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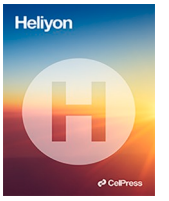

Research article

Immunogenicity and protective efficacy of Ag85A and truncation of PstS1 fusion protein vaccines against tuberculosis

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ABSTRACT

Tuberculosis (TB) is an important public health problem, and the One Health approach is essential for controlling zoonotic tuberculosis. Therefore, a rationally designed and more effective TB vaccine is urgently needed. To enhance vaccine efficacy, it is important to design vaccine candidates that stimulate both cellular and humoral immunity against TB. In this study, we fused the secreted protein Ag85A as the T cell antigen with truncated forms of the mycobacterial cell wall protein PstS1 with B cell epitopes to generate vaccine candidates, Ag85A-tnPstS1 (AP1, AP2, and AP3), and tested their immunogenicity and protective efficacy in mice. The three vaccine candidates induced a significant increase in the levels of T cell-related cytokines such as IFN-γ and IL-17, and AP1 and AP2 can induce more balanced Th1/Th2 responses than AP3. Strong humoral immune responses were also observed in which the production of IgG antibodies including its subclasses IgG1, IgG2c, and IgG3 was tremendously stimulated. AP1 and AP2 induced early antibody responses and more IgG3 isotype antibodies than AP3. Importantly, the mice immunised with the subunit vaccine candidates, particularly AP1 and AP2, had lower bacterial burdens than the control mice. Moreover, the serum from immunised mice can enhance phagocytosis and phagosome-lysosome fusion in macrophages, which can help to eradicate intracellular bacteria. These results indicate that the subunit vaccines Ag85A-tnPstS1 can be promising vaccine candidates for tuberculosis prevention.

1. Introduction

Tuberculosis (TB) is the leading killer infectious disease in the world (above HIV/AIDS and COVID-19), with an estimated 10 million new TB cases and 1.5 million deaths in 2021 [1,2]. *Mycobacterium tuberculosis* (*Mtb*) and *Mycobacterium bovis* (*M.bovis*) are the two main species of the *Mycobacterium tuberculosis* complex, both of which can infect humans and animals. It is estimated that approximately 140,000 TB cases and 11,400 deaths were caused by *M. bovis* infection in 2019 [1,2]. Bovine TB symptoms are clinically similar to those of human TB [3]. The *Bacille Calmette-Guérin* (BCG) vaccine is the only licensed TB vaccine for humans, and badgers in the United Kingdom. However, BCG cannot confer protection in adults and has shown variable efficacies in both human and animal field trials worldwide [4,5]. Therefore, it is necessary to develop novel and effective vaccines to prevent tuberculosis and strengthen the One Health approach. This is essential for controlling tuberculosis in both humans and animals.

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Most TB vaccine candidates are designed to induce cellular immunity against *Mtb*. However, emerging evidence suggests that humoral immunity plays an important role in *Mtb* infection [4,6–12]. Therefore, designing and developing TB vaccines that stimulate both T and B cells is a rational strategy. Subunit vaccines are non-replicating and refractory to prior mycobacterial sensitization and are different from BCG and other live vaccine candidates. Another advantage of subunit vaccines is their well-established safety since they can significantly reduce the live-vaccine vaccination risk in immunosuppressed individuals such as patients with HIV [13].

The Ag85 complex is a 30–32 kDa family of three proteins (Ag85A, Ag85B, and Ag85C) that are involved in cord factor biogenesis and the coupling of mycolic acids to arabinogalactan in the mycobacterial cell wall [14]. Moreover, the Ag85 complex is one of the most secreted proteins of *Mtb* and can strongly induce T cell proliferation and IFN-γ production in BCG-vaccinated mice and healthy individuals exposed to *Mtb* [15]. Therefore, Ag85 complexes have been included as important antigens for TB vaccine candidates, such as the modified vaccinia virus Ankara expressing Ag85A (MVA85A), Ag85A-overexpressing BCG, subunit and DNA vaccines [15–18]. Because of the robust T-cell responses induced by Ag85A, we chose it as the T-cell antigen for our subunit vaccine candidates.

PstS1 is a 38 kDa phosphate-binding protein located in the *Mtb* cell wall and is an immune-dominant marker of active tuberculosis [19,20]. Previous studies have indicated that anti-PstS1 monoclonal antibodies (mAbs) isolated from patients protect against *Mtb* infection in mice [9]. Epitopes recognised by protective mAbs have also been identified [9]. Moreover, PstS1 variation plays an important role in *Mtb* immune evasion and has a highly conserved *Mtb* T cell epitope 259-AAAGFASKTPANQAISMIDG-280 domain [21,22]. This suggests that PstS1 is an antigen that stimulates both B and T cells and is important for vaccine research and development [9,21,22]. Adjuvants are important in vaccine development as they augment immune responses to the given vaccines and reduce the number of required boosters [23,24]. Aluminum salts have been clinically approved and are widely used in human vaccines which can primarily evoke innate immunity and enhance antibody responses by stimulating B cell differentiation [23,24]. Hence, we used aluminum as an adjuvant for the vaccine candidates in this study.

The goal of this study was to design TB subunit vaccines composed of T and B cell antigens and to investigate the immunogenicity and protective efficacy of these vaccine candidates. In this study, the T cell-inducing antigen, Ag85A, was fused with three truncated forms of PstS1 with B cell epitopes to generate the fusion proteins AP1, AP2, and AP3. The fusion proteins were mixed with aluminum to produce vaccine candidates. We evaluated the immunogenicity and protection provided by each of the three vaccine candidates. Strong T and B cell responses were observed, and AP1 or AP2 vaccination prevented *M. bovis* infection in a mouse model. These results highlight the importance of designing and developing TB vaccine candidates based on T and B cell immune responses.

2. Materials and methods

2.1. Ethics statement

Animal experiments were performed in a Biosafety Level-3 Laboratory (BSL-3) at China Agricultural University following the "3R" principles of the Animal Welfare Law. All animal experiments were approved by the Laboratory Animal Ethical Committee of China Agricultural University (license No.AW70211202-2-1).

2.2. Mice

Female C57BL/6J mice (6–8 weeks old) were obtained from SiPeiFu Biotechnology (Beijing, China). Six mice were assigned to each cage upon their arrival. The animal feed and bedding were also purchased from SiPeiFu Biotechnology. Before the experiments, the mice were kept in specific pathogen-free cages for 1 week to recover and acclimatise to the environment.

2.3. Preparation of the Ag85A-tnPstS1

Based on the epitopes of PstS1 verified in previous studies [9,21,22,25,26], three sections of PstS1 were selected to generate fusion proteins with the antigen Ag85A. Basic Local Alignment Search Tool (BLAST) and Vector NTI Software were used for sequence analysis. The three truncated fragments of PstS1 were PstS1(AA84-167), PstS1(AA184-284), and PstS1(AA84-284). To better distinguish between the three fusion proteins of the Ag85A and PstS1 truncations (Ag85A-tnPstS1), the abbreviations AP1, AP2, and AP3 were used. The *Ag85A* and *Psts1* truncation genes were separately amplified from the H37Rv genome and fused with the (GGGGS)3 linker by overlapping PCR. The fused genes with *his tag* were inserted into the pET-21a(+) plasmid and the recombinant plasmids were transfected into *E. coli* BL21 (DE3) strain for fusion protein expression. IPTG (1 mM/L) was added to induce protein expression when the OD_{600} of the bacterial culture was between 0.6 and 0.8. After 4 h of induction, bacterial cells were harvested by centrifugation and sonicated for protein identification and purification. All three fusion proteins were expressed as aggregated inclusion bodies, which were washed and dissolved in a binding buffer containing 8 M urea (pH 8.0) for Ni-agarose resin purification. The eluted fusion proteins in imidazole were gradually dialyzed with 0–8 M urea and finally dialyzed in PBS (pH 7.4). Endotoxin levels in purified proteins were tested using the Tachypleus amebocyte lysate (TAL) assay (Chinese Horseshoe Crab Reagent Manufactory, Xiamen, China). The purified protein concentration was tested using a BCA protein assay, and the purified proteins were stored at −80 °C.

2.4. Bacterial culture

M. bovis BCG Pasteur 1173P2 strain and *M. bovis* C68004 strain [27] were cultured in Middlebrook 7H9 broth medium (Difco, USA) supplemented with 10% oleic acid-albumin-dextrose-catalase (OADC), 0.05% Tween 80 and 0.5% glycerol at 37 °C.

2.5. Mouse challenge model

Two vaccination mouse models were established and used for vaccine candidate evaluation in this study according to previous studies [28–32]. For the prevention model, five groups of mice were used, and each group had six mice. The three trial groups of mice were subcutaneously immuniszed with 100 μL subunit vaccines including 75 μL (20 μg) fusion proteins AP1, AP2, or AP3 in PBS and 25 μL Imject™ Alum Adjuvant (Thermo fisher) individually thrice with 2-week intervals. The BCG and PBS control groups were administered subcutaneously with 5×10^5 colony-forming units (CFU) of BCG Pasteur (1173P2) in 100 μL PBS or 100 μL PBS individually. For the prime-boost model, 30 C57BL/6J mice were divided into five groups using random number tables, including the BCG control, AP1, AP2, AP3, and PBS control groups with six mice per group. The BCG control and three trial groups were immunised with 5×10^5 CFU of BCG Pasteur (1173P2). The PBS control group was administered 100 μL PBS. After 4 weeks, all three trial groups were subcutaneously immunised thrice with 100 μL subunit vaccines, at 2-week intervals. The mice were anesthetised with a subcutaneous injection of PBS-diluted Zoletil 50 (50 mg/kg; Virbac, France) before the *M.bovis* challenge. Mice were challenged with virulent *M.bovis* (C68004) 2 weeks after the last immunisation via the intranasal route at 500 CFU for chronic infections or 2000 CFU for acute infections. Three mice were sacrificed to determine the initial lung infection dose in chronic and acute infection models separately in 24 h. Four weeks after the challenge, all remaining mice were sacrificed. The lungs and spleens were collected with two grinding beads in bead-beating tubes, and then were homogenized in PBS plus 0.05% Tween-80 by a tissue homogeniser (Kangtao Tech, China). The homogenates were diluted and plated on 7H10 agar supplemented with 10% OADC, amphotericin B (50 μg/mL), and polymyxin B (50 μg/mL). The CFUs on plates were counted after approximately 4 weeks of incubation at 37 °C.

2.6. Preparation of single-cell suspensions

Three mice from each group were sacrificed 2 weeks after the last immunisation. Spleens were aseptically harvested from the euthanised mice and processed to extract single-cell suspensions as previously described [33]. The splenocytes of each experimental group were harvested, counted, and cultured with 10 μg/mL of a specific protein (AP1, AP2, or AP3) in 12-well plates at a density of 1×10^7 cells/well. Splenocytes from the PBS and BCG control groups were stimulated with 10 μg/mL BCG lysates [34,35]. The culture supernatants of the groups were collected for cytokine detection, and the cells were harvested for surface marker staining to detect cell-mediated immune responses.

2.7. Enzyme-linked immunosorbent assay (ELISA)

Antibody titers and cytokines were detected by Enzyme-linked immunosorbent assay (ELISA) and performed as previously described [6]; briefly, 1×10^7 CFU heat-inactivated *M. bovis* dissolved in water was plated in each well of a 96-well Maxisorp plate (Thermo, Denmark). ELISA plates were dried at 60 °C and incubated with pre-cooled methanol (−20 °C) for 15 min at room temperature. The plates were washed thrice with PBST (PBS containing 0.5% Tween 20). The wells were blocked with 5% (w/v) skimmed milk (Difco, USA) in PBS for 2 h at room temperature. Then the plates were incubated with diluted serum from immunised mice (1:1000) for 1 h at 37 °C. Horseradish peroxidase (HRP)-conjugated IgG, IgG1, IgG2c, IgG3, IgM, and IgA secondary antibodies (Abcam, Cambridge, UK) were added separately. For cytokine detection, the splenocytes were stimulated with 10 μg/mL specific fusion antigens in 12-well plates for 10 h at 37 °C. The supernatants were collected, and IFN-γ, TNF-α, IL-2, IL-10, and IL-17 cytokines were detected using ELISA kits (Neobioscience, China) according to the manufacturer's instructions.

2.8. Flow cytometry

For the cell subset analysis, splenocytes stimulated with specific fusion antigens were harvested by centrifugation at 1000 rpm for 5 min. The cells were then stained with CD3-FITC, CD4-PE-Cy7, and CD8-APC-Cy7 (MultiSciences, Beijing, China) antibodies and detected using a BD C6 cytometer. The phagocytosis assay was performed as previously described [6]. Briefly, the log phase BCG was collected and stained with 1 mg/mL FITC for 2 h at 37 °C. RAW264.7 macrophages were infected with FITC-stained BCG for 3 h at an MOI of 10:1 at 37 °C. Serum harvested from euthanised mice was diluted at a 1:10 ratio in blank Dulbecco's Modified Eagle Medium (DMEM) medium and added to each well. Non-internalised bacteria were removed by washing thrice with ice-cold PBS. RAW264.7 macrophages were then fixed with 4% paraformaldehyde (PFA) and analysed using a BD C6 cytometer. Raw data were analysed using FlowJo software.

2.9. Histopathological analysis

Lung tissues from different mice were fixed in 10% formalin, embedded in paraffin, and cut into 3-μm sections. Sections were mounted, deparaffinised, and stained with hematoxylin and eosin (H&E). The H&E-stained slides were scanned and analysed using a VENTANA DP 200 slide scanner (Roche Diagnostics, Switzerland). Three mice from each group were subjected to histopathological analyses. The inflamed areas were qualitatively analysed using ImageJ software.

2.10. Phagosome-lysosome (P-L) fusion assessment using confocal microscopy

Coverslips (Solarbio, China) were placed into specific wells of 24-well plates, and 3×10^5 RAW264.7 cells were cultured in each

well in DMEM medium without FBS overnight. Then the RAW264.7 cells were infected with the log phase BCG and stained with FITC at an MOI of 10:1 for 3 h with 10% (v/v) mouse serum in the medium. Serum was collected from the mice 4 weeks after the challenge. The infected cells were washed by blank DMEM thrice and stained with 1:20000 LysoTracker® Red DND-99 (Beyotime, China) for 1 h at 37 °C. After washing, the cells were fixed with 4% PFA for 2 h at room temperature. Coverslips were mounted on glass slides using an antifade mounting medium (Solarbio, China). Images were captured using a Nikon A1 confocal microscope (Nikon, Japan). Ten random fields were selected and counted per condition; at least 250 cells were counted in each field. The percentage of P-L fusions was calculated. The BCG CFUs in every 100 phagolysosomes were counted and calculated for each condition.

2.11. Statistical analysis

Statistical analyses were performed using GraphPad Prism 9 software. Data are expressed as an absolute number or mean ± SD. Flow cytometry data were analysed using FlowJo software. Data from the experiments were analysed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. Statistical significance was set at $P < 0.05$.

3. Results

3.1. PstS1 immune epitopes mapping and preparation of Ag85A-tnPstS1 fusion proteins

The protective epitopes were mapped in the Immune Epitope Database and the three PstS1 truncations covering the B and T cell epitopes were selected and fused with the robust T cell antigen Ag85A based on previous studies [9,21,22,25,26] (Fig. 1a, Supplementary Fig. S1a). AP1, AP2 and AP3 were designed by fusion of the Ag85A and PstS1 epitopes (AA84-167), AP2 PstS1(AA184-284), and AP3 PstS1(AA84-284), respectively. PstS1 epitopes (AA84-167) contain B cell epitopes targeted by the P4-36 protective antibody, and PstS1 (AA184-284) contains B cell epitopes targeted by a P4-163 protective antibody, and the conserved *Mtb* T cell epitope 259-AAAGFASKTPANQAISMIDG-280 domain. PstS1(AA84-284) contains both the P4-36 and P4-163 antibodies targeting epitopes and conserved *Mtb* T-cell epitope 259-AAAGFASKTPANQAISMIDG-280 domain (Fig. 1a, Supplementary Fig. S1a). The *Ag85A* and *Psts1* truncation genes were joined by a flexible $(GGGGS)_3$ linker using the overlapping fusion PCR [36]. The fusion genes were inserted into the pET-21a (+) plasmid and transfected into the *E.coli* expression system for protein expression and purification (Fig. 1a and b). Purified proteins were identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting (Fig. 1c, Supplementary Figure S2ãd). Notably, the fusion protein AP2 had two bands in the denaturing gel; however, one band remained in the non-denaturing gel. This may be due to the structural and conformational changes during protein denaturation (Fig. 1c, Supplementary Fig. S1b, Supplementary Figure S2e∼f). Moreover, endotoxin levels in the purified proteins used for animal vaccination were <20 EU/mL which is recommended for subunit vaccine development [37]. These results suggest that we obtained the correct purified fusion proteins for subsequent research.

3.2. Ag85A-tnPstS1 vaccination protects against M.bovis infection in mice

Mice were challenged with virulent *M. bovis* at 500 CFU/mouse 2 weeks after the third dose of Ag85A-tnPstS1 via the intranasal

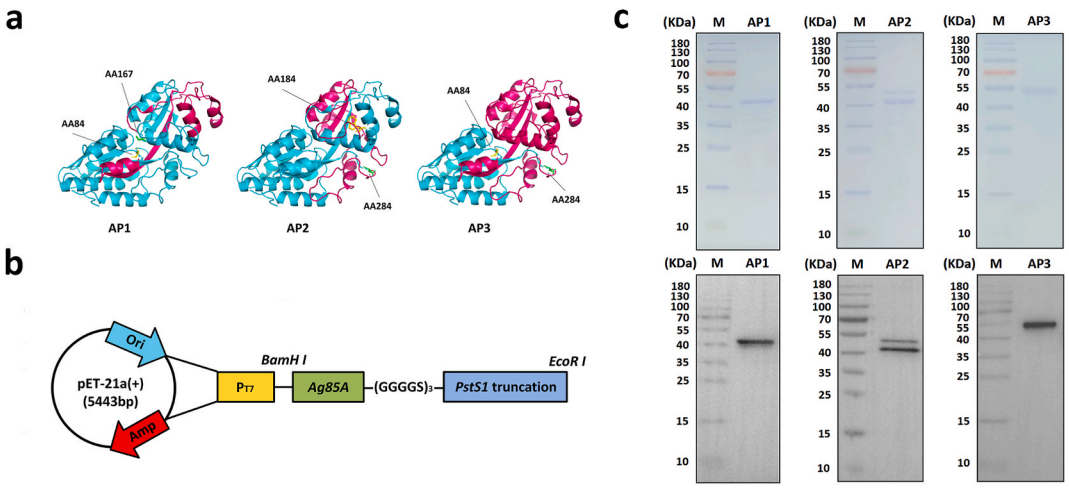

Fig. 1. Expression, Purification, and identification of the recombinant proteins AP1, AP2, and AP3. a: The PstS1 structure and its epitopes selected for subunit vaccine development were analysed using YASARA software. The yellow and green amino acids in the PstS1 structure indicate the start and end amino acids of the selected truncations, respectively. The structures marked in pink are truncated fragments of PstS1. b: Principles of recombinant Ag85A-tnPstS1 design and construction. c: Purification and identification of recombinant AP1, AP2, and AP3 by SDS-PAGE and Western blotting. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

route and were sacrificed 4 weeks after infection (Fig. 2a). According to the H&E staining results, the lungs of Ag85A-tnPstS1 immunised mice presented fewer and milder nodular lesions at 4 weeks post-challenge compared to the PBS- and BCG-vaccinated groups (Fig. 2b). The bacterial burden in the organs was evaluated using a CFU assay. According to the results, BCG, AP1, and AP2 significantly reduced the lung bacterial burden in the prevention vaccination model compared to the PBS group. However, no significant differences were observed between AP1 or AP2 with the BCG group. Moreover, the results indicated that AP3 has no effects on organs' bacterial burden reduction in the prevention mouse model (Fig. 2c). Of all three subunit vaccines, only AP1 showed inhibitory effects in mouse spleens (Fig. 2d), suggesting that AP1 could be the best vaccine candidate for TB prevention. In the prime-boost mouse model, there were no differences in the bacterial burden reduction between the BCG and boost groups (Supplementary Figs. S3a and c). However, the reduction in splenic bacterial burden was only observed in the AP1 boost group, which further supports the better efficacy of AP1 in TB prevention (Supplementary Fig. S3d).

To further identify the protective effects of Ag85A-tnPstS1, we established an acute mouse infection model in which each mouse was challenged with 2000 CFU. H&E staining analysis indicated that more inflammatory areas were present in the PBS group than in the BCG, AP1, and AP2 groups (Supplementary Fig. S4a). The CFU counts in the animals indicated that BCG and the three fusion proteins all provide protection against *M.bovis* in the mice with acute infections in the prevention model, and AP1 and AP2 showed better efficacy than AP3 (Supplementary Figs. S4b and c).

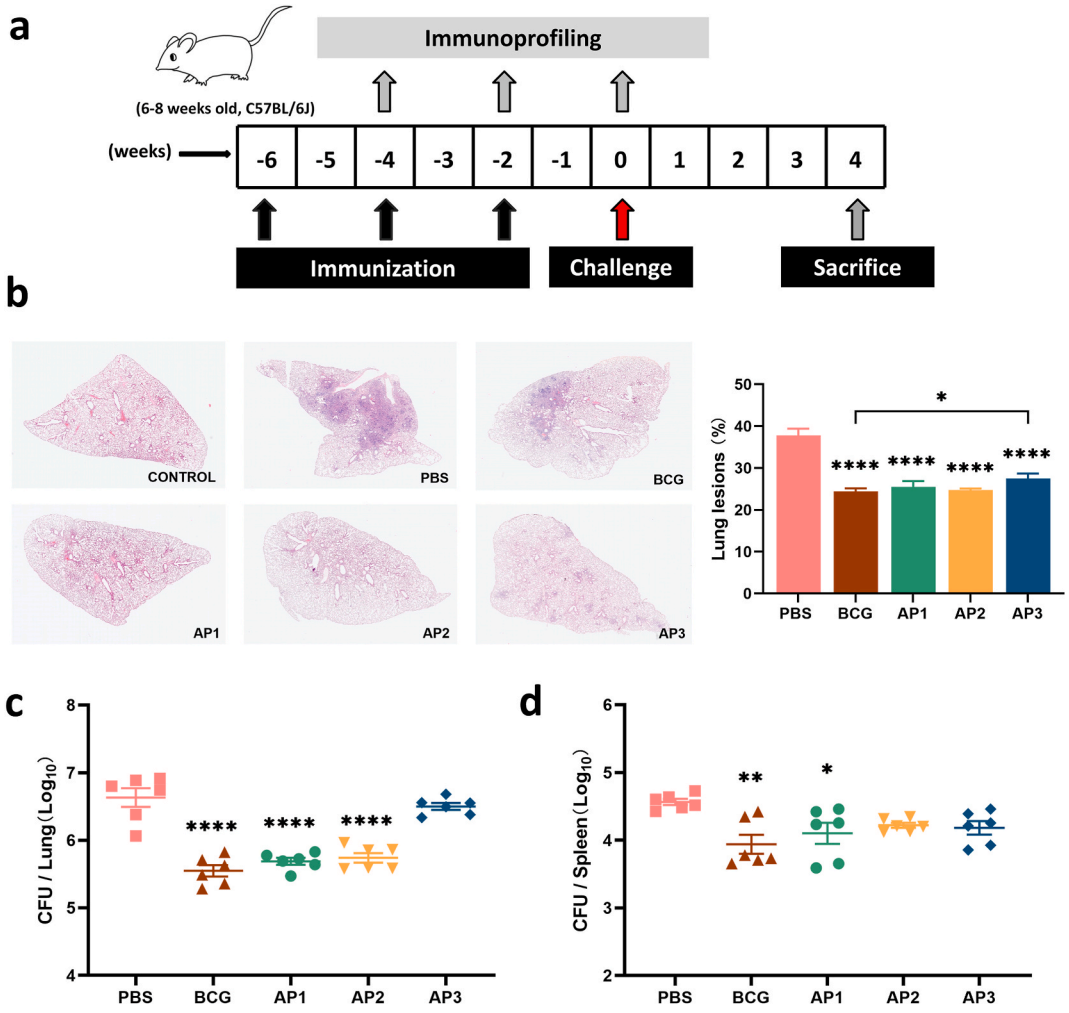

Fig. 2. Ag85A-tnPstS1 vaccination has protection against *M.bovis* infection in the prevention mouse model. a: Schematic representation of the prevention vaccination schedule. b: Representative lung pathological changes in the different vaccination groups by H&E staining. The inflammatory areas were qualitatively analysed using ImageJ software in the prevention mouse model. c and d: C57BL/6J mice were immunised subcutaneously with 20 μg individual recombinant proteins, and then challenged with 500 CFU M.*bovis* via the intranasal route. Lung and spleen bacterial counts were determined 4 weeks after the challenge. Statistical analyses were performed using one-way ANOVA (ns: not significant; *P < 0.05; ****P < 0.0001).

3.3. T cell responses and cytokines profiles to Ag85A-tnPstS1 vaccination

Cellular immunity plays an important role in tuberculosis [38]. According to the results, we observed that in the prevention mouse model, the AP3 vaccination group had lower $CD4^+$ T cells than the BCG group, and there were no differences between the AP1, AP2, and control groups (Fig. 3a, Supplementary Fig. S5). For $CD8^+$ T cell responses, the AP2 and AP3 groups showed a significant decrease in $CD8^+$ T cell numbers compared to the PBS or BCG groups, while there are no differences observed in the AP1 group (Fig. 3b, Supplementary Fig. S5).

In this study, we also tested the levels of Th1 cytokines IFN-γ, IL-2, and TNF-α; Th2 cytokine IL-10; and Th17 cytokine IL-17 in all the groups using ELISA (Fig. 3c). In the prevention mouse model, BCG and the three subunit vaccine groups showed significant increases in IFN-γ compared with the PBS group. In terms of TNF-α, all three subunit vaccine groups had higher concentrations compared with the PBS group, and AP3 was significantly higher compared with the BCG group. BCG, AP2, and AP3 vaccination groups showed a higher increase in IL-2 than the PBS group, whereas AP1 did not. Significant increases in the IL-17 were observed in the AP1 and AP2 groups compared with the PBS and BCG groups, but not in the AP3 group. For IL-10 detection, a higher concentration was observed in three subunit vaccine groups, and AP2 was higher than that in the BCG group.

3.4. Ag85A-tnPstS1 vaccination induces robust antibodies and immune responses

To investigate humoral immunity induced by Ag85A-tnPstS1 vaccination, mouse antibody isotypes IgM, IgA, and IgG, including the subclasses IgG1, IgG2c, and IgG3, were detected after vaccination in the prevention mouse model. Antibody titers were tested against heat-inactivated *M. bovis* using ELISA.

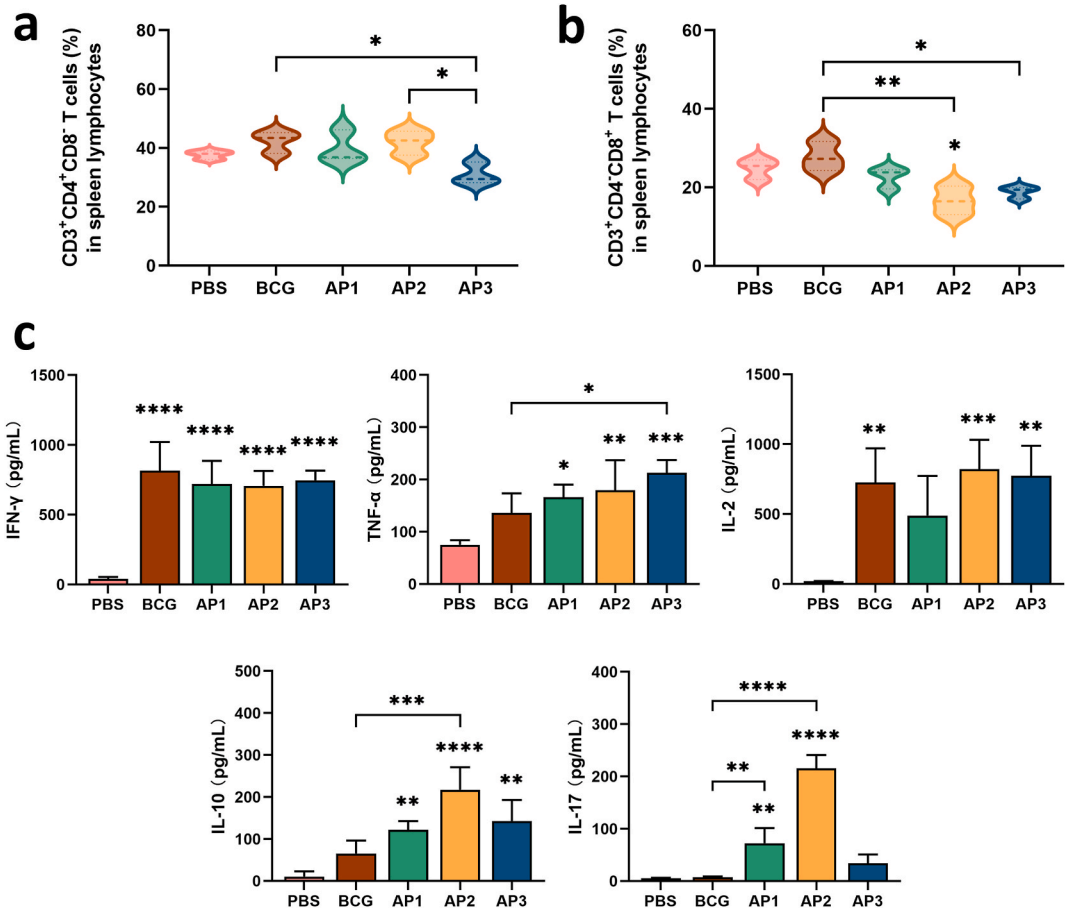

Fig. 3. Detection of T cells and cytokines change in the mice vaccinated by BCG or the subunit vaccines. a and b: Percentages of $CD4^+$ (a) and $CD8^+$ T cells (b) in splenic lymphocytes after vaccination. Statistical analyses were performed using one-way ANOVA (ns: not significant; *P < 0.05). c: The concentration of cytokines IFN-γ, TNF-α, IL-2, IL-17 and IL-10 were detected after subunit vaccines prevention vaccination in C57BL/6J mice. 10^7 splenocytes from subunit vaccines vaccinated and control mice (PBS and BCG) were separated and stimulated with antigens AP1, AP2, AP3, or BCG lysates, and cytokine levels were measured by ELISA. Statistical analyses were performed using one-way ANOVA (ns: not significant; *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001).

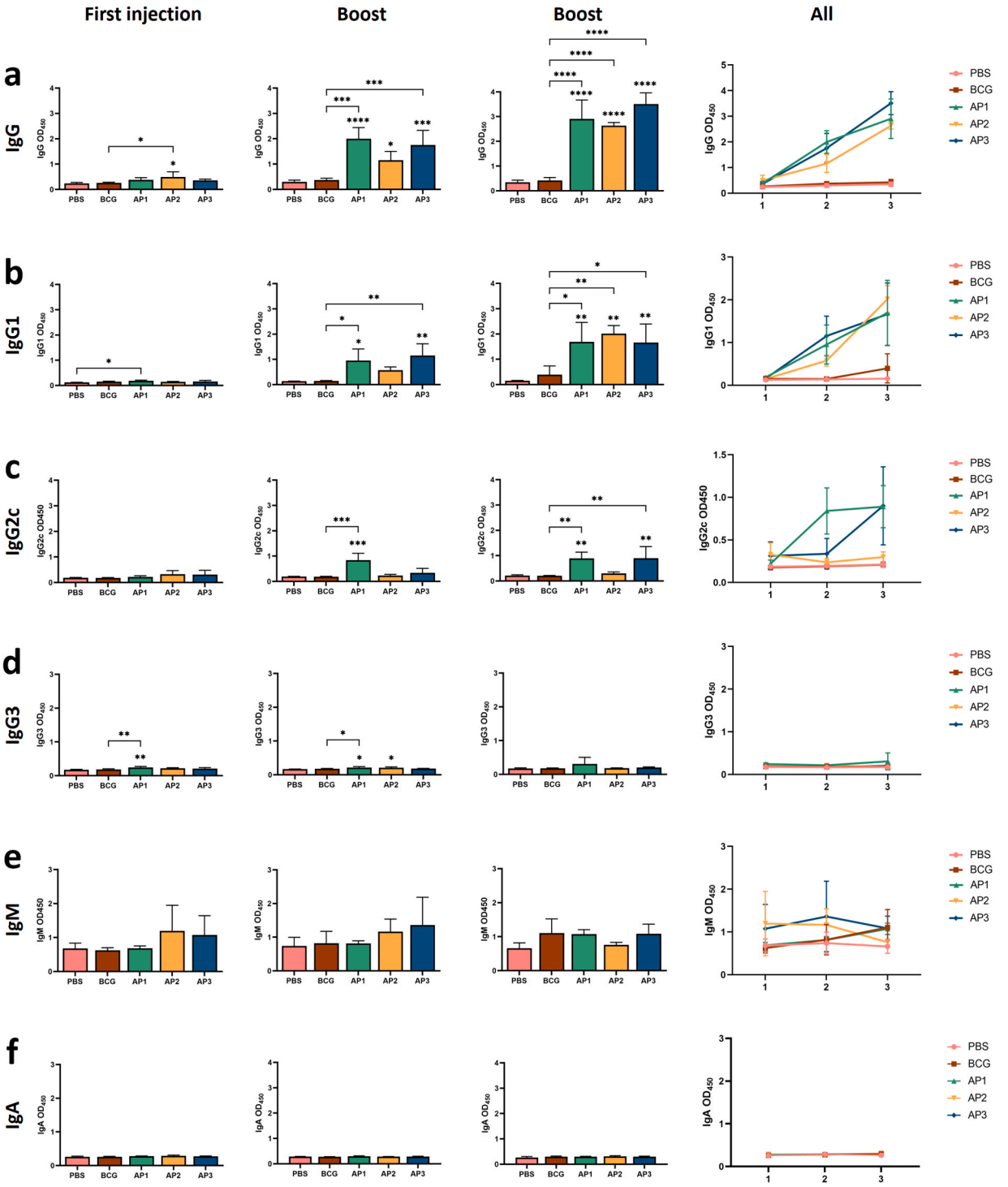

Fig. 4. The antibody responses to Ag85A-tnPstS1 vaccination in mice Mouse antibody isotypes IgM, IgA, and IgG including the subclasses IgG1, IgG2c, and IgG3 were tested by ELISA (ãf). Heat-inactivated *M.bovis* were plated on the Maxisorp plate. Mouse serum from each vaccination was used as the primary antibody, and HRP-conjugated IgG, IgG1, IgG2c, IgG3, IgM, and IgA were used as secondary antibodies. OD450 was measured to determine the antibody titers of IgG (a), IgG1 (b), IgG2c (c), IgG3 (d), IgM (e), and IgA (f) after each immunization, along with a summary of the overall trends in antibody titers' changes (ãf). The statistical analyses were performed using one-way ANOVA (ns: not significant; *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001).

AP1 induced higher antibody titers of IgG1 and IgG3 compared to the control groups, and AP2 induced higher total IgG levels, whereas AP3 had no effects on antibody production after the first vaccination. AP1 induced stronger total IgG, IgG1, IgG2c, and IgG3 after the second vaccination compared to the control groups, whereas AP2 induced higher IgG and IgG3, and AP3 induced higher IgG1 compared to the control. After the third vaccination, the results indicated that all three fusion proteins could induce a significant increase in IgG and IgG1, and AP1 can AP3 can induce higher IgG2c levels compared to the BCG group. Increasing antibody titers of IgG and IgG1 were observed in AP1, AP2, and AP3 after all three vaccinations (Fig. 4).

3.5. *Serum from Ag85A-tnPstS1 immunised mice can enhance macrophage phagocytosis and P-L fusion*

To investigate how the antibody mediates protection during vaccination, RAW264.7 cells were infected with BCG to identify macrophage phagocytosis and P-L fusion. The results indicated that serum from the three groups immunised with fusion proteins significantly enhanced the phagocytosis of BCG by macrophages at an MOI of 10:1 for 3 h (Fig. 5a and c). All vaccination groups, including the BCG group, showed enhanced P-L fusion with the vaccination serum (Fig. 5b and d). Moreover, after treatment with serum from Ag85A-tnPstS1 immunised mice, more bacteria were observed in the macrophage phagolysosomes, where the bacteria

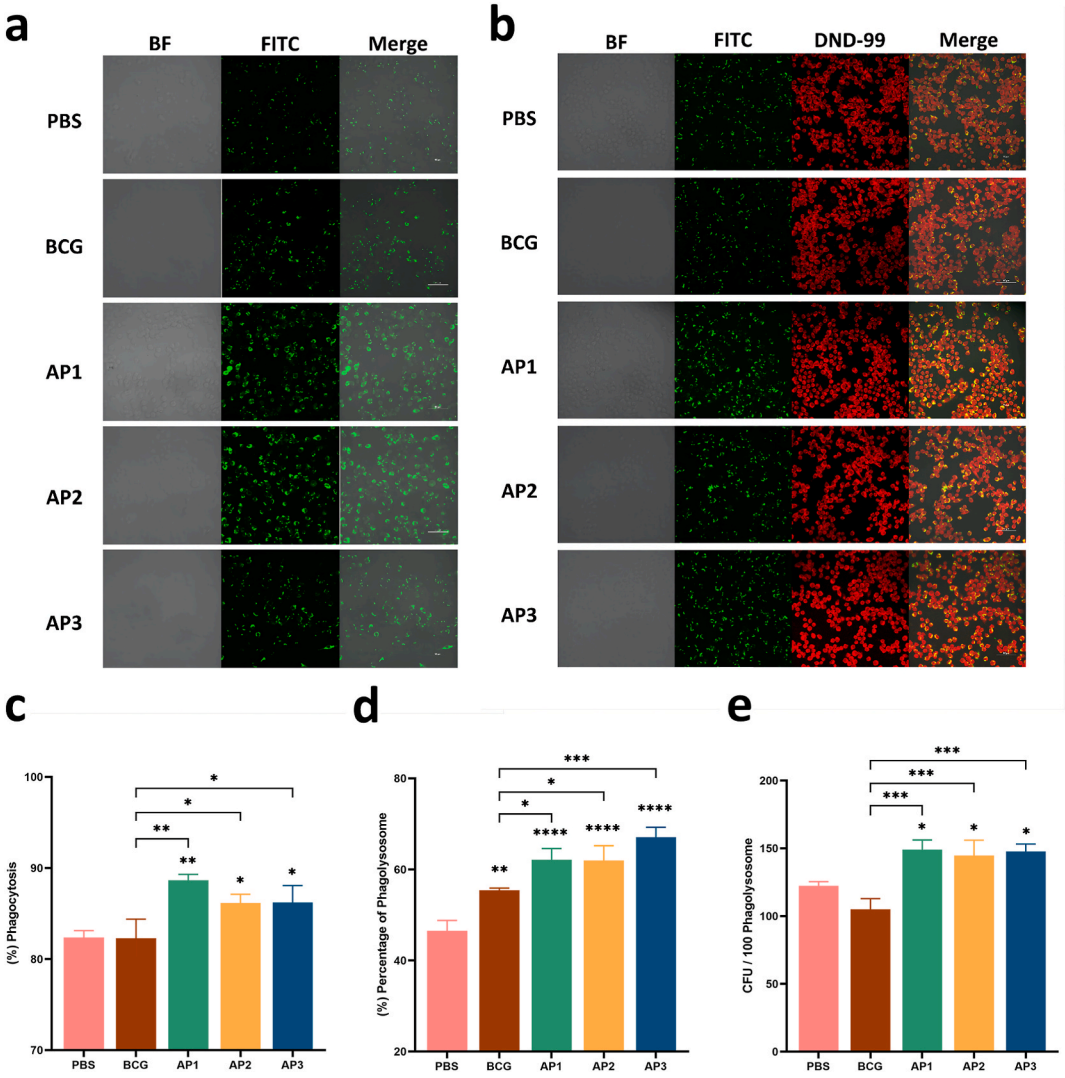

Fig. 5. Serum from Ag85A-tnPstS1 immunised mice can increase macrophage phagocytosis and phagosome-lysosome (P–L) fusion RAW264.7 cells were infected with FITC-conjugated BCG at an MOI of 10:1 for 3 h, andmacrophage phagocytosis was tested using confocal microscopy (a) and flow cytometry (c). b: The co-localisation of endogenous BCG/phagosomes (green) with lysosomes (red) in RAW264.7 cells was analysed by confocal microscopy. d: Quantification of cells with colocalization of BCG/phagosomes and lysosomes in RAW264.7. At least 2500 cells in ten fields were counted for each sample, 3 samples in one group. e: Phagocytosed BCG in every 100 phagolysosomes in Raw264.7 cells. Statistical analyses were performed using one-way ANOVA (ns: not significant; *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

were digested (Fig. 5e). These results suggested that antibody-mediated phagocytosis plays an important role in fusion protein-mediated protection.

4. Discussion

Tuberculosis is an important public health problem worldwide, and the development of new vaccines against TB is urgently required. Both B and T cell-mediated immunity are essential for the successful clearance of *Mtb* [10,39,40]. Ag85A is a robust antigen for T cells and is promising for TB vaccine design [15–17]. PstS1, a mycobacterial cell wall protein, also induces protective antibody responses against *Mtb* infection [9]. PstS1 protective B and conserved T cell epitopes have been identified and mapped [9,21,22,25,26]. In this study, fusion proteins of Ag85A and three truncations of PstS1 with immune-dominant T and B cell epitopes were constructed based on previous studies. The immunogenicity and protective ability of these vaccines were also evaluated. These results indicate that AP1 and AP2 can induce protective T and B cell immunity, and significantly decrease the bacterial burdens in the prevention mouse model.

T cells are required for protective immunity against TB [41,42]. In this study, the $CD4^+$ and $CD8^+$ T cell percentages in activated splenocytes were tested, and the cytokine profiles suggested that different T cell immune responses were induced by the subunit vaccines. During infection, naïve $CD4^+$ T cells are activated and differentiated into various subsets, including Th1, Th2, and Th17 cells, which are important for bacterial control after *Mtb* infection [40,43]. In this study, the AP3 vaccination group had lower $CD4^+$ and $CD8^+$ T cells responses than the BCG group which may explain why mice injected with AP3 did not show a significant reduction in bacterial burden. The multifunctional Th1 cells that secrete IFN-γ, TNF-α, and IL-2 are accepted as the major population mediating protective immunity against TB [44]. In this study, the three subunit vaccines induced robust production of Th1 cytokines. However, it is noticeable that AP3, which induced most TNF-α, did not provide protection in the prevention mice model. This may be explained by the fact that excessive TNF-α production causes the development of tissue-damaging immunopathology [45,46]. Further evidence suggests that Th1/Th2 balance is crucial for controlling the progression of active TB disease [47]. IL-10 is an anti-inflammatory cytokine strongly expressed in the prevention models. Th17 cells have been shown to have protective immunity against TB [48]. IL-17 produced by Th17 cells can drive Th1 responses by overcoming IL-10-mediated inhibition. After BCG vaccination, lung-resident IL-17 -producing Th17 cells can recruit IFN-γ-producing $CD4^+$ T cells during infection [49,50]. In this study, the subunit vaccines AP1 and AP2 induced a significant increase of IL-17 in mice, suggesting that Th17 cell responses may play an important role in vaccine efficacy. Taken together, compared to AP3, AP1 and AP2 can induce more balanced T cell cytokines to exert protective effects against TB infection in mice.

Antibodies targeting PstS1 have been shown to confer protection against *Mtb* infection [9], and the subunit vaccines used in this study contained protective B cell epitopes of PstS1. Previous studies have indicated that murine isotypes (IgA, IgM, and IgG) exert protective effects against *Mtb* infection in a mouse model [51]. To investigate the antibody response against *M. bovis*, we tested antibody isotypes IgA, IgM, and IgG including the subclasses IgG1, IgG2c, and IgG3. IgG and its subclasses, IgG1 and IgG2c, were robustly induced by three doses of the fusion proteins (AP1, AP2, and AP3). Notably, only AP1 or AP2 induced a significant increase of IgG, IgG1, and IgG3 isotype antibody responses after the first vaccination. Moreover, compared to PBS or BCG, AP3 did not induce more IgG3 isotype antibodies, whereas AP1 and AP2 did. These results may explain why AP3 did not provide sufficient protection against *M. bovis* infection. Because early antibody responses are related to the control and eradication of pathogens during infection or vaccination, rapid antibody responses can be beneficial to the host against infections [52,53]. If B cell responses cannot be initiated in time or the B cell response is not sufficient, the host cannot obtain proper protection against infection [54,55]. Moreover, IgG3 and IgG1 isotypes antibody levels are associated with chronic and recurrent infections in the lower airway in humans [56]. In mice models, high levels of Th1 cytokines IFN-γ and IgG2c are usually associated with Th1 responses [57]. IgG3 antibodies are related to the upregulation of the anti-inflammatory cytokine IL-10 which helps balance the Th1/Th2 immune responses [58]. In our study, AP1 was the best vaccine candidate to induce IFN-γ, IgG2c, and IgG3 secretion, and had the best protective effects in reducing lung pathology and bacterial burden in the prevention mouse model. Regarding the vaccine design strategy in this study, AP1 contains only protective B cell epitopes, and AP2 and AP3 contain both the previously identified protective B cells epitopes and PstS1 conserved *Mtb* T cell epitopes [9], and the results in our study support that AP1 has the best vaccine efficacy, which further suggests that the immune balance is crucial to control the progression of active TB disease.

Previous studies have indicated that anti-mycobacterial antibodies may act by increasing macrophage phagocytosis and P-L fusion [59,60]. In our study, the sera from mice immunised with AP1, AP2, and AP3 showed enhanced phagocytosis and P-L fusion [59,60]. These results suggest that the antibodies induced by vaccination with the subunit vaccines provide protection by mediating macrophage phagocytosis and increasing P-L fusion, leading to intracellular bacteria killing.

TB Antigens such as Ag85, ESAT6, and CFP10 have been extensively selected for TB subunit vaccine development based on their ability to induce robust T cell immune responses [61]. It is well established that T cell responses against *Mtb* are essential; however, they may not be sufficient to obtain a fully protective immune response against TB [62,63]. Moreover, *Mtb* contains approximately 4000 genes and has a complicated antigen repertoire for selecting the suitable protective antigens for subunit vaccine development [62]. In our study, we selected Ag85A and PstS1 as immunogenic antigens for vaccine development, and the results indicated that the vaccine candidates could induce balanced T cell and protective antibody responses. Combined with the prime-boost strategy with BCG vaccination, subunit vaccines may promote robust immune responses which can significantly increase BCG vaccination efficacy [64–66]. However, in our study, BCG-vaccinated mice that received three boost vaccinations with Ag85A-tnPstS1 did not have a lower bacterial burden than the BCG vaccination control group. Previous studies have shown that subunit vaccines containing shared BCG antigens boost vaccination can inhibit BCG colonisation and vaccine efficacy, while BCG is still replicating in mice [28,65]. Ag85A and

PstS1 are antigens shared by BCG and *M.bovis/Mtb*. Therefore, the Ag85A-tnPstS1 boost vaccination may exhibit *in vivo* cross-reactivity with BCG, which does not reflect the advantage of the prime-boost strategy. For the prime-boost strategy, it is valuable to evaluate Ag85A-tnPstS1's boost effects in improved mouse models, such as by extending the intervals between initial BCG and boost vaccinations and other animal infection models, because different vaccination intervals and animal models may affect the protective effects of subunit vaccines [67,68].

In conclusion, three subunit vaccines were evaluated in the prevention and prime-boost mouse models. These immunological and pathological parameters indicate that Ag85A-tnPstS1 is a promising subunit vaccine candidate. Moreover, this study suggests that it is rational to consider both T and B cell-mediated immunity when designing TB vaccines.

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Ethics approval

All studies involving mice were approved by the Laboratory Animal Ethical Committee of China Agricultural University (license No.AW70211202-2-1).

Data availability statement

Data will be made available on request.

CRediT authorship contribution statement

Lingyuan Zeng: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Xiuling Ma:** Methodology, Investigation. **Mengjin Qu:** Methodology, Investigation. **Minghui Tang:** Resources, Methodology. **Huoming Li:** Software, Resources, Methodology. **Chengrui Lei:** Resources, Methodology. **Jiahong Ji:** Resources, Methodology. **Hao Li:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e27034.

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