Journal of Infection 78 (2019) 275–280

Contents lists available at ScienceDirect

Journal of Infection

journal homepage: www.elsevier.com/locate/jinf

Differential T cell responses against DosR-associated antigen Rv2028c in BCG-vaccinated populations with tuberculosis infection

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ARTICLE INFO

Article history:
Accepted 31 October 2018
Available online 5 December 2018

Keywords:
Mycobacterium tuberculosis
IFN-γ release assay
Rv2028c
Protective immunity
Latency antigen
DosR regulon

SUMMARY

The IFN-γ release assays (IGRAs) based on region of difference 1 (RD1) antigens have improved diagnosis of *Mycobacterium tuberculosis* (*Mtb*) infection. However, IGRAs with these antigens could not distinguish latent tuberculosis infection (LTBI) from active tuberculosis (ATB). DosR regulon genes are thought to be important for *Mtb* dormancy, and their products have higher immunogenicity in LTBI than ATB individuals, suggesting protective immunity mediated by DosR regulon-encoded antigens and potential utility of them for differential diagnostics of *Mtb*-infected populations or development of therapeutic vaccines against tuberculosis (TB). Among them, Rv2028c is a dormancy-related antigen that has demonstrated potential use in TB control, but its immunological characteristics in the BCG-vaccinated Chinese population are unknown. In this study, a total of 148 individuals, including 98 patients with ATB, 20 cases with LTBI and 30 healthy controls, were tested for Rv2028c-specific T cell responses by using an IFN-γ ELISA assay. The results showed that the T-cell responses in LTBI individuals were almost always higher than those in ATB patients, regardless of the site of infection or the results of bacteriological examination in the patients. This allowed for good differentiation between these two groups of *Mtb*-infected individuals even in the BCG-vaccinated high TB-incidence setting that pertains in China. In addition, the diagnostic efficacy for ATB was enhanced by combining the results from Rv2028c and RD1 antigen-based IFN-γ ELISA assays. In conclusion, Rv2028c-specific T-cell responses might contribute to natural protection against dormant *Mtb* infection, and the determination of these responses can aid discrimination between healthy LTBI individuals and ATB patients in the *Mtb*-infected populations.

Introduction

Tuberculosis (TB), an epidemic disease caused by *Mycobacterium tuberculosis* (*Mtb*), remains a persistent global health crisis, especially in developing countries. About 5–10% of people infected with *Mtb* develop active TB (ATB) at some point during their lifetime, while the infection remains latent and has no clinical symptoms in the majority of individuals.[1,2] Individuals with latent TB infection (LTBI) are the main source of active cases; therefore effective diagnosis distinguishing LTBI is critical for TB control.[3] In individuals with LTBI, *Mtb* can persist anywhere in the body, especially in lung granulomas, and can survive in a reversible, metabolically inactive state for a long time.[4] If the host cell-mediated immune response, which is crucial to protective immunity against TB, becomes impaired, some LTBI individuals will develop ATB as a result of *Mtb* reactivation. This can be caused by aging, human immunodeficiency virus (HIV) infection or immunosuppressive therapy. Reactivation of persisting *Mtb* is the major cause of adult pulmonary TB.[5]

However, the existing methods for differential diagnosis of LTBI and ATB are not effective, neither the specificity nor the sensitivity. For example, although the tuberculin skin test (TST) has been in use for about a century, the results might be influenced by many factors, such as the quality of the test antigen (purified protein derivative, PPD), previous vaccination with Bacillus Calmette-Guérin (BCG) or exposure to non-TB mycobacteria.[6–8]

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https://doi.org/10.1016/j.jinf.2018.10.016
0163-4453/

Table 1
Characteristics of the subject groups tested for cellular responses.

Characteristics	ATB	LTBI	HC
Participants(n)	98	20	30
Age, years (mean ± SD)	42.65 ± 20.62	33.45 ± 6.21	32.12 ± 6.95
Gender (male: female)	62:36	9:11	14:16

ATB: active TB; LTBI: latent TB infection; HC: uninfected healthy controls.

Interferon-γ (IFN-γ) release assays (IGRAs) are newer detection methods based on the release of IFN-γ from T lymphocytes stimulated by *Mtb*-specific antigens. The IGRAs can have significantly higher specificity than TST, and be not affected by BCG vaccination.[9,10] However, neither TST nor IGRAs can distinguish between ATB and LTBI. This drawback is particularly serious in countries with a high incidence, where 85.6% of TB patients are smear or culture-negative, and this together with subclinical symptoms, leads to misdiagnosis with other diseases.[11] Therefore, the study of *Mtb* antigens associated with LTBI may be a priority in the determination of immune diagnostic candidates for improving the specificity and sensitivity of the diagnosis of TB infection. Studies of healthy adults with LTBI have identified T cell responses to latency associated proteins produced by *Mtb*, indicating a role for these antigens in maintaining asymptomatic latent infection.[12–14]

In mycobacteria, stresses such as low pH, hypoxia, nitric oxide and carbon monoxide are *in vitro* stimuli that induce the expression of transcription regulator, resulting in an up-regulation of 48 gene-set called DosR-regulon.[15] The evidence suggests that the DosR regulator plays a vital role in adapting to these pressures; however, the detailed functions of most of the proteins encoded in this regulator are not fully understood yet. Recent studies have shown that, although both ATB and LTBI patients can specifically recognize some of the DosR proteins, latent infections induce higher responses both in the peripheral blood and bronchoalveolar lavage.[16–18] So, it is reasonable to use DosR antigens as LTBI biomarkers, which might also be suitable targets for vaccines to control the resurrection of dormant *Mtb*.

Rv2028c is one of the latency-associated proteins encoded in the DosR regulon[19] and mutations in it result in a hypoxic survival defect,[20] suggesting it is crucial in dormant *Mtb*. Since it was shown that active TB infection induced low Rv2028c-specific IFN-γ mediated responses,[21,22] a small cohort study showed that Rv2028c induced stronger T cell responses in LTBI compared with active pulmonary TB (PTB) in a low TB burden setting.[5] However, the diagnostic efficacy of Rv2028c in other forms of TB and in high burden countries is still to be defined. In order to expand our current understanding about the Rv2028c protein and to better understand its potential usage in TB control, we prospectively compared T-cell responses against Rv2028c antigen in different populations in China. In particular, we sought to identify effective biomarkers for diagnosis of TB infection.

Materials and methods

Study subjects

ATB patients ($n = 98$), LTBI subjects ($n = 20$) and healthy volunteers ($n = 30$) were enrolled from Shanghai Public Health Clinical Center (SPHCC, Shanghai, China) to investigate T-cell responses against Rv2028c antigen (Table 1). Study protocols were approved by the Institutional Review Board of SPHCC, and written informed consent was obtained from all participants. All participants were of Chinese Han ethnicity, so they had similar general genetic background. All the participants were BCG vaccinated. The diagnosis of active pulmonary TB was confirmed according to the following criteria.[23] 1) Typical 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) detected TB lesion on chest X-ray or computed tomography; 3) smear and/or culture positive; 4) TST positive; 5) evidence from bronchoscopy; 6) evidence from pathological examination and 7) effective anti-TB treatment. Exclusion criteria were cases without definitive diagnosis, or the presence of immunodeficiency diseases, including HIV infection, hematological or autoimmune diseases, and patients receiving immunosuppressive therapy. All LTBI cases were asymptomatic volunteers who gave a positive reaction in the T-SPOT.*TB* assay (Oxford Immunotec, Abingdon, UK), negative chest X-ray and had no evidence of active TB in microbiological, clinical and other examinations. The healthy volunteers were defined as T-SPOT.*TB* assay negative, with no disease symptoms and medical history of TB (including blood test, serum chemistry and chest X-ray). After this main investigation, to investigate the effect of anti-TB therapy on Rv2028c-specific IFN-γ responses, 28 cured patients who had successfully completed the 6-month standard regimen for drug-susceptible TB, which consisted of 2 months of HRZE, followed by 4 months of HR, were recruited. Their clinical symptoms disappeared and were negative for two consecutive bacteriological tests. Anti-TB treatment was provided according to Chinese national CDC guidelines.

Whole blood culture assay

Whole blood cultures were performed essentially as previously described.[22] Peripheral venous blood (2 mL) was collected in vacuum-trainers containing sodium heparin. Blood was diluted 1:2 in RPMI-1640 supplemented with 2 mM L-glutamine, 100 U/mL penicillin and 100 mg/mL streptomycin (complete medium) and 200 µL of this dilution was plated in 96-well plates (Corning Costar Inc, Corning, USA), and incubated with medium as negative control, or phytohemagglutinin (2.5 mg/mL; Sigma, Germany) as positive control, or with recombinant ESAT-6/CFP10 protein (5 µg/ml) or recombinant Rv2028c protein (5 µg/ml) at 37 °C, 5% CO_2, 95% relative humidity. After 48-h stimulation, culture supernatants were collected and stored at −80°C until further processing. The results used for analysis were subtracted from negative control.

Determination of IFN-γ by ELISA

Supernatants were analyzed by IFN-γ ELISA as previously described.[22] Briefly, 96-well plates were coated overnight with mouse anti-human IFN-γ monoclonal antibody (2 µg/mL; Becton-Dickinson, USA) at 4 °C. Following washing with PBS-Tween 20, wells were blocked using phosphate buffer saline (PBS) containing 1% albumin from bovine serum (BSA) for 2 h. Samples, controls and standards were then added and the plates were incubated overnight at 37°C. After washing, biotinylated anti-rabbit detection antibody (1 µg/mL; BD Biosciences, USA) was added, plates were left for 45 min at room temperature and a final colorogenic step performed by addition of avidin-peroxidase. The reaction was stopped with 2M H_2SO_4 and the plates were read at OD_{490}. The same batches of antibodies and standards were used in all assays with a standardized protocol.

Statistical methods

Data are represented as mean ± SEM. GraphPad Prism 5 (GraphPad Software Inc., CA, USA) was used to generate plots and perform statistical analyses on the data. The statistical analysis was completed using SPSS. First, the data were analyzed by a one-way analysis of variance (ANOVA). When the variance between samples was equal, a Tukey's HSD or LSD post-hoc test was performed.

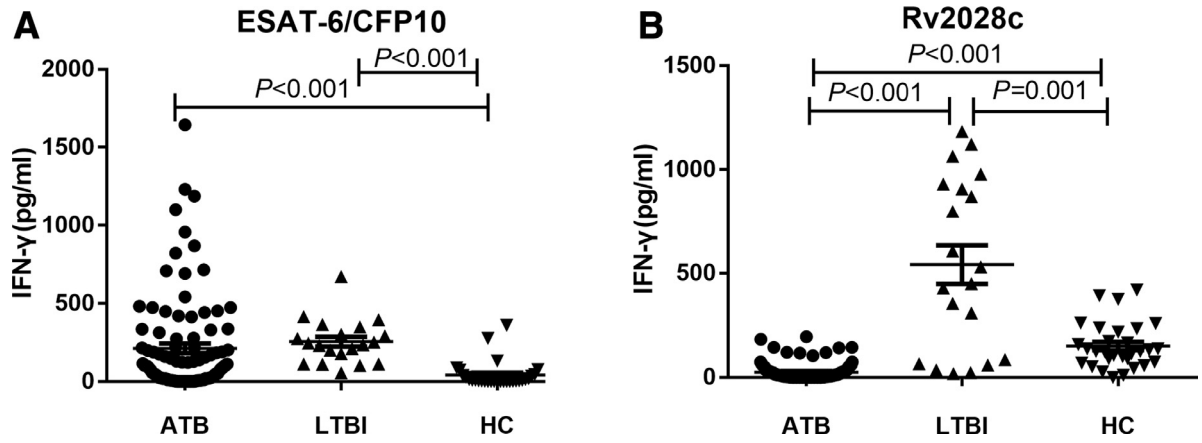

Fig. 1. Antigen-specific release of IFN-γ from T cells responding to ESAT-6/CFP10 and Rv2028c. Samples of whole blood from ATB patients ($n = 98$), LTBI individuals ($n = 20$) and healthy controls ($n = 30$) were stimulated with ESAT-6/CFP10 (A) or Rv2028c (B) in parallel, and IFN-γ was quantified by ELISA assay. The results are expressed in pg/mL and were shown the mean value ± SEM. Each symbol represents an individual subject, and the background values of negative controls were subtracted from each individual donor. *P* value was shown for comparisons between 2 groups, by one-way analysis of variance (ANOVA).

When the variance was not equal, the Tamhane's T2 or Dunnett'S T3 method was used to determine whether there were significant differences between two groups. Receiver operating characteristic (ROC) curve analysis was conducted to analyze the predictive value of IFN-γ response to Rv2028c or ESAT-6/CFP10, calculating the area under the curve (AUC) and the 95% confidence interval (CI). $P < 0.05$ was considered statistically significant.

Results

Comparison of cellular responses against Rv2028c and ESAT-6/CFP10 in active and latent TB infection individuals

Ex vivo IFN-γ ELISA assay showed that antigen-specific responses against ESAT-6/CFP10 were significantly stronger in blood samples from both active and latently infected individuals (ATB patients and LTBI individuals) than in those from healthy control (HC) subjects, and there was no statistical difference between these two *Mtb*-infected groups (Fig. 1A). This was consistent with the IGRAs detection results widely found in clinical practice. However, when stimulated with Rv2028c, the responses in LTBI cases were significantly stronger than those in ATB and HC subjects. This allowed discrimination between ATB patients and LTBI cases (Fig. 1B).

Rv2028c-specific responses from active TB patients were low regardless of infection sites or bacteriological status

When ATB patients were subdivided into PTB and extrapulmonary TB (EPTB) according to the infection sites, no differences were found between the three groups in ESAT-6/CFP10-specific responses (Fig. 2A). However, Rv2028c-specific responses were always lower in ATB patients compared to the LTBI cases regardless of infection site (Fig. 2B). When the ATB group was subdivided according to acid-fast bacilli (AFB) results, the response of the bacteria-positive cases to ESAT-6/CFP 10 was significantly higher than that of the bacteria-negative as expected (Fig. 2C). The responses to Rv2028c stimulation were very low and were not statistically different between the two groups (Fig. 2D).

The differential diagnostic performance of Rv2028c antigen

Since Rv2028c induced relatively high IFN-γ responses in LTBI cases and low responses in ATB patients, ROC curves were constructed to discriminate between LTBI and ATB *Mtb*-infected individuals. The IFN-γ values of LTBI individuals were taken as the positive group, and the ATB patients were taken as the control group. The AUC was up to 0.9388 (95% CI: 0.8865-0.9910) upon Rv2028c stimulation (Fig. 3B), and the AUC was only 0.6735 (95% CI: 0.5615-0.7855) upon ESAT-6/CFP10 stimulation (Fig. 3A); this indicated that the IFN-γ response to Rv2028c might be the more effective to differentiate between LTBI and ATB individuals. By using the Youden index,[24] an optimal cut-off point to Rv2028c of 58.72 pg/mL gave 85% sensitivity and 86.7% specificity to distinguish between the *Mtb*-infected populations. But the optimal cut-off point to ESAT-6/CFP10 was 177.89 pg/mL, which gave 73.7% sensitivity and 66.3% specificity.

IFN-γ responses to Rv208c were selectively elevated after successful treatment of ATB

Antigen-specific IFN-γ responses in blood from individuals who had successfully completed treatment were compared with those in blood from ATB and LTBI individuals (Fig. 4). The levels of Rv2028c-specific IFN-γ were significantly higher in the treated ATB group compared with individuals with untreated ATB and reached or approached the levels seen in the LTBI group (Fig. 4B). However, the responses to ESAT-6/CFP10 were indistinguishable between treated ATB, LTBI and HC groups (Fig. 4A).

Combination of Rv2028c and ESAT-6/CFP10 IFN-γ ELISA assays enhanced the diagnostic efficacy for active TB cases

When active pulmonary TB patients were to be detected by ESAT-6/CFP10 ELISA, 54 of 62 PTB cases and 23 of 36 EPTB cases were diagnosed to be positive, which resulted in 78.57% (77/98) sensitivity. When we used Rv2028c as stimulus in the parallel assay, and used the optimal Rv2028c-specific IFN-γ levels < 58.72 pg/ml as diagnostic of ATB cases, 83 of 98 were diagnosed (84.7%) and 53 of 62 PTB cases and 30 of 36 EPTB cases were diagnosed to be negative. By combining the results using these two antigens in the separate IFN-γ ELISA assays, a total of 95 cases were ESAT-6/CFP10 positive or Rv2028c negative, which gave 96.94% diagnostic efficacy for active TB cases (Table 2).

Discussion

TB remains a continuing global health crisis. The ability of *Mtb* to adapt to become dormant in hypoxic and nutritionally impaired conditions is one of the major contributory factors.[25] The effective diagnosis distinguishing latent from active TB infection is thought

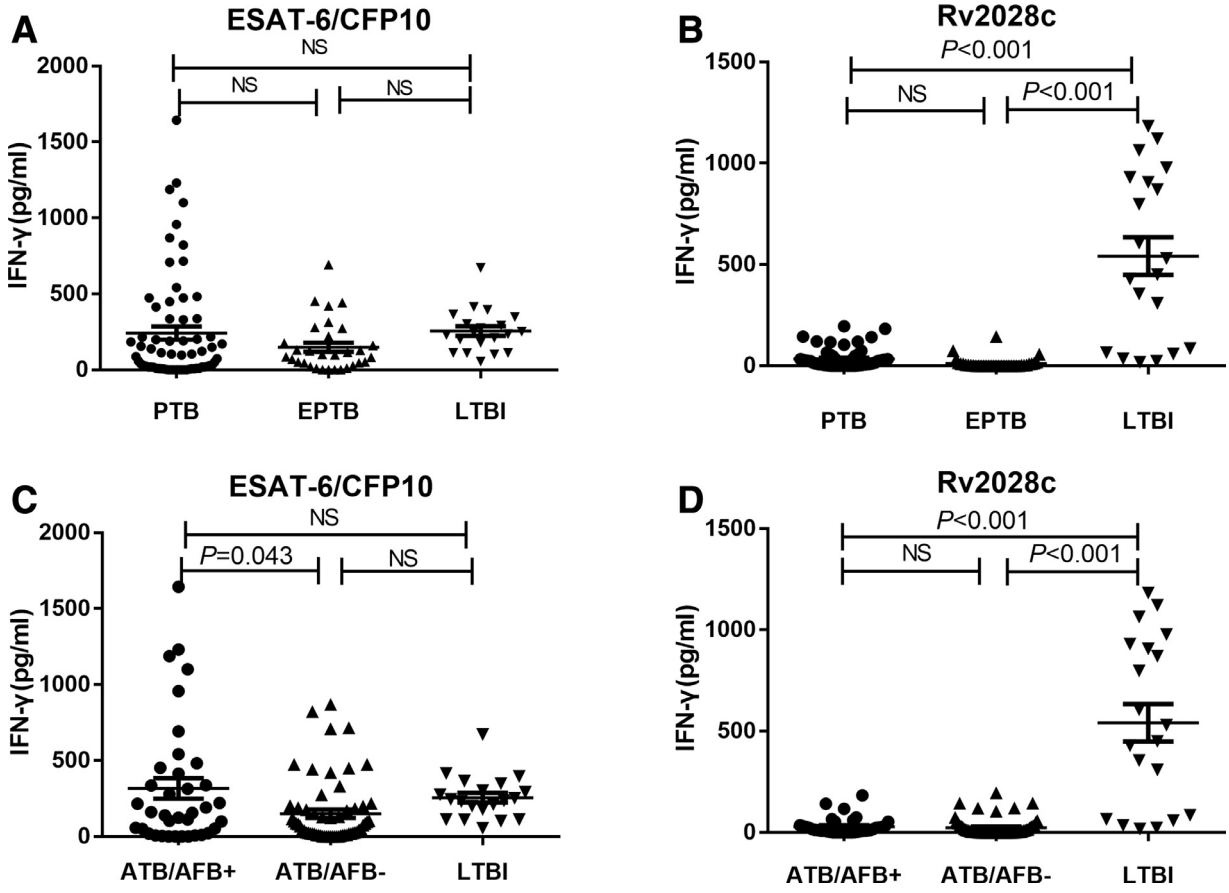

Fig. 2. Antigen-specific IFN-γ responses against ESAT-6/CFP10 and Rv2028c in different subgroups. ATB patients were divided into pulmonary TB (PTB, $n=62$) and extra-pulmonary TB (EPTB, $n=36$) (A, B) or into ATB subgroups with positive ($n=37$) and negative AFB results ($n=61$) (C, D). The results are expressed in pg/mL and were shown the mean value ± SEM. Each symbol represents an individual subject, and the background values of negative controls were subtracted from each individual donor. *P* value was shown for comparisons between 2 groups, by one-way analysis of variance (ANOVA). *NS*: not significant.

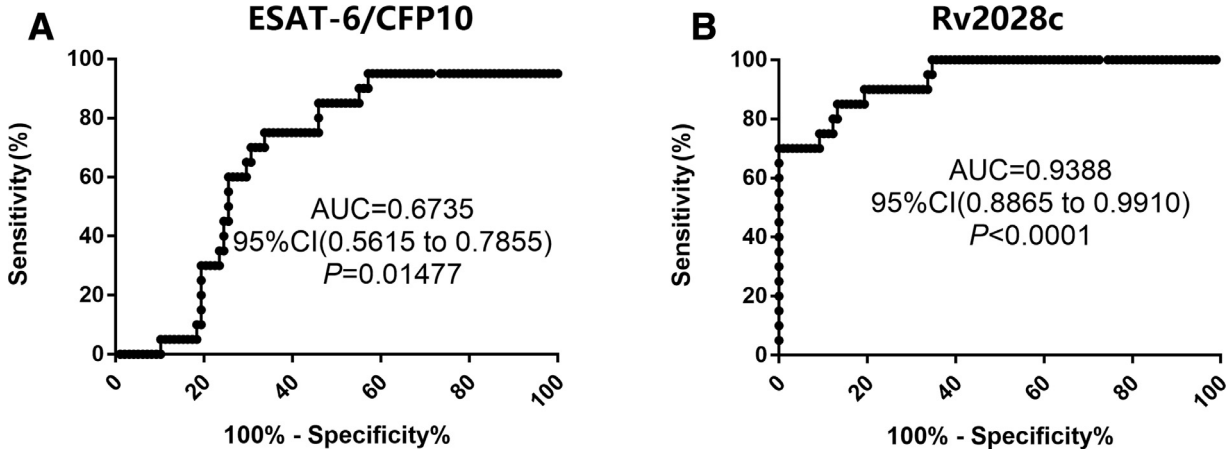

Fig. 3. ROC curves evaluating IFN-γ responses to ESAT-6/CFP10 and Rv2028c to discriminate between LTBI cases and ATB patients. AUC represents area under the ROC curve.

Table 2
Detection of ATB cases by Rv2028c-specific response in the ELISA assay.

Rv2028c *n* (%)	ESAT-6/CFP10					
	ATB ($n=98$)		PTB ($n=62$)		EPTB ($n=36$)	
	Positive ($n=77$)	Negative ($n=21$)	Positive ($n=54$)	Negative ($n=8$)	Positive ($n=23$)	Negative ($n=13$)
Positive	12 (15.58)	3 (14.29)	7 (12.96)	2 (25)	5 (21.74)	1 (7.69)
Negative	65 (84.42)	18 (85.71)	47 (87.04)	6 (75)	18 (78.26)	12 (92.31)

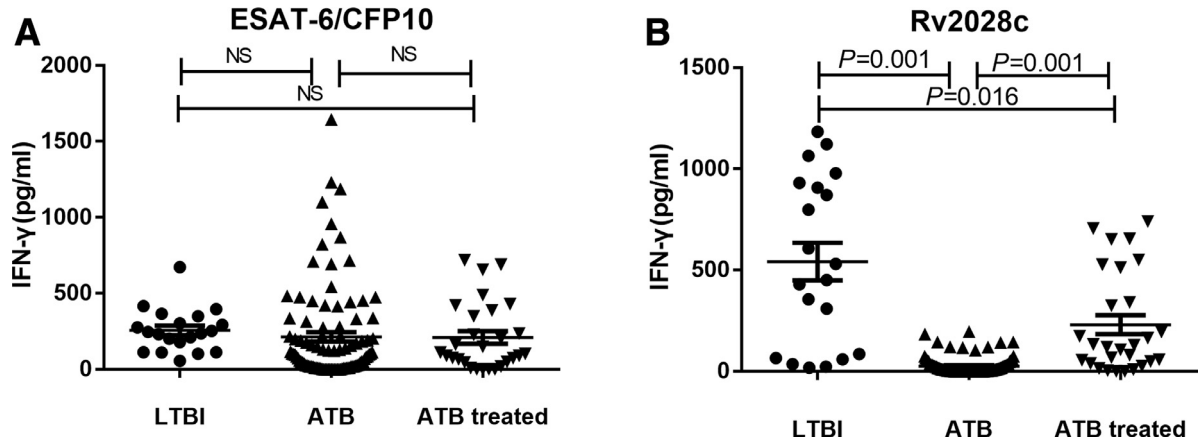

Fig. 4. Elevated responses to Rv2028c but not ESAT-6/CFP10 after successful treatment of ATB. The ATB-treated group contained 5 individuals from the ATB group and 23 additional cases (ATB-treated, $n = 28$). The results are expressed in pg/mL and were shown the mean value ± SEM. Each symbol represents an individual subject, and the background values of negative controls were subtracted from each individual donor. *P* value was shown for comparisons between 2 groups, by one-way analysis of variance (ANOVA). *NS*: not significant.

to be critical for execution of efficient measures to control TB. However, there is no gold standard for the diagnosis of LTBI yet. IGRAs using ESAT-6 and CFP10 antigens have been shown to distinguish between active TB infection and non-TB disease with high diagnostic sensitivity and specificity, but were found ineffective for discrimination between ATB and LTBI.[26]

Due to unique expression profiles and function in dormant *Mtb*, proteins encoded by DosR regulators are considered to be potential targets for novel vaccines to prevent the reactivation of *Mtb*.[15] Mushtaq et al. found that Rv2028–Rv2031 is important in response to stress and dormant state of *Mtb*, and proteins encoded by this operon may lead to expression of latency associated genes, nucleoside metabolism, and protein folding.[27] Leyten et al. found that healthy TST positive individuals had stronger T-cell responses to DosR regulon-encoded antigens than patients with ATB.[14] In this study, we found that human T-cell responses to Rv2028c proteins were stronger in LTBI than ATB patients in a Chinese population. These observations suggest that the immune responses to the antigens encoded by the DosR regulatory gene may contribute to the natural protection against dormant *Mtb*, and the lack or reduction of this response may be related to the development of ATB. Therefore, DosR regulon-encoded proteins which can effectively induce T cell response in LTBI individuals, may be potential candidate antigens to prevent the reactivation of *Mtb*.

This study used IFN-γ ELISA to screen individuals with *Mtb* infection and healthy individuals and found that the response of T cells to Rv2028c protein was stronger in LTBI than ATB patients. The level of IFN-γ stimulated in whole blood by Rv2028c protein in the LTBI group was significantly higher than that stimulated by ESAT-6/CFP10 fusion protein used as control antigen (Figs. 1 and S1). In patients with ATB, Rv2028c-specific IFN-γ responses were seen to be consistently low regardless of the location of infection (Fig. 2C) or the patient's bacteriological status (Fig. 2D). In addition, the level of Rv2028c-specific IFN-γ in the TB patients receiving effective anti-TB drug treatment was significantly higher (Fig. 3B), indicating that a low response may result from active disease. These observations may be of etiological significance and relevance for vaccine development: most latently infected people do not develop disease throughout their life period, so the immune response to natural infection in these people can be regarded as conferring protection against the disease. In contrast, the immune response of active TB patients to infection is not protective by definition. However, we must keep in mind that although antigen Rv2028c has clear potential for use in vaccines that aim to eliminate latent infection, deployment in a vaccine would be likely to preclude use in diagnostic applications.

Discrimination between confirmed cases of ATB and LTBI was superior with Rv2028c compared to ESAT-6/CFP10 antigens. The ROC curve analysis showed an AUC that was up to 0.9388 upon Rv2028c stimulation while upon ESAT-6/CFP10 stimulation it was 0.6735 (Fig. 4). The greater differentiation ability of the Rv2028c response might be exploited in diagnosis by using assays that detect this antigen in addition to others. Consistent with this, when the results of two ELISA assays against different antigens were combined, a total of 95 from 98 confirmed ATB cases were ESAT-6/CFP10 positive or Rv2028c negative, indicating that they were really in a state of active TB disease and the efficacy for diagnosis of active TB was up to 96.94% (Table 2). Therefore, the combination of ELISAs for these antigens might be clinically helpful to improve the detection of active TB cases and to provide clues for the diagnosis of LTBI.

In conclusion, the recombinant Rv2028c protein was obtained and used to demonstrate high T cell responses in individuals with LTBI in a selective immunogenicity. Measuring these responses can help distinguish between LTBI individuals and ATB patients with *Mtb*-infection even in the context of the BCG vaccination and high TB incidence setting that is China.

Conflict of interest

No potential conflicts of interest declared.

Acknowledgments

This work was supported by the grants from Chinese National Mega Science and Technology Program on Infectious Diseases (2018ZX10302301, 2018ZX10731301), National Key R&D Program of China (2018YFD0500900), National Science Foundation of China (31771004), and Shanghai Science and Technology Commission (18411970700, 17ZR1423900).

Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jinf.2018.10.016.

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