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Systematic Evaluation of *Mycobacterium tuberculosis* Proteins for Antigenic Properties Identifies Rv1485 and Rv1705c as Potential Protective Subunit Vaccine Candidates

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ABSTRACT The lack of efficacious vaccines against *Mycobacterium tuberculosis* (MTB) infection is a limiting factor in the prevention and control of tuberculosis (TB), the leading cause of death from an infectious agent. Improvement or replacement of the BCG vaccine with one that reliably protects all age groups is urgent. Concerns exist that antigens currently being evaluated are too homogeneous. To identify new protective antigens, we screened 1,781 proteins from a high-throughput proteome-wide protein purification study for antigenic activity. Forty-nine antigens (34 previously unreported) induced antigen-specific gamma interferon (IFN-γ) release from peripheral blood mononuclear cells (PBMCs) derived from 4,452 TB and suspected TB patients and 167 healthy donors. Three (Rv1485, Rv1705c, and Rv1802) of the 20 antigens evaluated in a BALB/c mouse challenge model showed protective efficacy, reducing lung CFU counts by 66.2%, 75.8%, and 60%, respectively. Evaluation of IgG2a/IgG1 ratios and cytokine release indicated that Rv1485 and Rv1705c induce a protective Th1 immune response. Epitope analysis of PE/PPE protein Rv1705c, the strongest candidate, identified a dominant epitope in its extreme N-terminal domain accounting for 90% of its immune response. Systematic preclinical assessment of antigens Rv1485 and Rv1705c is warranted.

KEYWORDS *Mycobacterium tuberculosis*, TB vaccine, antigen, protective efficacy, Th1 immune response, IFN-γ, ELISpot assay, bacillus Calmette-Guérin vaccine, PE/PPE protein

Realization of the WHO End TB strategy by 2030 requires new momentum in every aspect of tuberculosis (TB) prevention and control, including development of new vaccines, diagnostics, and drugs. Low efficacy of the bacillus Calmette-Guérin (BCG) vaccine and increasing resistance to antitubercular drugs have led to the reemergence of TB as a global health threat (1). TB is the leading cause of death from an infectious agent, with ~10 million new cases and ~1.41 million deaths occurring worldwide in 2019 (1). Furthermore, the STOP TB Partnership and other global bodies have indicated

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that the COVID-19 pandemic has heavily impacted national TB programs and will likely lead to significant increases in TB cases and deaths (2, 3). Development of improved vaccines is the most efficient strategy for achieving the elimination of TB. While significant investments in vaccine research have yielded a pipeline of 14 vaccine candidates in different stages of clinical trials (reviewed in depth elsewhere [4, 5]), concerns exist that antigens currently being evaluated are too homogeneous. A licensed universally effective tuberculosis vaccine is still unlikely to emerge in the near future (6).

The BCG vaccine prevents severe forms of TB in children (7) but has weak and inconsistent efficacy against adult pulmonary TB (8). The goal of the heterologous "prime-boost" vaccine development strategy is to enhance and extend the immune response induced by BCG inoculations given to infants before exposure to *Mycobacterium tuberculosis (MTB)*, by administering a booster subunit vaccine (comprised of proteins, peptides, or DNA) later in childhood or in adolescence, when BCG's protective effect begins to wane (9, 10). The protective efficacy of antigens used as subunits is key to the success of this strategy; however, to date, only 11 antigens that induce a protective immune response (PepA, PPE18, Ag85A, Ag85B, TB10.4, PPE42, EsxV, EsxW, Rv1813, ESAT-6, and Rv2660c) are widely utilized (11–16). The most recent vaccine candidate to complete phase IIb trials, M72/AS01E, showed an efficacy of 49.7% 36 months postimmunization in adults with MTB infection in southern Africa, providing the first proof-of-concept evidence of the biological feasibility of developing an efficacious vaccine against TB (17). However, a recent study comparing the antigen-specific T cell responses of six current vaccine candidates and BCG found that while T cell responses vary in magnitude among candidates, M72/AS01E being the strongest, the induced cytokine coexpression profiles of memory CD4 T cells were alike, and functional profiles revealed a lack of response diversity (18). System-wide antigen screening studies to identify and evaluate a wider range of antigens with protective efficacy are thus needed (19, 20).

Poor understanding of TB immune responses is an additional challenge, particularly as there are no well-established immunological correlates of protection or biomarkers for efficacy (20, 21). BCG inoculation induces a T-helper cell 1 (Th1) type response, involving gamma interferon (IFN-γ) production by $CD4^+$ T cells (21). To be effective as boosters, ideal vaccine candidates should induce a cell immune response during both initial infection with *M. tuberculosis* and during already established latent TB infection in order to prevent disease progression. Achieving control of *M. tuberculosis* requires eliciting a balanced immune response encompassing multiple components of both the pulmonary mucosal and systemic responses. Effective vaccine candidates will boost Th1 responses and T lymphocytes (particularly CD4 Th1 lymphocytes), with IFN-γ produced by these cells playing a key role in controlling *M. tuberculosis* (22, 23). Given its important role, IFN-γ release in response to MTB antigen stimulation (24) is a practical and convenient marker for preliminary antigen screening to identify biomarkers for MTB infection or candidates for development into protein subunit vaccines (25).

Here, to screen for MTB antigens with protective efficacy, we performed high-throughput expression and purification of the MTB proteome and then used IFN-γ enzyme-linked immunosorbent spot (ELISpot) assays and peripheral blood mononuclear cell (PBMC) samples from patients with active TB and suspected TB to perform a preliminary screen for proteins that induce antigen-specific IFN-γ release. Further evaluation of candidate antigens in murine challenge and cell biology experiments showed that proteins Rv1485 and Rv1705c have promising protective efficacy and induce protective Th1 cytokine responses. Our results suggest that Rv1485 and Rv1705c are protective antigens deserving further investigation as potential subunits for TB vaccines.

RESULTS

High-throughput purification of 1,781 *M. tuberculosis* proteins. We performed a preliminary systems-level screen for MTB protein antigens using a high-throughput approach, screening for the ability to induce IFN-γ release from PBMCs isolated from TB patients. We then evaluated the protective efficacy of promising candidates in a

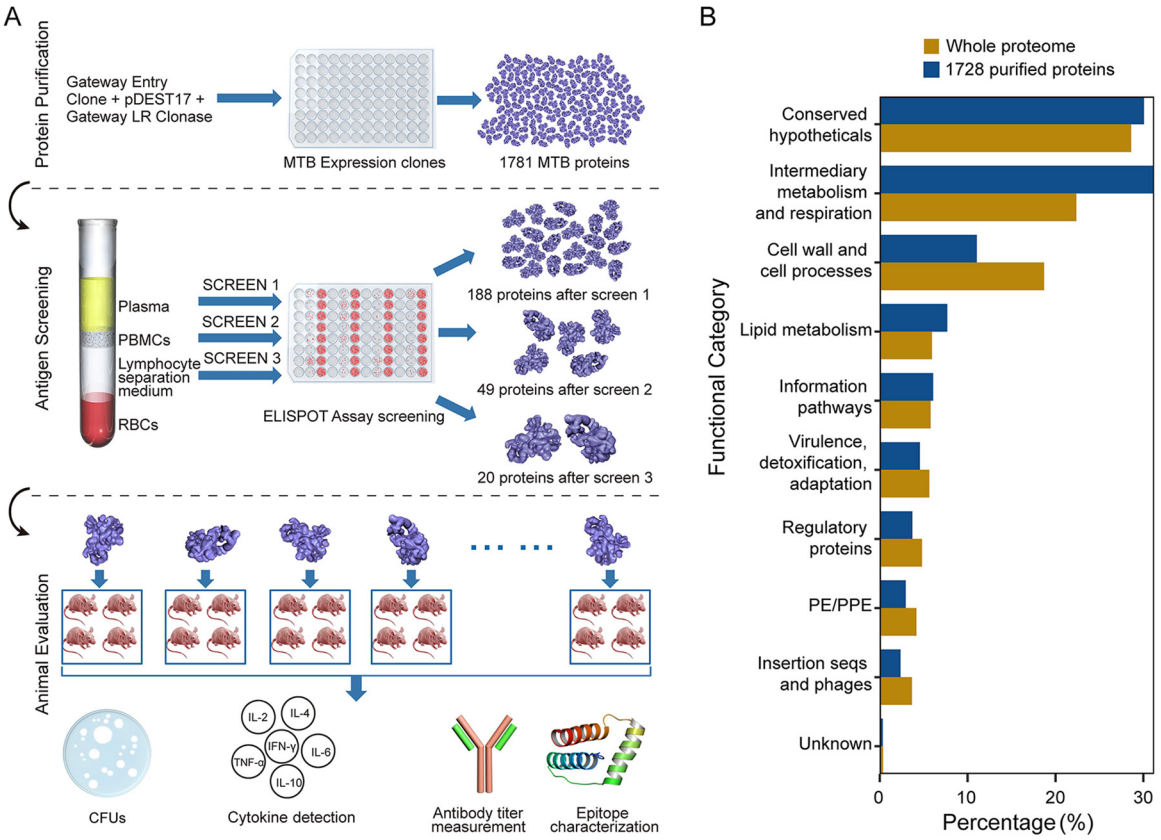

FIG 1 Systematic screening for antigenic *M. tuberculosis* proteins and evaluation of their host protective efficacy. (A) Strategy for screening for MTB antigenic proteins and validating their protective efficacy. A total of 1,781 recombinant MTB proteins were evaluated with IFN-γ ELISpot assays using PBMC samples from hospitalized TB patients (screen 1, 8 PBMC samples from active TB/suspected TB patients; screen 2, 20 PBMC samples from active TB/suspected TB patients; screen 3, 20 PBMC samples from patients with active TB and 20 from healthy donors). BALB/c mice vaccinated with the top 20 candidate antigens were challenged with MTB H37Rv, and protective efficacy was evaluated by lung bacterial burdens. Results for Rv1705c and Rv1485 were confirmed in a second experiment using six mice per antigen. Host immune responses were evaluated by assessing cytokine secretion from splenocytes and IgG2a/IgG1 ratios, and epitope characterization was performed on Rv1705c. (B) Functional category annotation of 1,728 H37Rv proteins. The distribution of the 1,728 H37Rv proteins evaluated here among functional annotation categories (TubercuList) is compared with that of the whole MTB proteome.

BALB/c murine challenge model and investigated host immune responses induced by the strongest candidates by assessing cytokine secretion from splenocytes and determining IgG2a/IgG1 ratios (Fig. 1A).

Using a previously constructed MTB entry clone library (26), we first expressed 3,829 protein-encoding genes from the H37Rv strain and 433 from the CDC1551 strain in *Escherichia coli* Rosetta(DE3) cells. While use of an *E. coli* expression system results in expression of recombinant proteins in inclusion bodies, this strategy was necessary to ensure sufficient quantities of proteins for subsequent evaluation. We confirmed the suitability of proteins purified from inclusion bodies (using standard denaturation/renaturation techniques [27]) for our purposes by comparing them with corresponding solubly expressed proteins in IFN-γ release assays, with equivalent results being obtained (see Fig. S2A in the supplemental material). Lipopolysaccharide (LPS) levels in the purified proteins were low ($<$10 endotoxin units [EU]/ml), and these levels did not interfere with the results of the IFN-γ release assays (Fig. S2B). Molecular weights and purity of the 1,781 proteins (1,728 H37Rv proteins and 53 CDC1551 proteins) suitable for further analysis ($>$80% purity, concentration of $\geq$0.5 mg/ml; LPS, $<$10 EU/ml) were assessed by SDS-PAGE (Table S2; Fig. S3). Functional category analysis (TubercuList [28]) indicated that the 1,728 purified MTB H37Rv proteins evaluated can be considered representative of the whole proteome (Table S3; Fig. 1B).

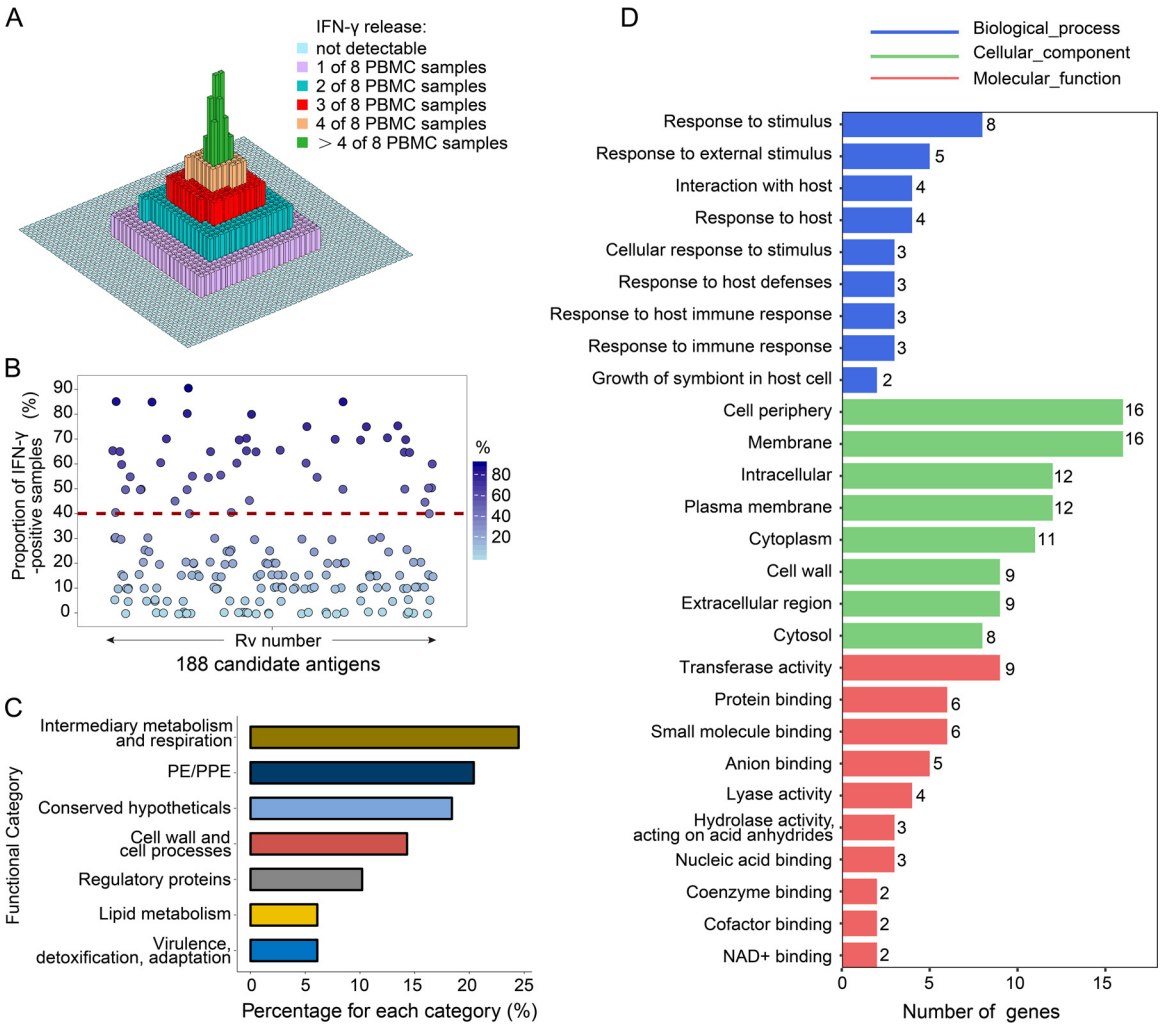

FIG 2 Selection of antigens that provoke host immune responses by screening for antigen-induced IFN-γ release from PBMC samples from hospitalized TB patients. (A) Antigenic activity of the 1,781 MTB proteins in screen 1. Detection of IFN-γ release from PBMC samples from 8 suspected TB patients stimulated with each protein is shown. Each column represents one MTB protein. (B) Screen 2, selection against 20 PBMC samples from suspected TB patients. Forty-nine of 188 putative antigenic proteins identified in screen 1 provoked IFN-γ release in $\geq$40% PBMC samples from clinically confirmed active TB patients. (C) Functional category annotation (TubercuList) of the 49 candidate antigens from screen 2. (D) GO analysis (DAVID) of the 29 of 49 candidate MTB antigens for which data were available.

Screening for T cell antigenic activity. During MTB infection, T cell responses to MTB antigens are important in host-pathogen interactions, promoting immunity to infection (29). T cells sensitized to MTB antigens and activated T cells, including $CD4^+$ and $CD8^+$ T cells, release IFN-γ when stimulated by MTB–specific antigens *in vitro*, with IFN-γ levels reflecting the strength of the antigen-induced immune response (30). $CD4^+$ T cells are crucial for the generation of a strong cell-mediated immune defense response against both intracellular and extracellular pathogens, and while their central role is not restricted to the secretion of IFN-γ to control MTB infection, IFN-γ is nonetheless an important marker validating their activation (31). Here, we evaluated the capacity of the above-mentioned 1,781 MTB proteins to provoke IFN-γ release in PBMCs from hospitalized TB patients using two commercial IFN-γ ELISpot assays (Table S4; Fig. S1). In the preliminary screen, 369 MTB proteins provoked detectable levels of IFN-γ in more than one of eight PBMC samples from patients suspected of having TB at the Beijing Chest Hospital (Table S5; Fig. 2A). A subset (188) of these samples that provoked IFN-γ release in $\geq$2 patients later confirmed clinically to have active TB (Table S5) was screened against an additional 20 PBMC samples from active TB/suspected TB

patients, with those showing the strongest antigenic activity (49 proteins; cutoff, IFN-γ release in $\geq$40% of PBMC samples from confirmed TB patients) being carried forward for further analysis (Table S6; Fig. 2B).

Functional category analysis assigned 12 of the 49 candidate antigens to the intermediary metabolism and respiration category and 10 to the PE/PPE protein family (Table S7; Fig. 2C), categories viewed as potential sources of new TB vaccine candidates (32–35). PE/PPE family proteins have important roles in host immune responses to MTB; PPE42 and PPE18, for example, are important components of ID93+GLA-SE and M72/AS01$_E$, vaccine candidates currently in clinical trials (36, 37). Gene ontology (GO) analysis (DAVID [38]) indicated that the 29 candidate antigens with available information (Table S7; Fig. 2D) fell mainly into the "response to stimulus," "cellular response to stimulus," "response to host immune response," and "interaction with host" biological process categories. Many of the proteins were assigned to the "cell periphery" and "membrane and cell wall" categories, as might be expected of antigens located in extracellular regions that induce a host immune response. In summary, bioinformatic analyses provided supporting evidence for the association of these 49 candidate antigens with host immune responses.

The 49 candidate antigens were screened further against PBMC samples from 20 active TB patients and 20 healthy donors to select antigens that consistently provoked a significant IFN-γ immune response in active TB patients ($\geq$40%) and a limited response among healthy donors ($\leq$30%). The top 20 candidate antigens (Table S8), namely, 6 PPE proteins (PPE22, PPE23, PPE26, PPE28, PPE30, and PPE32), 5 intermediary metabolism and respiration proteins (HemZ, Rv0082, AdhA, NadA, and Rv0187), 4 conserved hypothetical proteins (Rv1352, Rv1341, Rv1482, and Rv1147), 2 regulatory proteins (Rv3095 and Rv1151c), 2 proteins involved in lipid metabolism (Rv1867 and FadD11.1), and 1 member of the virulence, detoxification, and adaptation protein category (Rv2303c), were then evaluated in a BALB/c murine challenge model.

Candidate T cell antigens Rv1485, Rv1705c, and Rv1802 have comparable protective efficacies. The protective efficacies of the 20 candidate MTB antigens were evaluated by immunizing 7- to 9-week-old BALB/c mice (4 or 5 per group) by subcutaneous injection (3 injections, 2-week intervals) with 10 μg (39) candidate antigen (in Freund's incomplete adjuvant), with an intravenous challenge (via the lateral tail vein) 6 weeks later with MTB H37Rv (5×10^5 CFU/mouse) (40) and then euthanasia after 6 weeks. Intravenous injection of MTB H37Rv gave a disease model characterized by more severe disease and a high lung bacterial burden (41). Comparing lung bacterial burdens in mice vaccinated with candidate antigens, saline, or *Mycobacterium bovis* BCG (Fig. 3A; Table S9) indicated that Rv1485, Rv1705c, and Rv1802 had significant protective efficacy (Rv1485 versus saline, 4.86 $\pm$ 0.06 $\log_{10}$ CFU versus 5.33 $\pm$ 0.04 $\log_{10}$ CFU, respectively, $P < 0.01$; Rv1705c versus saline, 4.72 $\pm$ 0.07 $\log_{10}$ CFU versus 5.33 $\pm$ 0.11 $\log_{10}$ CFU, respectively, $P = 0.013$; Rv1802 versus saline, 4.90 $\pm$ 0.05 $\log_{10}$ CFU versus 5.30 $\pm$ 0.07 $\log_{10}$ CFU, respectively, $P < 0.01$) (Fig. 3A; Table S9). Although the performances of Rv1485 and Rv1802 were comparable, due to practical constraints, only one of these candidates (Rv1485) was carried forward. Essentially similar results were obtained when the experiment was repeated with 6 mice/group to confirm the protective efficacies of Rv1485 and Rv1705c (Table S10; Fig. 3D).

Rv1485 and Rv1705c induce a protective Th1 immune response. We evaluated the humoral response elicited by the most promising candidates, Rv1485 and Rv1705c, in a separate experiment by immunizing BALB/c mice with 30 μg Rv1485/Rv1705c (in Freund's incomplete adjuvant, 3 subcutaneous injections, 2-week intervals) and measuring serum titers of Rv1485/Rv1705c-specific IgG, IgG1, and IgG2a 3 weeks later. High titers of Rv1485-specific and Rv1705c-specific IgG, IgG1, and IgG2a were detected, and no IgG, IgG1, or IgG2a was detected in the negative controls (Fig. 4A; Table S11). Th1 immune responses provide important protection against MTB infection (42), while Th2 immune responses are believed to weaken host protective immunity (43). Here, the IgG2a/IgG1 (Th1/Th2) ratios in the Rv1485 and Rv1705c groups (Fig. 4A) were 1.5 and

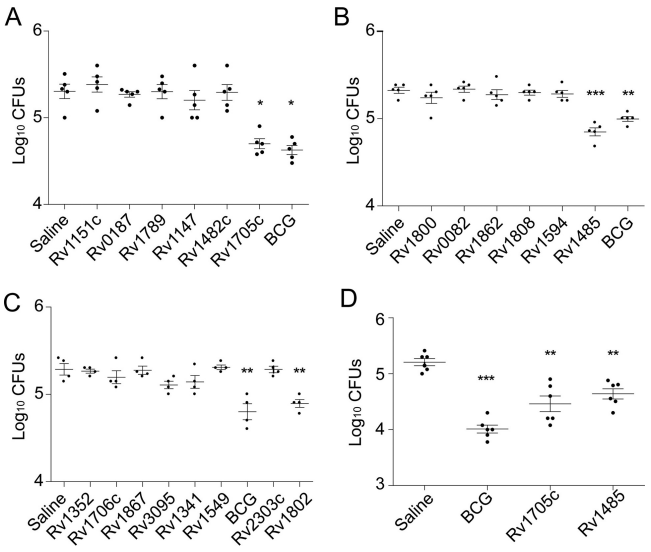

FIG 3 Evaluation of the protective efficacy of 20 candidate *M. tuberculosis* T cell antigens in a BALB/c mouse challenge model. (A to C) Lung bacterial loads in mice immunized with MTB candidate antigens 6 weeks after challenge with MTB H37Rv ($n = 4$ or 5 per group). Panels A to C represent separate experiments, each including the antigens indicated and performed under the same conditions. Positive control, BCG; negative control, saline. Data shown are $\log_{10}$ means ± standard errors of the mean (SEM). Each candidate antigen was evaluated once. (D) Validation of the protective efficacy of candidate antigens Rv1485 and Rv1705c. This experiment was performed once, essentially as described for panel A, except that 6 mice were tested per group. Lung bacterial loads ($\log_{10}$ means ± SEM) are shown. Mann-Whitney U tests were used for comparisons between groups. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

3.33, respectively, compared to 1.33 for BCG, suggesting that Rv1485 and Rv1705c elicited a protective Th1-type immune response.

In the case of intracellular pathogens such as MTB, a strong cellular response is also key. Th1 cytokines, like interleukin 2 (IL-2), tumor necrosis factor alpha (TNF-α), and IFN-γ, play essential roles in combatting pathogens, controlling TB, and effectively protecting the host (44, 45). We immunized a further set of mice (3 subcutaneous injections, 2-week intervals) with Rv1485/Rv1705c and harvested splenocytes 4 weeks later. Levels of Th1 cytokines IL-2, IL-6, TNF-α, and IFN-γ, which activate macrophages and promote the polarization of effector Th1 cells, were high after stimulation of splenocytes with either Rv1485 or Rv1705c, while Th2 cytokines IL-4 and IL-10, which inhibit macrophage functions and promote antibody responses, could not be detected (Fig. 4B; Table S12). Cytokine levels detected were significantly higher in Rv1485/Rv1705c-immunized mice than in BCG-immunized mice, presumably because (i) the latter received only one BCG immunization, (ii) the time between BCG immunization and euthanization was 8 weeks rather than 4 weeks, and (iii) splenocytes were not stimulated with immunogens before cytokine release was measured.

Our results indicate that Rv1485 and Rv1705c both elicited protective Th1-type humoral and cellular immune responses that are beneficial for TB control.

Rv1705c epitope analysis. Given the importance of PE/PPE proteins in inducing host immune responses, we characterized the epitopes of Rv1705c (PPE22), the most promising antigen candidate. Twenty-five 30-mer overlapping (10-mer) peptides, covering the whole Rv1705c sequence (Table S13), were divided into 3 pools (pool 1, N-terminal residues 1 to 175: P1, P4, P6 to P8, P11; pool 2, residues 166 to 295: P12, P14, P16 to P19; pool 3, residues 286 to 385: P20 to P25 [P2, P3, P5, P9, P10, P13, and P15 could not be synthesized]) and tested against PBMCs from 10 active TB patients in IFN-γ ELISpot assays. Peptide pool 1 provoked a significant increase in IFN-γ release, and when tested individually against PBMCs from a further 10 active TB patients, peptide 1 was the only peptide that provoked significant IFN-γ release (Fig. 5A). A BLAST

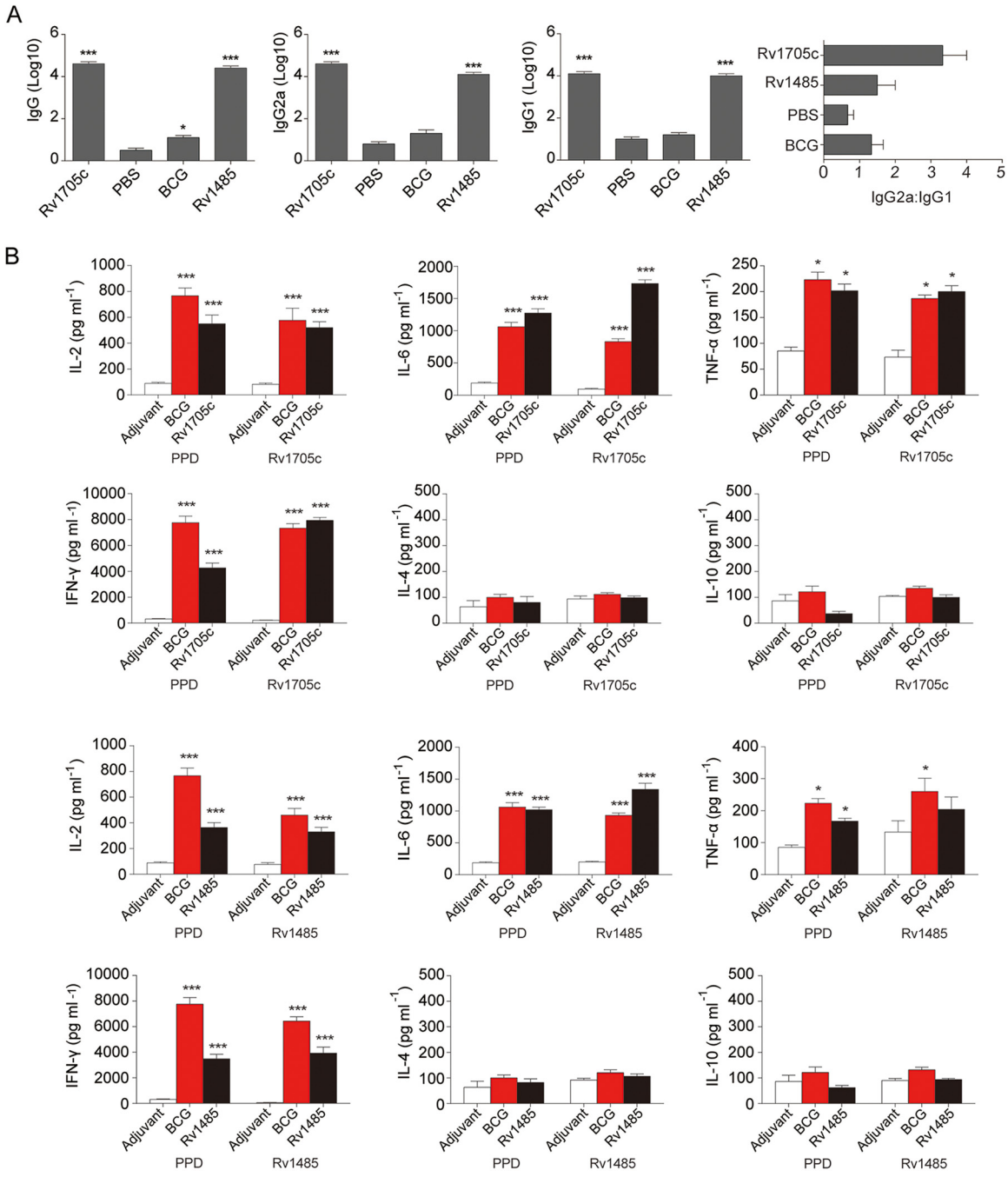

FIG 4 Immune responses of Rv1485- and Rv1705c-immunized mice. (A) Rv1705c- and Rv1485-specific IgG, IgG1, and IgG2a antibody levels in sera collected 3 weeks after the final immunization. Results shown are mean $\log_{10}$ endpoint titers (±SEM), $n = 3$ (experiment performed once). Right-hand panel, ratio of IgG2a to IgG1. (B) Cytokine (IL-2, IL-6, TNF-α, IFN-γ, IL-4, IL-10) release from splenocytes isolated from mice immunized with adjuvant, BCG, Rv1485, or Rv1705c 4 weeks after the final immunization. Cells seeded into 24-well plates were incubated with 10 μg/ml Rv1705c (upper two rows) or Rv1485 (lower two rows) or with PPD (purified protein derivative; 10 μg/ml, positive control) for 24 h at 37°C. Means ± SEM (two independent experiments) are shown. All comparisons were made using Student's *t* tests. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

comparison with Rv1705c homologues from other mycobacterial species indicated that pool 1 sequences from the N-terminal domain are highly conserved (Fig. 5B). SWISS-MODEL (http://swissmodel.expasy.org/) modeling of the N-terminal region structure (173 amino acids) using default parameters showed that the Rv1705c N-terminal domain consists of 5 α-helices (α1, amino acids [aa] 8 to 13; α2, aa 22 to 55; α3, aa 59 to 103; α4, aa 107 to 122; α5, aa 130 to 161) (Fig. 5C). Peptide 1 maps to

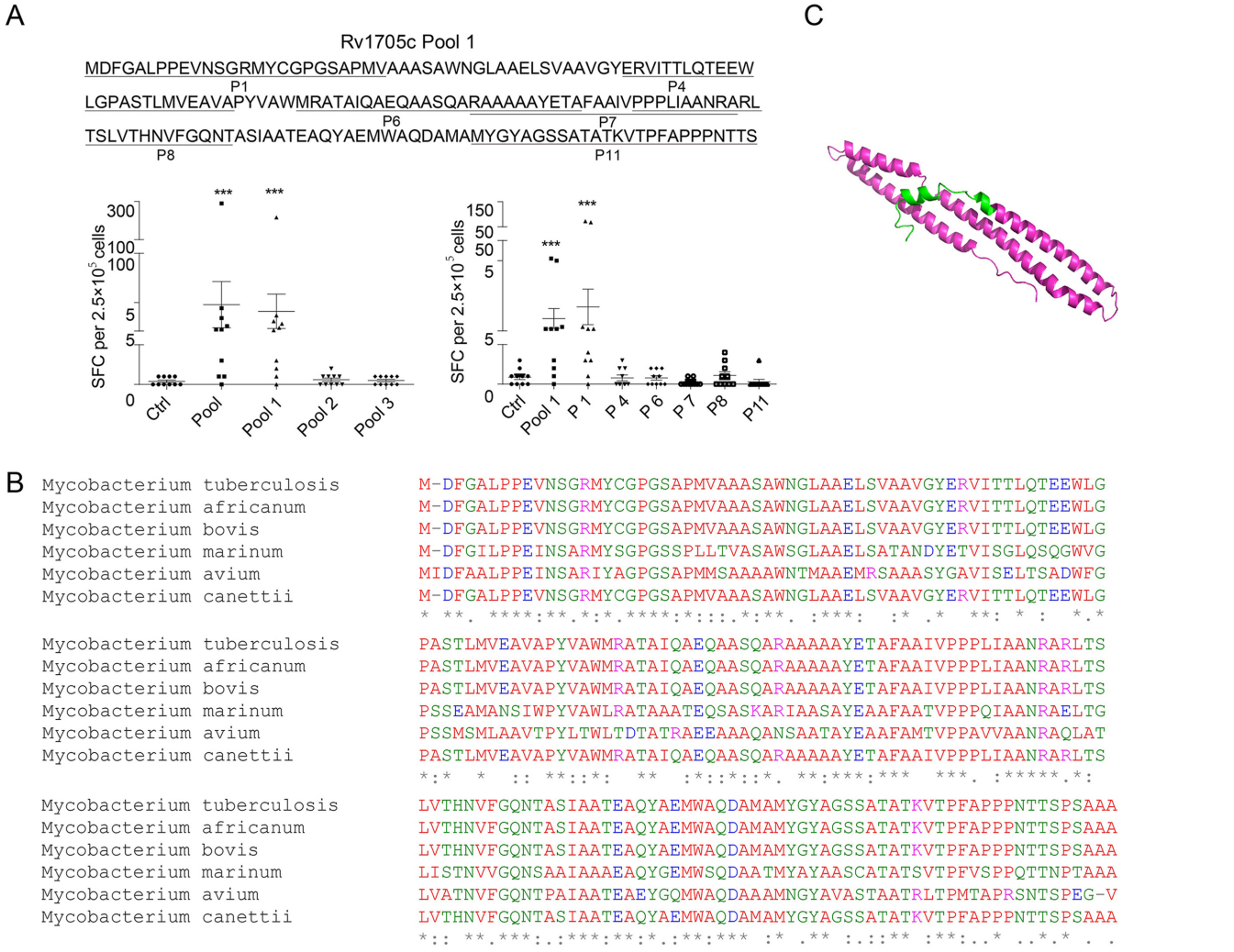

FIG 5 Rv1705c (PPE22) epitope characterization. (A) Upper panel, peptide design. Sequences of 30-mer peptides in pool 1 covering PPE22 N-terminal residues 1 to 175. Peptide sequences tested for their capacity to provoke IFN-γ release are underlined and labeled. Lower left panel, pool 1 peptides provoked an IFN-γ response. Pool, all 18 peptides synthesized; pool 1, peptides P1, P4, P6 to P8, P11; pool 2, peptides P12, P14, P16 to P19; pool 3, peptides P20 to P25. Control, peptides from Rv1789. Lower right panel, of the pool 1 peptides, only peptide P1 provoked an IFN-γ response. Control, peptides from Rv1789. Results were compared with Mann-Whitney U tests. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. (B) Alignment of the N-terminal domain of Rv1705c (PPE22) from different mycobacterial species. (C) Three-dimensional structure of the Rv1705c (PPE22) N-terminal domain, modeled using SWISS-MODEL. Magenta, N-terminal domain α-helixes; green: peptide P1.

the extreme end of the N-terminal domain and consists of two short α-helical sections (α1 and the N-terminal end of α2) connected by a short flexible loop, with another flexible loop at its N-terminal end.

DISCUSSION

Lack of a broad range of antigens that reliably induce a robust protective immune response is a bottleneck in TB vaccine development. Here, by screening 1,781 recombinant MTB proteins, representing ~45% of the MTB proteome, for antigen-specific IFN-γ release using 4,452 hospitalized TB patient-derived PBMC samples (and 167 from healthy donors), we identified 49 MTB proteins that provoked a robust immune response (32 reported here as antigens for the first time), providing a useful resource for further in-depth studies on MTB antigens. After further evaluation of these candidate antigens, we identified two proteins, Rv1485 (HemZ) and Rv1705c (PPE22), that elicited a promising protective immune response in BALB/c mice and warrant further investigation as candidate subunit vaccines to boost BCG protection.

About 400 MTB proteins have been shown to exhibit some immunogenicity (16), but only 15 to 20% of these have been reported in more than one study, and few have been extensively investigated or shown to induce a protective immune response in animal models (4, 46). Common shortcomings of genome-wide studies include difficulty in expressing/purifying sufficient protein to adequately evaluate protective efficacy in animal/immunological experiments and limited availability of suitable clinical samples for evaluating human immune responses (47). Here, we assembled a library of 1,781 purified MTB recombinant proteins using a high-throughput *E. coli* expression system to ensure sufficient quantities of protein. The large number of proteins involved meant it was not practical to optimize expression and purification conditions for each protein, and proteins were expressed in inclusion bodies. We confirmed, however, that the performance of proteins renatured from inclusion bodies in the IFN-γ release ELIspot assays used in our screen for antigenicity was equivalent to solubly expressed proteins (see Fig. S2 in the supplemental material). We tested the 1,781 proteins for antigenic activity using 4,452 PBMC samples derived from hospitalized TB patients and 176 from healthy donors. Samples used were fresh blood samples obtained from hospitalized TB patients awaiting a final diagnosis prior to the onset of TB treatment. The TB disease status of the sample cohort used in screens 1 and 2 was thus complex and included patients later confirmed to have active TB and those with other chest diseases with clinical symptoms resembling TB (some of whom had latent TB infection [LTBI]). Our screening criteria, however, ensured that decisions to carry a given protein forward for further screening were based on their performance against PBMC samples from clinically diagnosed active TB patients (screen 1, positive IFN-γ response in $\geq$2 samples from confirmed active TB patients; screen 2, positive IFN-γ response in $\geq$40% samples from confirmed active TB patients). As active TB is a clearly defined clinical disease state, samples from patients with a final clinical diagnosis of active TB thus represent a relatively uniform population.

Our aim was to identify novel MTB antigens that induce a robust host immune response, and we monitored antigen-specific T cell activation using ELIspot assays of antigen-specific IFN-γ release from patient-derived PBMC samples (48). While a broader screen that monitors the concomitant production of multiple effectors, such as IFN-γ, TNF-α, and IL-17 (44), would likely have been more specific, antigen-specific IFN-γ release ELIspot assays nonetheless proved to be a rapid, practical, and effective method for standardizing screening for T cell antigens in the large number of proteins included in our study. Candidates that provoke a strong IFN-γ release *in vitro* do not necessarily perform well as vaccine candidates (49), and our data may thus include some false-positive/negative results that require further validation.

Forty-nine proteins demonstrated antigen-specific IFN-γ release strongly associated with active TB after three rounds of screening (Fig. 1A; Table S9), with a number of them provoking IFN-γ release in a high proportion of the samples in screen 3; for example, Rv1705c, Rv1789, and Rv1808 provoked IFN-γ release in 75% of PBMC samples from active TB patients (Table S9). Screen 3 introduced PBMC samples from healthy donors to assess antigen specificity for active TB. Although all healthy donors were T. SPOT-TB and chest X-ray negative, with no apparent TB symptoms, positive IFN-γ responses to antigens tested were relatively high, ranging from 15 to 30% for the top 20 antigens that were subsequently selected for further evaluation and 35 to 50% for excluded antigens. These high responses may reflect at least two factors: (i) the T. SPOT-TB assay has variable sensitivity, ranging from 60 to 80% (50), and (ii) rates of LTBI within the Chinese population are high (51). It is possible, then, that some "healthy donors"' were individuals with LTBI, explaining positive IFN-γ responses. Our goal was to screen for proteins with strong stable antigenic activity, i.e., antigens with potential for development as subunit vaccines. As such, many of the proteins not selected for further evaluation may still merit further study. For example, Rv0442c (PPE10) provoked IFN-γ release in 90% of TB patients in screen 3 but was not evaluated further as it provoked a response in 35% of healthy donors. Rv3620c and

Rv1196, antigens that have been investigated as vaccine candidates in other studies (52, 53), likewise performed well here but were not carried forward for the same reason. Going forward, it will be important to test this new library of T cell antigens on a larger population of healthy (non-LTBI) and LTBI patient samples to further evaluate their TB specificity and also to evaluate their antigenicity more thoroughly by screening for antigen-specific responses with a broader range of cytokines known to be involved in conferring protection against TB.

The top 20 antigens from screen 3 were evaluated for protective efficacy in a small BALB/c mouse MTB (H37Rv) challenge study, with antigens Rv1705c, Rv1485, and Rv1802 showing promising protective efficacy, each significantly reducing lung bacterial burdens relative to a saline control (Tables S9 and S10; Fig. 3). Our results require further validation; we chose here to use an intravenous infection model (to establish relatively severe disease with high lung bacterial burdens), to keep the number of animals per experimental group small, and to evaluate only one adjuvant, Freund's incomplete adjuvant, known for its ability to strengthen TH-1 immune responses (54). We expect that antigen performance might be improved by selecting a more optimal adjuvant. Our purpose was to identify some (not necessarily all) antigens with protective efficacy; some of the other antigens tested may demonstrate protective efficacy in larger experiments or with other mouse strains that display different susceptibility patterns to TB.

Rv1485 (HemZ), an essential protein for MTB growth (55, 56), Rv1705c (PPE22), and Rv1802 (PPE30), all underinvestigated proteins, are reported here as MTB antigens for the first time (55, 56). PE/PPE proteins have innate advantages in inducing host immune responses and are effective immunogens (33, 35). PPE26, for example, drives Th1 T cell immunity (57), and PPE32 can induce cytokine production and host cell apoptosis (58). PPE18 is one of two antigens included in the M72/AS01 vaccine candidate that has already completed phase IIb clinical trials (17, 59), and PPE42 is one of four MTB antigens in ID93/GLA-SE, a subunit vaccine candidate that has exhibited good protection in animal models and has been shown to be safe and immunogenic in a phase Ia trial (5, 60, 61). Rv1705c is known to be present in MTB H37Rv-infected guinea pig lungs at 30 days postinfection (62). *Ex vivo* production of IFN-γ by PBMCs from LTBI donors stimulated with synthesized peptides derived from Rv1705c has been reported (63), but the peptides used (and the TB status of the PBMC donors) were different from those shown here to be immunogenic. Our finding is consistent with detailed analyses of other PE/PPE proteins indicating that T cell epitopes are found mainly in the conserved N-terminal domain (63, 64).

Current vaccine strategies emphasize the importance of identifying and evaluating a broader panel of effective antigens and antigen combinations in a range of animal models and of gaining a better fundamental understanding of what constitutes a protective immune response. Here, we have identified 34 previously unreported antigens and shown that two of these antigens, Rv1485 and Rv1705c, have promising protective efficacy and induce a protective Th1 immune response. A more comprehensive preclinical assessment of these two antigens in other animal models, either alone or with other previously reported antigens, using a range of adjuvants, is warranted.

MATERIALS AND METHODS

Bacterial culture. *M. bovis* BCG Pasteur (ATCC 35734) and *M. tuberculosis* H37Rv were cultured in liquid Middlebrook 7H9 medium (Difco Laboratories, Detroit, MI) containing 0.5 glycerol, 0.05% Tween 80, and 10% ADC (bovine albumin, dextrose, and catalase) or solid Middlebrook 7H10 medium (Difco, Franklin Lakes, NJ) containing ADC.

MTB gene expression vectors. An MTB Gateway entry clone library (26) containing 3,404 H37Rv and 437 CDC1551 sequenced open reading frame (ORF) clones was used. Expression plasmids were constructed via an LR recombination reaction between each entry clone and a Gateway destination vector (pDEST17: Invitrogen, USA) using the Gateway LR Clonase enzyme mix (Invitrogen, USA).

Protein expression and purification. Proteins were expressed by adding 0.4 mM isopropyl-β-D-thiogalactoside (IPTG) to cultures of *E. coli* Rosetta(DE3) cells containing pDEST17 constructs grown in LB medium for 6 h at 37°C, 200 rpm. After overnight culture, bacterial cell suspensions were sonicated and centrifuged (12,000 rpm, 10 min), and pellets were washed twice in a washing buffer (50 mM Tris-HCl,

0.05 M EDTA [pH 8.0] containing 2% deoxycholate [DOC], 1 M urea) before resuspension in 50 mM Tris-HCl, 1 mM EDTA (pH 8.0) containing 15 mM DTT, 8 M urea. To reconstitute proteins from inclusion bodies, dissolved proteins were dialyzed overnight against 200 mM Tris-HCl (pH 10.0) containing 8 M urea, 20 mM L-arginine, using a pump to add water into the buffer slowly until it was diluted 10 times, and then against 20 mM Tris-HCl (pH 8.0) containing 20 mM NaCl. Endotoxins were removed during the purification process by the addition of 2% DOC. Protein concentration was determined using modified bicinchoninic acid (BCA) protein assay kits (Sangon Biotech, Shanghai, China) according to the manufacturer's instructions.

Clinical sample collection and antigen screening. Clinical sample collection was approved by the Ethics Committee of Beijing Chest Hospital, Capital Medical University, Beijing. Blood samples were provided by Beijing Chest Hospital (2015 to 2018) from 4,452 hospitalized patients suspected to have TB, 2,803 of which were later confirmed clinically to have active TB. All blood samples were collected before the onset of TB treatment. In addition, 167 blood samples were obtained from healthy donors (BCG-vaccinated university age students) who had no obvious symptoms of active TB and were confirmed to be TB negative by chest X-ray and IFN-γ ELISpot assays. As the blood samples provided were left over after other clinically requested blood tests, and associated medical information on patients was provided anonymously, informed consent was not sought. Clinicians determined patient TB status based on clinical symptoms, chest X-rays, sputum smear microscopy, bacterial culture results, results from IFN-γ ELISpot assays, and history of prior contact with TB patients. Blood samples were used as they became available, 8 samples per protein being used in screen 1 and 20 in screen 2. Twenty samples from confirmed active TB patients and 20 from healthy donors were used in screen 3. Demographic information on the patient population is given in Table S1 in the supplemental material.

IFN-γ ELISpot assays. IFN-γ ELISpot assays were performed for each protein using commercial T-SPOT.TB (Oxford Immunotec, UK) and X.DOT-TB (TB Healthcare, China) kits, previously shown to have the same performance (Table S2; Fig. S1), according to the manufacturer's instructions. Briefly, PBMCs were collected by Ficoll-Paque density gradient centrifugation of blood samples, and 2.5×10^5 cells per well were seeded into 96-well plates. Cells were incubated with MTB proteins (200 pmol) or ESAT6/CFP10 (50 μl) or with medium or phytohemagglutinin (PHA) (as negative and positive controls, respectively) at 37°C. Secreted IFN-γ was captured by specific antibodies on a polyvinylidene difluoride (PVDF) membrane on the well surface. Spots formed as a result of this reaction (spot-forming cells [SFCs]) were counted. Positive responses were defined as $\geq$6 spots for the T-SPOT.TB kit and $\geq$11 spots for the X.DOT-TB kit.

MTB antigen immunization. All animal procedures were approved by the Ethics Committee of Beijing Chest Hospital, Capital Medical University, Beijing, China, and were performed in accordance with the Chinese Council on Animal Care Guidelines. Candidate MTB antigens were evaluated in a BALB/c mouse model. After 1 week of acclimatization, 6- to 8-week-old BALB/c female mice (Vital River Laboratory Animal Technology, Beijing, China), randomly assigned to experimental and control groups (4 or 5 per group), were immunized subcutaneously (three times, 2 weeks apart) either with 10 μg recombinant MTB protein (39) formulated in 200 μl incomplete Freund's adjuvant or with 200 μl saline and adjuvant (negative control). Mice in BCG-positive control groups were immunized subcutaneously with one dose of 1×10^5 CFU BCG. Mice were monitored daily by animal care staff not associated with the study until the experiments were terminated. Space restrictions in the animal facility made it necessary to divide the 20 candidate antigens randomly into three groups for evaluation in three successive experiments, with each antigen being evaluated once. Saline and BCG controls were included in each experiment, providing a measure of the experimental error associated with each experiment. The two most promising antigens, Rv1705c and Rv1485, were subsequently validated in a separate experiment that was identical to the one described above, except that 6 mice were used per group.

Murine MTB H37Rv challenge model. Immunized mice were challenged with MTB H37Rv 6 weeks after the last immunization by injecting a mycobacterial suspension (5×10^6 CFU/ml, 100 μl/mouse, i.e., ~5×10^5 CFU/mouse) into the lateral tail vein (65–67). Lungs were harvested 6 weeks later (mice were euthanized by dislocation of cervical vertebrae) and transferred to plastic Tekmar bags with 10 ml phosphate-buffered saline (PBS)–0.1% Tween 80. One lobe of each lung was homogenized in sterile 0.05% PBS–Tween 80 in a FastPrep-24 (MP Biomedicals). Homogenized lung suspensions were plated in 10-fold serial dilutions on Middlebrook 7H10 medium. The numbers of CFU were scored after the plates were incubated at 37°C for 3 to 4 weeks.

Mouse splenocyte culture and cytokine detection. Spleens were harvested from mice (immunized as described above) 4 weeks after the final immunization, homogenized, and passed through a 100-μm nylon cell strainer (BD Pharmingen). Splenocytes obtained were washed twice in RPMI 1640 (Invitrogen) and centrifuged (1,000 rpm, 5 min), and cell pellets were resuspended in medium (10% fetal bovine serum [FBS] in RPMI 1640) and seeded in 24-well plates (Costar) at 2.5×10^5 cells/well. After incubation with recombinant proteins (10 μg/ml) or purified protein derivative (PPD) (10 μg/ml) for 24 h (IL-2) at 37°C, supernatants were collected, and IFN-γ, TNF-α, IL-2, IL-4, IL-6, and IL-10 were detected using ELISA kits (BioLegend, San Diego, CA).

Antigen-specific antibody titers. Antigen-specific serum IgG2a and IgG1 antibody responses in blood samples drawn from mice (immunized as described above) 1 week after the final immunization were evaluated by ELISA. Ninety-six-well plates, coated overnight (4°C) with each recombinant protein (1 μg/ml) in coating buffer (0.05 M carbonate buffer, pH 9.6), were washed three times with PBS-T (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, and 0.05% Tween 20), blocked in PBS-T–3% BSA (37°C, 2 h), and washed again (three times) with PBS-T. Serial dilutions of mouse sera (1:1,000) in PBS-T–1% BSA were added to the plates before incubation for 1 h at 37°C. After further washing (with

PBS-T, three times), horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG1 and IgG2a antibodies (Bethyl Laboratories, Montgomery, TX) (1:10,000 dilution in PBS-T–1% BSA) were added to each well. After incubation for 1 h at 37°C and washing with PBS-T (three times), TMB (3,3′,5,5′-tetramethylbenzidine) solution was added and the plates were incubated for 15 min (room temperature). Light emission (450 nm) was measured using a microplate reader (Tecan, Switzerland) after the addition of stop solution (2N H_2SO_4), and IgG2a/IgG1 endpoint ratios were calculated.

Statistical analysis. Student's *t* tests (normally distributed data) or Mann-Whitney U tests (non-normally distributed data) were used to compare continuous data, and the chi-square or Fisher's exact test was used for comparing categorical variables. The cutoff for statistical significance was set at a P of <0.05. P values were adjusted using Benjamini and Hochberg's approach for controlling the false discovery rate. All data analyses were performed with R statistical software (v3.6.1) and related packages.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.8 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.1 MB.

SUPPLEMENTAL FILE 3, XLSX file, 0.02 MB.

SUPPLEMENTAL FILE 4, XLSX file, 0.5 MB.

SUPPLEMENTAL FILE 5, XLSX file, 0.1 MB.

SUPPLEMENTAL FILE 6, XLSX file, 0.1 MB.

SUPPLEMENTAL FILE 7, XLSX file, 0.02 MB.

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