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Potential novel markers to discriminate between active and latent tuberculosis infection in Chinese individuals

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ABSTRACT

Latent tuberculosis infection (LTBI) constitutes the main reservoir for reactivation tuberculosis. The finding of potential biomarkers for differentiating between TB and LTBI is very necessary. In this study, the immunological characteristics and potential diagnostic utility of Rv2029c, Rv2628 and Rv1813c proteins were assessed. These three proteins stimulated PBMCs from ELISPOT-positive LTBI subjects produced higher levels of IFN-γ in comparison with TB patients and ELISPOT-negative healthy subjects ($p < 0.05$). BCG vaccination and non-TB respiratory disease had little influence on the immunological responses of Rv2029c and Rv2628 proteins ($p > 0.05$). The LTBI diagnostic performance of Rv2029c was higher than Rv2628 and Rv1813c by ROC evaluation. But Rv2628 had much higher specificity than Rv2029c in active TB patients and uninfected healthy subjects. The IgG level against Rv1813c was higher in the TB group than in LTBI and uninfected healthy subjects ($p < 0.05$). These results suggest that T cell response to Rv2628 and antibody against Rv1813c might be applicable as biomarkers to distinguish TB from LTBI and uninfected individuals.

1. Introduction

Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis* (*M.tb*). TB has been a serious public health problem worldwide for a long time. However, it is estimated that at least 2 billion healthy people have been latently infected with *M.tb.* which are classified as latent TB infection (LTBI). 90–95% of these *M.tb*-infected individuals are in a latent asymptomatic infection state for a long time, but 5–10% of them will progress to active TB during their life time. LTBI constitutes the main reservoir for reactivation TB [1] and presents a major challenge in a high burden country such as China. The diagnosis of LTBI is a critical step to control the disease. Currently there is no accepted standard for the diagnosis of LTBI. The tuberculin skin test (TST) that depends on purified protein derivative (PPD) and Interferon-γ release assays (IGRA) that depend on ESAT6 and CFP10 antigens cannot differentiate between LTBI and active TB [2,3]. New biomarkers are urgently needed to facilitate the diagnosis of LTBI.

Some antigens are expressed more strongly under the stresses imposed by latency. DosR regulon-encoded genes have been found to be up-regulated by stresses such as hypoxia, low pH, nitric oxide and carbon monoxide [4], and some of the DosR antigens can elicit stronger immune T-cell responses in LTBI than in patients with active TB in European (low-burden), African (high-burden) and Japan (intermediate) settings [5–8]. Rv2029c, Rv2628 and Rv1813c were among them, but the immune response profiles were somewhat different in different areas [5–9]. In order to gain better insight into protection against TB and to expand our current understanding about DosR protein variations, we prospectively compared T-cell and antibody responses against these three antigens in different populations in China to identify effective diagnosis biomarkers. This is the first research to evaluate humoral immune response to Rv1813c and to study the effect of BCG vaccination on immune responses of Rv2029c and Rv2628.

2. Patients and methods

2.1. Patient population

Our primary goal was to assess the differential diagnostic performance of latency antigens Rv2029c, Rv2628 and Rv1813c in

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Table 1
Characteristics of the subject groups tested for T cell or humoral responses.

Groups	ELISPOT assay			ELISA assay		
	Subject numbers	Age Mean ± SD	Gender Male:female	Subject numbers	Age Mean ± SD	Gender male:female
Uninfected healthy	103	21.0 ± 4.9	79:24	72	18.4 ± 1.5	72:0
LTBI	51	22.1 ± 6.2	39:12	36	19.2 ± 1.3	36:0
Active TB	116	43.9 ± 20.4	66:50	62	42.6 ± 19.3	32:30
Non-tuberculosis respiratory disease	57	62.2 ± 16.5	30:27			
BCG vaccination	49	19.5 ± 1.5	49:0			

Chinese populations. Therefore, we selected a wide spectrum of individuals mentioned below. In a T-cell immune response study, the peripheral blood mononuclear cells (PBMCs) derived from 5 groups as follow: (1) uninfected healthy control group: 103 healthy subjects detected were negative by enzyme-linked immunospot (ELISPOT) assay with recombinant CFP10-ESAT6 fusion protein (rCFP10-ESAT6) as a stimulus [10,11]; (2) LTBI group: 51 healthy subjects detected were positive by rCFP10-ESAT6 ELISPOT test [10,11]; (3) TB group: 116 patients were diagnosed definitely as active TB; (4) BCG group: 49 subjects with negative PPD tests were performed BCG vaccination, and then repeated PPD tests at 3 months after BCG vaccination; (5) non-TB respiratory disease group: 57 patients were diagnosed definitely as non-TB respiratory disease [12,13]. In a humoral immune response study, the sera derived from 3 groups as follow: (1) 72 uninfected healthy subjects with negative rCFP10-ESAT6 ELISPOT test [10,11]; (2) 36 LTBI subjects with positive rCFP10-ESAT6 ELISPOT test [10,11]; (3) 62 active TB patients. The TB patients were composed of chest TB, bone TB and a few with TB in other organs. The TB was diagnosed according to the following standards: (a) TB symptoms and signs, including cough, cough producing phlegm, coughing up blood, fever, night sweats, fatigue, loss of appetite and weight, thoracalgia, dyspnea, etc.; (b) TB lesions by imaging examination; (c) there may be positive-smear or culture; (d) there may be strongly positive PPD skin test; (e) maybe find TB lesions by bronchoscopy examination and biopsy or histopathologic examination of the affected tissue; and (f) anti-TB treatment must be effective, which were proposed by the Respiratory Disease Branch of the Chinese Medical Association in 2001 [14]. Cases without definitive diagnosis were excluded. All uninfected healthy subjects and LTBI subjects were asymptomatic volunteers. All subjects in this study were negative for HIV antibodies. Details of all groups are given in Table 1. Study protocols were approved by the PLA 309th Hospital Ethics Committee. All samples were collected after written informed consent was given.

2.2. Recombinant antigen preparation

Recombinant proteins were produced as described previously [15]. In short, nucleotide sequences of the Rv2029c, Rv2628 and Rv1813c genes were obtained from *M.tb* H37Rv genome database (http://www.tbdb.org). PCR was used to amplify the selected genes from genomic H37Rv DNA. The PCR products were finally cloned into pET30a plasmid (Invitrogen, San Diego, CA, USA), a bacterial expression vector containing a N-terminal 6 × histidine-tag for rapid protein purification with nickel-chelating resin. PCR of bacterial clony, enzyme digestion and sequencing were performed to confirm the identity of the cloned DNA fragments. *Escherichia coli* BL21 (DE3) was used to over-express the *M.tb* proteins. Recombinant proteins were further purified as described previously [15] and tested in quality control assays including size and purity check with Bandscan5.0, determination of residual endotoxin levels by the Limulus Amebocyte Lysate (LAL) Assay (Associates of Cape Cod Inc., East Falmouth, MA, USA). Fusion protein CFP10-ESAT6 was used as control. Recombinant antigens were freeze-dried and stored at −80 °C.

2.3. PPD tuberculin skin tests

PPD (batch S10960016) was purchased from Beijing Sanroad Biological Products Co., Ltd (Beijing, China). TST was performed by the Mantoux method, with the size of induration read at 72 h. Intradermal administration of PPD and reaction reading were always performed by the same trained professionals.

2.4. ELISPOT assay

Blood samples were obtained from the patients with active TB within 1 week of the initiation of treatment. MultiScreen 96-well filtrater plates (Millipore, Bedford, MA, USA) were coated with 15 μg/mL anti-human IFN-γmAb 1-D1K (MABTECH AB, Sweden) and incubated overnight at 4 °C and then blocked with 2% bovine serum albumin (BSA) at room temperature for 2 h. 4–5 mL venous blood sample of each subject was collected in a heparinized glass tube, and then PBMCs were isolated and quantified in serum-free medium. PBMCs were plated at 2.5×10^5 cells/well, and stimulated with medium as the negative control, PHA (20 μg/mL; Sigma, St. Louis, MO, USA) as positive control, or recombinant antigens Rv2029c, Rv2628 and Rv1813c (20 μg/mL) with 5% CO_2 for 18–20 h at 37 °C, respectively. Then the plates were incubated with a biotin-conjugated anti-human IFN-γ and developed using streptavidin-ALP conjugate and BCIP/NBT plus (MABTECH AB, Sweden) according to the manufacturer's protocol. Spot-forming cells (SFC) were counted on an automated ELISPOT reader (CTL-Immuno-Spot S5 Versa Analyzer; Cellular Technology Ltd., Ohio, USA) and analyzed with Immunospot® (CTL Analyzer LLC).

2.5. Antibody ELISA assay

Levels of IgG antibodies against Rv2029c, Rv2628 and Rv1813c were determined by an enzyme-linked immunosorbent assay (ELISA). Serum was collected from venous blood. 96-well plates (Costar, Washington, DC, USA) were pre-coated with recombinant protein Rv2029c, Rv2628 or Rv1813c (5 μg/well, 10 μg/well, 10 μg/well, respectively), blocked with 10% fetal calf serum (FBS), then 100 μL of 1:10 dilution of test serum was added, followed by a secondary sheep anti-human light and heavy chain antibody (1:10,000; CWBIO, Biotech Co., Ltd., Beijing, China), and TMB Substrate Reagent Set (BD Bioscience, Becton Dr Franklin Lakes, NJ, USA) for revelation. Data are presented as the optical density (OD) at 450 nm.

2.6. Statistical methods

Antigen-specific SFC values of stimulated wells were determined by subtracting the un-stimulated well values. The SFC values of unstimulated wells should be less than 20. Antigen-specific IgG levels were determined by subtracting values from blank wells.