly-IC was investigated. Our data showed that the AEC/Al/poly-IC vaccine induced robust humoral and cellular

immune responses in mice and afforded effective protection of guinea pigs latently infected with *Mtb*.

Previous studies suggested that humoral immune response was less relevant for the protective immunity to *Mtb*, given that *Mtb*, an intracellular pathogen, replicates predominantly inside macrophage. However, recent studies indicate that antibody response plays an important role in protecting against *Mtb* infection [36,37], for example, the protective functions *in vitro* of LTBI IgG, including antibody-dependent cellular phagocytosis and cytotoxicity associated with IgG Fc-FcγR interactions, have been demonstrated [38]. Here, we found that all AEC/Al/poly-IC-immunized mice were seropositive, and the levels of Ag85b and EC-specific IgG, IgG1 and IgG2a antibodies induced by AEC/Al/poly-IC were significantly higher than those elicited by AEC/BC02 (a subunit vaccine consisted of AEC proteins and BC02 adjuvant, data unpublished). We infer that the difference in antibody titers between these two vaccines might result from the effects of different adjuvant components. The adjuvant effects of Al/poly-IC might make more contributions than BC02 (Al/BCG-derived CpG), as poly-IC is well known to enhance the production of antibodies *in vivo* [28]. Moreover, the two vaccines consistently elicit an Ag85b-specific Th2 type response as demonstrated by the IgG2a/IgG1 ratio (<1), which is likely due to the Al in the adjuvant mixture [39,40]. Additionally, a balanced EC-specific Th1/Th2 type response has been found with the value of IgG2a/IgG1 closing to 1, suggesting the EC-specific cellular-mediated and antibody-mediated immunity act synergistically in AEC/Al/poly-IC-vaccinated mice. Nevertheless, an EC-specific Th1 type response had been induced by AEC/BC02, which likely resulted from the effect of Th1-biased CpG. Further study is needed to elucidate the mechanisms involving adjuvant components and the protective effects of Ag85b- or EC-specific antibodies in both AEC/BC02 and AEC/Al/poly-IC vaccinations.

It has been well documented that CMI plays critical roles in protection of humans and animals against *Mtb* infections [41,42]. Consistent with previous studies, we observed elevated levels of antigen-specific Th1-type cellular immune response in mice [17,18]. AEC/Al/poly-IC was shown to induce strongest Ag85b-, ESAT6-, CFP10- and EC-specific IFN-γ-secreting cell responses among each group detected by ELISPOT. The levels of these responses in AEC/Al/poly-IC-immunized mice were comparable to those in AEC/BC02-immunized mice reported in a previous study [32]. Furthermore, AEC/Al/poly-IC was also shown to induce strongest antigen-specific Th1-type CD4$^+$ T cell response among each group detected by ICS. This is also true with respect to stimulation of CD8$^+$ T cells which is also known to be important in containing *Mtb* infection, especially the latent *Mtb* infection [23,43,44]. Notably, AEC/Al/poly-IC vaccination predominantly induced CD8$^+$ T cell response, which is different from other subunit vaccines, such as H56:CAF01 vaccination in mice [45], adjuvanted M72 vaccination in TB-naïve (M72/AS01, M72/AS02, M72F/AS02 and M72/AS01$_E$) or TB-treatment (LTBI, M72/AS01$_E$) cohorts [46,47]. It is likely that the difference among these vaccines may be due to the difference in their compositions. Nevertheless, it is noted that balanced CD4$^+$/CD8$^+$ T cell response was found in M72 vaccines. Moreover, the magnitude of the increased Th1-type CD4$^+$/CD8$^+$ T cell response elicited by AEC/Al/poly-IC cannot be compared with that of AEC/BC02, given relevant data of the later one is unreported. Additionally, AEC/Al/poly-IC was shown to induce significant antigen-specific IFN-γ$^+$TNF-α$^+$ or IFN-γ$^+$IL-2$^+$ or TNF-α$^+$IL-2$^+$ bi-functional CD4$^+$ and CD8$^+$ T cell responses, suggesting that both the short-lived and long-lived memory Th1 responses have been established after AEC/Al/poly-IC vaccination, as the IFN-γ$^+$TNF-α$^+$ cells are known to be effector memory T cells and the TNF-α$^+$IL-2$^+$ cells are central memory T cells [48,49].

The ideal TB vaccines with good immunogenicity would prevent or control *Mtb* infection. The protective capacity of AEC/Al/poly-IC was evaluated in both preventive and LTBI animal models. It is found that AEC/Al/poly-IC is not suitable for pre-exposure prophylaxis since no difference was found between AEC/Al/poly-IC and BCG with regard to organ lesions or bacterial loads in the spleen (data not shown), which is in line with previous findings to the preventive effect of AEC/BC02 [32]. However, AEC/Al/poly-IC is appropriate for preventive therapy for LTBI as AEC/BC02, H56: CAF01 and M72/AS01$_E$ vaccines. The AEC/Al/poly-IC-immunized guinea pigs were effectively protected against LTBI at 4 weeks after the last vaccination, with the protective effects of AEC/Al/poly-IC clearly better than that of AEC/BC02 and H56:CAF01 as demonstrated by the reduced bacterial loads in organs. It is mostly likely that the large production of antibodies and the robust Th1-type CD8$^+$ T cell elicited by AEC/Al/poly-IC immunization led to containment of latent *Mtb* infection, largely in agreement with previous observations that the control of latent *Mtb* infection may be largely dependent on CD8$^+$ T cells [50]. These speculations need to be further clarified. It is of note that the AEC/Al/poly-IC vaccination was only evaluated 4 weeks later, where a strong effector response could play a role in containing the infection, future studies should be considered to evaluate the effect of AEC/Al/poly-IC for a longer period. Additionally, M72/AS01$_E$ affords 54% protection for LTBI adults in a randomized, double-blind, placebo-controlled, phase 2b trial [51]. Further studies are needed for the evaluation of similar candidate subunit vaccines, including AEC/Al/poly-IC.

In summary, we demonstrated that AEC adjuvanted by combined Al and poly-IC is more effective than either adjuvant alone in eliciting robust antigen-specific humoral immune response and Th1-type CD4$^+$ and CD8$^+$ T cell responses in mice; importantly, AEC/Al/poly-IC also afforded effective protection of guinea pigs latently infected by *Mtb*.

Declaration of Competing Interest

The authors declare no financial or commercial conflict of interest.

Acknowledgements

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2019.06.078.

References

[1] Naghavi M, Abajobir AA, Abbafati C, Abbas KM, Abd-Allah F, Abera SF, et al. Global, regional, and national age-sex specific mortality for 264 causes of death, 1980–2016: a systematic analysis for the Global Burden of Disease Study 2016. Lancet 2017;390(10100):1151–210.

[2] World Health Organization. Global tuberculosis report 2018, http://www.who.int/tb/publications/global_report/en/; 2018 [accessed 18 September 2018].

[3] Chen L, Xu M, Wang ZY, Chen BW, Du WX, Su C, et al. The development and preliminary evaluation of a new Mycobacterium tuberculosis vaccine comprising Ag85b, HspX and CFP-10:ESAT-6 fusion protein with CpG DNA and aluminum hydroxide adjuvants. FEMS Immunol Med Microbiol 2010;59(1):42–52.

[4] Brewer TF, Colditz GA. Relationship between bacille Calmette-Guérin (BCG) strains and the efficacy of BCG vaccine in the prevention of tuberculosis. Clin Infect Dis 1995;20(1):126–35.

[5] Colditz GA, Brewer TF, Berkey CS, Wilson ME, Burdick E, Fineberg HV, et al. Efficacy of BCG vaccine in the prevention of tuberculosis. Meta-analysis of the published literature. JAMA 1994;271(9):698–702.

[6] Fine PE. Variation in protection by BCG: implications of and for heterologous immunity. Lancet 1995;346(8986):1339–45.

[7] Ginsberg AM. The tuberculosis epidemic: scientific challenges and opportunities. Public Health Rep 1998;113(2):128–36.

[8] Trunz BB, Fine P, Dye C. Effect of BCG vaccination on childhood tuberculous meningitis and miliary tuberculosis worldwide: a meta-analysis and assessment of cost-effectiveness. Lancet 2006;367(9517):1173–80.

[9] Roy A, Eisenhut M, Harris RJ, Rodrigues LC, Sridhar S, Habermann S, et al. Effect of BCG vaccination against Mycobacterium tuberculosis infection in children: systematic review and meta-analysis. BMJ 2014;349:g4643.

[10] Abubakar I, Pimpin L, Ariti C, Beynon R, Mangtani P, Sterne JA, et al. Systematic review and meta-analysis of the current evidence on the duration of protection by bacillus Calmette-Guerin vaccination against tuberculosis. Health Technol Assess 2013;17(37):1–372.

[11] Colditz GA, Berkey CS, Mosteller F, Brewer TF, Wilson ME, Burdick E, et al. The efficacy of bacillus Calmette-Guerin vaccination of newborn and infants in the prevention of tuberculosis: meta-analyses of the published literature. Pediatrics 1995;96(1):29–35.

[12] Mangtani P, Abubakar I, Ariti C, Beynon R, Pimpin L, Fine PE, et al. Protection by BCG vaccine against tuberculosis: a systematic review of randomized controlled trials. Clin Infect Dis 2014;58(4):470e80.

[13] Gong W, Liang Y, Wu X. The current status, challenges, and future developments of new tuberculosis vaccines. Hum Vaccin Immunother 2018;14(7):1697–716.

[14] Zhu B, Dockrell HM, Ottenhoff THM, Evans TG, Zhang Y. Tuberculosis vaccines: opportunities and challenges. Respirology 2018;23(4):359–68.

[15] Kaufmann SH, Weiner J, von Reyn CF. Novel approaches to tuberculosis vaccine development. Int J Infect Dis 2017;56:263–7.

[16] Orme IM. Induction of nonspecific acquired resistance and delayed-type hypersensitivity, but not specific acquired resistance in mice inoculated with killed mycobacterial vaccines. Infect Immun 1988;56(12):3310–2.

[17] Horwitz MA, Lee BW, Dillon BJ, Harth G. Protective immunity against tuberculosis induced by vaccination with major extracellular proteins of Mycobacterium tuberculosis. Proc Natl Acad Sci USA 1995;92(5):1530–4.

[18] Weinrich Olsen A, van Pinxteren LA, Meng Okkels L, Birk Rasmussen P, Andersen P. Protection of mice with a tuberculosis subunit vaccine based on a fusion protein of antigen 85b and esat-6. Infect Immun 2001;69(5):2773–8.

[19] Berthet FX, Rasmussen PB, Rosenkrands I, Andersen P, Gicquel B. A Mycobacterium tuberculosis operon encoding ESAT-6 and a novel low-molecular-mass culture filtrate protein (CFP-10). Microbiology 1998;144(Pt11):3195–203.

[20] Renshaw PS, Panagiotidou P, Whelan A, Gordon SV, Hewinson RG, Williamson RA, et al. Conclusive evidence that the major T-cell antigens of the Mycobacterium tuberculosis complex ESAT-6 and CFP-10 form a tight, 1:1 complex and characterization of the structural properties of ESAT-6,

CFP-10, and the ESAT-6*CFP-10 complex. J Biol Chem 2002;277 (24):21598–603.

[21] Pym AS, Brodin P, Brosch R, Huerre M, Cole ST. Loss of RD1 contributed to the attenuation of the live tuberculosis vaccines Mycobacterium bovis BCG and Mycobacterium microti. Mol Microbiol 2002;46:709–17.

[22] Hoang T, Aagaard C, Dietrich J, Cassidy JP, Dolganov G, Schoolnik GK, et al. TB10.4 (EsxH) based vaccines for pre- and post-exposure tuberculosis vaccination. PLoS One 2013;8(12):e80579.

[23] Kamath AB, Woodworth J, Xiong X, Taylor C, Weng Y, Behar SM. Cytolytic CD8+ T cells recognizing CFP10 are recruited to the lung after Mycobacterium tuberculosis infection. J Exp Med 2004;200(11):1479–89.

[24] Andersen P. Effective vaccination of mice against Mycobacterium tuberculosis infection with a soluble mixture of secreted mycobacterial proteins. Infect Immun 1994;62(6):2536–44.

[25] Roberts AD, Sonnenberg MG, Ordway DJ, Furney SK, Brennan PJ, Belisle JT, et al. Characteristics of protective immunity engendered by vaccination of mice with purified culture filtrate protein antigens of Mycobacterium tuberculosis. Immunology 1995;85:502–8.

[26] Pashine A, Valiante NM, Ulmer JB. Targeting the innate immune response with improved vaccine adjuvants. Nat Med 2005;11(4 Suppl):S63–8.

[27] O'Hagan DT, Valiante NM. Recent advances in the discovery and delivery of vaccine adjuvants. Nat Rev Drug Discov 2003;2(9):727–35.

[28] Kumar H, Koyama S, Ishii KJ, Kawai T, Akira S. Cutting edge: cooperation of IPS-1- and TRIF-dependent pathways in poly IC-enhanced antibody production and cytotoxic T cell responses. J Immunol 2008;180(2):683–7.

[29] Stahl-Hennig C, Eisenblätter M, Jasny E, Rzehak T, Tenner-Racz K, Trumpfheller C, et al. Synthetic double-stranded RNAs are adjuvants for the induction of T helper 1 and humoral immune responses to human papillomavirus in rhesus macaques. PLoS Pathog 2009;5(4):e1000373.

[30] Moschos SA, Bramwell VW, Somavarapu S, Alpar HO. Adjuvant synergy: the effects of nasal coadministration of adjuvants. Immunol Cell Biol 2004;82 (6):628–37.

[31] Skeiky YA, Alderson MR, Ovendale PJ, Guderian JA, Brandt L, Dillon DC, et al. Differential immune responses and protective efficacy induced by components of a tuberculosis polyprotein vaccine, Mtb72F, delivered as naked DNA or recombinant protein. J Immunol 2004;172(12):7618–28.

[32] Lu JB, Chen BW, Wang GZ, Fu LL, Shen XB, Su C, et al. Recombinant tuberculosis vaccine AEC/BC02 induces antigen-specific cellular responses in mice and protects guinea pigs in a model of latent infection. J Microbiol Immunol Infect 2015;48(6):597–603.

[33] Lu JB, Deng HQ, Chen BW, Du WX, Yang L, Shen XB, et al. Establishment and validation of a guinea pig model of latent Mycobacterium tuberculosis infection. Chin J Microbiol Immunol 2013;12:900–5.

[34] Xie HA, Yang GT, Lin SZ, Wang XF, Xiao CZ. Modern tuberculosis. 1st ed. China: Beijing; 2000.

[35] Floyd K, Glaziou P, Zumla A, Raviglione M. The global tuberculosis epidemic and progress in care, prevention, and research: an overview in year 3 of the End TB era. Lancet Respir Med 2018;6(4):299–314.

[36] Borrero R, García Mde L, Canet L, Zayas C, Reyes F, Prieto JL, et al. Evaluation of the humoral immune response and cross reactivity against Mycobacterium tuberculosis of mice immunized with liposomes containing glycolipids of Mycobacterium smegmatis. BMC Immunol 2013;14(Suppl 1):S13.

[37] Achkar JM, Prados-Rosales R. Updates on antibody functions in Mycobacterium tuberculosis infection and their relevance for developing a vaccine against tuberculosis. Curr Opin Immunol 2018;53:30–7.

[38] Lu LL, Chung AW, Rosebrock TR, Ghebremichael M, Yu WH, Grace PS, et al. A functional role for antibodies in tuberculosis. Cell 2016;167(2):433–43.

[39] McKee AS, Munks MW, Marrack P. How do adjuvants work? Important considerations for new generation adjuvants. Immunity 2007;27(5):687–90.

[40] Bungener L, Geeraedts F, Ter Veer W, Medema J, Wilschut J, Huckriede A. Alum boosts TH2-type antibody responses to whole-inactivated virus influenza vaccine in mice but does not confer superior protection. Vaccine 2008;26(19):2350–9.

[41] Kaufmann SH, Hussey G, Lambert PH. New vaccines for tuberculosis. Lancet 2010;375:2110–9.

[42] Baldwin SL, D'Souza C, Roberts AD, Kelly BP, Frank AA, Lui MA, et al. Evaluation of new vaccines in the mouse and guinea pig model of tuberculosis. Infect Immun 1998;66(6):2951–9.

[43] Feng CG, Bean AG, Hooi H, Briscoe H, Britton WJ. Increase in gamma interferon-secreting CD8(+), as well as CD4(+), T cells in lungs following aerosol infection with Mycobacterium tuberculosis. Infect Immun 1999;67 (7):3242–7.

[44] Smith SM, Dockrell HM. Role of CD8 T cells in mycobacterial infections. Immunol Cell Biol 2000;78:325–33.

[45] Aagaard C, Hoang T, Dietrich J, Cardona PJ, Izzo A, Dolganov G, et al. A multistage tuberculosis vaccine that confers efficient protection before and after exposure. Nat Med 2011;17(2):189–94.

[46] Gillard P, Yang PC, Danilovits M, Su WJ, Cheng SL, Pehme L, et al. Safety and immunogenicity of the M72/AS01E candidate tuberculosis vaccine in adults with tuberculosis: a phase II randomised study. Tuberculosis (Edinb) 2016;100:118–27.

[47] Leroux-Roels I, Forgus S, De Boever F, Clement F, Demoitié MA, Mettens P, et al. Improved CD4+ T cell responses to Mycobacterium tuberculosis in PPD-negative adults by M72/AS01 as compared to the M72/AS02 and Mtb72F/AS02 tuberculosis candidate vaccine formulations: a randomized trial. Vaccine 2013;31(17):2196–206.

[48] Darrah PA, Patel DT, DeLuca PM, Lindsay RW, Davey DF, Flynn BJ, et al. Multifunctional TH1 cells define a correlate of vaccine-mediated protection against Leishmania major. Nat Med 2007;13:843–50.

[49] Andersen P, Novel Kaufmann SH. Vaccination strategies against tuberculosis. Cold Spring Harb Perspect Med 2014(6):4.

[50] van Pinxteren LA, Cassidy JP, Smedegaard BH, Agger EM, Andersen P. Control of latent Mycobacterium tuberculosis infection is dependent on CD8 T cells. Eur J Immunol 2000;30(12):3689–98.

[51] Van Der Meeren O, Hatherill M, Nduba V, Wilkinson RJ, Muyoyeta M, Van Brakel E, et al. Phase 2b controlled trial of M72/AS01E vaccine to prevent tuberculosis. N Engl J Med 2018;379(17):1621–34.