Protection against *Mycobacterium tuberculosis* Infection Offered by a New Multistage Subunit Vaccine Correlates with Increased Number of IFN-γ⁺IL-2⁺ CD4⁺ and IFN-γ⁺ CD8⁺ T Cells

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Abstract

Protein subunit vaccines present a compelling new area of research for control of tuberculosis (TB). Based on the interaction between *Mycobacterium tuberculosis* and its host, five stage-specific antigens of *M. tuberculosis* that participate in TB pathogenesis—Rv1813, Rv2660c, Ag85B, Rv2623, and HspX—were selected. These antigens were verified to be recognized by T cells from a total of 42 whole blood samples obtained from active TB patients, patients with latent TB infections (LTBIs), and healthy control donors. The multistage polyprotein A1D4 was developed using the selected five antigens as a potentially more effective novel subunit vaccine. The immunogenicity and protective efficacy of A1D4 emulsified in the adjuvant MTO [monophosphoryl lipid A (MPL), trehalose-6,6'-dibehenate (TDB), components of MF59] was compared with Bacillus Calmette-Guerin (BCG) in C57BL/6 mice. Our results demonstrated that A1D4/MTO could provide more significant protection against *M. tuberculosis* infection than the PBS control or MTO adjuvant alone judging from the A1D4-specific Th1-type immune response; however, its efficacy was inferior to BCG as demonstrated by the bacterial load in the lung and spleen, and by the pathological changes in the lung. Antigen-specific single IL-2-secreting cells and different combinations with IL-2-secreting CD4⁺ T cells were beneficial and correlated with BCG vaccine-induced protection against TB. Antigen-specific IFN-γ⁺IL-2⁺ CD4⁺ T cells were the only effective biomarker significantly induced by A1D4/MTO. Among all groups, A1D4/MTO immunization also conferred the highest number of antigen-specific single IFN-γ⁺ and IFN-γ⁺TNF-α⁺ CD4⁺ T cells, which might be related to the antigen load in vivo, and single IFN-γ⁺ CD8⁺ T cells by mimicking the immune patterns of LTBIs or curable TB patients. Our strategy seems promising for the development of a TB vaccine based on multistage antigens, and subunit antigen A1D4 suspended in MTO adjuvant warrants preclinical evaluation in animal models of latent infection and may boost BCG vaccination.
Introduction

Protein subunit vaccines, consisting of defined immunodominant antigens of *Mycobacterium tuberculosis* (*M. tuberculosis*), are sufficiently safe as a stand-alone prophylactic vaccine in a population where Bacillus Calmette-Guerin (BCG) immunization is not recommended, or as a boost to the currently used BCG vaccine. Half of twelve tuberculosis (TB) vaccine candidates that are currently entering clinical trials belong to the protein subunit category, demonstrating the important role of this type of vaccine in the next generation of TB vaccination [1, 2]. Nevertheless, the selection of protective antigens has mainly focused on proteins released from the growing *M. tuberculosis* bacteria into the culture medium *in vitro* [3, 4], and only a few antigens have been identified so far as promising targets for TB subunit vaccines [1, 2]. Suitable antigens are mainly selected on the basis of antigenicity, immunogenicity, and anti-infection protection in various animal models [5]. These methods may face inherent limitations to antigen screening due to the complex pathogenesis of *M. tuberculosis* and its interaction with the host as demonstrated in a recent review [6], thereby significantly hindering the development of effective subunit vaccines.

The pathogenesis of *M. tuberculosis* progresses successively through primary infection, latency, and reactivation. During these infection stages, the metabolism and gene transcriptional profiles of *M. tuberculosis* show significant differences and stage-specific features *in vivo* [7–10]. Once inhaled into the pulmonary alveoli, *M. tuberculosis* can reside and even replicate in the macrophages, thereby evading the innate immune response against infection of the host through immunosuppression [11, 12] and immune evasion [13, 14]. In a mouse model of primary infection, *M. tuberculosis* grew exponentially over 2 to 3 weeks following infection *in vivo* [15] and mainly secreted antigens such as the Ag85 complex [16] and the ESX protein family [17] when grown in liquid medium *in vitro*. Subsequently, T cells, especially CD4+ Th1-type cells that were sensitized in the local lymph node and homing the infected lesion via the pulmonary circulation, can secrete Th1-typed cytokines such as IFN-γ, which activates macrophage phagocytosis, thereby killing intracellular bacteria, and TNF-α, which attracts more infiltrating macrophages and lymphocytes and promotes granuloma formation at the site of infection, ultimately leading to latent TB infection (LTBI). During this chronic phase, the metabolic status of *M. tuberculosis* aggregating in the granuloma transfers from growing replication to dormancy *in vivo* with limited antigen presentation [18]. In addition, several mycobacterial genes involved in metabolism conversion such as Rv2660c [19], and 48 DosR-regulon genes [10] including HspX [20], Rv1813 [21], and Rv2623 [22], were significantly up-regulated. Dormant *M. tuberculosis* persisting in the host can reactivate under conditions of suppressed immunity of the host such as co-infection with HIV, thereby resulting in the formation of endogenous infection and the development of active pulmonary TB in the adult. Although the BCG vaccine confers the CD4+ Th1 response and protects effectively against serious forms of TB during childhood, the protective efficacy gradually decreases over time and does not protect against pulmonary TB in adults or against reactivation from LTBI [23, 24]. This might be attributed to low or masked IFN-γ responses to latency antigens induced by BCG vaccination [21, 22, 25], although the genes encoding latency antigens are highly homologous between BCG and *M. tuberculosis*. Promising targets for TB vaccines were previously focused on antigens from the Ag85 complex and the ESX protein family secreted by *M. tuberculosis* during primary infection. More recently, several latency antigens such as Rv2660c [19], Rv1733 [26], Rv1813 [21], and HspX [27] were used to construct fusion multistage vaccine candidates such as H56 [19, 28] and ID93 [29], which were shown to provide protection against primary and latent infection and even MDR-TB in animal vaccination models. These studies partially confirm the vaccine potential of stage-specific antigens expressed by *M. tuberculosis*. 

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The aim of the present study was to develop a more effective multistage subunit vaccine. Antigens expressed during different stages of *M. tuberculosis* were screened and confirmed on the basis of immune recognition by T cells from TB patients and LTBIIs. A multistage polyprotein based on five selected antigens was constructed and its immunogenicity and protective efficacy was compared with BCG in a C57BL/6 mouse model.

**Materials and Methods**

**Prokaryotic expression of recombinant proteins in *E. coli***

The coding sequence of the A1D4 fusion protein consisting of antigens Rv1813, Rv2660c, Ag85B, Rv2623, and HspX, was PCR-amplified using primers specific for the recombinant plasmid pDC316-A1D4, which was commercially synthesized by Life Invitrogen (Shanghai, China). Genes encoding the antigens Rv1813, Rv2660c, and Rv2623 were amplified by PCR with their respective primers from the genomic DNA of *M. tuberculosis* H37Rv (S1 Table). All PCR products were digested with *Nde*I and *Xho*I restriction enzymes and then inserted into the corresponding sites of the prokaryotic expression vectors pET-30b or pET28a, respectively. Recombinant prokaryotic expression plasmids were verified by enzyme digestion, and the inserted DNA fragments were confirmed by DNA sequencing. The recombinant plasmids were transformed into the *E. coli* strain BL21 (DE3), and the expression of the target proteins was induced by incubation with isopropyl thiо-β-D-galactoside (IPTG) at a final concentration of 1 mM for 4 h. Recombinant proteins were purified using NTA-metal ion affinity chromatography according to the manufacturer’s instructions (GE Healthcare, NJ, USA). Successful expression and purification was monitored by 15% SDS-PAGE. The specificity of recombinant proteins was confirmed by western blotting with an anti-His 6 mouse monoclonal antibody (mAb, diluted 1/2000; Tiangen, Beijing, China) or anti-A1D4 protein mouse serum as the primary antibody and peroxidase-conjugated goat anti-mouse IgG (diluted 1/5000; Proteintech Biotech, Wuhan, China) as the secondary antibody. The protein bands on immunoblots were visualized using enhanced chemiluminescence (ECL) technology (Tiangen, China). Recombinant proteins were dialyzed against sterilized PBS with a urea concentration gradient (from 8 M to 0 M) for 48 h at 6 h intervals. Proteins were lyophilized, diluted in phosphate-buffered saline (PBS) using pyrogen-free reagents, aliquoted, and stored at -20°C. Residual endotoxin contamination was verified to be below 0.1 EU/mL of protein. The protein concentration was determined using a BCA Protein Assay Kit (Beyotime, Shanghai, China). The recombinant proteins Ag85B [30], HspX [30], and rCM (CFP21-MPT64) [31] were prepared as previously described.

**Ethics statement**

The study protocol was approved by the Ethics Committee of Tongji Medical College (Permit Number: 201201). Patients were enrolled from Xinxiang Institute of TB Prevention and Treatment (Xinxiang, Henan Province, China), and written informed consent was obtained from all subjects; for children the informed consent was provided by their parents. Animal experiments were performed in accordance with the guidelines of the Chinese Council on Animal Care. The research protocol was approved by the ABSL-3 Lab of Wuhan University (Wuhan, China) and the Committee on the Ethics of Animal Experiments of Wuhan University (Permit Number: S01312122Q). All surgery was performed under sodium pentobarbital anesthesia, and every effort was made to minimize suffering.
Human clinical samples

All participants were recruited for screening based on the diagnostic standards listed in S2 Table. Active TB patients were diagnosed by history, clinical symptoms, positive sputum smear microscopy, and aberrant lung X-ray. Healthy LTBI patients had a history of household contact with sputum-positive TB patients in the preceding six months and a tuberculin skin test (TST) result of ≥5 mm, while clinical symptoms and aberrant lung X-ray were absent in these patients. In order to avoid any effects of BCG vaccination on the results, all TB infections were further screened by recombinant CFP21-MPT64-whole blood IFN-γ assay (rCM-WBIA) as rCM-WBIA-positive TB patients and LTBI patients based on rCM-specific IFN-γ concentration with a cut-off point of 398.5 pg/mL [31]. Healthy donors were diagnosed with no history of prior M. tuberculosis contact or exposure, and negative results of both TST and rCM-WBIA. All subjects were seronegative for HIV infection.

Whole blood IFN-γ assay based on rCM, A1D4, and subfractions of A1D4

Before treatment, 5 mL of fresh heparinized whole blood was collected from each donor, and 0.5 mL was seeded in each well of a sterile 24-well plate. rCM (20 μL), A1D4, and subfractions of A1D4 (10 μg/mL each) were used in each well for stimulation and incubated for 18 to 24 h at 37°C. PHA was used as a positive control at a final concentration of 20 μg/mL. Saline solution served as the negative control. Following stimulation, 200 μL of supernatant were collected from each well and stored at -20°C until further detection. The IFN-γ concentration was quantified using a commercial human IFN-γ assay kit according to the manufacturer’s instructions (Dakewe Biotech, Shenzhen, China). The antigen-specific concentration of IFN-γ in each sample was calculated by subtracting the value of the saline control from the value of the sample stimulated with the respective antigen. The detection limit of the ELISA was 4 pg/mL and the assay was set arbitrarily at 100 pg/mL for PHA-WBIA. The cut-off value for a positive response to each multistage antigen was set arbitrarily as 3X the mean of the negative control value of healthy controls. The results were analyzed as previously described [32].

Animals and immunization protocol

Specific pathogen-free, 6- to 8-week-old female C57BL/6 mice were obtained from Wuhan University Center for Animal Experiment and were bred in separate cages on an animal feeding cabinet (VentiRack, CA, USA). The protein subunit vaccine contained 20 μg of A1D4 emulsified in the adjuvant MTO composed of MPL (50 μg/dose; Avanti Polar Lipids Inc., AL, USA), TDB (50 μg/dose; Avanti Polar Lipids Inc., USA), and components of MF59 including squalene oil (1%, v/v), Tween 80 (0.4%, v/v), and Span 85 (1%, v/v, all purchased from Sigma-Aldrich, MO, USA). For this study, 0.2 mL of vaccine preparation was given to mice subcutaneously (s.c.) three times at 3-week intervals. BCG China was used as a positive control and inoculated s.c. once with 1.67 × 10^6 CFU at the time of the first vaccination. PBS and adjuvant MTO alone were separately used as negative controls.

Antigen-specific antibody titers

Three weeks after the last immunization, sera were collected from each mouse and A1D4-specific endpoint titers for IgG1, IgG2c, and IgG were detected by enzyme-linked immunosorbent assay (ELISA). Microtiter plates were precoated with 100 μL A1D4 protein (5 μg/mL) or BSA (irrelevant control) in carbonate/bicarbonate buffer (pH 9.6) overnight at 4°C and blocked with 1% BSA in PBST for 1 h at 37°C. Sera from mice treated with PBS were used as a negative
control. Serial dilutions of serum samples were added, followed by addition of horseradish peroxidase-conjugated rabbit anti-mouse IgG (Ab6789, 1/5000), IgG1 (Ab97240, 1/10000), or IgG2c (Ab97255, 1/10000, all antibodies from Abcam, Cambridge, UK). Colors were developed with 3,3',5,5'-tetramethylbenzidine (TMB) substrate and the enzymatic reaction was stopped with 1 N H2SO4. Plates were read at 450 nm using a microplate ELISA reader with the detection limit set at 0.05 at OD 450 nm. Antibody titers in each group were expressed as reciprocal end point titers by comparison with the PBS control with the value of P/N ≤ 2.1. The results were expressed as the mean (± SEM) log10 endpoint titers per group and the ratio of IgG2c/IgG1 in the different vaccination groups (n = 3).

Antigen-specific cytokines secreted by splenocytes

Splenocytes (2.5 × 10^6 cells) from three individual mice of each group were incubated in a 24-well microtiter plate for 24 h (for IL-2 detection) and 72 h (for IFN-γ and TNF-α detection) at 37°C with either RPMI1640 medium (negative control), PPD (10 μg/mL, positive control, Statens Serum Institut, Copenhagen, Denmark), or A1D4 (10 μg/mL). Supernatants from three separate wells were collected for each group and stored at -20°C until further assay. Mouse IFN-γ, TNF-α, and IL-2 ELISA kits (Multi Sciences LTD., Hangzhou, China) were employed according to the manufacturer’s instructions with the detection limits of IFN-γ, TNF-α, and IL-2 of 5 pg/mL, 8 pg/mL, and 0.22 pg/mL, respectively. The results were expressed as the mean ± SD (pg/mL) for each group (n = 3).

Intracellular flow cytometry analysis

Splenocytes from three individual mice per group were counted and plated at 5 × 10^6 cells/well in a 24-well plate, stimulated with A1D4 (10 μg/mL) in the presence of 1 μg/mL anti-CD28/CD49d (eBioscience, CA, USA) for 16 h, and were incubated for another 4 to 6 h after the addition of 3 μg/mL brefeldin A and 2 μM monensin solution (eBioscience). PPD (10 μg/mL) served as a positive control and cell stimulation cocktail (1 μg/mL, eBioscience) as a monitoring control. Either BSA (10 μg/mL) or RPMI1640 medium was used as a negative control. Cells were washed in FACS buffer (1% FCS-PBS), and stained for 30 min in the dark at room temperature with the following surface markers: anti-CD3-FITC (clone 17A2, eBioscience), anti-CD4-allophycocyanin-Cy7 (clone GK1.5, BD Pharmingen, CA, USA), and anti-CD8a-PE (clone53-6.7, eBioscience) mAbs. Cells were then washed again, permeabilized using the Cytofix/Cytoperm kit (BD Pharmingen) according to the manufacturer’s instructions, and stained intracellularly for 30 min using anti-IFN-PerCP-Cy5.5 (clone XMG1.2; eBioscience), anti-TNF-PE Cy7 (clone MP6-XT2; BD Pharmingen), and anti-IL-2-allophycocyanin (clone JES6-5H4; eBioscience) mAbs. Cells were subsequently washed, fixed in 1% paraformaldehyde-PBS, and then resuspended in FACS buffer. Viable lymphocytes were gated by forward and side scatter, and 1 × 10^6 CD3+ events were acquired for each sample. The absolute number of PPD- or A1D4-specific CD4+ T and CD8+ T lymphocytes secreting single, double, or triple cytokines per spleen of the differently vaccinated mice was analyzed using a LSRII multicolor flow cytometer (BD Biosciences) (S1 Fig). The results were analyzed with FlowJo software (Tree Star Inc., OH, USA) and represented as the mean ± SEM per group (n = 3).

Challenge of vaccinated mice with M. tuberculosis H37Rv

Three weeks after the last immunization, six mice in each group were challenged via the lateral vein with 1.2 × 10^6 CFU of the virulent M. tuberculosis H37Rv strain. Four weeks later, animals were sacrificed and lungs and spleens were removed aseptically. Organs were homogenized in sterile 0.05% PBS-Tween80, and plated at 10-fold serial dilutions on Middlebrook 7H11 agar
(BD, MD, USA). When required, plates were supplemented with 2-thiophenecarboxylic acid hydrazide (2 μg/mL) to selectively inhibit the possible growth of residual BCG in BCG-vaccinated mice. Plates were incubated at 37°C for 3 to 4 weeks and the protection was evaluated by assessing the bacterial load represented as mean (log_{10} CFU) ± SEM per organ for each group (n = 5).

**Lung histopathological analysis**

Right lung lobes from the differently vaccinated groups were fixed in 10% formalin in PBS and embedded in paraffin. Tissue sections were prepared and stained with hematoxylin and eosin (HE) or acid-fast (AF) staining. Pathological changes were analyzed by a pathologist under a light microscope (Nikon ECLIPSE E100, Nikon, Tokyo, Japan) in a blinded fashion. The score was obtained by measuring the consolidation area of the whole field of vision (amplification 40) and calculated by the following formula: total area of consolidation/area of whole field of vision × 100%. The results were expressed as the mean of five fields of vision from each different group (n = 3).

**Statistical analysis**

Nonparametric Mann-Whitney U test was used to analyze the antigen-specific IFN-γ levels in the different groups of human subjects. One-way ANOVA analysis was employed to compare the levels of antigen-specific cytokines, the numbers of polyfunctional T lymphocytes, antibody titers, and organ burdens between the different groups. A p-value of <0.05 was considered significant.

**Results**

**Purification and identification of A1D4 polyprotein and multistage antigens**

The recombinant fusion protein A1D4 was constructed by tandem-linking the five *M. tuberculosis* antigens Rv1813, Rv2660c, Ag85B, Rv2623, and HspX, and expression in the plasmid pET30b-A1D4 (Fig 1), and the successful plasmid generation was verified by enzyme digestion (Fig 1). The recombinant protein A1D4 with a C-terminal His-tag was efficiently expressed in *E. coli* BL21 (DE3) as inclusion bodies, and purification was performed under denaturing conditions (Fig 1). The recombinant plasmid expressing *M. tuberculosis* antigens Rv1813, Rv2660c, Ag85B, Rv2623, and HspX, was constructed with an N- or C-terminal His-tag. SDS-PAGE analysis of all purified proteins revealed a single major band with the expected molecular weight (Fig 1). The identity of the major expected bands was confirmed by immunoblotting with an anti-His 6 mouse monoclonal antibody (Fig 1) or mouse polyclonal serum against A1D4 (Fig 1). Notably, polyclonal sera raised against A1D4 were also recognized by each of the single proteins, which confirms the presence of five antigens within the fusion polyprotein (Fig 1).

**IFN-γ profiles in response to A1D4 polyprotein and multistage antigens in human whole blood**

In order to compare the immunogenicity of the recombinant proteins, 10 active TB patients, 17 healthy LTBI, and 15 healthy controls were selected based on the established diagnostic standard, and WBIA technology was used to evaluate the levels of IFN-γ response to purified A1D4 polyprotein and multistage antigens. As shown in Fig 2, there were no statistically significant differences between the three groups in the whole blood IFN-γ response to the positive
control PHA. Interestingly, although China has a high coverage of BCG vaccination, less than 15% of healthy donors responded to antigen Ag85B and the other four latency antigens (Fig 2). Moreover, lower IFN-γ levels ranging from 0 to 390 pg/mL were induced by different multi-stage antigens in the healthy controls than in the TB and LTBI groups. Out of the five multi-stage antigens, HspX (94%) was recognized by T cells of LTBIs with the highest frequency, followed by Rv1883 and Rv2660c. T cells from all TB patients reacted easily to HspX, and more than 70% responded to Rv1883, Ag85B, and Rv2623. Notably, the five stage-specific antigens could be more easily recognized by T cells of both active TB patients and LTBIs, indicating the complex status of *M. tuberculosis* after infection in vivo. In addition, there were no statistically significant differences in the levels of IFN-γ responses to Rv1883, Rv2660c, or A1D4 in TB patients and LTBIs. Most importantly, 41% of the healthy donors, 94% of LTBIs, and 90% of TB patients responded to A1D4 (Fig 2). Our results indicate that the fusion protein A1D4 synergized the immunogenicity of the five individual antigens.

**Decreased bacterial load and lung pathological changes in A1D4-immunized mice**

In order to determine the protective efficacy of A1D4, vaccinated C57BL/6 mice were challenged with the live *M. tuberculosis* H37Rv strain. Four weeks after infection, the bacterial load in different organs and the lung pathology were assessed and compared between the groups.
Whole blood IFN-γ profiles in response to A1D4 polyprotein and multistage antigens in active TB patients, LTBI, and healthy controls. Whole blood samples were obtained from each subject of the active TB patient (n = 10) (A), LTBI (n = 17) (B), and healthy control (n = 15) groups (C), and stimulated with A1D4, Rv1813, Rv2660c, Rv2623, Ag85B, and HspX for 18 to 24 h. PHA was used as a positive control with a cut-off value of 100 pg/mL, and rCM (CFP21-MPT64) was used to confirm the different population characteristics based on the cut-off value of 398.5 pg/mL after clinical diagnosis. The antigen-specific concentration of IFN-γ in each sample was determined with a commercial ELISA kit with a detection limit of 4 pg/mL. Each spot represents the concentration in a sample, and median values for the different groups are shown by horizontal lines. The dotted line represents the cut-off value for a positive response to each.
multistage antigen, which was set arbitrarily at 3 X the mean of the negative control value of healthy controls. The nonparametric Mann-Whitney U-test was used to analyze the IFN-γ levels between the groups or different proteins. \( P < 0.05 \) was considered statistically significant.

The highest bacterial load in the lung and spleen was obtained in the PBS control group (Fig 3) as expected. Importantly, vaccination with BCG or A1D4/MTO strongly reduced the bacterial load in both organs of C57BL/6 mice. A1D4/MTO vaccination significantly decreased the bacterial load in the lung (-1.05 log) and the spleen (-0.99 log) compared to the PBS control (\( p < 0.05 \)). In addition, although mice vaccinated with MTO also evoked a certain protection in comparison to the control group (\( p < 0.05 \)), the protection by MTO was inferior to A1D4/MTO multistage antigen, which was set arbitrarily at 3 X the mean of the negative control value of healthy controls. The nonparametric Mann-Whitney U-test was used to analyze the IFN-γ levels between the groups or different proteins. \( P < 0.05 \) was considered statistically significant.

![Bacterial organ load and pathological changes in the lung.](image)

Fig 3. Bacterial organ load and pathological changes in the lung. Three weeks after the last immunization, C57BL/6 mice (n = 5) were challenged i.v. with 1.2 \( \times 10^6 \) CFU of the \( M. \) tuberculosis H37Rv strain. Four weeks after the challenge, spleens and lungs were harvested and the CFU numbers per organ were counted. The bacterial load in the lung (A) and spleen (B) of the different groups is represented as the mean (±SEM) log10 CFU/organ (n = 5). Lung pathological scores (C) and representative lung pathological changes from the different groups (HE, scale bar, 400 \( \mu \)m) are also shown (D). AF staining (scale bar, 50 \( \mu \)m) of lung tissue section also supported the hierarchy of lung bacterial load in the different groups (D) and arrows indicate AF-positive bacteria in the lung tissue. Challenge experiments were repeated twice with similar results.
immunization (p<0.05). Nevertheless, mice vaccinated with BCG experienced the strongest protective effect of all groups (p<0.05) (Fig 3).

Consistent with the observed varying bacterial loads in the lung, HE- and acid-fast stained lung sections showed clear differences between the groups (Fig 3). Acid-fast bacilli (AFB) were present throughout the whole lung tissue section of control mice, which exhibited the most serious lung pathology with extensive fibrosis, perivasculitis, and alveolitis (Fig 3) and also had the highest pathological score (Fig 3). Lungs from mice of the MTO group showed damage of the alveolar tissues to a lesser extent, and dispersed AFB could be detected. In contrast, lungs from mice vaccinated with A1D4/MTO featured interstitial pneumonia that was accompanied by less pronounced lung inflammation, and much fewer AFB were observed in the tissue. Only a few AFB could be detected in the alveolar tissue from BCG-vaccinated mice, which exhibited limited lung inflammation (Fig 3) and the lowest pathological score (Fig 3).

Antibody responses induced by A1D4 in adjuvant of MTO

The immunogenicity of A1D4 in the adjuvant of MTO was evaluated in C57BL/6 mice. A1D4-specific antibodies, including IgG, IgG1, and IgG2c, were determined by ELISA in the 9th week. As expected, there was no induction of antibodies in either the MTO alone or the PBS control group. Only mice vaccinated with A1D4/MTO induced significantly higher levels of A1D4-specific IgG, IgG1, and IgG2c antibodies (Fig 4) than the BCG group (p<0.05). Moreover, the ratio of IgG2c/IgG1 in the A1D4/MTO group was much higher than that of the BCG group, which indicates a switch of the IgG subclass to a Th1-based response (Fig 4).

Antigen-specific Th1 cytokines induced by A1D4/MTO immunization

The Th1-type response plays a fundamental role in the protection against TB [2]. Th1-type cytokines, such as IFN-γ and TNF-α, which synergize to activate the microbicidal mechanism of macrophages, and IL-2, which promotes the proliferation and maturation of T cells [33], are produced in the Th1-type response. The important effects of IL-2 on the development of TB vaccine were confirmed in our [34] and other [35] previous reports. Splenocytes from the PBS

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**Fig 4. A1D4-specific IgG, IgG1, and IgG2c antibodies in immunized mice (n = 3).** Three weeks after the last immunization, sera were collected from each C57BL/6 mouse of the different groups. IgG, IgG1, and IgG2c (replaced by IgG2a during the assay) antibodies against A1D4 in the immunized mice were detected by ELISA as described in detail in the Materials and Methods section. Results are shown as mean (±SEM) log10 endpoint titer (A) and the ratio of IgG2a/IgG1 (B) in the differently vaccinated mice (n = 3). *P*<0.05 was considered statistically significant and is indicated by the bar. This experiment was repeated twice with similar results.

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control only produced very low levels of the cytokines IL-2, IFN-γ, and TNF-α, when stimulated with either PPD or A1D4 protein (Fig 5). Compared with the control, the BCG vaccine induced higher levels of IL-2, IFN-γ, and TNF-α responses to PPD ($p<0.05$) (Fig 5), which confirms the essential role of these cytokines in the BCG-induced protection against TB. Among all the groups, the highest levels of TNF-α and IFN-γ responses to PPD were induced in the BCG group, and those to A1D4 protein were conferred in the A1D4/MTO group ($p<0.001$). In addition, there were no statistically significant differences in the levels of IL-2 responses to PPD or A1D4 between the A1D4/MTO, BCG, and MTO groups. Taken together with previous results, our study suggests that the protection conferred by A1D4/MTO is also largely dependent on A1D4-specific Th1-type responses.

Polyfunctional T cells in response to PPD and A1D4

In order to obtain protective biomarkers for clinical or experimental evaluation of TB vaccine candidates, seven combinations of three cytokines in CD4+ or CD8+ T cells were compared between the different groups. Relative to the control, the BCG vaccine only significantly increased

![Fig 5. The levels of IFN-γ, IL-2, and TNF-α secreted by splenocytes of differently immunized mice.](https://example.com/fig5.png)

Three weeks after the last immunization, splenocytes were obtained from each mouse of the differently vaccinated groups ($n=3$). $2.5 \times 10^6$ cells were added into each well of 24-well microtiter plates and incubated with A1D4 protein (10 μg/mL), PPD (10 μg/mL, positive control), or complete RPMI1640 medium (negative control) for 24 h (for IL-2 detection) or 72 h (for IFN-γ and TNF-α detection) at 37°C, respectively. The cytokine concentrations in the suspension were detected with commercial ELISA kits. The results are shown as mean ± SD (pg/mL) and the bar indicates $p<0.05$. Similar results were obtained from two independent experiments.

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the absolute number of PPD-specific single IL-2+ CD4+ T cells and IL-2+IFN-γ+TNF-α+ IL-2+IFN-γ+, and IL-2+TNF-α+ CD4+ T cells \( (p<0.05) \). More than half of the seven combinations of CD4+ T cell responses to PPD or A1D4 in BCG-vaccinated mice belonged to single IL-2+ and IFN-γ+IL-2+TNF-α+ cells (Fig 6), which could be the key factors correlating with BCG-induced protection. Among these four combinations, only PPD-specific IL-2+IFN-γ+ CD4+ T cells were more significantly increased in the A1D4/MTO group (only 0.98% of the seven combinations in CD4+ T cells) \( (p<0.05) \), and especially increased to 2.21% in response to A1D4 \( (p<0.05) \) compared with the PBS and MTO controls (Fig 6). In contrast, A1D4/MTO immunization mainly increased PPD- or A1D4-specific single IFN-γ+ CD4+ T cells and the following IFN-γ+TNF-α+ CD4+ T cells \( (p<0.05) \). On the other hand, the BCG vaccine evoked mainly PPD-specific single IFN-γ+ and single IL-2+ CD8+ T cells, and the following IL-2+IFN-γ+TNF-α+, IFN-γ+TNF-α+, and IL-2+IFN-γ+ CD8+ T cells, when compared with control mice \( (p<0.05) \). Among these five combinations in CD8+ T cells, the highest absolute number of single IFN-γ+ and IFN-γ+TNF-α+ CD8+ T cells response to either PPD or A1D4 protein were obtained only in A1D4/MTO-vaccinated mice \( (p<0.05) \) (Fig 6) followed by A1D4-specific IL-2+IFN-γ+ and IL-2+IFN-γ+TNF-α+ CD8+ T cells, which also increased more significantly in the A1D4/MTO group compared to the PBS control \( (p<0.05) \).

Discussion

Due to the serious threat of a TB epidemic to public health [36] and the lack of protection by the current BCG vaccine, protein subunit vaccines present a compelling area of research for improved control of TB. Nevertheless, it is very difficult to screen promising antigens encoded by the genome of \( M. \) \( \text{tuberculosis} \) that contains 4018 ORFs [37]. The approach of combining clinical diagnosis with WBIA has been presented in our previous studies [38, 39]. This method was reconﬁrmed as a reasonable, simple, and effective way to screen target antigens with vaccine potential, in comparison with other methods such as biochemical separation [3, 4], comparative [40] or immune proteomics [41], comparative genomics [42], transcriptomics [43], or even direct animal challenge experiments [21]. In this study, five multistage antigens were selected based on the interaction between \( M. \) \( \text{tuberculosis} \) and its host, and were conﬁrmed to be recognized by T cells of human TB subjects. The polyprotein A1D4 consisting of five multistage antigens was constructed and its immunogenicity and protective efficacy in adjuvant of MTO was evaluated in vaccinated C57BL/6 mice. Our results demonstrated that the subunit vaccine A1D4/MTO could provide more significant protection against \( M. \) \( \text{tuberculosis} \) infection than the PBS control and adjuvant MTO alone, as evidenced by lower bacterial load in the lung and spleen, less pathological changes in the lung, and more significantly increased A1D4-specific Th1-type responses.

The BCG vaccine has been used as the gold standard to evaluate the efficacy of vaccine candidates in TB animal models. Nonetheless, it has proven difficult to find a new subunit vaccine that provides stronger protective efficacy than BCG, because BCG is an attenuated strain derived from \( M. \) \( \text{bovis} \) and secretes at least 800 proteins [44]. Naturally, BCG infects APCs including macrophages and DCs as a way of presenting antigens after immunization [45]. Although protection by A1D4/MTO is not as good as BCG, differential immunogenicity and protective efficacy observed in the BCG- and A1D4/MTO-immunized mice suggest that this is a good model for finding effective biomarkers associated with protection, which would help assess vaccine-induced protection and develop the next generation of vaccines. The occurrence and progression of TB infection is mainly associated with cell-mediated immunity of the host. BCG is a stronger inducer of CD4+ Th1 cells than CD8+ T cells, partially because BCG lacks the ESX-1 mechanism [46] and is unable to translocate from the phagolysosome to the cytosol. Antigens
Fig 6. Polyfunctional expression of IFN-γ, TNF-α, and IL-2 by CD4+ or CD8+ T cells in response to PPD or A1D4 analyzed by multicolor flow cytometry. Splenocytes of each mouse from the different groups were collected and counted. 5 x 10^6 cells/well in a 24-well plate were stimulated with A1D4 (10 μg/mL) or PPD (10 μg/mL) as described in detail in the Materials and Methods section. Intracellular cytokine profiles for IFN-γ, TNF-α, and IL-2 in individual cells were measured by multicolor flow cytometry by gating for CD4+ or CD8+ T cells. Every possible combination of cytokines is shown on the x-axis of the bar chart and the absolute number of antigen-specific T cells expressing any combination of cytokines is shown as mean ± SEM in the different groups (n = 3). Similar results were obtained from two independent experiments (the bar indicates p<0.05).

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localized to the phagosome would therefore be processed and presented mainly via MHC II molecules. Identifying and understanding effective biomarkers with protection capacity is of extreme significance, because the BCG vaccine provides only limited protection against adult TB, and further enhanced Th1 response boosted by MVA85A [47] fails to provide enhanced efficacy in vaccinated infants [48].

Polyfunctional analysis was first reported in HBV vaccine-induced T cell responses [49] and was highlighted in a few recent clinical trials of TB vaccine candidates [50, 51] and in a mouse model using Leishmania [52]. Although polyfunctional T cells may be involved in the protection against TB, effective biomarkers of polyfunctional T cells with vaccine-induced protection remain to be defined. It is worth noting that polyfunctional T cell responses to PPD or A1D4 in BCG- and A1D4/MTO-vaccinated mice were quite different. Our results clearly demonstrate the phenotypic and functional heterogeneity of antigen-specific CD4+ T cells and CD8+ T cells induced by BCG or A1D4/MTO. In particular, the number of single IL-2+ and IFN-γ IL-2 TNF-α+ CD4+ T cells was significantly increased in BCG-vaccinated mice. Although the highest absolute number of single IFN-γ CD4+ T cell responses to PPD or A1D4 was conferred by A1D4/MTO-immunized mice, it did not positively correlate with the strongest protection against infection among all the groups, which supports the previous conclusion [53]. In addition, H56 in adjuvant of CAF01-vaccinated mice also significantly evoked IL-2 IFN-γ TNF-α- and IL-2 IFN-γ- secreting CD4+ T cells and provided a protection against M. tuberculosis infection comparative with BCG [19], while mainly the number of IFN-γ TNF-α+ CD4+ T cells increased in ID93/GLA-SE-immunized mice with protection inferior to BCG [29]. The same observation was demonstrated in A1D4/MTO-vaccinated mice, which also significantly elicited PPD- and A1D4-specific single IFN-γ- and IFN-γ TNF-α- secreting CD4+ T cells in this study. Single IFN-γ+ CD4+ T cells are associated with viral primary infection with high viral load, while chronic viral infection is related to single IL-2+ or IL-2 IFN-γ+ CD4+ T cell responses [54]. Polyfunctional analysis in HIV and M. tuberculosis co-infections revealed that HIV viral load positively correlated with single IFN-γ CD4+ T cells yet negatively correlated with M. tuberculosis-specific IL-2- secreting CD4+ T cells [54]. Antiretroviral therapy promotes significant increases of IL-2 IFN-γ- and IL-2 TNF-α- secreting CD4+ T cells and hinders the progression from infection to active TB, while single IFN-γ+ CD4+ T cells declined significantly [55]. In addition, IL-2 IFN-γ TNF-α- secreting CD4+ T cells in response to antigen Ag85B or HspX were also more easily detected in active TB patients than LTBI subjects [56,57]. Compared with pretreatment, there was a clear increase in the proportions of IL-2 IFN-γ secreting CD4+ T cells following treatment, which is similar to the pattern observed in the LTBI population [56]. All of these findings indicate that antigen-specific single IL-2 secreting cells and different combinations with IL-2 secreting CD4+ T cells are positively correlated with vaccine-induced protection against TB. Therefore, the increased number of IL-2 IFN-γ CD4+ T cells should be closely related to the protection in A1D4/MTO-vaccinated mice, whereas the absence of single IL-2-, IFN-γ IL-2 TNF-α-, and IL-2 TNF-α+ CD4+ T cells might explain the reason for a protection by A1D4/MTO inferior to BCG.

CD8+ T cells were confirmed to play an important role in the protection in mice or non-human models, which might take part in the process of preventing the reactivation of LTBI and compensate the role of CD4+ Th1 response against primary infection [58]. The optimal protection induced by vaccines may be obtained by combination of CD4+ Th1 and CD8+ T cell responses. However, the role of polyfunctional CD8+ T cells in the vaccine induced-protection against TB infection remains unclear. In this study, the frequency of single IFN-γ CD8+ T cells was increased more significantly in A1D4/MTO- than BCG-vaccinated mice. Previous studies have confirmed the protective role of IFN-γ secreting CD8+ T cells in a mouse TB model [59] and in humans [60]. In addition, much more PPD- or A1D4-specific IFN-γ TNF-α+ CD8+ T cells...
T cells were induced in A1D4/MTO-vaccinated mice than in those vaccinated with BCG. IFN-γ+TNF-α+ CD8+ T cells in response to latency antigens including HspX was the most frequently identified in long-term latently infected individuals that did not progress to active TB [61]. Therefore, these CD8+ T cell types might present the advantages of A1D4/MTO over BCG to protect against latent infection, providing indirect evidence to support the popular strategy of BCG priming and heterologous boosting with A1D4/MTO in future studies in order to remedy CD8+ T-cell responses after BCG vaccination and limit infection and progression to active TB.

Conclusions
The observations of the present study suggest that vaccination with A1D4/MTO, consisting of four antigens related to the phase of latency and one secreted antigen related to the phase of primary infection, could provide effective protection against *M. tuberculosis* infection in vaccinated mice and present an effective strategy for the development of the next generation of vaccines. Our results warrant further evaluation of A1D4/MTO in different animal models, including latent infection and BCG vaccination boosting.

Supporting Information
S1 Fig. Representative flow cytometry showing the gating strategy with the FlowJo 7.61 software for the identification of single and polyfunctional CD4+ and CD8+ T cells per spleen from differently vaccinated mice.

S1 Table. Primers and thermal cycle parameters for cloning of *M. tuberculosis* antigens.

S2 Table. Characteristics of human subjects.

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Author Contributions
Conceived and designed the experiments: XLF XCW YZ. Performed the experiments: XCW JYZ JPL. Analyzed the data: XCW JYZ JPL XDT XLF XFY. Contributed reagents/materials/analysis tools: XFY YZ. Wrote the paper: XLF XCW YZ.

References
1. Wang CC, Zhu B, Fan X, Gicquel B, Zhang Y (2013) Systems approach to tuberculosis vaccine development. Respirology 18: 412–420. doi: 10.1111/resp.12052 PMID: 23331331
2. Ottenhoff TH, Kaufmann SH (2012) Vaccines against tuberculosis: where are we and where do we need to go? PLoS Pathog 8: e1002607. doi: 10.1371/journal.ppat.1002607 PMID: 22589713
3. Andersen P, Askgaard D, Ljungqvist L, Bennedsen J, Heron I (1991) Proteins released from Mycobacterium tuberculosis during growth. Infect Immun 59: 1905–1910. PMID: 19037688
4. Nagai S, Wiker HG, Harboe M, Kinomoto M (1991) Isolation and partial characterization of major protein antigens in the culture fluid of Mycobacterium tuberculosis. Infect Immun 59: 372–382. PMID: 1898899
5. Sable SB, Kalra M, Verma I, Khuller GK (2007) Tuberculosis subunit vaccine design: the conflict of antigenicity and immunogenicity. Clin Immunol 122: 239–251. PMID: 17208519
6. Geluk A, van Meijgaarden KE, Joosten SA, Commandeur S, Ottenhoff TH (2014) Innovative Strategies to Identify M. tuberculosis Antigens and Epitopes Using Genome-Wide Analyses. Front Immunol 5: 256. doi: 10.3389/fimmu.2014.00256 PMID: 25099541
7. Betts JC, Lukey PT, Robb LC, McAdam RA, Duncan K (2002) Evaluation of a nutrient starvation model of Mycobacterium tuberculosis persistence by gene and protein expression profiling. Mol Microbiol 43: 717–731. PMID: 11929527
8. Voskuil MI, Visconti KC, Schoolnik GK (2004) Mycobacterium tuberculosis gene expression during adaptation to stationary phase and low-oxygen dormancy. Tuberculosis (Edinb) 84: 218–227. PMID: 15207491
9. Voskuil MI, Schnappinger D, Visconti KC, Harrell MI, Dolganov GM, Sherman DR, et al. (2003) Inhibition of respiration by nitric oxide induces a Mycobacterium tuberculosis dormancy program. J Exp Med 198: 705–713. PMID: 12953092
10. Selvaraj S, Sambandam V, Sardar D, Anishetty S (2012) In silico analysis of DosR regulon proteins of Mycobacterium tuberculosis. Gene 506: 233–241. doi: 10.1016/j.gene.2012.06.033 PMID: 22759512
11. Sturgill-Koszycki S, Schlesinger PH, Chakraborty P, Haddix PL, Collins HL, Fok AK, et al. (1994) Lack of acidification in Mycobacterium phagosomes produced by exclusion of the vesicular proton-ATPase. Science 263: 678–681. PMID: 8303277
12. Gringhuis SI, den Dunnen J, Litjens M, van der Vlist M, Geijtenbeek TB (2009) Carbohydrate-specific signaling through the DC-SIGN signalosome tailors immunity to Mycobacterium tuberculosis, HIV-1 and Helicobacter pylori. Nat Immunol 10: 1081–1088. doi: 10.1038/ni.1778 PMID: 19718030
13. Gutierrez MG, Master SS, Singh SB, Taylor GA, Colombo MI, Deretic V, et al. (2004) Autophagy is a defense mechanism inhibiting BCG and Mycobacterium tuberculosis survival in infected macrophages. Cell 119: 753–766. PMID: 15060793
14. Keane J, Remold HG, Komfeld H (2000) Virulent Mycobacterium tuberculosis strains evade apoptosis of infected alveolar macrophages. J Immunol 164: 2016–2020. PMID: 10657653
15. Glickman MS, Jacobs WJ (2001) Microbial pathogenesis of Mycobacterium tuberculosis: dawn of a discipline. Cell 104: 477–485. PMID: 11239406
16. D’Souza S, Rosseels V, Romano M, Tanghe A, Denis O, Jurion F, et al. (2003) Mapping of murine Th1 helper T-Cell epitopes of mycolyl transferases Ag85A, Ag85B, and Ag85C from Mycobacterium tuberculosis. Infect Immun 71: 483–493. PMID: 12496199
17. Lange S, Rosenkranz J, Stein R, Andersen P, Kaufmann SH, Jungblut PR, et al. (2014) Analysis of protein species differentiation among mycobacterial low-Mr-secreted proteins by narrow pH range Immobiline gel 2-DE-MALDI-MS. J Proteomics 97: 235–244. doi: 10.1016/j.jprot.2013.06.036 PMID: 23865608
18. Egen JG, Rothfluchs AG, Feng CG, Horwitz MA, Sher A, Germain RN, et al. (2011) Intravital imaging reveals limited antigen presentation and T cell effector function in mycobacterial granulomas. Immunity 34: 807–819. doi: 10.1016/j.immuni.2011.03.022 PMID: 21569592
19. Aagaard C, Hoang T, Dietrich J, Cardona PJ, Izzo A, Dolganov G, et al. (2011) A multistage tuberculosis vaccine that confers efficient protection before and after exposure. Nat Med 17: 189–194. doi: 10.1038/nm.2285 PMID: 21258338
20. Vasudeva-Rao HM, McDonough KA (2008) Expression of the Mycobacterium tuberculosis acr-coregulated genes from the DevR (DosR) regulon is controlled by multiple levels of regulation. Infect Immun 76: 2478–2489. doi: 10.1128/IAI.01443-07 PMID: 18391009
21. Bertholet S, Ireton GC, Kahn M, Guderian J, Mohamath R, Stride N, et al. (2008) Identification of human T cell antigens for the development of vaccines against Mycobacterium tuberculosis. J Immunol 181: 7948–7957. PMID: 19017986
22. Leyten EM, Lin MY, Franken KL, Friggen AH, Prins C, Van Meijgaarden KE, et al. (2006) Human T-cell responses to 25 novel antigens encoded by genes of the dormancy regulon of Mycobacterium tuberculosis. Microbes Infect 8: 2052–2060. PMID: 16931093
23. Andersen P, Doherty TM (2005) The success and failure of BCG—implications for a novel tuberculosis vaccine. Nat Rev Microbiol 3: 656–662. PMID: 16012514
24. Mangtani P, Abubakar I, Arifi A, Beynon R, Pimpin L, Fine PE, et al. (2014) Protection by BCG vaccine against tuberculosis: a systematic review of randomized controlled trials. Clin Infect Dis 58: 470–480. doi: 10.1093/cid/cit790 PMID: 24336911
25. Geluk A, Lin MY, van Meijgaarden KE, Leyten EM, Franken KL, Ottenhoff TH, et al. (2007) T-cell recognition of the HspX protein of Mycobacterium tuberculosis correlates with latent M. tuberculosis infection but not with M. bovis BCG vaccination. Infect Immun 75: 2914–2921. PMID: 17387166
26. Geluk A, van den Eeden SJ, van Meijgaard KE, Dijkman K, Franken KL, Ottenhoff TH, et al. (2012) A multistage-polyepitope vaccine protects against Mycobacterium tuberculosis infection in HLA-DR3 transgenic mice. Vaccine 30: 7513–7521. doi: 10.1016/j.vaccine.2012.10.045 PMID: 23103299

27. Xin Q, Niu H, Li Z, Zhang G, Hu L, Wang B, et al. (2013) Subunit vaccine consisting of multi-stage antigens has high protective efficacy against Mycobacterium tuberculosis infection in mice. PLoS One 8: e72745. doi: 10.1371/journal.pone.0072745 PMID: 23967337

28. Lin PL, Dietrich J, Tan E, Abalos RM, Burgos J, Bigbee C, et al. (2012) The multistage vaccine H56 boosts the effects of BCG to protect cynomolgus macaques against active tuberculosis and reactivation of latent Mycobacterium tuberculosis infection. J Clin Invest 122: 303–314. doi: 10.1172/JCI46252 PMID: 22133873

29. Bertholet S, Iretton GC, Ordway DJ, Windish HP, Pine SO, Kahn M, et al. (2010) A defined tuberculosis vaccine candidate boosts BCG and protects against multidrug-resistant Mycobacterium tuberculosis. Sci Transl Med 2: 53r–74r.

30. Shi C, Chen L, Chen Z, Zhang Y, Zhou Z, Lu J, et al. (2010) Enhanced protection against tuberculosis by vaccination with recombinant BCG over-expressing HspX protein. Vaccine 28: 5237–5244. doi: 10.1016/j.vaccine.2010.05.063 PMID: 20538090

31. Fu R, Wang C, Shi C, Lu M, Fang Z, Lu J, et al. (2009) An improved whole-blood gamma interferon assay based on the CFP21-MPT64 fusion protein. Clin Vaccine Immunol 16: 686–691. doi: 10.1128/CVI.00486-08 PMID: 19279170

32. Commandeur S, van Meijgaarden KE, Prins C, Pichugin AV, Dijkman K, van den Eeden SJ, et al. (2013) An unbiased genome-wide Mycobacterium tuberculosis gene expression approach to discover antigens targeted by human T cells expressed during pulmonary infection. J Immunol 190: 1659–1671. doi: 10.4049/jimmunol.1201593 PMID: 23319735

33. Smith KA (1988) Interleukin-2: inception, impact, and implications. Science 240: 1169–1176. PMID: 3131876

34. Fan XL, Yu TH, Gao Q, Yao W (2006) Immunological properties of recombinant Mycobacterium bovis bacillus Calmette-Guerin strain expressing fusion protein IL-2-ESAT-6. Acta Biochim Biophys Sin (Shanghai) 38: 683–690.

35. Young SL, O’Donnell MA, Buchan GS (2002) IL-2-secreting recombinant bacillus Calmette Guerin can overcome a Type 2 immune response and corticosteroid-induced immunosuppression to elicit a Type 1 immune response. Int Immunol 14: 793–800. PMID:12096039

36. World Health Organization (WHO) Global tuberculosis report 2013. Geneva: WHO; 23 Oct 2013.

37. Tuberculist. Available: http://tuberculist.epfl.ch/. Accessed 2015 Mar 15.

38. Wang C, Chen Z, Fu R, Zhang Y, Chen L, Huang L, et al. (2011) A DNA vaccine expressing CFP21 and MPT64 fusion protein enhances BCG-induced protective immunity against Mycobacterium tuberculosis infection in mice. Med Microbiol Immunol 200: 165–175. doi: 10.1007/s00430-011-0188-z PMID: 21340709

39. Lu J, Wang C, Zhou Z, Zhang Y, Cao T, Shi C, et al. (2011) Immunogenicity and protective efficacy against murine tuberculosis of a prime-boost regimen with BCG and a DNA vaccine expressing ESAT-6 and Ag85A fusion protein. Clin Dev Immunol 2011: 617892. doi: 10.1155/2011/617892 PMID: 21461375

40. Mollenkopf HJ, Grode L, Mattow J, Stein M, Mann P, Knapp B, et al. (2004) Application of mycobacterial proteomics to vaccine design: improved protection by Mycobacterium bovis BCG prime-Rv3407 DNA boost vaccination against tuberculosis. Infect Immun 72: 6471–6479. PMID:15501778

41. Kunnath-Velayudhan S, Salamon H, Wang HY, Davidow AL, Molina DM, Huynh VT, et al. (2010) Dynamic antibody responses to the Mycobacterium tuberculosis proteome. Proc Natl Acad Sci U S A 107: 14703–14708. doi: 10.1073/pnas.1009080107 PMID: 20668240

42. Behr MA, Wilson MA, Gill WP, Salamon H, Schoolnik GK, Rane S, et al. (1999) Comparative genomics of BCG vaccines by whole-genome DNA microarray. Science 284: 1520–1523. PMID: 10348738

43. Golby P, Hatch KA, Bacon J, Cooney R, Riley P, Allnutt J, et al. (2007) Comparative transcriptomics reveals key gene expression differences between the human and bovine pathogens of the Mycobacterium tuberculosis complex. Microbiology 153: 3323–3336. PMID: 17906132

44. Jungblut PR, Schäible UE, Mollenkopf HJ, Jimney-Arndt U, Raupach B, Mattow J, et al. (1999) Comparative proteome analysis of Mycobacterium tuberculosis and Mycobacterium bovis BCG strains: towards functional genomics of microbial pathogens. Mol Microbiol 33: 1103–1117. PMID: 10540122

45. Kumari M, Saxena RK (2011) Relative efficacy of uptake and presentation of Mycobacterium bovis BCG antigens by type I mouse lung epithelial cells and peritoneal macrophages. Infect Immun 79: 3159–3167. doi: 10.1128/IAI.05406-11 PMID: 21646448
46. van der Wel N, Hava D, Houben D, Fluitsma D, van Zon M, Pierson J, et al. (2007) M. tuberculosis and M. leprae translocate from the phagolysosome to the cytosol in myeloid cells. Cell 129: 1287–1298. PMID: 17604718

47. Tameris M, Geldenhuys H, Luabeya AK, Smit E, Hughes JE, Vermaak S, et al. (2014) The candidate TB vaccine, MVA85A, induces highly durable Th1 responses. PLoS One 9: e87340. doi: 10.1371/journal.pone.0087340 PMID: 24498312

48. Satti I, Meyer J, Harris SA, Manjaly TZ, Griffiths K, Antrobus RD, et al. (2014) Safety and immunogenicity of a candidate tuberculosis vaccine MVA85A delivered by aerosol in BCG-vaccinated healthy adults: a phase 1, double-blind, randomised controlled trial. Lancet Infect Dis 14: 939–946. doi: 10.1016/S1473-3099(14)70845-X PMID: 25151225

49. Harari A, Dutoit V, Cellerei C, Bart PA, Du Pasquier RA, Pantaleo G, et al. (2006) Functional signatures of protective antiviral T-cell immunity in human virus infections. Immunol Rev 211: 236–254. PMID: 16824132

50. Scriba TJ, Tameris M, Mansoor N, Smit E, van der Merwe L, Isaacs F, et al. (2010) Modified vaccinia Ankara-expressing Ag85A, a novel tuberculosis vaccine, is safe in adolescents and children, and induces polyfunctional CD4+ T cells. Eur J Immunol 40: 279–290. doi: 10.1002/eji.200939754 PMID: 20017188

51. Leroux-Roels I, Forgus S, De Boever F, Clement F, Demolite MA, Mettens P, et al. (2013) 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 31: 2196–2206. doi: 10.1016/j.vaccine.2012.05.035 PMID: 22643213

52. Darrah PA, Patel DT, De Luca PM, Lindsay RW, Davey DF, Flynn BJ, et al. (2007) Multifunctional TH1 cells define a correlate of vaccine-mediated protection against Leishmania major. Nat Med 13: 843–850. PMID: 17558415

53. Mittrucker HW, Steinhoff U, Kohler A, Krause M, Lazar D, Mex P, et al. (2007) Poor correlation between BCG vaccination-induced T cell responses and protection against tuberculosis. Proc Natl Acad Sci U S A 104: 12434–12439. PMID: 17640915

54. Day CL, Mkhwanazi N, Reddy S, Mncube Z, van der Stok M, Klenerman P, et al. (2008) Detection of polyfunctional Mycobacterium tuberculosis-specific T cells and association with viral load in HIV-1-infected persons. J Infect Dis. 197: 990–999. doi: 10.1086/529048 PMID: 18419535

55. Sutherland JS, Young JM, Peterson KL, Sanneh B, Whittle HC, Rowland-Jones SL, et al. (2010) Polyfunctional CD4(+) and CD8(+) T cell responses to tuberculosis antigens in HIV-1-infected patients before and after anti-retroviral treatment. J Immunol 184: 6537–6544. doi: 10.4049/jimmunol.1000399 PMID: 20435929

56. Caccamo N, Guggino G, Joosten SA, Gelsomino G, Di Carlo P, Titone L, et al. (2010) Multifunctional CD4(+) T cells correlate with active Mycobacterium tuberculosis infection. Eur J Immunol 40: 2211–2220. doi: 10.1002/eji.201040455 PMID: 20540114

57. Sutherland JS, Adetifa IM, Hill PC, Adegbola RA, Ota MO (2009) Pattern and diversity of cytokine production differentiates between Mycobacterium tuberculosis infection and disease. Eur J Immunol 39: 723–729. doi: 10.1002/eji.200838693 PMID: 19224636

58. Woodworth JS, Behar SM (2006) Mycobacterium tuberculosis-specific CD8+ T cells and their role in immunity. Crit Rev Immunol 26: 317–352. PMID: 17073557

59. Tascon RE, Stavropoulos E, Lukacs KV, Colston MJ (1998) Protection against Mycobacterium tuberculosis infection by CD8+ T cells requires the production of gamma interferon. Infect Immun 66: 830–834. PMID: 9453650

60. Lalvani A, Brookes R, Wilkinson RJ, Malin AS, Pathan AA, Anderson P, et al. (1998) Human cytolytic and interferon gamma-secreting CD8+ T lymphocytes specific for Mycobacterium tuberculosis. Proc Natl Acad Sci U S A 95: 270–275. PMID: 9419365

61. Commandeur S, Lin MY, van Meijgaard en KE, Friggen AH, Franken KL, Drijfhout JW, et al. (2011) Double- and monofunctional CD4(+) and CD8(+) T-cell responses to Mycobacterium tuberculosis DosR antigens and peptides in long-term latently infected individuals. Eur J Immunol 41: 2925–2936. doi: 10.1002/eji.201114602 PMID: 21728172