



ORIGINAL ARTICLE

Immune responses to latent tuberculosis antigen Rv2659c in Chinese populations



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KEYWORDS

latency antigen;
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Background: The latency-associated antigen Rv2659c is a starvation-related protein of *Mycobacterium tuberculosis* (*M. tuberculosis*). It has potential use in tuberculosis (TB) control, but its immunological characteristics in Chinese populations are unclear.

Methods: In this study, immunological characteristics and potential diagnostic use of recombinant Rv2659c protein were assessed. Interferon- γ (IFN- γ) production from peripheral blood mononuclear cells (PBMC) was assayed by enzyme-linked immunospot (ELISPOT) in TB patients (80 cases), individuals who were purified protein derivative (PPD)-positive after Bacillus Calmette-Guérin (BCG) vaccination (27 cases), nontuberculous respiratory disease patients (30 cases), individuals who were identified by standard techniques as having latent TB infection (LTBI) (37 cases), and uninfected healthy individuals (75 cases). Serum immunoglobulin G (IgG) levels were assayed by enzyme-linked immunosorbent assay (ELISA) in TB patients (43 cases), LTBI individuals (36 cases) and uninfected healthy individuals (66 cases).

Results: When stimulated by rRv2659c, PBMC from LTBI individuals gave ELISPOT counts that were significantly higher than those from TB patients, BCG vaccinated individuals, non-TB respiratory disease patients and uninfected healthy individuals ($p < 0.05$). The rRv2659c stimulation gave detectable IFN- γ production in a higher proportion of persons with LTBI compared with TB patients and uninfected healthy individuals. BCG vaccination and non-TB respiratory disease had little influence on the PBMC response to rRv2659c. The levels of serum IgG specific for rRv2659c were not significantly different between LTBI individuals and TB patients ($p > 0.05$).

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Conclusion: These results suggest that rRv2659c has potential for the diagnosis of LTBI. This is the first clinical report of human immune recognition of Rv2659c in Chinese populations. Copyright © 2014, Taiwan Society of Microbiology. Published by Elsevier Taiwan LLC. All rights reserved.

Introduction

Tuberculosis (TB) has been a serious health problem globally for a long time, especially in the developing countries. It has been estimated that one-third of the world's population is latently infected with *Mycobacterium tuberculosis* (*M. tuberculosis*).¹ China is one of the 22 highest TB burden countries in the world. According to the report of the fifth national epidemiological sampling survey of TB in China in 2010, the incidence of pulmonary TB was 459/100,000 population; the incidence of smear-positive or culture-positive pulmonary TB (active TB) was 66/100,000 population.² Active TB patients are a major source of infection, so diagnostic differentiation between active TB patients and the latently infected individuals is a critical step to control TB.³

Our current methods of diagnosing LTBI are not highly effective, with either low specificity or low sensitivity or both. Latent TB infection (LTBI) diagnosis has been made using the tuberculin skin test (TST) for about a century. The TST result is influenced by many factors such as quality of purified protein derivative (PPD), Bacillus Calmette-Guérin (BCG) vaccination and previous exposure to non-TB mycobacteria. Specificity of the TST is diminished in consequence.^{4–7} Interferon- γ (IFN- γ) release assays (IGRA) provide a new approach in the detection of LTBI, which is based on the release of IFN- γ from T lymphocytes upon stimulation with *M. tuberculosis* specific antigens. IGRAs have much higher specificity than TST and are not affected by BCG vaccination.^{8–10} However, neither TST nor IGRA can differentiate active TB from LTBI. The problem is especially important in countries with a high TB incidence, where 85.6% of TB patients may have smear-negative and culture-negative pulmonary TB, subclinical disease and latent infection are widespread, and reinfection happens frequently.¹¹ Therefore, the study of antigens of *M. tuberculosis* that may be associated with LTBI is a priority to define immunodiagnostic candidates that can enhance the specificity and sensitivity of diagnosis of TB infection. Studies of healthy adults with LTBI have identified T cell responses to selected latency related proteins encoded by *M. tuberculosis*, suggesting a role in maintenance of the asymptomatic phase of latent infection.^{12–15} Variations in the immunogenicity of the latency proteins have been observed in European, South American, African, and Japanese populations, but no data have been published from other geographic areas.^{12–14,16} In order to gain better insight into TB latency and to identify specific antigens to differentiate LTBI from active TB, we focus here on latency antigen Rv2659c (probable PhiRv2 prophage integrase) encoded within the RD11 region of *M. tuberculosis*. It is encoded by one of the *M. tuberculosis* starvation-related genes present in a "starvation stimulon" that is induced by hypoxia and upregulated

after starvation in nutrient deficient medium and is absent from all *M. bovis* strains including BCG.^{17–21} China is a country with a high TB incidence and high TB infection rate, and it is estimated that 76% of infants have received BCG vaccination at birth.² It is therefore difficult to differentiate *M. tuberculosis* infection from BCG vaccination. We hypothesized that antigen Rv2659c may be a promising biomarker for differentiating LTBI from TB and would not be affected by BCG vaccination. The aims of this study were to obtain a recombinant protein Rv2659c, to analyze its immunological characteristics, and to evaluate its potential utility. To our knowledge, this is the first clinical evaluation of immune recognition of the starvation stimulon gene product Rv2659c in Chinese people.

Materials and methods

Study participants

Because our primary goal was to assess the T-cell and humoral responses to latency antigen Rv2659c in China, we selected a wide spectrum of individuals. In a T-cell response study: (1) the TB patient group ($n = 80$) consisted of 38 patients with pulmonary TB and 42 with extrapulmonary TB. The mean age was 46.9 years (range, 12–89 years) and 50 were male; (2) the healthy group consisted of 112 new male People's Liberation Army recruits; the mean age was 19 years (range, 17–21 years); (3) the PPD-positive BCG-vaccinated group consisted of 27 new male PLA recruits whose PPD test result was positive 3 months after receiving BCG vaccination; (4) non-TB respiratory disease individuals consisted of 30 patients aged from 23 years to 80 years, diagnosed as having pneumonia (23 cases), bronchitis (1 case) or pulmonary carcinoma (6 cases). In a humoral response study, the TB patient group ($n = 43$) consisted of 36 patients with pulmonary TB and seven with extrapulmonary TB. The mean age was 41 years (range, 19–81 years) and 26 were male. The TB patients were diagnosed according to the following criteria: (1) having TB symptoms and signs, including cough, cough producing phlegm, coughing up blood, fever, night sweats, fatigue, loss of appetite, weight loss, chest or head pain, breathing difficulty, etc.; (2) having a TB lesion on chest X-ray or CT; (3) maybe having a positive smear or positive culture; (4) maybe having a strongly positive PPD skin test; (5) maybe having evidence of bronchoscopy; (6) maybe having evidence of pathological examination; and (7) the anti-TB treatment was effective, TB was confirmed (Respiratory Disease Branch of Chinese Medical Association 2001). Cases without definitive diagnosis were excluded. All individuals in this study were negative for HIV antibodies. Details of TB patients and control groups are given in [Table 1](#).

Table 1 Characteristics of tuberculosis (TB) patients and healthy recruits for T cell responses and humoral responses

Groups	T cell responses			Humoral immune responses		
	Number	Mean age (y) \pm SD	Sex (male:female)	Number	Mean age (y) \pm SD	Sex (male:female)
TB patients						
Total	80	46.9 \pm 20.3	50:30	43	52 \pm 23.2	2:17
Smear- or culture-positive TB	12	47.3 \pm 24.4	8:4	8	45.6 \pm 21.1	4:4
Smear- and culture-negative TB	68	46.8 \pm 19.7	42:26	35	49.2 \pm 23.5	21:14
Pulmonary TB or pulmonary TB with endobronchial TB	36	49.5 \pm 22.0	23:13	28	40.0 \pm 19.0	15:13
Tuberculous pleurisy	5	65.0 \pm 18.6	2:3	5	30.2 \pm 11.3	4:1
Pulmonary TB complicated with tuberculous pleurisy	2	25.0 \pm 2.8	1:1	3	60.0 \pm 21.7	2:1
Bone TB	28	43.3 \pm 16.7	17:11			
Others including TB in multiple organ and tuberculous pericarditis, tuberculous peritonitis, etc.	9	42.4 \pm 17.2	5:4	7	45.4 \pm 15.0	4:3
Healthy recruits	112	18.3 \pm 1.3	112:0	102	18.8 \pm 1.2	102:0
BCG vaccination group	27	19.3 \pm 1.0	27:0			
Non-tuberculosis respiratory disease individuals	30	60.6 \pm 17.9	14:16			

Recombinant protein preparation

The nucleotide sequence of Rv2659c was obtained from TubercuList (<http://www.tbdb.org>). The procedures for cloning, expression and purification of Rv2659c were briefly as follows²²: a 1154 bp gene fragment containing the gene encoding Rv2659c protein, with the sites of restrictive enzymes Nhe I (upstream) and EcoR I (downstream) was amplified by Polymerase chain reaction from genomic DNA of *M. tuberculosis* H37Rv, and then cloned into pGEM-T vector (Promega Corporation, Madison, WI, USA). Positive colonies were identified by PCR, enzyme digestion, and sequencing. The pGEM-T plasmid DNA was digested by restrictive enzymes Nhe I and EcoR I (Promega Corporation). The trimmed 1128 bp fragment was ligated with T4 DNA ligase into Nhe I and EcoR I-digested pET30a plasmid vector (Novagen, San Diego, CA, USA), a bacterial expression vector containing an N-terminal hexahistidine tag for rapid purification with nickel-chelating resin, and transferred into competent *Escherichia coli* (*E. coli*) BL21(DE3). The recombinant plasmid Rv2659c/pET30a was extracted and sequenced with T7 and T7t universal primers by Beijing Liuhe BGI Inc, China.

For the expression of the protein, *E. coli* BL21 cells transfected with plasmid Rv2659c/pET30a were grown overnight at 37°C in LB medium containing kanamycin (50 μ g/mL). Then, the culture was inoculated into the same medium with the same kanamycin concentration and grown at 37°C until the optical density at 600 nm (OD₆₀₀) reached between 0.8 and 1.0, then induced with 0.1 mM/L Isopropyl β -D-1 thiogalactopyranoside at 37°C for 3–4 hours and the inclusion bodies were extracted. The expression level and form of Rv2659c protein were checked by gel electrophoresis of 15% SDS-PAGE.

Since the recombinant Rv2659c protein was expressed with an N-terminal 6 \times histidine-tag in inclusion bodies, it was purified by metal chelate affinity chromatography under

denaturing conditions according to the manufacturer's protocol (GE Healthcare Life Sciences Pittsburgh, PA, USA). The purified Rv2659c protein was dialyzed to remove urea, and then its molecular mass was determined by 15% SDS-PAGE. Gels were stained with Coomassie brilliant blue. The molecular mass and purity of Rv2659c protein were analyzed and calculated with BandsCan 5.0. Residual endotoxin levels were determined by the Limulus Amebocyte Lysate (LAL) Assay (Associates of Cape Cod Inc., East Falmouth, MA, USA). Recombinant antigens were freeze-dried and stored at -80° C until further use.

Recombinant *M. tuberculosis* CFP10-ESAT6 (rCFP10-ESAT6) cloning, expression and purification were performed as described previously.²³

Enzyme-linked immunospot assay

MultiScreen 96-well plates (Millipore, Bedford, MA, USA) were coated with 15 μ g/mL anti-human IFN- γ Ab 1-D1K (MABTECH AB), incubated overnight at 4°C and then blocked with 2% BSA at room temperature for 2 hours. A 4–5 mL venous blood sample from each study participant was collected in a heparinized glass tube, and then peripheral blood mononuclear cells (PBMC) were isolated and quantified in serum free medium. PBMCs of every individual were plated in duplicate at 2.5×10^5 cells/well, and stimulated with medium as negative control, or phytohemagglutinin (PHA, 20 μ g/mL; Sigma, St. Louis, MO, USA) as positive control, or with recombinant CFP10-ESAT6 protein (20 μ g/mL) or recombinant Rv2659c protein (20 μ g/mL) at 37°C with 5% CO₂ for 20 hours. The optimum concentration of Rv2659c protein had been determined in preliminary studies to be 20 μ g/mL. The plates were incubated with a biotin-conjugated anti-human IFN- γ and were developed using the streptavidin-ALP conjugate and BCIP/NBT plus (MABTECH AB) according to the manufacturer's protocol. The number of spot-forming cells (SFC) in each well was

counted with an automated enzyme-linked immunospot (ELISPOT) reader, CTL-Immuno-Spot S5 Versa Analyzer (Cellular Technology Ltd., Shaker Heights, Ohio, USA) and analyzed with Immunospot (CTL Analyzer LLC).

Antibody level by enzyme-linked immunosorbent assay

Serum was collected from venous blood of each study participant and stored at -20°C until further use. The level of immunoglobulin G (IgG) antibodies specific for recombinant Rv2659c was determined by an enzyme-linked immunosorbent assay (ELISA). In brief, $100\ \mu\text{L}$ of recombinant Rv2659c protein ($5\ \mu\text{g}/\text{mL}$) were coated in 96-well microplate (Costar, Washington, D.C., USA) overnight at 4°C . After washing three times with wash buffer, the plate was blocked with PBS containing 10% fetal bovine serum (FBS) for 1 hour at 37°C . Subsequently, individual sera at 1:10 dilution were added to the wells and incubated for 40 minutes at 37°C . After three washes, horseradish peroxidase-labeled goat anti-human IgG (1:10000, CWBIO, Biotech Co., Ltd., Beijing, China) was added to the wells and incubated at 37°C for 30 minutes. Then the plate was washed six times. The solutions A and B of TMB Substrate Reagent Set (BD Bioscience, Becton Dr Franklin Lakes, NJ, USA) mixed with a ratio of 1:1 were added ($100\ \mu\text{L}$) to each well, and then the plate was kept for 10 minutes in the dark at room temperature. Data are presented as the optical density at 450 nm determined with a microplate photometer (Multiskan Ascent, Thermo Labsystems, Wyman St Waltham, MA, USA).

Data analysis

All data were stored in Excel files. Antigen-specific SFC values of IFN- γ -secreting T cells were determined by subtracting values from unstimulated wells. A positive IFN- γ response to CFP10-ESAT6 protein after background subtraction was defined as 13.²⁴ Since the SFC data were not normally distributed, non-parametric statistical data descriptions and analyses were used. The mean and standard deviation of IgG value differences among the different groups were calculated and the difference between each group was assessed by Student's *t* test. A *p* values <0.05 was considered significant. A receiver operating characteristic (ROC) curve was plotted by the SPSS18 (Chicago, IL, USA), and the sensitivity and specificity were calculated.

Ethical approval

Ethical approval for the study was granted by the 309th Hospital of the Chinese PLA Research Ethics Committees and informed consent was obtained from all participants.

Results

Gene sequencing, expression and purification of recombinant Rv2659c protein

DNA sequencing of recombinant plasmid Rv2659c/pET30a showed that its nucleotide sequence was completely

consistent with the design. A recombinant expression plasmid for Rv2659c protein had been successfully constructed.

When a transfected culture was induced with $0.1\ \text{mM}/\text{L}$ IPTG at 37°C for 3–4 hours, Rv2659c protein was expressed at a high level in *E. coli* BL21 cells, and had a yield of $10\ \text{mg}/\text{L}$ culture. The protein was found to be primarily located in inclusion bodies, and amounted to 54.5% of total bacterial protein. SDS-PAGE revealed that the molecular weight of the protein was around 40.76 kDa; the purified protein showed only one band with the expected molecular mass and 96.6% purity (Fig. 1). Residual endotoxin levels were found to be below $50\ \text{IU}/\text{mg}$ recombinant protein.

Comparison of cellular immune responses to recombinant Rv2659c protein in TB patients grouped by different age and sex

The healthy group consisted of young male soldiers, whereas the TB group age range was much wider and included both males and females. We separated the TB patients' results according to age and sex. From Table 2, we can conclude that there was no significant difference in ELISPOT results among three age groups or between males and females ($p > 0.05$).

Comparison of cellular immune response to recombinant Rv2659c protein between TB, LTBI, and uninfected healthy individuals

A positive IFN- γ SFC response to CFP10-ESAT6 protein had been defined as 13 SFC after background subtraction.²⁴ Therefore, the 112 healthy recruits were divided into

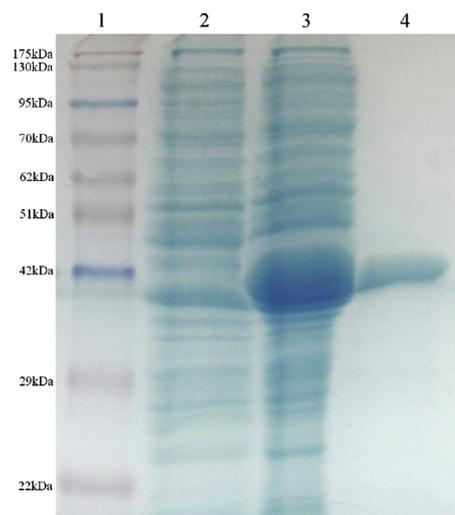


Figure 1. Expression and purification of recombinant Rv2659c protein as determined by SDS-PAGE electrophoresis. Lane 1, protein molecular weight marker. Lane 2, *Escherichia coli* (*E. coli*) extracts before Isopropyl β -D-1 thiogalactopyranoside induction. Lane 3, *E. coli* extracts 4 hours after induction with $0.1\ \text{mM}$ IPTG. Lane 4, the purified recombinant Rv2659c protein. The gel was subjected to electrophoresis followed by Coomassie blue staining.

Table 2 Comparison of enzyme-linked immunospot (ELISPOT) assays to rCFP10-ESAT6 and Rv2659c antigens among different individuals in the Chinese population

Groups	Cases	ELISPOT responses to rCFP10-ESAT6			ELISPOT responses to Rv2659c			
		SFC number			SFC number			
		Median	Max	IQR	Median	Max	IQR	<i>p</i>
TB patients 10–30 y	24	43.5	481	89.75	0	22	2	<i>p</i> 1 = 0.886
31–60 y	35	19	311	67.0	0	12	2	
61–90 y	21	15	338	26	0	20	1	
Male	50	25	395	55.25	0	20	2	<i>p</i> 2 = 0.945
Female	30	30.5	481	156	0	22	2	
All	80	25.5	481	73	0	22	2	
LTBI	37	64	460	88	5	297	36	<i>p</i> 3 = 0.003; <i>p</i> 4 = 0.000
Uninfected healthy individuals	75	0	12	2	0	76	1.5	
BCG vaccinated individuals	27	0	15	5.5	0	258	0.5	<i>p</i> 6 = 0.768
Non-TB, respiratory disease	30	1	159	8.5	0	96	1	<i>p</i> 7 = 0.003; <i>p</i> 8 = 0.655

BCG = Bacillus Calmette-Guérin; ELISPOT = Enzyme-linked immunospot assay; LTBI = latent tuberculosis infection; SFCs = spot-forming cells; TB = tuberculosis.

LTBI (rCFP10-ESAT6 SFCs ≥ 13); uninfected healthy individuals (rCFP10-ESAT6 SFCs < 13); BCG vaccination, positive conversion in individuals of purified protein derivative (PPD) after BCG vaccination.

*p*1, 10–30 (years) vs. 31–60 (years) vs. 61–90 (years) in TB patients stimulated by rRv2659c.

*p*2, male vs. female in TB patients stimulated by rRv2659c.

*p*3, 10–30 years TB patients vs. LTBI stimulated by rRv2659c.

*p*4, TB patients vs. LTBI stimulated by rRv2659c.

*p*5, LTBI vs. uninfected healthy individuals stimulated by rRv2659c.

*p*6, BCG vaccination group vs. uninfected healthy individuals stimulated by rRv2659c.

*p*7, non-TB respiratory system disease individuals vs. LTBI stimulated by rRv2659c.

*p*8, non-TB respiratory system disease individuals vs. uninfected healthy individuals stimulated by rRv2659c.

LTBI individuals and uninfected healthy individuals according to their SFC response (≥ 13 and < 13 , respectively), to CFP10-ESAT6 protein. We compared the SFC responses of LTBI individuals to rRv2659c with those of TB patients aged 10–30 years old, all TB patients, and uninfected healthy individuals. We found that the SFC values from LTBI were significantly higher than those from TB patients of either the 10–30 years old group or the whole TB group ($p < 0.05$) and the response from LTBI individuals was significantly higher than from uninfected healthy individuals ($p < 0.05$). These results show that rRv2659c induced T cell production to a higher level in LTBI individuals and are summarized in Table 2. Representative scanning results of the ELISPOT assay in TB patients and healthy recruits are shown in Fig. 2. The scatter diagram showing the distribution tendency of SFC values of different groups is seen in Fig. 3.

Comparison of cellular immune responses to recombinant Rv2659c protein between the PPD positive conversion group after BCG vaccination and in uninfected healthy individuals

In order to evaluate whether BCG vaccination influenced the response to Rv2659c protein, we compared the SFC values between the PPD positive conversion group after BCG vaccination and the uninfected healthy individuals. The difference was not significant ($p > 0.05$), as seen in Table 2, implying that cellular immune responses of antigen Rv2659c could be a promising biomarker for differentiating LTBI from TB and unaffected by BCG vaccination.

Comparison of cellular immune responses to recombinant Rv2659c protein between patients with non-TB respiratory disease and LTBI, and uninfected healthy individuals, respectively

To evaluate whether non-TB inflammation of the lungs would affect the PBMC responses to Rv2659c protein, we compared the SFC values of non-TB respiratory disease individuals with LTBI and uninfected healthy individuals. The response of the respiratory disease group was lower than in LTBI ($p < 0.05$), and was not significantly different from uninfected healthy individuals ($p > 0.05$) (Table 2), implying that nonspecific immune stimulation did not affect the cellular immune response of Rv2659c.

The performance of Rv2659c ELISPOT assay

To define an ROC curve, the SFC values of recombinant Rv2659c protein ELISPOT assay in LTBI individuals were taken as the positive group, and the active TB patients were used as a control group (Fig. 4). The area under the curve was 0.728 (95% confidence intervals: 0.619–0.836). According to the ROC curve, culture wells with more than three SFCs (specificity 91.2%) were scored as positive results and more than eight SFCs (specificity 95.0%) as strongly positive results. The positive and strongly positive rates by Rv2659c ELISPOT assay of the different groups of individuals are shown in Table 3. Compared to active TB patients, a greater proportion of healthy persons, and especially the LTBI individuals, had T cells that recognized Rv2659c antigen.

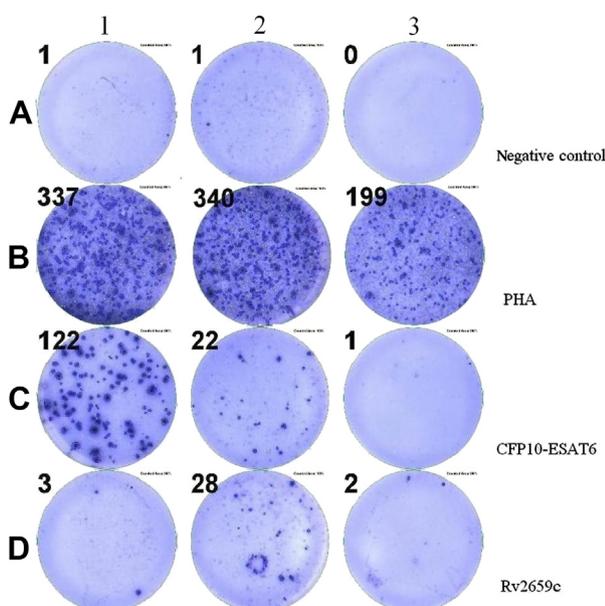


Figure 2. Representative results of enzyme-linked immunospot (ELISPOT) assay in tuberculosis (TB) patients and healthy recruits. The values of spot-forming cells (SFC) obtained from the negative control wells were subtracted from the SFC in the stimulated wells. Column 1 shows the results from a TB patient. Column 2 shows the results from a healthy recruit with latent TB infection (LTBI). Column 3 shows the results from a healthy recruit without LTBI. Row A: negative control; Row B: positive control; Row C: test well 1, in which peripheral blood mononuclear cells (PBMCs) were stimulated by rCFP10-ESAT6 antigen; Row D: test well 2, in which PBMCs were stimulated by Rv2659c antigen.

Humoral immune responses to recombinant Rv2659c protein in different population

Serum IgG antibody levels were measured by ELISA in 43 cases of TB patients and 102 army recruits and the means and standard deviations are shown in Table 4. The levels of IgG antibody against recombinant Rv2659c protein did not show significant differences ($p > 0.05$).

Discussion

TB remains an ongoing health crisis of global dimensions that may be ascribed to *M. tuberculosis* being able to adapt to a hypoxic and nutritionally compromised environment in LTBI lesions.²⁵ The differential diagnosis between TB and LTBI seems critical to control TB prevalence.

ELISPOT assays using CFP10 and ESAT6 antigens have previously been shown to distinguish between active TB infection and non-TB diseases with a high diagnostic sensitivity and specificity, but were found inadequate for discrimination between active TB and LTBI.^{26,27} In our previous studies, the ELISPOT assay using rCFP10-ESAT6 fusion protein was used to detect active TB with 60–70% sensitivity and >80% specificity when 13 SFCs was used as the optimal cut-off value for positivity.^{24,26} Therefore, in

this study we used this assay to screen persons with TB infection and to divide healthy persons into an rCFP10-ESAT6-positive group (LTBI, SFCs ≥ 13) and an rCFP10-ESAT6-negative group (uninfected healthy individuals, SFCs < 13). The positive rate of the rCFP10-ESAT6 ELISPOT assay was 75.0% in this study, which was in agreement with our previous results.²⁶ The T cells stimulated by Rv2659c protein in the LTBI group produced significantly fewer spots than those stimulated by CFP10-ESAT6 fusion protein (Table 2 and Fig. 3). Furthermore, there was no clear antibody response to this antigen seen in the sera from TB patients or LTBI persons, suggesting that the antigenicity of Rv2659c protein for both T cells and B cells may be weaker than CFP10 and ESAT6.

Our results with Chinese people confirmed and extended the previous reports that T lymphocyte recognition of Rv2659c antigen differs in different populations and may have diagnostic utility.^{28,29} Our observations that SFCs values induced by recombinant Rv2659c protein were significantly higher in LTBI individuals than in TB patients, and that a greater proportion of persons with LTBI recognized Rv2659c antigen compared with TB patients, are consistent with the findings of Govender et al.²⁸ and Gideon et al.²⁹ Govender et al used Rv2659c peptide pools to stimulate the PBMCs from 25 persons with LTBI and 25 TB patients in the Western Cape region of South Africa for 6 days, and showed that an IFN- γ response to Rv2659c was present in a greater proportion of persons with LTBI compared to TB patients.²⁸ Gideon et al found that a peptide pool from Rv2659c was preferentially recognized by 20 latently infected persons compared with 20 TB patients by ELISPOT assay of IFN- γ production at the Ubuntu Clinic at Khayelitsha site B of South Africa, but these responses were very modest.²⁹ In contrast, Riaño et al¹³ found that Rv2659c antigen in a whole-blood IFN- γ release assay did not stimulate T cell response in 30 LTBI persons and 20 recently diagnosed pulmonary TB patients in Colombia. The different T cell responses of Rv2659c in different populations may have various causes: one may be polymorphisms in the HLA type in the host populations; a second may be differences in *M. tuberculosis* strains present in different countries; the third may be experimental factors, since there is much heterogeneity in the IFN- γ release assays, for example format, type and source of antigen, cut-off value used for positivity, type of blood specimen, incubation time etc. Here, IFN- γ production was assayed by ELISPOT and, since this is a single cell level detection method, its result may be more accurate, informative, and intuitive compared with ELISA.

In this research, there were distinct differences in response between the groups. However, the healthy group (including the LTBI group) comprised only young males. The TB group had an age range that was much wider and it included both males and females. Therefore, we compared the cellular immune responses to recombinant Rv2659c protein in different age and sex groups of TB patients. The results showed that there was no difference between males and females or among the three different age groups ($p < 0.05$, Table 2). This suggests that sex and age had little impact on our research.

When we evaluated Rv2659c ELISPOT assay using confirmed cases of TB and healthy controls with LTBI, we

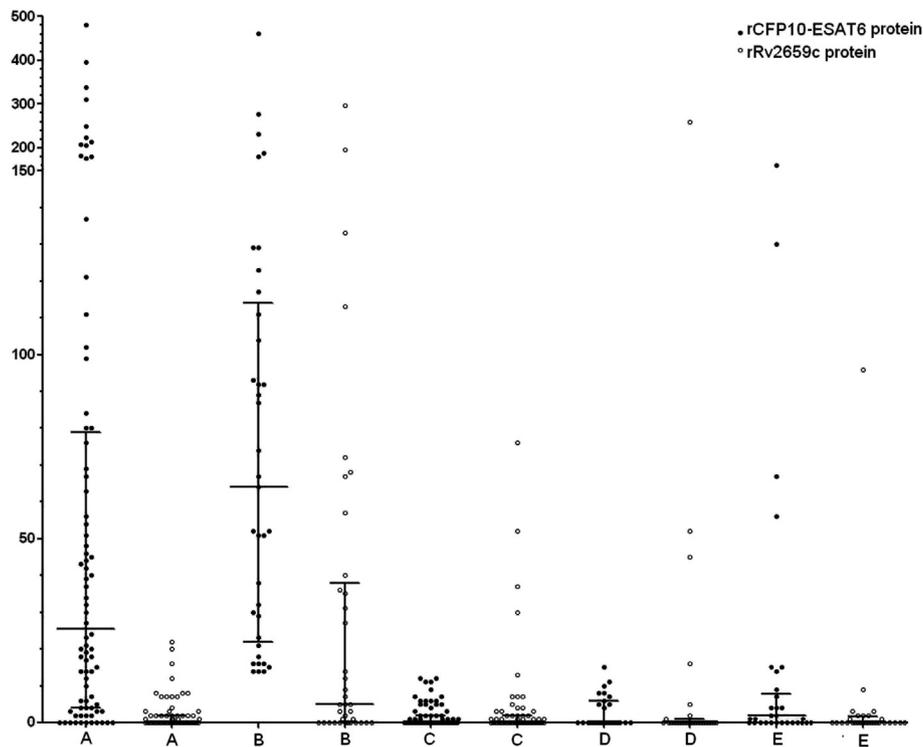


Figure 3. The scatter diagram of spot-forming cell (SFC) values stimulated by rCFP10-ESAT6 or rRv2659c. The median and interquartile ranges of each group are indicated by lines. (A) Tuberculosis (TB) patients; (B) latent TB infection (LTBI) individuals, (rCFP10-ESAT6 SFCs ≥ 13); (C) uninfected healthy individuals (rCFP10-ESAT6 SFCs < 13); (D) individuals converting to purified protein derivative (PPD) positivity after Bacillus Calmette-Guérin (BCG) vaccination; (E) non-TB respiratory system disease patients.

defined the cut-off points of specificity of diagnosing LTBI as 81.2% and 91.2% (sensitivity 59.5.2% and 43.2%), respectively. A total of 59.5% LTBI and 8.8% TB patients were positive Rv2659c ELISPOT. This greater discriminatory

power of the Rv2659c response might, therefore, be useful clinically to improve detection of the presence of LTBI and provide clues to exclude the diagnosis of active TB. As expected, our results showed that BCG vaccination and non-TB inflammation had little impact on the cellular immune response of antigen Rv2659c. Consequently, we speculate that a combination of rCFP10-ESAT6 ELISPOT and Rv2659c ELISPOT assays may have potential utility in the diagnosis of TB and LTBI. Thus, when rCFP10-ESAT6 ELISPOT and Rv2659c ELISPOT are both positive, the person tested may be indicated as having LTBI, when rCFP10-ESAT6 ELISPOT is positive and Rv2659c ELISPOT is negative, the person tested may be indicated as having active TB infection, and when rCFP10-ESAT6 ELISPOT and Rv2659c ELISPOT are both negative, the person tested may be without TB infection. However, 8.8% of TB patients had positive Rv2659c ELISPOT. This might be either a false positive or indicate that some latent *M. tuberculosis* is present. The latter seems more likely in view of the evolving concept that active TB in humans has diverse pathological presentations within the lungs of an individual patient. Lesions can range from small granulomas and caseous lesions that may contain substantial numbers of bacteria in a relatively hypoxic or starving environment, to liquefied cavities that may contain large numbers of replicating bacteria.^{11,25} Strikingly, 18.7% of uninfected healthy controls (rCFP10-ESAT6 negative) had a positive Rv2659c ELISPOT result, perhaps indicating that Rv2659c is expressed more than CFP10 and ESAT6 in the latent or resting phase and vice versa in the proliferating stage, as previously speculated.²⁶ However, we plan to further evaluate the diagnostic value of the combination of

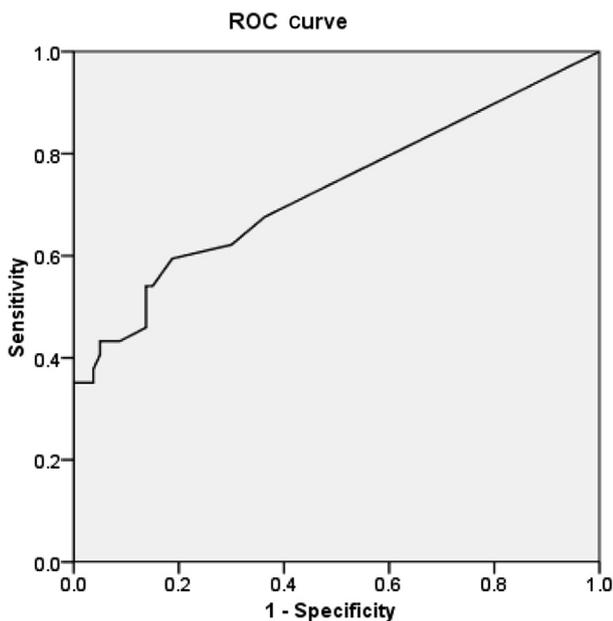


Figure 4. The receiver operating characteristic (ROC) curve of spot-forming cell (SFC) responses to Rv2659c from tuberculosis (TB) patients and latent TB infection (LTBI) plotted by the SPSS18. Area under the curve is 0.728.

Table 3 The performance of enzyme-linked immunospot (ELISPOT) assays to rCFP10-ESAT6 and Rv2659c antigens among 80 tuberculosis (TB) patients and 112 healthy recruits in the Chinese population

Groups	Cases	rCFP10-ESAT6 ELISPOT assay		Rv2659c ELISPOT assay	
		Positive cases (rate %) (SFCs \geq 13)	Strong positive cases (rate %) (SFCs \geq 30)	Positive cases (rate %) (SFCs \geq 3)	Strong positive cases (rate %) (SFCs \geq 8)
Tuberculosis patients	80	60 (75.0%)	41 (51.3%)	15 (18.8%)	7 (8.8%)
LTBI	37	37 (100%)	11 (29.7%)	22 (59.5%)	16 (43.2%)
Uninfected healthy individuals	75	0	0	14 (18.7%)	5 (6.7%)

ELISPOT = Enzyme-linked immunospot assay; LTBI = latent TB infection; SFCs = spot-forming cells.

Table 4 Comparison of immunoglobulin G (IgG) to recombinant Rv2659c protein among 43 tuberculosis (TB) patients and 102 healthy recruits in the Chinese population by enzyme-linked immunosorbent assay (ELISA)

Groups	Cases	Rv2659c IgG optical density at 450 nm (X \pm SD)	p
TB patients	43	0.247 \pm 0.14	p1 = 0.157; p2 = 0.466
Healthy recruits	102	0.250 \pm 0.22	
LTBI	36	0.213 \pm 0.16	p3 = 0.104
Uninfected healthy individuals	66	0.270 \pm 0.25	

LTBI, latent TB infection (rCFP10-ESAT6 SFCs \geq 13); uninfected healthy individuals (rCFP10-ESAT6 SFCs $<$ 13).

p1, TB patients vs. healthy recruit.

p2, TB patients vs. LTBI.

p3, LTBI vs. uninfected healthy individuals.

rCFP10-ESAT6 ELISPOT and Rv2659c ELISPOT in larger numbers of TB cases and non-TB cases.

The role of humoral immunity against TB is still controversial. High levels of antibodies to some *M. tuberculosis* specific proteins have been noted in patients with active TB.^{30–32} However, there has been little study of the humoral immune response in LTBI persons and antibody-based methods are still experimental and not used in clinical practice for the detection of LTBI.^{16,33,34} Although the low level of immune responses to Rv2659c might imply a limited role of this antigen in protective immunity, particularly against active disease, it might play some role in eliminating latent TB or restricting LTBI progression into active disease. Irrespective of any role in protection, it appears to have use for detecting latent infection.

In summary, purified recombinant Rv2659c protein was obtained and shown to have selective immunogenicity in eliciting T cell responses in persons with LTBI. Accordingly, it has potential diagnostic value and might even be a vaccine candidate for use against LTBI in Chinese and other populations.

Conflicts of interest

All authors declare no conflicts of interest.

Acknowledgments

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