Diagnosis of Active Tuberculosis in China Using an In-House Gamma Interferon Enzyme-Linked Immunospot Assay

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Tuberculosis (TB) is a leading cause of morbidity and mortality throughout the world, with 95% of cases and 97% of all deaths occurring in high-prevalence countries, such as China, where the prevalence of active TB is as high as 367/100,000 population (10). For the effective and efficient control of TB in these countries, rapid diagnosis and treatment for active-TB patients are the mainstays of the TB control program. However, the current widely used tests, including acid-fast staining and culture methods, have not been proven to be useful in the diagnosis of active tuberculosis (TB) in Shenzhen, China, where the prevalence of tuberculosis is severe and mortality burden in the world (11), the aim of this study was to evaluate the usefulness of IFN-γ assays in China is scarce, and no data are available to evaluate the diagnostic value of the QFT-IT assay mainly because of the high cost of these kits. Since China ranks second on the list of 22 countries with the highest tuberculosis burden in the world (11), the aim of this study was to evaluate the usefulness of IFN-γ assays in the diagnosis of active tuberculosis in the Chinese population in mainland China. Thus, we developed and evaluated an in-house IFN-γ ELISPOT assay by using recombinant ESAT-6 protein and peptide pools derived from ESAT-6 and CFP-10 for diagnosis of active TB. We also compared the performance of our ELISPOT assay with that of the commercial QFT-IT assay and analyzed the influence of clinical manifestation on the accuracy of the ELISPOT assay.

MATERIALS AND METHODS

Participants. A total of 323 patients with suspected TB were prospectively enrolled at the Department of Tuberculosis of Shenzhen Donghu Hospital, from April 2007 to October 2008. Diagnosis of active TB was made on the basis of clinical, radiological, microbiological, and pathological information collected after recruitment and during follow-up/response to antituberculosis therapy for at least 3 months. Among the suspected TB cases, diagnoses of active TB have eventually been confirmed for 305 patients, while 18 were diagnosed with non-tuberculosis lung diseases (Table 1). For comparison, two hundred and two healthy individuals were recruited in...
using PET-11d expression vector and purified with Ni
peptide by 10 residues. Recombinant ESAT-6 was expressed in
spanned the length of ESAT-6 and CFP-10 proteins, and overlapped its adjacent
strate was added. The individual spots were counted by use of an automated
zolium-BCIP (5-bromo-4-chloro-3-indolylphosphate; Sigma) chromogenic sub-
Biotinylated anti-IFN-	ibody (eBioscience). Cells were stimulated with the different antigens for 24 h at
zhen, China) was 20 amino acids (aa) in length, had
tuberculosis (SPTB) patients and healthy controls. Each peptide (HyBio, Shen-
collected within 2 weeks of the initiation of antituberculosis treatment.
excluded from our study. Blood samples from patients with confirmed TB were
proved by the Institutional Review Board of Shenzhen Donghu Hospital. Writ-
Health workers occasionally exposed to sputum-positive TB patients were
years and for less than 2 years were categorized as class 1 and class 2, respec-
tochondrion antigens (protein, peptide, or combined peptide pools) of sputum-positive pulmonary
characteristics of patients plus healthy controls. The agreement between different tests was as-
characterized by lobe infiltrate, pleural
injuries, and cavities involved two or more lobes in one or both lungs (5).
Radiographic image examination and analysis. Chest radiographs of 89 pa-
tin patients with SPTB were reviewed by two experienced, board-certified radiologists
on the volar surface of the forearm, and the size of the induration was evaluated
after 72 h. The TST responses were categorized as follows based on the trans-
verse diameter of skin induration in millimeters: TST induration of ≤5 mm (−),
from participants were screened for human immunodeficiency virus, hepatitis B virus, and hepatitis C
infection using an enzyme-linked immunosorbent assay diagnostic kit pro-
vided by Wantai Company (Beijing, China), and patients found positive were
excluded from our study. Blood samples from patients with confirmed TB were
collected within 2 weeks of the initiation of antituberculosis treatment.
ELISPOT assay. For development of an ELISPOT assay to detect specific
IFN-γ responses against M. tuberculosis, recombinant M. tuberculosis antigens and peptides were screened based on the differentiated responses to each antigen
(protein, peptide, or combined peptide pools) of sputum-positive pulmonary tuberculosis (SPTB) patients and healthy controls. Each peptide (HyBio, Shenz-
zen, China) was 20 amino acids (aa) in length, had >95% purity, sequentially
spanned the length of ESAT-6 and CFP-10 proteins, and overlapped its adjacent peptide by 10 residues. Recombinant ESAT-6 was expressed in Escherichia coli
using PET-11d expression vector and purified with Ni²⁺ affinity chromatography. Recombinant M. tuberculosis Ag85A and Eis were provided by Haiying Liu
(Chinese Academy of Medical Sciences) and Richard Friedman (University of
Arizona), respectively. Recombinant ESAT-6 protein, peptide pool A (ESAT-6 aa 21 to 40, aa 51 to 70, and aa 71 to 90), and peptide pool B (CFP-10 aa 21 to 40,
aa 51 to 70, and aa 66 to 85) were eventually selected for the ELISPOT assay. The final concentration of each antigen (protein or peptide) used in the
ELISPOT assay was 10 μg/ml, as optimized by titration.
For IFN-γ ELISPOT assays, peripheral blood mononuclear cells (PBMC) from participants were obtained from whole blood by centrifugation over Ficoll-
Hypaque density gradient (Ficoll-Paque Plus; Amersham Biosciences). Cells were
resuspended in Lympho-Spot medium (U-CyTech Bioscience, The Neth-
erlands). A total of 2 × 10^6 cells/well were seeded in duplicate in 96-well plates
(MultiScreen-IP; Millipore) precoated with anti-IFN-γ capture monoclonal antibody (eBioscience), Cells were stimulated with the different antigens for 24 h at
37°C, 5% CO2. PBMC in medium alone or stimulated with phytohemagglutinin (Sigma) at 2.5 μg/ml were used as negative or positive controls, respectively.
Biotinylated anti-IFN-γ detection monoclonal antibody (eBioscience) was added for 4 h, and followed by the addition of streptavidin-alkaline phosphataseotate conjugate (Pierce Biotechnology) for 1 h. After a washing step, the nitroblue tetra-
zolumin-BCIP (5-bromo-4-chloro-3-indolylphosphate; Sigma) chromogenic sub-
strate was added. The individual spots were counted by use of an automated
image analysis system ELISPOT reader (BioReader 4000 Pro-X; Biosys, Ger-
many).
QFT-IT assay. The QFT-IT assay was performed in two stages, according to
the manufacturer’s instructions (6). The cutoff value for a positive test was ≥0.35
IU/ml IFN-γ, as recommended in accordance with previous studies (6).
TST. The standard tuberculin skin test (TST) was performed using five tuber-
culin units (0.1 ml) of purified protein derivative administered intradermally in the
volar surface of the forearm, and the size of the induration was evaluated
after 72 h. The TST responses were categorized as follows based on the trans-
verse diameter of skin induration in millimeters: TST induration of ≤5 mm (−),
6 to 9 mm (+), 10 to 14 mm (++), and ≥15 mm (+++), and a TST induration of
≥15 mm was considered caused by TB infection other than BCG vaccination
(17).

### Table 1. Characteristics of different groups of subjects in the study

| Characteristic                      | No. of subjects | Males/females | Mean age ± SD (yr) | No. of positive ELISPOT results (%) | Sensitivity of ELISPOT assay (%) |
|-------------------------------------|-----------------|---------------|-------------------|-------------------------------------|---------------------------------|
| PTB                                 | 221             | 168/53        | 39.73 ± 17.57     | 187 (84.6)                          | 84.6                            |
| Sputum positive                     | 89              | 72/17         | 38.7 ± 17.12      | 80 (89.89)                          | 89.89                           |
| Sputum negative                     | 132             | 96/36         | 40.46 ± 17.93     | 107 (81.06)                         | 81.06                           |
| Tuberculous pleurisy               | 53              | 37/16         | 37.68 ± 16.16     | 46 (86.79)                          | 86.79                           |
| Tuberculous meningitis             | 16              | 11/5          | 29.07 ± 15.02     | 10 (62.5)                           | 62.5                            |
| Extrapulmonary tuberculosis        | 15              | 6/9           | 26.92 ± 11.77     | 13 (86.67)                          | 86.67                           |
| Tuberculous lymphadenitis           | 9               |               |                   |                                     |                                 |
| Pelvic tuberculosis                 | 3               |               |                   |                                     |                                 |
| Renal tuberculosis                  | 2               |               |                   |                                     |                                 |
| Spinal tuberculosis                 | 1               |               |                   |                                     |                                 |
| Nontuberculosis lung disease        | 18              | 12/6          | 43.73 ± 19.45     | 7 (38.89)                           |                                 |
| Bacterial pneumonia                | 13              |               |                   |                                     |                                 |
| Chronic obstructive pulmonary disease | 2            |               |                   |                                     |                                 |
| Pneumothorax                        | 2               |               |                   |                                     |                                 |
| Lung cancer                         | 1               |               |                   |                                     |                                 |
| Healthy controls                    | 202             | 152/50        |                   | 31.00 ± 10.23                       | 45 (22.28)                      |
| TB exposure class 1                 | 20              | 11/9          | 38.32 ± 9.53      | 11 (55.00)                          |                                 |
| TB exposure class 2                 | 21              | 6/15          | 32.86 ± 9.134     | 8 (38.09)                           |                                 |
| TB exposure class 3                 | 40              | 35/5          | 34.6 ± 9.40       | 9 (22.50)                           |                                 |
| TB exposure class 4                 | 121             | 100/21        | 28.33 ± 9.868     | 17 (14.05)                          |                                 |

To determine the specificity and sensitivity of ELISPOT assay responses, the Pearson χ² test was used to analyze the correlation between the IFN-γ ELISPOT responses and TB exposure. Differences were considered significant when the P value was less than 0.05.

### Results

Sensitivity and specificity of ELISPOT assay in diagnosis of active tuberculosis. To determine the specificity and sensitivity of the ELISPOT assay in diagnosis of active tuberculosis, we first compared the IFN-γ ELISPOT responses from 89 patients...
with SPTB with those from 121 healthy controls who had no experience of TB exposure (class 4). Receiver operating characteristic curve analysis was performed to determine the optimal cutoff value of the ELISPOT assay for different antigens (ESAT-6 and peptide pool A and pool B), balanced with the highest possible positive rate for patients with SPTB and the lowest positive rate for healthy controls (data not shown). Based on this concept, the cutoff values for ESAT-6 and peptide pool A and pool B were 30, 18, and 11 spot-forming cells (SFC) per 2 × 10^5 cells, respectively. A positive response was scored as IFN-γ responses to any one of the above antigens higher than the cutoff value. Using this standard, we found that the positive rates of IFN-γ ELISPOT responses were 83.60% (255/305), 38.39% (7/18), and 22.82% (45/202) for patients with active tuberculosis, patients with nontuberculosis diseases and healthy controls, respectively. Therefore, the specificity and sensitivity of the ELISPOT assay for diagnosis of active TB were 83.6% and 76.36%, respectively (Table 1). The sensitivities of the assay for different forms of TB are depicted in Table 1.

Comparison of ELISPOT and QFT-IT assays. Previous data have demonstrated that the QFT-IT assay has higher specificity than T-SPOT.TB in the diagnosis of TB infection (11). Since specificity is a priority for using the ELISPOT assay to diagnose active TB, the performances of the ELISPOT and QFT-IT assays were compared. PBMC from a total of 156 individuals (49 TB patients and 107 healthy controls) were tested by ELISPOT and QFT-IT assays simultaneously. Eight individuals (two TB patients and six healthy controls) who displayed indeterminate results by the QFT-IT assay were excluded, and 148 samples were finally subjected to analysis. Our results indicated that the sensitivity (80.85%) and specificity (73.26%) of the QFT-IT and ELISPOT assays were comparable. The agreement between the two assays was 81.08%, and the concordance (κ) was 0.75 (Table 2).

Clinical characteristics associated with false-negative ELISPOT results in patients with sputum-positive TB. The ELISPOT assay gave false-negative results for nine patients with sputum-positive tuberculosis. Compared to sputum-positive patients with positive IFN-γ ELISPOT responses, those with false-negative ELISPOT results were older (P < 0.01), had fewer bacterial DNA copies in sputum specimens (measured by real-time PCR) (P < 0.001), and had proportionally more severe cases (although not significantly so), as defined by image examination (P = 0.091) (Table 3).

IFN-γ ELISPOT responses in relation to TST reactivity and TB exposure of healthy controls. To evaluate the performance of the ELISPOT assay in diagnosis of latent TB, healthy controls with different TB exposures (Table 1) were subjected to ELISPOT and TST assays simultaneously (18). A 15-mm induration was chosen as the cutoff of TST results caused by TB infection rather than BCG vaccination. Based on this cutoff value, TSTs were positive in 7 of 20 cases (35.00%) in class 1 (healthy donors), 7 of 21 cases (33.30%) in class 2, 5 of 40 (12.50%) in class 3, and 15 of 121 (12.4%) in class 4. The agreement between the TST and ELISPOT assays was low (concordance κ = 0.25). Neither the positive rate of TST responses nor the diameter of TST induration correlated with TB exposure. In contrast, both the positive rate (r = −0.9910, P = 0.009) and magnitude of IFN-γ responses (SFC numbers) to ESAT-6 determined by the ELISPOT assay were significantly correlated with TB exposure (r = −0.6040, P < 0.0001) (Fig. 1).

IFN-γ responses in relation to the clinical manifestation of TB. It has been suggested that different forms of TB may lead to different immune response profiles and therefore may affect the sensitivity of IFN-γ release assays (IGRA) (13). To address this question, we compared the IFN-γ responses of patients with different forms of TB. The magnitudes of IFN-γ responses to all three antigens (ESAT-6, pool A, and pool B) of PBMC from patients with tuberculous meningitis were significantly lower than those of PBMC from patients with PTB. In con-
patients with extrapulmonary TB had higher IFN-γ responses than those with PTB (Fig. 2). Similarly, the positive rate of the ELISPOT assay was also the lowest in patients with tuberculous meningitis (Table 1).

Influence of antituberculosis treatment on the sensitivity of ELISPOT assay. A previous report showed no difference in the sensitivities of IGRA performed with PBMC from TB patients within 7 days of treatment and with PBMC from patients at 8 to 14 days of treatment (3). However, since the magnitude of IFN-γ responses to ESAT-6 decreased after prolonged antituberculosis treatment, there is a concern that longer antituberculosis treatment may influence the sensitivity of such IGRA (12, 15). To evaluate the influence of antituberculosis treatment on the sensitivity of our ELISPOT assay, we monitored the IFN-γ responses in 46 patients with active TB subjected to antituberculosis therapy. Although there was a trend toward a decrease in the magnitude of IFN-γ responses in treated patients with active tuberculosis, no significant differences were observed during the 6-month follow-up (Fig. 3). Therefore, the sensitivities of the ELISPOT assay performed within 2 weeks, 1 month, 3 months, and 6 months after antituberculosis treatment was initiated were not significantly different (Fig. 3).

DISCUSSION

In this study, we used an in-house ELISPOT assay to detect antigen-specific IFN-γ responses of PBMC from patients with tuberculosis and evaluate the assay’s usefulness in diagnosis of tuberculosis infection in Shenzhen, China, where BCG vaccination is mandatory at birth and tuberculosis is highly prevalent.

At least three factors, including TB endemics, host genetic background, and immune status, have been recognized as influencing the performance of IGRA in the diagnosis of TB infection and/or active TB (2, 3). While there are a few studies that have investigated the performance of IGRA with the Chinese population, none of them have been performed in mainland China (3, 16). In this study, we investigate the performance of IGRA in a clinical setting in Shenzhen, China. Due to the difficulty in defining LTBI and given that the priority for tuberculosis control in China is to identify and treat patients with active tuberculosis, we sought to evaluate the potential of the clinical utilization of IGRA, particularly our cost-effective in-house ELISPOT assay. To achieve this aim, we first evalu-

FIG. 2. The magnitude of IFN-γ responses in relation to different forms of TB. The number of IFN-γ SFC per 2 × 10^5 PBMC (minus the values of the negative control wells) in response to ESAT-6, pool A, and pool B, which were determined in patients with different forms of TB, was compared by a one-way analysis of variance Newman-Keuls multiple comparison test. Data were expressed as means ± standard errors of the means. * indicates significant difference (P < 0.05) compared to secondary PTB. Mtb., Mycobacterium tuberculosis.

FIG. 3. The influence of anti-TB treatment on the magnitude of IFN-γ responses and sensitivity of ELISPOT assay. The number of IFN-γ SFC per 2 × 10^5 PBMC (minus the values of the negative control wells) in response to ESAT-6 (A), pool A (B), and pool B (C) determined at different time points after anti-TB treatment in patients with active TB (n = 46). (D) The sensitivities of the ELISPOT assay (calculated as responses to composite antigens) at different time points after anti-TB treatment. 2w, with 2 weeks; 1m, 1 month; 3m, 3 months; 6m, 6 months.
ated the sensitivities of the ELISPOT and QFT-IT assays with patients with SPTB. Our results indicate that the sensitivity of our in-house ELISPOT assay was 89.89%, which is comparable to the reported sensitivity of T-SPOT.TB (3, 11). Using control patients with nontuberculous lung diseases and control healthy donors (regardless of their exposure to active tuberculosis), we determined that the specificity of the ELISPOT assay in diagnosing active tuberculosis was 76.36%. This result is not consistent with the study from Kang et al. (using patients with nontuberculous lung diseases as controls), indicating that the specificity of T-SPOT.TB is 47% for the diagnosis of active TB (8). While the exact reasons underlying this substantial difference are uncertain, one possibility is that a high proportion of controls recruited in that study had a history of TB (13). Supporting this possibility, we observed a high frequency (85%) of positive IFN-γ ELISPOT responses in recovered TB patients who had completed antituberculosis treatment 2 years before, although the magnitude of the response of PBMC from these patients was significantly lower compared to that from patients with active TB (data not shown). Therefore, an ELISPOT result should be interpreted appropriately, with respect to the patient’s history of TB.

In contrast to a previous report, which demonstrated statistically significant differences in the performances of T-SPOT.TB and the QFT-IT assay (3), our results, in agreement with other studies (8, 11), showed that comparable results were obtained using the ELISPOT assay versus the QFT-IT assay with both patients with active TB and healthy donors. This could be due to the different compositions of the studied populations, as suggested by a previous report indicating that Chinese patients are more likely to be positive with the T-SPOT.TB assay (but not the QFT-IT assay) than those of Malay and Indian descent (3).

Although the main purpose of this study is to evaluate the sensitivity and specificity of IGRA in the diagnosis of active tuberculosis in a Shenzhen Chinese population, it is still necessary to investigate the accuracy of IGRA in diagnosis of LTBI amid the high prevalence of TB infection in China. It is plausible that the positive responses of control individuals could be due to LTBI. However, evaluating the accuracy of IGRA in diagnosing LTBI remains a problem since there is no “gold standard” for such diagnoses. To address this issue, we analyzed IFN-γ responses in healthy controls according to their exposure to TB, since it is well established that people who have spent time in close contact with contagious TB patients are at the highest risk of TB infection. The significant correlation of IFN-γ responses determined by the ELISPOT assay (but not the TST) with TB exposure suggests that these ELISPOT-positive healthy controls were subjected to LTBI. This further suggests that the TST has limited utility in diagnosing LTBI. Supporting this hypothesis, the prevalence (22.3%) of LTBI in Shenzhen determined by our homemade ELISPOT assay is much lower than the overall prevalence (44.5%) of TB infection in China (reported by the China CDC and using the TST as a diagnostic tool) (10). This may not be due to regional difference, since the prevalence of active TB in Shenzhen is even higher than the overall prevalence in China.

It should be noted that the sensitivity of IGRA may vary among different forms of tuberculosis. Consistent with a recent study reporting a higher sensitivity of the QFT-IT assay for diagnosis of extrapulmonary tuberculosis compared with PTB (13), we also observed a higher magnitude of IFN-γ responses in patients with extrapulmonary TB than in those with PTB. Our results also indicate that patients with tuberculous meningitis had the lowest IFN-γ responses compared to other forms of TB. A similar positive rate (<60%) was observed in a report studying Vietnamese patients, which argued against the use of the IFN-γ ELISPOT assay for diagnosis of tuberculous meningitis (14). While this study reported no differences in IFN-γ ELISPOT responses between healthy controls and patients with tuberculous meningitis, our current results argue for significantly increased IFN-γ ELISPOT responses in the latter group compared to controls. This disparity cannot be explained only by LTBI in healthy donors since prevalence of TB infection is not significantly different in Vietnam and China (7). It may be related to the low specificity of the ELISPOT assay used in their study due to the different antigens used. IGRA may thus be useful adjuncts to the current tests for diagnosis of tuberculous meningitis, provided that the specificity of the assay can be improved to the level of the QFT-IT or T-SPOT.TB assay. At the same time, the low IFN-γ responses of patients with severe TB indicated the complex roles of IFN-γ in immunity and the pathogenesis of TB. A comprehensive understanding of the role of IFN-γ responses is warranted for better clinical indications, applications, and utilization of IFN-γ as a biomarker (1).

Consistent with previous reports, we found that the magnitude of IFN-γ responses gradually decreased after antituberculosis treatment (12, 15). However, both the magnitude and frequency of IFN-γ ELISPOT responses were not significantly different for patients tested within 6 months after antituberculosis treatment was initiated. Thus, antituberculosis treatments shorter than at least 3 months did not affect the sensitivity of the ELISPOT assay. Nevertheless, the decrease of IFN-γ responses measured by the ELISPOT assay after antituberculosis treatment suggested the potential of using the ELISPOT assay to monitor the efficacy of antituberculosis treatment. Overall, our findings indicate that this particular ELISPOT assay may have a useful application for the diagnostic of TB.

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