Identification and evaluation of the novel immunodominant antigen Rv2351c from *Mycobacterium tuberculosis*

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There is an urgent need for new immunodominant antigens to improve the diagnosis of tuberculosis (TB) and the efficacy of the TB vaccine to control the disease worldwide. In this study, we evaluated the diagnostic potential of a novel *Mycobacterium tuberculosis* (MTB)-specific antigen, Rv2351c, from region of difference (RD) 7 of the MTB genome, and investigated the potency of the vaccine by identifying its immunological function in human and animal immunological experiments. Twenty T-cell epitopes were identified using TEPredict and prediction tools from the Immune Epitope Database and Analysis Resource. A total of 159 subjects, including 61 patients with pulmonary TB, 38 patients with no TB and 55 healthy donors, were recruited and analyzed with an enzyme-linked immunospot (ELISpot) assay. The ELISpot assay using Rv2351c to detect TB infection, as compared with bacteriological tests as the gold standard, had a sensitivity and specificity of 61.4% (35/57) and 91.4% (85/93), respectively. The ELISpot assay using Rv2351c had a good conformance ($\kappa = 0.554$) as compared with the bacteriological test. Rv2351c also elicited a potent cellular immune response with a high expression of cytokines (IFN-$\gamma$ (4978 ± 596.7 μg/mL) and IL-4 (68.3 ± 15.5 μg/mL)) and a potent humoral immune response with a high concentration of IgG (1:2.2 × 10^6), IgG1 (1:4.5 × 10^5) and IgG2a (1:1.6 × 10^5) in immunized BALB/c mice. In addition, the ratio of IgG2a/IgG1 indicated that Rv2351c induced cellular immunity in the mice. The results of this study indicated that Rv2351c is an antigen with good immunogenicity that may potentially be used to develop diagnostic techniques and new TB vaccines.

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INTRODUCTION

Despite the widespread use of the Bacille Calmette-Guerin (BCG) vaccine, tuberculosis (TB) continues to be a burden to public health. One-third of the world’s population is asymptomatically infected with *Mycobacterium tuberculosis* (MTB), and 10.4 million new TB cases, 1.4 million deaths from TB and an additional 0.4 million deaths resulting from TB among patients with HIV were reported in 2015 worldwide.\(^1\) The increased rate of multidrug-resistant and extensively drug-resistant TB and co-infection with HIV/AIDS has worsened the TB burden. Therefore, the rapid diagnosis of latent and active TB and early treatment are the key steps to decreasing TB prevalence. In addition to the development of rapid diagnostic techniques for TB, novel and effective vaccines that may provide long-lasting protection or immunological therapy for individuals immunized with BCG are crucial for controlling and eliminating TB globally.

Because MTB is an intracellular bacterial pathogen surviving and proliferating within the macrophage, both protection and pathogenesis are mediated primarily by cellular responses, which involve interactions of lymphocytes (mainly T cells) and monocyte/macrophage lineages.\(^2\) Protective immunity against TB is mediated by Th1 CD4$^+$ and effector CD8$^+$ T cells.\(^3\) Currently, the identification of MTB-specific antigens is mainly focused on region of difference (RD) genes that are absent from the BCG strains.\(^4\) Detection based on T-cell immune responses against early secreted antigenic target 6 (ESAT-6) and culture filtrate protein 10 (CFP-10) from RD1 of MTB has been proposed as a tool for the diagnosis of TB infection and used in an enzyme-linked immunospot (ELISpot) assay.\(^5,6\) Many other antigens, such as EspC (Rv3615c), MPT-64(Rv1980c), TB7.7 (Rv2654c), HspX (Rv2031c) and Essl (Rv1038c), have been identified as immunodiagnostics, but they still cannot meet clinical needs.\(^7,10\) Phospholipases are important virulence factors in many bacteria, including *Pseudomonas*...
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The genome of MTB H37Rv contains three contiguous genes, plcA, plcB and plcC, which are similar to the phospholipase C (plc) gene from P. aeruginosa. PlcA (512 aa) is a membrane-associated phospholipase C1 that was originally identified by mass spectrometry of MTB H37Rv and is encoded by Rv2351c; the expression of the native plcA has been demonstrated through immunoblotting of MTB. PlcA is essential in the pathogenic MTB complex and is found in both MTB and M. africanaum, but is absent in all the BCG strains. Because plcA is an antigen located in RD7 of MTB, we evaluated its potential in the diagnosis of TB infection and the development of TB vaccine. In recent years, many in silico epitope prediction tools have been applied to successfully predict epitopes in bacteria and viruses and have facilitated the progress of vaccine development. The quality of predictions can be improved by combining multiple approaches. TEpredict is an efficient epitope prediction tool that can predict the interaction between oligopeptides and the transporters associated with antigen processing and can estimate the coverage of peptides in the population on the basis of data on the HLA allele genotypic frequencies. The Immune Epitope Database and Analysis Resource (IEDB-AR) is a database of experimentally characterized immune epitopes, including T-cell and B-cell epitopes of humans, non-human primates (chimpanzee, gorilla and macaque), rodents (mice and rats) and other species. In this study, to improve the accuracy and efficiency of epitope prediction, we utilized TEpredict and T-cell epitope prediction tools at the IEDB-AR (http://tools.iedb.org/mhci/) to predict the existing linear T-cell epitopes in Rv2351c, because T-cell responses are the basis of the diagnosis of TB infection and the development of TB vaccines. Finally, 20 common T-cell epitopes of Rv2351c were obtained from both TEpredict and IEDB (Table 1), and the results indicated that Rv2351c might have the potential to elicit a T-cell response in patients with TB. Therefore, we constructed a recombinant vector, pET32a-Rv2351c, purified the recombinant Rv2351c protein, and evaluated the diagnostic potential and immunogenicity of Rv2351c in human and animal immunological experiments. Ag85B is an immunodominant antigen that elicits a strong Th1 immune response against MTB challenge and an increased humoral IgG antibody production; hence, the Ag85B antigen was used as a positive control in animal experiments.

MATERIALS AND METHODS

T-cell epitope prediction

T-cell epitopes were identified using TEpredict and other prediction tools from the IEDB-AR. The FASTA format of the amino-acid sequence of Rv2351c has been submitted to TEpredict and IEDB-AR (http://tools.iedb.org/mhci/). The human major histocompatibility complex (MHC) HLA-A and HLA-B alleles were used for the MHC class I antigen peptide binding. The binding affinity for the human MHC alleles that were obtained from prediction tools at IEDB-AR used the half-maximal inhibitory concentration of a biological substance (IC50) as the unit of measure. An IC50 < 50 nM indicates high affinity; IC50 < 500 nM indicates medium affinity; and IC50 < 5000 nM indicates low-affinity binding. A lower IC50 indicates a stronger binding affinity to the host MHC. The negative logarithm of IC50, pIC50, was used by TEpredict as the unit of measure to describe the strength of the binding affinity between the peptides and MHC molecules. A pIC50 < 6.3 (IC50 > 500 nM) indicates a low-binding affinity, a pIC50 in the range from 6.3 to 7.3 (50 nM < IC50 < 500 nM) indicates a medium binding affinity, and a pIC50 > 7.3 (IC50 < 50 nM) indicates high-binding affinity. Nine-mer MHC class I T-cell epitope prediction was performed using the consensus method. Every epitope was predicted with an immunogenicity score using the T-cell pMHC class I immunogenicity predictor (http://tools.immuneepitope.org/immunogenicity/).

Construction of the recombinant plasmid pET-32a-Rv2351c

A fragment of Rv2351c was amplified from MTB H37Rv DNA by the polymerase chain reaction (PCR) using the following two primers: (forward): 5' GGATCC GGG AAT CAT CTC GAC AGA GTG TTT G -3', (reverse): 5' ATAGCT GCT TCA GGT GCA CGC GGT-3'. The PCR was performed in a 25-μL solution containing 1 μL DNA, 1 U Ex Taq HS (TaKaRa Biomedical Technology, Beijing, China), 1 μL forward primer, 1 μL reverse primer, 2.5 μL 10× Ex Taq buffer and 8.5 μL ddH2O. The PCR procedure consisted of an initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 3 s, annealing at 60 °C for 1 min and extension at 72 °C for 1 min, and a final extension at 72 °C for 5 min. The PCR amplicons were purified using a DNA purification kit (TIANGEN, Beijing, China). The fragment was then cloned into a pET-32a vector after digestion with BamHI and HindIII, and the recombinant plasmid was transformed into Escherichia coli DH5α cells. The recombinant plasmid pET-32a-Rv2351c was isolated from the E. coli DH5α cells and chemically transformed into E. coli BL21(DE3) cells after the fragment’s identity was confirmed by endonuclease restriction digestion and DNA sequencing.

Expression and purification of the recombinant Rv2351c protein

The E. coli BL21(DE3) cells with the recombinant plasmid were cultured in Luria–Bertani medium overnight at 37 °C. When OD600 was in the range of 0.6–0.8, isopropyl β-D-1-thiogalactopyranoside was then added to the Luria–Bertani medium to a concentration of 1.0 mmol/L, and the culture was incubated for 3 h at 37 °C. The cells were then collected by centrifugation at 8000 g for 20 min, and the supernatant and cell pellet were analyzed using 12% sodium dodecyl sulfate-polyacrylamide gels after the cells were processed by ultrasonication. The sodium dodecyl sulfate-polyacrylamide gel

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Table 1 T-cell epitopes predicted by TEpredict and IEDB

| Peptide ID | Location (start-end) | Amino-acid sequence | Immunogenicity score | Number of bound HLA alleles |
|------------|----------------------|---------------------|---------------------|-----------------------------|
| 1          | 6-14                 | FLTHTGAG            | -0.1272             | 2                           |
| 2          | 18-26                | FMLMDWAPV           | 0.24439             | 8                           |
| 3          | 46-54                | IVMOLGIER           | -0.21408            | 4                           |
| 4          | 76-84                | FOIAGWNPM           | 0.01527             | 6                           |
| 5          | 134-142              | WLPQATTR            | 0.01524             | 2                           |
| 6          | 146-152              | WPLMTMYY            | 0.01582             | 4                           |
| 7          | 163-171              | YLADTFITU           | 0.21539             | 7                           |
| 8          | 169-177              | FTIDCGYHC           | 0.05823             | 4                           |
| 9          | 180-188              | LGTLPLNR            | 0.0536              | 2                           |
| 10         | 236-244              | YQNKKGLGRF          | -0.1377             | 4                           |
| 11         | 281-288              | FAADVRANR           | 0.15318             | 4                           |
| 12         | 295-303              | WLPVNIOS           | 0.04418             | 2                           |
| 13         | 315-323              | SVMVALRNI          | 0.08005             | 3                           |
| 14         | 317-325              | MVTALRII            | 0.19573             | 4                           |
| 15         | 323-331              | ILLSNPAW           | -0.12967            | 3                           |
| 16         | 363-371              | FVTVPNIDA           | 0.16253             | 2                           |
| 17         | 390-398              | CVISYPYSR          | -0.124              | 3                           |
| 18         | 434-442              | VQGDMTSAF           | -0.2179             | 6                           |
| 19         | 473-481              | WVLTDGDGA           | 0.14336             | 2                           |
| 20         | 485-493              | IPYRVPYQQ           | 0.07104             | 6                           |
electrophoresis (SDS-PAGE) was carried out using 1.5-mm thick 10.1 cm × 7.3 cm glass plates, and the electrophoresis was performed for 30 min at 80 V until the tracer dye reached the end of the gel. The proteins were visualized by staining with Coomassie blue. The results of the SDS-PAGE indicated that the Rv2351c protein was expressed in the form of inclusion bodies. The protein inclusion bodies were washed twice with Tris-HCl buffer containing 1 M NaCl, 2 M urea and 0.5% Triton X-100 and then dissolved in binding buffer (8 M urea, 0.5 M NaCl, 20 mM Tris-HCl and 5 mM imidazole).

The recombinant Rv2351c protein was purified using nickel column chromatography, and the purified lysate was loaded onto a 5-mL Ni-NTA column (His Trap HP, GE Life Sciences, Pittsburgh, PA, USA). The column was washed with wash buffer (8 M urea, 0.5 M NaCl, 20 mM Tris-HCl and 60 mM imidazole), the protein was eluted with elution buffer (8 M urea, 0.5 mM NaCl, 20 mM Tris-HCl and 1 M imidazole) and the column was then stripped with stripping buffer (8 M urea, 0.5 M NaCl, 20 mM Tris-HCl and 10 mM EDTA). The fractions that contained the Rv2351c protein were pooled and dialyzed in phosphate buffer containing 0.2 mM EDTA, 0.9 mM GSH, 0.18 mM GSSG and different concentrations of urea (6, 4, 2, 1 and 0.5 M, and no urea). The refolded protein was concentrated to 1 mg/mL after being analyzed using a BCA protein assay kit (Thermo, Waltham, MA, USA). The purified Rv2351c protein was analyzed by SDS-PAGE and Western blot.

**Study subjects**

To evaluate the diagnostic potential of the Rv2351c protein, 159 eligible subjects including patients with and without TB from Fujian and healthy donors from Beijing were enrolled and subjected to analysis with the ELISpot assay using the Rv2351c protein and T-SPOT.TB, along with clinical, microbiological and radiographical examinations. The criteria for enrollment included the following: (1) The patients with active TB were those with clinical and radiographical features of TB concomitant with bacteriological evidence of MTB infection. (2) The patients with no TB were those with other pulmonary diseases than TB. (3) The healthy donors included those with no known history of pulmonary diseases.

**IFN-γ ELISpot assays**

A diagnostic kit for MTB-specific T cells (ELISpot) (QuanBio, Beijing, China) was used to evaluate the magnitude of the response in each case under Rv2351c stimulation. The peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll–Hypaque density gradient centrifugation from 5 to 10 mL of heparinized peripheral blood obtained from each participant. The number of PBMCs was counted using an automatic hematologic analyzer before the sample was obtained from each participant. The number of PBMCs was counted after centrifugation from 5 to 10 mL of heparinized peripheral blood cells (PBMCs) were isolated by Ficoll–Hypaque density gradient centrifugation from 5 to 10 mL of heparinized peripheral blood

**Animals and immunization protocol**

Thirty-six 6-week-old, specific-pathogen-free female BALB/c mice were obtained and raised at the Wuhan Institute of Biological Products Co. Ltd. The mice were randomly divided into six groups (n = 6). To determine the optimal dose that would elicit better immune responses against MTB infection and decrease the adverse effects, each antigen was used at two doses: 20 μg/mouse (low dose) and 50 μg/mouse (high dose). The groups immunized with 20 μg Rv2351c, 50 μg Rv2351c, 20 μg Ag85B and 50 μg Ag85B were denoted Rv2351c-L, Rv2351c-H, Ag85B-L and Ag85B-H, respectively, whereas two groups were immunized with PBS or dimethyl-dioctyldecylammonium bromide (DDA)/poly (I:C) were denoted the PBS and DP groups and were maintained as negative controls. DDA is an adjuvant that elicits a mixed Th1/Th2 immune response. To determine the humoral response elicited by the Rv2351c protein, we measured the titer of Rv2351c-specific IgG, IgG1 and IgG2a, because IgG1 is a Th2-type antibody, and IgG2a is a Th1-type antibody. The ratio of IgG2a/IgG1 was plotted to indicate whether either a Th1 or Th2 profile was induced by the Rv2351c protein.

**Humoral immunity test**

To determine the humoral response elicited by the Rv2351c protein, we measured the titer of Rv2351c-specific IgG, IgG1 and IgG2a, because IgG1 is a Th2-type antibody, and IgG2a is a Th1-type antibody. The ratio of IgG2a/IgG1 was plotted to indicate whether either a Th1 or Th2 profile was induced by the Rv2351c protein.
H₂SO₄, and the absorbance was measured at 450 nm. The mean absorbance of the diluted (1:100) negative mouse serum plus 3 SD was noted as the cutoff absorbance for determining antibody titers.

**Cellular immunity test**

Four weeks after the last immunization, the mice were killed by cervical dislocation, and the spleens were aseptically removed from the mice. The spleens were ground and passed through a cell strainer. The cell suspensions were concentrated by centrifugation at 1000 r/min for 5 min, and the erythrocytes were lysed using ACK lysis buffer. The erythrocytes were removed, and the splenocytes were washed twice with RPMI-1640 (Gibco, Waltham, MA, USA) medium and then diluted to 1 × 10⁶ cells/mL in RPMI-1640 medium supplemented with 10% FBS and 100 U/mL penicillin–streptomycin. Next, 0.5 mL of splenocytes (1 × 10⁶) were seeded in duplicate in 24-well tissue culture plates (Corning, New York, NY, USA); the cells were stimulated with 0.5 mL of purified Rv2351c protein (5 μg/mL) and Ag85B protein (5 μg/mL) and cells that were stimulated with phytohemagglutinin (1 μg/mL) and AIV medium served as the positive and negative controls, respectively, and were grown at 37 °C with 5% CO₂ for 72 h. The culture supernatants were collected, and the concentrations of IFN-γ, IL-2 and IL-4 in the supernatants were determined using the cytokine ELISA kit (BD Biosciences, NJ, USA).

**Statistical analysis**

The diagnostic performance of Rv2351c was evaluated on the basis of the assay’s sensitivity and specificity. The χ²-test was used to evaluate the differences between the methods. The coincidence rate between results was analyzed using Cohen’s kappa coefficients. According to the rules of Landis and Koch, κ < 0.4 indicates poor agreement, κ ≥ 0.4 indicates fair to good agreement and κ ≥ 0.75 indicates excellent agreement.

The experimental data of cytokine secretion were expressed as the mean ± SD. The data were analyzed using the IBM SPSS statistical software package (version 21.0; IBM Corp, Armonk, NY, USA). The t-test was used to evaluate the difference between the cytokine level and the antibody titer. P < 0.05 and P < 0.001 were considered significant and highly significant differences, respectively, between the experimental groups.

**RESULTS**

**T-cell epitope prediction**

Rv2351c was predicted to have many medium-affinity (IC₅₀ < 500 nM or pIC₅₀: 6.3–7.3) and a small number of high-affinity epitopes (IC₅₀ < 50 nM or pIC₅₀ > 7.3), on the basis of IEDB-AR and TEpredict (data not shown). Twenty overlapping nine-mer T-cell epitopes were identified by TEpredict and IEDB-AR (Table 1). Peptides with a higher score and more HLA alleles to bind were more likely to be immunogenic. As shown in Table 1, peptides 2 and 7 were more likely to be human T-cell epitopes and to elicit a T-cell response in humans.

**Expression and purification of recombinant Rv2351c protein**

A 1539-bp fragment was successfully inserted into the pET32a vector (Solarbio, Beijing, China) and confirmed by DNA sequencing. As shown in Figure 1, the result of the SDS-PAGE analysis indicated that the Rv2351c was expressed in the form of inclusion bodies (Figure 1C) and was purified as an approximately 69.35 kD recombinant protein (Figure 1F). Western blot analysis was performed using an anti-His antibody to specifically confirm the presence of the recombinant Rv2351c protein (Figure 1F).

**Characteristics of the subjects**

From 15 August 2015 to 21 September 2015, a total of 159 subjects including 61 patients with pulmonary TB and 38 patients with no TB were recruited from the Fuzhou Pulmonary Hospital, Fujian, and 60 healthy donors were recruited from the Chinese Center for Disease Control and Prevention, Beijing, China. A total of 154 subjects with valid results and diagnostic information were enrolled for the statistical analyses, whereas five healthy donors were excluded because of invalid ELISPOT results in which the number of SFCs in the positive control was less than 20. All the subjects were vaccinated with BCG. The
patients in the TB group included microbiologically positive subjects with clinically diagnosed pulmonary TB. The 38 patients with no TB included patients with diseases, such as chronic obstructive pulmonary disease, pneumonia, bronchiectasis and lung abscess. The 55 healthy donors showed normal chest X-rays. The 38 patients with no TB and the 55 healthy donors were considered negative controls. The baseline information for the 159 participants is given in Table 2. There were no significant differences in the sex ratio among the three groups (P > 0.05). The median age of healthy donors was significantly lower than that of patients with no TB and those with pulmonary TB (Table 2).

**Diagnostic performance of the Rv2351c protein**

To evaluate the diagnostic performance of Rv2351c, PBMCs from patients with active TB, patients with no TB and healthy donors were stimulated with the Rv2351c protein for 20 h. Among the 61 patients with clinically diagnosed TB, 37 were positive for Rv2351c among the 38 patients with no TB and the 55 healthy donors, 4 from each group were positive for Rv2351c. Among the 61 patients with TB, 57 were bacteriologically positive, and all the patients with no TB and healthy donors were bacteriologically negative; therefore, the bacteriological tests could be considered the 'gold standard'; the sensitivity and specificity of Rv2351c detection by the T-SPOT.TB assay were 61.4% (35/57) and 98.2% (56/57), respectively. There was a significant difference between the results of the T-SPOT.TB and ELISpot assays using Rv2351c (P < 0.05) (Table 3). However, the results of the ELISpot assay using Rv2351c had a moderate overall agreement (78%) with the results of the T-SPOT.TB assay using the 3Ag peptide cocktail (Rv3615c, ESAT-6 and CFP-10). Subsequently, the response magnitude of different groups of patients against the cocktail peptides and Rv2351c was analyzed with the T-SPOT.TB assay (Figure 2). The results revealed that the magnitude of the response against Rv2351c in patients with active TB was significantly lower than that against cocktail peptides (P < 0.01), whereas the magnitude of response of the patients with no TB and the healthy donors against the cocktail peptides and Rv2351c was significantly lower than that of patients with active TB. No significant differences were observed between patients with no TB and healthy donors, thus indicating that the immune responses against MTB did not differ between patients with different pulmonary diseases and healthy donors. This result also indicated that the difference in median age among the three groups had no influence on the ELISpot results. TB infection, but not age, influenced the T-cell response among patients with active TB, patients with no TB and healthy donors.

Collectively, the Rv2351c protein distinguish patients with active TB from those with no TB and healthy donors. The ELISpot assay results, after the samples were stimulated with the Rv2351c protein, also showed good agreement with the gold standard results and a moderate overall agreement (78%) with the results of T-SPOT.TB assay.

**Humoral immunity**

To determine the ability of Rv2351c to elicit a humoral immune response in BALB/c mice, the level of Ag85B/Rv2351c-specific IgG and the levels of the isotypes IgG1 and IgG2a were tested in the serum using ELISA when the mice were killed four weeks after their last immunization.

As shown in Figure 3, a significantly higher titer of Rv2351c-specific and Ag85B-specific IgG, IgG1 and IgG2a (Figure 3) was observed in the Rv2351c-L (P < 0.001), Rv2351c-H (P < 0.001), Ag85B-L (P < 0.001) and Ag85B-H (P < 0.001) groups, whereas no IgG1/IgG1/IgG2a was detected in the negative control groups (PBS and DP groups). A significantly higher titer of IgG (P < 0.001) was found in the groups immunized with Rv2351c at a dose similar to that of Ag85B.

The IgG2a/IgG1 ratio in the Rv2351c-L, Rv2351c-H, Ag85B-L and Ag85B-H groups was 2.5, 3.5, 3.1 and 2.2, respectively, thus suggesting a moderate extent of Th1-type immune response to Rv2351c and Ag85B.

As a result, Rv2351c, rather than Ag85B, elicits potent humoral and Th1-type immune responses that are crucial to the control of MTB infection.

**Cellular immunity**

To characterize the cellular immune responses elicited by Rv2351c and Ag85B, the concentration of IFN-γ, IL-2 and IL-4 was determined in culture supernatants of splenocytes that were isolated from immunized mice stimulated with Ag85B or Rv2351c. A high concentration of IFN-γ (Figure 4A) was observed in all the groups except the PBS and DP groups. A significantly high production of IFN-γ was observed in the Rv2351c-H (P < 0.001) and Ag85B-H (P < 0.001) groups compared with that in the Rv2351c-L and Ag85B-L groups. IL-2 is produced by activated CD4+ T cells and CD8+ T cells and is involved in immune responses in MTB infection. However, the Rv2351c-L and Rv2351c-H groups showed low production of IL-2 (Figure 4B), whereas the Ag85B-L and Ag85B-H groups showed a significantly higher level of IL-2 compared with the PBS and DP groups (Figure 4B).

IL-4 is produced by Th2 cells and aids in directing naïve T lymphocytes in Th2 polarization. All the groups except the PBS and DP groups showed high production of IL-4 (Figure 4C), Rv2351c-L and Rv2351c-H (P < 0.05) induced higher production of IL-4 than did Ag85B-L and Ag85B-H. The Rv2351c elicited a Th1-dominant immune response with IFN-γ:IL-4 ratios of 105.4 and 73.2 in the Rv2351c-L and Rv2351c-H groups, respectively.

In summary, Rv2351c elicited a cellular immune response in BALB/c mice with high concentrations of IFN-γ and IL-4.

**DISCUSSION**

TB has high morbidity and mortality rates, thus leading to severe economic losses in both developing and developed countries. Rapid diagnosis and prompt treatment are the best strategies to control TB. However, conventional detection methods, such as acid-fast staining and sputum bacterial culture, show poor sensitivity, thus leading to a failure to detect some patients with active TB; moreover, radiographic tests with low specificity cannot distinguish between TB and other pulmonary diseases. The tuberculin skin test sometimes displays false-negative and false-positive results and cannot distinguish between MTB infection and BCG vaccination. The ELISpot assay is now increasingly being used for TB diagnosis because of its high specificity.
diseases and healthy donors. To determine the diagnostic value of patients with TB, patients with no TB but with other pulmonary diagnostic performance of Rv2351c in a study cohort including genes from the plcABC and/or plcD loci in clinical MTB isolates.31 Genomic deletions resulting in the loss of parts of genes or complete polymorphisms in the to have had a latent infection. Previous studies on the genetic assay using Rv2351c, thus indicating that these cases were more likely induced by the Rv2351c protein or a latent infection. However, two of four patients with no TB tested positive with the T-SPOT.TB assay using Rv2351c, thus indicating that these cases were more likely to have had a latent infection. Previous studies on the genetic polymorphisms in the plc genes, including plcA, have reported genomic deletions resulting in the loss of parts of genes or complete genes from the plcABC and/or plcD loci in clinical MTB isolates.31 Because the exact data regarding Rv2351c deletion among MTB clinical isolates are unavailable, more research on the deletion of Rv2351c in clinical isolates should be undertaken before its use in the diagnosis of TB infection. Given the polymorphism of the Rv2351c gene, Rv2351c might not be the best choice, as compared with other conserved genes to be used as a TB diagnostic tool. However, according to our results, its good diagnostic performance in TB infection was clear. Use of a combination of different antigens can improve diagnostic performance in ELISpot assays.7 Therefore, we speculated that the diagnostic performance of Rv2351c might also be improved by combining it with other widely used antigens, such as ESAT-6, CFP-10 or Rv3615c. Hence, Rv2351c has the potential to be used as a supplement for other immunodominant antigens.

Because rapid diagnosis increases the treatment duration of TB, developing an effective vaccine that protects against TB is also important to limit the disease. The BCG vaccine is currently the only TB vaccine used in humans that can protect children from severe disseminated diseases, such as tubercular meningitis and hematogenously disseminated TB, but it cannot protect adults against ’reactivation’ TB later in life.32 A TB subunit vaccine has the potential to replace BCG or to be used as a complement to boost BCG-elicited immune responses when it is combined with a recombinant fusion protein that induces effective protective immunity against TB infection.33-36 Presently, increasing attention is being focused on TB subunit protein vaccines, because they are safe, can be used in immunocompromised individuals and their immune reactivity is not influenced by previous exposure to environmental mycobacterium.37

More detailed studies on immunoprotection against MTB infection are crucial for effective vaccine development. Undoubtedly, cellular immunity plays a crucial role in the control of MTB infection, because MTB is an intracellular bacterial pathogen that survives and proliferates within macrophages.2 Cytokines are also an important factor determining the immune response toward the desired Th1 and Th2 bias. Cytokines, such as IFN-γ, IL-2, IL-12 and TNF-α, are responsible for cellular immunity against MTB and are produced by Th1 cells, not only activate macrophages, but also promote polarization of effector Th1 cells.38 In contrast, IL-4, IL-5, IL-10 and IL-13, which are produced by Th2 cells, are responsible for inhibiting macrophage functions and promoting antibody responses.39 IFN-γ is the most important cytokine marker in T-cell stimulation assays and is

Table 3 Comparison of results using the bacteriological test results as ‘gold standard’ for TB diagnosis

|                  | T-SPOT.TB | ELISpot-2351c | P-value |
|------------------|-----------|---------------|---------|
| Sensitivity      | 98.2%     | 61.4%         | 0.000   |
| Specificity      | 94.6%     | 91.4%         | 0.388   |
| Youden index     | 0.928     | 0.528         |         |
| Kappa value      | 0.916     | 0.554         |         |

Note: 57 TB patients were diagnosed positive and 93 were diagnosed negative with the ‘gold standard’.

Figure 2 Response magnitude of different subjects against cocktail peptides and Rv2351c in the ELISpot assay. Sixty-one patients with active TB, 38 patients with no TB and 55 healthy donors were enrolled to evaluate the T-cell response to cocktail peptides (Rv3615c, ESAT-6 and CFP-10) and the Rv2351c protein. Responses against the cocktail peptides and the Rv2351c protein were obtained through the T-SPOT.TB assay. The dots represent the response in each case under stimulation with cocktail peptides and Rv2351c. The thick line represents the average response of each group. *P < 0.05; **P < 0.001.

Figure 3 Antibody response against Ag85B and Rv2351c in BALB/c mice immunized with Ag85B or Rv2351c conjugate in DDA/poly (I:C) adjuvant. Serum samples were analyzed for the presence of anti-Ag85B and anti-Rv2351c antibodies via ELISA. The isotype profile of the antibodies was characterized using conjugated secondary antibodies specific for IgG, IgG1 and IgG2a. The data are plotted as geometric mean ± SD log10 end point titer. P was calculated by t-test to evaluate the statistically significant differences (*P < 0.05, **P < 0.001).
involved in protective immunity of the host toward mycobacterial antigens, because it activates macrophages in conjunction with TNF-α, thereby facilitating the killing of intracellular mycobacteria. As expected, Rv2351c induced a significantly higher level of specific IFN-γ and IL-4 production, and the IFN-γ/IL-4 ratio indicated a Th1-dominant immune response. Compared with a DNA vaccine of M. bovis that is administered with the Ag85B antigen, Rv2351c elicited a more potent cellular immune response with much higher concentrations of IFN-γ and IL-4.

The role of humoral immune responses in MTB infection has been ambiguous. However, much research has indicated that adaptive immune responses contribute to the outcome of MTB infection, because antibodies can affect the interaction between mycobacteria and other components of the immune system. Therefore, it is important to consider humoral responses, especially given that understanding of the pathogenic mechanisms underlying MTB infection is still insufficient. Many studies have shown that several classical functions, such as opsonization and complement activation, which are mediated by antibodies, play an essential role in the defense against MTB.

Human IgG enhances complement activation and increases the phagocytosis of MTB by macrophages. In a previous study, increased complement activation by BCG has been observed among patients with TB with a high IgG2 to LAM ratio. Moreover, IgM, IgG1, IgG3 and IgA have been confirmed to be protective antibodies against MTB.

In our study, significantly higher levels of anti-Rv2351c IgG, IgG2a and IgG1 were observed in mice immunized with the Rv2351c protein. The efficacy of antibodies primarily relies on three aspects: the titer of the antibody available, the structural features of the immunoglobulin molecule and the immunological state of the host. Therefore, Rv2351c, which induces high-level production of IgG, IgG1 and IgG2a, has great potential to protect against MTB. Because cellular and humoral immunities interact with and influence each other, both should be considered in designing new vaccines. The recombinant BCG strain expressing protein from RD1 induced increased protective efficacy, thus suggesting that supplementation of BCG with a subunit Rv2351c vaccine may be a good vaccination strategy.

An optimal adjuvant can be of equal importance as an antigen in vaccine development. Adjuvants not only increase opportunities for the antigen to be recognized by the immune system by inhibiting the antigen's degradation and extending the time that the antigen exists in vivo, but also change the type of immune response elicited by the antigens. Because proteins alone are poorly immunogenic and prone to degradation, adjuvants, such as DDA and poly (I:C), have been considered in animal experiments. Our study indicated that DDA/poly (I:C) are promising adjuvant candidates to supplement the induction of potent cellular and humoral immunity by Rv2351c and Ag85B. However, more attention should be given to the safety and stability of poly (I:C) before its clinical application because it has the potential to induce excessive immune and autoimmune disorders.

Figure 4  Evaluation of cytokine secretion by splenocytes from immunized mice, isolated and co-cultured with Ag85B or Rv2351c. The splenocytes were prepared four weeks after the mice were immunized with Ag85B or Rv2351c (three times, 2-week intervals). The splenocytes (1×10⁶) were then co-cultured with Ag85B (5 μg/mL) or Rv2351c (5 μg/mL) for 72 h before the levels of the cytokines (A) IFN-γ, (B) IL-2 and (C) IL-4 were measured in the culture supernatants using commercial ELISA kits. The data for cytokine secretion are presented as the mean ± SD of two independent experiments. The level of statistical significance for differences between the negative control groups and the Ag85B or Rv2351c groups was determined using the t-test (*P<0.05; **P<0.001).
In summary, we confirmed that Rv2351c is an immunodiagnostic antigen that can distinguish patients with TB from those without infection and healthy donors. This study also revealed that Rv2351c is an immunodominant antigen that elicits potent cellular and humoral immune responses in Rv2351c-immunized mice. Therefore, Rv2351c has potential for use in the diagnosis of TB and in a subunit vaccine. This study lays a foundation for the application of Rv2351c in TB vaccine development.

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1 World Health Organization. Global Tuberculosis Report 2016. Geneva: WHO, 2016. Available at http://www.who.int/tb/publications/global_report/en/ (accessed 1 October 2016).
2 North RJ, Jung YJ. Immunity to tuberculosis. Annu Rev Immunol 2008; 25: 177–184.
3 Marel A, Ghemmaghami A, Ranesha P et al. Superior T cell activation by ESAT-6 as compared with the ESAT-6-CFP-10 complex. Int Immunol 2005; 17: 1439–1446.
4 Millington KA, Fortune SM, Low J et al. Rv3615c is a highly immunodominant RD1 (region of difference 1)-dependent secreted antigen specific for Mycobacterium tuberculosis infection. Proc Natl Acad Sci USA 2011; 108: 5730–5735.
5 Acheampong IK, Siddique M, Henderson R et al. Dissecting mechanisms of immunodominance to the common tuberculosis antigens ESAT-6, CFP10, Rv2031c (hspX), Rv2654c (TB7.7), and Rv1038c (EsuX). J Immunol 2012; 188: 5020–5031.
6 Koroll V, Bosue S, Gideon HP et al. Population tailored modification of tuberculosis specific interferon-gamma release assay. J Infect 2016; 72: 179–188.
7 Khalid R, Atzal M, Khurshid S et al. Fusion molecules of heat shock protein HSPX with other antigens of Mycobacterium tuberculosis show high serodiagnosticity of tuberculosis. PLoS One 2016; 11: e0163349.
8 Goldie H, Wintell B, Vasi ML et al. Mutations in the hemolytic-phospholipase C operon result in decreased virulence of Pseudomonas aeruginosa PA01 grown under phosphate-limiting conditions. Infect Immun 1989; 57: 1369–1373.
9 Rahme LG, Stevens EJ, Wolfart SF et al. Common virulence factors for bacterial pathogens in plants and animals. Science 1995; 268: 1899–1902.
10 Camilli A, Goldfine H, Portnoy DA. Listeria monocytogenes mutants lacking the hemolytic-phospholipase C-specific phospholipase C are avirulent. J Exp Med 1991; 173: 751–754.
11 Kaminsky S, Sato H, Murata R. The role of alpha-toxin of Clostridium perfringens in experimental gas gangrene in guinea pigs. Jpn J Med Sci Biol 1972; 25: 200.
12 Raynaud C, Guilloh C, Rauzier J et al. Phospholipases C are involved in the virulence of Mycobacterium tuberculosis. Mol Microbiol 2002; 45: 203–217.
13 de Souza GA, Leversen NA, Mamen H et al. Bacterial proteins with cleaved or uncleaved signal peptides of the general secretory pathway. J Proteomics 2011; 75: 502–510.
14 Matsu T, Carneiro CR, Leao SC. Evidence for the expression of native Mycobacterium tuberculosis phospholipase C recognition by immune sera and detection of promoter activity, Br J Med Biol Res 2000; 33: 1275–1282.
15 Parkash O, Singh BP, Pai M. Regions of differences encoded antigens as targets for immunodiagnosis of tuberculosis in humans. Scand J Immunol 2009; 70: 345–357.
16 Lundegaard C, Lund O, Buus S et al. Major histocompatibility complex class I binding predictions as a tool in epitope discovery. Immunology 2010; 130: 309–318.
17 Lahaut EM, Reche PA. Prediction of MHC-peptide binding, a systematic and comprehensive overview. Curr Pharm Des 2009; 15: 3209–3220.
18 Chen P, Rayner S, Hu KH. Advances of bioinformatics tools applied in virus epitopes prediction. Virol Sin 2011; 26: 1–7.
19 Zang XW. A combination of epitope prediction and molecular docking allows for good identification of MHC class I restricted T-cell epitopes. Comput Biol Chem 2013; 45: 30–35.
20 Xu Y, Zhu B, Wang Q et al. Recombinant BCG coexpressing Ag85B, ESAT-6 and mouse-IFN-gamma confers effective protection against Mycobacterium tuberculosis in C57BL/6 mice. FEMS Immunol Med Microbiol 2007; 51: 480–487.
21 Huguen K, The immunodominant T-cell epitopes of the mycolyl-transferases of the antigen 85 complex of M. tuberculosis. Front Immunol 2014; 5: 321.
22 Lin CW, Su LJ, Chang JR et al. Recombinant BCG coexpressing Ag85B, CFP10, and interferon-12 induces multifunctional Th1 and memory T cells in mice Appiim 2012; 120: 72–82.
23 Diehrch J, Aagaard C, Leah R et al. Exchanging ESAT6 with TB10.4 in an Ag85B fusion molecule-based tuberculosis subunit vaccine: efficient protection and ESAT6-based sensitive monitoring of vaccine efficacy. J Immunol 2009; 181: 6332–6339.
24 Klingue-Hamour C, Libon C, Potrnik-Gilquin H et al. DDA adjuvant induces a mixed Th1/Th2 immune response when associated with BBG2Na, a respiratory syncytial virus potential vaccine. Vaccine 2020; 27: 2743–2751.
25 Cole ST, Brosch R, Parkhill J et al. Deciphering the biology of Mycobacterium tuberculosis from the complete genome sequence. Nature 1998; 393: 537–544.
26 Ramos A, Harries AD. The clinical value of new diagnostic tools for tuberculosis. F1000 Med Rep 2009; 1: 36.
27 Viana-Niero C, de Haas PE, van Sooijen D et al. Analysis of genetic polymorphisms affecting the four phospholipase C (plc) genes in Mycobacterium tuberculosis complex clinical isolates. Microbiology 2004; 150 (Pt I): 967–978.
28 Andersen P, Doherty TM. The success and failure of BCG – implications for a novel tuberculosis vaccine. Nat Rev Microbiol 2005; 3: 656–662.
29 Bertholet S, Iton GZ, Owicky DJ et al. A defined tuberculosis vaccine candidate boosts BCG and protects against multidrug-resistant Mycobacterium tuberculosis. Sci Transl Med 2010; 2: 63ra74.
30 Ottenhoff TH, Doherty TM, van Dissel JT et al. First in humans: a new molecularly defined vaccine shows excellent safety and strong induction of long-lived Mycobacterium tuberculosis-specific Th1 cell-like responses. Hum Vaccin 2010; 6: 1007–1015.
31 Vindish HP, Duthee MS, Misquith A et al. Protection of mice from Mycobacterium tuberculosis by ID8710LA-SE, a novel subunit tuberculosis vaccine candidate. Vaccine 2011; 29: 7842–7848.
32 Huang Q, Yu W, Hu T. Potent antigen-adjuvant delivery system by conjugation of Mycobacterium tuberculosis Ag85B-Hsp fusion protein with arboviralagcan-Lpoly(C) conjugate. Bioconjug Chem 2016; 27: 1165–1174.
33 Derrick SC, Yabe JM, Yang A et al. Immunogenicity and protective efficacy of novel Mycobacterium tuberculosis antigens. Vaccine 2013; 31: 4641–4646.
34 Matusci, Magg I, Vuillaggio A. Cellular and humoral immune responses during tuberculosis infection: useful knowledge in the era of biological agents. J Rheumatol Suppl 2014; 91: 17–23.
35 Romagnani P, Annunziato F, Piccinini MP et al. Th1/Th2 cells, their associated molecules and role in pathophysiology. Eur Cytokine Netw 2000; 11: 510–511.
36 Teixeira FM, Teixeira HC, Ferreira AP et al. DNA vaccine using Mycobacterium bovis Ag85B antigen induces partial protection against experimental infection in BALB/c mice. Clin Vaccine Immunol 2006; 13: 930–935.
37 Achkar JM, Casaldeval A. Antibody-mediated immunity against tuberculosis: implications for vaccine development. Cell Host Microbe 2013; 13: 250–262.
38 Manivannan S, Rao NV, Ramananathan VD. Role of complement activation and antibody in the interaction between Mycobacterium tuberculosis and human macrophages. Indian J Exp Biol 2012; 50: 542–550.
39 Helland G, Wiker HG, Hogansen K et al. Involvement of anti-poliovirusimmunoglobulins antibodies in classical complement activation in tuberculosis. Clin Diagn Lab Immunol 1998; 5: 211–217.
40 Casaldeval A, Pirizski LA. A new synthesis for antibody-mediated immunity. Nat Immunol 2011; 13: 21–28.
41 Bottai D, Frigioli W, Clark S et al. Increased protective efficacy of recombinant BCG strains expressing virulence-neutral proteins of the ESX-1 secretion system. Vaccine 2015; 33: 2710–2718.
42 Hafner AM, Corteyse B, Merkle HP. Clinical trials for the development of polycl(C) vaccine. Adv Drug Deliv Rev 2013; 65: 1386–1399.