Interferon-Gamma Release Assay Performance in Pulmonary and Extrapulmonary Tuberculosis

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Abstract

Background: The diagnosis of tuberculosis remains difficult. This study aimed to assess performance of interferon-gamma release assay (IGRA) in diagnosis of active tuberculosis (ATB) with pulmonary and extrapulmonary involvements, and to determine the diagnostic role of IGRA (T-SPOT.TB) and tuberculin skin test (TST) in BCG-vaccinated population.

Methods and Findings: Two hundred twenty-six ATB suspects were recruited and examined with T-SPOT.TB. Among them, fifty-two and seventy-six subjects were simultaneously tested by TST with 5TU or 1TU of purified protein derivative (PPD). The sensitivity of T-SPOT.TB was 94.7% (71/75), comparable in pulmonary and extrapulmonary disease groups (95.6% vs. 93.3%, P=0.05), while the specificity was 84.10% (90/107) but differed in two groups (69.2% vs. 88.9%, P=0.02). Compared to T-SPOT.TB, TST with 5TU-PPD showed less sensitivity (92.3% vs. 56.4%) and specificity (84.6% vs. 61.5%) (both P<0.01); the sensitivity of TST with 1TU-PPD was 27.8%, and despite its specificity identical to T-SPOT.TB (both 82.8%) positive predictive value (PPV) was only 33.3%. By combining T-SPOT.TB with TST (1TU), the specificity rose to 95%, but the PPV stayed unchanged.

Conclusions: IGRA could function as a powerful immunodiagnostic test to explore pulmonary and extrapulmonary TB, while TST failed to play a reliable or auxiliary role in identifying TB disease and infection in the BCG-vaccinated population.

Introduction

In recent decades, the burden of tuberculosis (TB) has been increasingly falling on developing countries. Although the TB vaccine Bacille Calmette-Guérin (BCG) is broadly vaccinated and DOTS (Directly Observed Treatment, Short-course) programme is well implemented, the incidence rate of active tuberculosis (ATB) in China has been doubled over ten years (39.03/100,000 in 1999 vs. 81.09/100,000 in 2009), with the death rate soaring 7-fold in this decade [1]. Despite incorporation of clinical, radiological, pathological and microbiological examinations, diagnosis of ATB can still be difficult. Conclusive diagnostic tests microbial culture and smear for acid-fast bacilli are not sensitive enough to identify all the active cases. Moreover, for extrapulmonary tuberculosis (EPTB), less specific clinical clues can be used and invasive procedures or low bacterial load leads to less chance to establish the pathological or microbiologic diagnosis [2,3].

Immunoassays capable of detecting the host’s immune response specific to TB causative agent Mycobacterium Tuberculosis (M.TB) has become an alternative diagnostic aid for ATB [2]. Long-time-used tuberculin skin test (TST) has encountered considerable difficulties, mainly due to the disability of its mixed antigens tuberculin purified protein derivative (PPD) to distinguish the true ATB patients from those vaccinated with BCG or sensitized with Nontuberculous Mycobacteria (NTM) [4]. Recently, interferon-gamma release assays (IGRAs) have shown their superior diagnostic performance over TST [4,5,6,7,8,9] by using at least two specific antigens (ESAT-6 & CFP 10) present exclusively in M.TB but absent in BCG strains and most NTM [9,10]. Herein, we put ELISPOT-based-IGRA (T-SPOT.TB) into test to examine how it works especially for identifying EPTB in comparison with pulmonary tuberculosis (PTB). Meanwhile, we compared the performance between IGRA and TST with two currently used doses (5TU; 1TU) to elaborate whether TST is still strong enough to carry on the diagnostic role in ATB for the TB-epidemic and BCG vaccinated populations.

Methods

This study got ethical approval from Huashan Institutional Review Board (HIRB), the ethics reviewing committee of Huashan Hospital, Fudan University. Informed consent was obtained from all the participants in the written form.
A prospective study was conducted in HIV-negative subjects with suspicion of active TB collected between September 2008 and September 2009. A total of 226 patients from China were tested with T-SPOT.TB at enrollment, together with routine clinical, microbiologic, pathological and radiographic examinations. Individuals were excluded if they have received >30 days of anti-tuberculosis therapy or if they have received the treatment within one year prior to enrollment; those treated for one year or longer before enrollment were otherwise involved. All patients were vaccinated with BCG at early childhood or during adolescence. Major clinical characteristics of recruited subjects were summarized in Table 1.

After a follow-up of at least 3 months, by January 2010, 44 patients were excluded from the study, among which 10 died before final diagnosis, 17 lost follow-up, and 17 had no final diagnosis. The remaining 182 patients were ultimately included for T-SPOT.TB analyses (Figure 1), of which 128 consented to perform TST concurrently, 76 with 1TU-PPD, and 52 with 5TU-PPD.

### Definitions and Diagnosis

TB suspects were defined as patients whose clinical or radiographic manifestations were consistent with active TB [11], but lack of culture or pathological evidence for confirmative diagnosis. Finally, they had one of three diagnoses: (1) ‘culture/biopsy-confirmed ATB’ if final diagnoses were made on the positive culture of M. TB from sputum or the presence of caseating granuloma in biopsy specimen; (2) ‘clinical ATB’ if patients, whose clinical presentations were consistent with ATB but lack of bacterial/pathological corroborative evidence, presented manifest clinical or radiographic responses to anti-TB treatment; (3) ‘no ATB’ if the patients did not meet the above two criteria and their clinical presentations diminished spontaneously or following non-TB-related treatment.

### T-SPOT.TB assay

The T-SPOT.TB test was performed following the instructions of the assay kit (Oxford Immunote Ltd., Oxford, UK). Briefly, peripheral blood mononuclear cells (PBMCs) were isolated and incubated with two antigens (ESAT-6 in panel A; CFP-10 in panel B). The procedure was performed in the plates pre-coated with anti-interferon-γ antibodies at 37°C for 16 to 20 hours. After

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**Table 1. Clinical characteristics of 182 patients with suspected active TB.**

| Characteristics                  | Total (n = 182) | ATB (n = 75) | No ATB (n = 107) |
|----------------------------------|----------------|-------------|------------------|
| Age, median (range), yr          | 52 (14–87)     | 41 (16–84)  | 51 (14–87)       |
| Male/Female                      | 87/95          | 43/32       | 44/63            |
| Presence of TB history           | 13             | 1           | 12               |
| Presence of TB contact           | 11             | 5           | 6                |
| TB scar in chest radiographs     | 23             | 8           | 15               |
| Immunocompromised conditions     | 13             | 1           | 12               |
| Liver cirrhosis                  | 1              | 0           | 1                |
| Chronic renal failure            | 2              | 1           | 1                |
| Leukemia                         | 2              | 0           | 2                |
| Idiopathic myelofibrosis         | 1              | 0           | 1                |
| Hemophagocytic syndrome          | 1              | 0           | 1                |
| Low CD4 count                    | 1              | 0           | 1                |
| Immunosuppressive drugs          | 5              | 0           | 5                |

ATB: active tuberculosis; No ATB: diagnosis other than active tuberculosis.

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**Figure 1. Flowchart of the study population.** A total of 226 subjects suspected to have active tuberculosis (ATB) were recruited and 182 were eligible to be included in the final analyses. The analyses were composed of two parts: a study on the diagnostic performance of the T-SPOT.TB on pulmonary and extrapulmonary ATB, and a study comparing the performance between T-SPOT.TB and TST with a dose of 1TU-PPD or 5TU-PPD. ATB, active tuberculosis; no ATB, final diagnosis excluded active tuberculosis.

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application of alkalinephosphatase-conjugated second antibody and chromogenic substrate, spots were scored using an automated ELISPOT plate reader (AID-Gmb-H, Germany).

TST

TST was tested on the patients' volar surface of a forearm, by intradermal injection of 1 tuberculin unit (TU) of PPD-S (Statens Seruminstitut, Copenhagen, Denmark) (n = 76) or 5TU of PPD (n = 52). The size of the induration was read at 72 h. Based on the transverse diameter of induration, the cut-off value was determined as follows: induration <10 mm denoted as negative (−); induration ≥10 mm as positive (+). The TST and T-SPOT.TB were all conducted simultaneously.

Statistical analyses

Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), likelihood ratio positive (LR+), and likelihood ratio negative (LR−) were calculated to evaluate diagnostic performance for the T-SPOT.TB and TST. Ninety-five percent confidence intervals (95%CI) were estimated according to the binomial distribution. Significance was inferred for \( P < 0.05 \). Concordance between the results of TST and T-SPOT.TB was assessed using \( \kappa \) coefficients (\( \kappa >0.75 \), excellent agreement; \( \kappa <0.4 \), poor agreement; \( 0.75 \leq \kappa \leq 0.4 \), fair to good agreement). Analyses were performed using statistical software packages (Stata version 9; StataCorp; College Station, TX).

Results

Clinical characteristics

Of 182 ATB suspects with valid T-SPOT.TB results, 71 were categorized into pulmonary disease group and 111 in extrapulmonary disease group (Figure 1). Patients with EPTB concurrently with documented PTB were included into PTB group. Eventually, 107 patients excluded ATB and 75 were diagnosed as ATB. Of the latter, 45 were confirmed with culture/biopsy evidences, and 30 as probable ATB cases with clinical evidences. Eventually, 107 patients excluded ATB and 75 were diagnosed as ATB. Of the latter, 45 were confirmed with culture/biopsy evidences, and 30 as probable ATB cases with clinical evidences. Clinical characteristics of 182 patients are shown in Table 1. The distribution of affected extrapulmonary organs was highly heterogeneous which involved central nervous system, peripheral lymph nodes, pleura, bones, genitourinary system, gastrointestinal tract, and skin (Table 2).

Diagnostic performance of T-SPOT.TB: overall and stratified by disease site

The diagnostic values of T-SPOT.TB for the 182 subjects are presented in Table 3. The overall sensitivity and specificity were 94.70% (95%CI, 86.9%–98.5%) and 84.10% (95%CI, 75.8%–90.5%), respectively. There was no significant difference in the sensitivity between the ‘confirmed ATB’ cases (95.3%, 42/45) and the ‘clinical ATB’ cases (96.7%, 29/30; \( P=0.05 \)). PPV, NPV, LR+, and LR− of the T-SPOT.TB were 80.70%, 95.70%, 5.96 and 0.06, respectively, and the prevalence was 41.2% in the cohort (Table 5).

The stratified performance by the site of disease is shown in table 2. Among the 71 patients with pulmonary involvement, T-SPOT.TB was positive in 43 of 45 ATB cases, with a sensitivity of 95.6% (95%CI, 84.9%–99.5%) which did not differ significantly from the sensitivity of 93.3% (95%CI, 77.9%–99.2%) in extrapulmonary disease group (28/30). However, the specificity was 69.2% (95%CI, 48.2%–85.7%) in pulmonary disease group, while a higher specificity was observed in extrapulmonary disease group (88.9%; 95%CI: 80.0%–94.8%; \( P = 0.017 \)). The results of extrapulmonary disease group were further stratified by affected sites. Notably, apart from pleura (50%; 1/2) and abdomen tuberculosis (33.3%; 5/6), the sensitivity of T-SPOT.TB was as high as 100% for the most affected sites, while the specificity ranged from 60% to 97% (Table 2).

Risk factors for false-positive outcomes in T-SPOT.TB: overall and stratified by disease site

A number of risk characteristics of patients associated with false-positive and false-negative results were evaluated by multivariate logistic regression. Age (median age (46-year) and ‘history of prior TB’ were turned out to be two independent risk factors related to false-positive outcomes. Odds ratio (OR) between false-positives and true-positives for overall, and in pulmonary and extrapulmonary groups are present individually in Table 4. For the risk factor of ‘age (median age)’, the overall OR was 5.09 (95%CI 1.26–20.25; \( P = 0.021 \)) whereas 10.71 (95%CI 1.21–

| Table 2. Comparison of performance of T-SPOT.TB assay in pulmonary and extrapulmonary tuberculosis. |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| **Site of disease** | **ATB n** | **No ATB n** | **T-SPOT.TB(+) n** | **T-SPOT.TB(−) n** | **Sensitivity** | **Specificity** |
|----------------|---------|-------------|-----------------|-----------------|-----------------|-----------------|
| Pulmonary disease | 45      | 26          | 43              | 18              | 95.6% (84.9%–99.5%)* | 69.2% (48.2%–85.7%)** |
| Extra-pulmonary disease | 30      | 81          | 28              | 72              | 93.3% (77.9%–99.2%) | 88.9% (80.0%–94.8%) |
| Central nervous system | 12      | 26          | 12              | 23              | 100%            | 88.5%          |
| Lymphadenitis | 3       | 0           | 3               | 0               | 100%            | N/A            |
| Pleurisy disease | 2       | 0           | 1               | 0               | 50%             | N/A            |
| Abdominal disease (liver, pancreas, spleen) | 6       | 4           | 5               | 4               | 83.3%           | 100%          |
| Genitourinary disease | 2       | 6           | 2               | 4               | 100%            | 66.7%          |
| Bone disease | 3       | 5           | 3               | 3               | 100%            | 60%            |
| Skin disease | 2       | 6           | 2               | 5               | 100%            | 83.3%          |
| Other sites | 0       | 33          | 0               | 34              | N/A             | 97.1%          |
| Total | 75      | 107         | 71              | 90              | 94.7% (86.9%–98.5%) | 84.1% (75.8%–90.5%) |

ATB, active tuberculosis; No ATB, diagnosis other than active tuberculosis; \*\( P < 0.05 \); **\( P = 0.017 \).
A statistically significant difference in sensitivity was found between either comparison, 67.11% for ‘T-SPOT.TB’ and ‘TST5TU-PPD’ (k = 0.28), and 63.46% for ‘T-SPOT.TB’ vs. TST5TU-PPD (k = 0.20).

Table 3. Diagnostic performance of T-SPOT.TB assay in 182 active tuberculosis suspects.

| Parameter            | Value          | 95%CI          |
|----------------------|----------------|---------------|
| Sensitivity, % (n)   | 94.70 (71/75)*| 86.9–98.5     |
| Specificity, % (n)   | 84.10 (90/107)| 75.8–90.5     |
| PPV, % (n)           | 80.70 (71/88) | 70.9–88.3     |
| NPV, % (n)           | 95.70 (90/94) | 89.5–98.8     |
| LR+                  | 5.96           | 3.84–9.24     |
| LR−                  | 0.06           | 0.02–0.17     |
| Prevalence, % (n)    | 41.2 (75/182)  | 34.0–48.7     |

PPV, positive predictive value; NPV, negative predictive value; LR+, likelihood ratio for positive test; LR−, likelihood ratio for negative test.

*The sensitivity for ‘culture/biopsy-confirmed’ subgroup was 93.3% (42/45), with a 95%CI of 81.7%–98.6%; for ‘clinical ATB group’ was 96.7% (29/30), with the 95%CI of 88.6%–99.9%; P > 0.05.

Table 4. Logistic regression analyses of risk factors leading to false-positive results in T-SPOT.TB assay.

| Risk factor          | Pulmonary disease | Extrapulmonary disease | Total          |
|----------------------|-------------------|------------------------|----------------|
|                      | OR                 | 95%CI                  | P value        | OR             | 95%CI                  | P value        | OR             | 95%CI                  | P value        |
| Age≥median age*      | 10.71              | 1.21–94.95             | 0.009          | 3.13           | 0.51–19.04       | 0.216          | 5.09           | 1.28–20.25       | 0.021          |
| History of prior TB  | N/A**              | 4.8                    | 0.35–65.76     | 0.240          | 10.06            | 1.61–62.75     | 0.013          |

OR: odds ratio of risk factors between false positive and true positive results.

*Median age: 46 years old, the median age calculated in patients positive for T-SPOT.TB.

**N/A: the value could not be calculated because no false positive subject had TB history in pulmonary disease group.

Discussion

Performance characteristics of T-SPOT.TB

Our study revealed a 94.7% overall sensitivity of T-SPOT.TB for detecting ATB which was parallel with our previously published data [12] and within the ranges recently reported elsewhere [8,13]. Extrapulmonary tuberculosis (EPTB) reported by different countries varies from 15% to 25% in the ATB cases [14]. However, the corroborative epidemiological data for EPTB is rarely available in most TB-endemic country due to diagnostic difficulties. We explored, for the first time, the role of the IGRA in detecting EPTB in Chinese patients. T-SPOT.TB turned out to be sensitive equally in determining PTB and EPTB (95.6% vs. 93.3%; P > 0.05). In the 111 patients with extrapulmonary involvement, of note was a 100% sensitivity seen in the most investigated sites (Table 2).
Our study revealed a little lower specificity (84.10%) and PPV (80.70%) than most data elicited from the developed countries [8,13] which may suggest relatively higher prevalence of latent tuberculosis infection (LTBI) in China. For better understanding the causes and likelihood of false-positives in the population tested, we analyzed risk factors leading to false-positives (Table 4). The

| Parameters      | TST<sup>STU</sup> PPD vs. T-SPOT.TB (n = 76) | TST<sup>STU</sup> PPD vs. T-SPOT.TB (n = 52) |
|-----------------|---------------------------------------------|---------------------------------------------|
|                 | TST<sup>STU</sup> PPD | T-SPOT.TB | TST<sup>STU</sup> PPD | T-SPOT.TB |
| Sensitivity, % (n) | 27.8 (5/18) | 94.4 (17/18)<sup>a</sup> | 56.4 (22/39) | 92.3 (36/39)<sup>c</sup> |
| Specificity, % (n)  | 82.8 (48/58) | 82.8 (48/58)<sup>b</sup> | 61.5 (8/13) | 84.6 (11/13)<sup>d</sup> |
| PPV              | 33.3 (5/15) | 63.0 (17/27) | 81.5 (22/27) | 94.7 (36/38) |
| NPV              | 78.7 (48/61) | 98.0 (48/49) | 32.0 (8/25) | 78.6 (11/14) |
| LR+ (95%CIs)     | 1.61 (0.63–4.10) | 5.48 (3.08–9.73) | 1.47 (0.70–3.08) | 6.00 (1.67–21.54) |
| LR– (95%CIs)     | 0.87 (0.64–1.19) | 0.07 (0.01–0.45) | 0.71 (0.41–1.24) | 0.09 (0.03–0.28) |
| Prevalence, %     | 23.7 (18/76) | 75.0 (39/52) |                     |               |
| Concordance, %     | 67.11 | 63.46 |                     |               |
| Kappa value       | 0.1866 | 0.2841 |                     |               |

TST: tuberculin skin test; PPV, positive predictive value; NPV, negative predictive value; LR+, likelihood ratio for positive test; LR–, likelihood ratio for negative test.

<sup>a</sup>: P = 0.0005;
<sup>b</sup>: P = 1.000;
<sup>c</sup>: P = 0.001;
<sup>d</sup>: P = 0.0078.

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Figure 2. The deviation features of the T-SPOT.TB, TST in single or combination way. The deviation from the gold standard test was compared between T-SPOT.TB and TST<sup>STU</sup> PPD (A), T-SPOT.TB and TST<sup>STU</sup> PPD (B), and between the combination in parallel and serial way for these two comparisons (C, D). The north, south, east and west poles in each panel represented 100% of the true-positive rate, true-negative rate, false-positive rate, and false-negative rate, respectively, and each observed rate located between the top poles of the axes (100%) and the central origin (0%). In A and B, the shape formed by connecting the diagnostic rates of T-SPOT.TB was outlined by the dark lines and the shape of TST were filled with grey color. In C and D, parallel testing was outlined by dashed lines, and serial testing by dotted lines.

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odds ‘over-median age’ or ‘having prior TB history’ leading to false-positives was about 5 or 10 times higher than the odds leading to true-positives. Agging and having TB history are known to be the important factors in the individualized clinical risk assessment for LTBI. Similarly, a discrepancy in specificity between pulmonary and extrapulmonary groups (69.2% vs. 88.9%; P = 0.017) can also be explained by the presence of significant difference in OR between groups. OR (10.71) for ‘over-median age’ factor in pulmonary group inferred a nearly 11-fold risk increase caused by the factor to yield a false-positive, whereas the risk factor of age was not related to false-positives in extrapulmonary group.

IGRAs were designed for identification of LTBI and high prevalence of TB may increase the ‘false-positives’ of IGRAs such that a considerable portion of cases with LTBI could be misrecognized as ATB if relying on IGRAs. Moreover, comparing with the specificity, PPV (80.70%) and LR+ (5.96), the sensitivity, NPV (95.7%) and LR− (0.06) strongly indicated IGRAs are better at ruling out ATB than ruling it in. Thus, the real role of IGRAs in high TB-prevalence countries would be appropriate for ruling out a diagnosis of TB.

### Comparing immunodiagnostic strategy for patients with the history of BCG vaccination

The TST suffers from the low specificity and sensitivity when tested on population with high BCG-vaccination coverage and high TB prevalence. TST using a standard dose of 5 tuberculin units (TU) PPD has been widely used, but there is always a disagreement about its role in vaccinated people. The US recommends the interpretation of TST irrespective of prior BCG vaccination, resulting in considerable overdiagnosis of LTBI, while the UK strategy probably misdiagnoses LTBI cases due to the recommendation that the serial TST be contraindicated for BCG-vaccinated persons and IFN-γ testing be used to help interpret positive TST results [15,16,17]. For a country having serious TB burden and urgent task to treat active TB, we have to know how much we can rely on this test and how to arrange our best immunodiagnostic strategy.

The study revealed that the sensitivity (56.4%) and specificity (61.5%) for TST with 5TU-PPD (TST5TU-PPD) were both beneath the performance of T-SPOT.TB (Table 5) and lower than a roughly 70% sensitivity and 66% specificity reported by two recent comprehensive reviews [8,18]. Meanwhile, a dose of 1 TU PPD (TST1TU-PPD) has long been used to rule in TB cases in some BCG-vaccinated counties like China. Its reliability faces the arguments for its strong specificity and against the weak sensitivity. They were both confirmed by our study (Table 5). Interestingly, a nearly symmetric ‘short’ and ‘fat’ figure for TST1TU-PPD and ‘shot’ in sensitivity but getting ‘fat’ toward false-negative for TST5TU-PPD were graphically presented in figure 2. By contrast, T-SPOT.TB remained ‘thin’ with only a slight growth in false-positive rate presumably reflecting the LTBI prevalence.

### Table 6. The effect of parallel and serial testing on sensitivity, specificity and predictive values for T-SPOT.TB and TST in two comparisons.

| Test                        | Sensitivity % (n) | Specificity % (n) | PPV % (n)  | NPV % (n)  |
|-----------------------------|-------------------|-------------------|------------|------------|
| TST5TU PPD                  | 27.8 (5/18)       | 82.8 (48/58)      | 33.3 (5/15) | 78.7 (48/61) |
| T-SPOT.TB                   | 94.4 (17/18)      | 82.8 (48/58)      | 63.0 (17/27) | 98.0 (48/49) |
| T-SPOT.TB and TST5TU PPD (parallel) | 94.4 (17/18) | 70.7 (41/58)      | 50.0 (17/34) | 97.6 (41/42) |
| T-SPOT.TB and TST5TU PPD (serial) | 27.8 (5/18) | 94.8 (55/58)      | 62.5 (5/8)  | 80.9 (55/68) |
| TST1TU PPD                  | 56.4 (22/39)      | 61.5 (8/13)       | 81.5 (22/27) | 32.0 (8/25)  |
| T-SPOT.TB                   | 92.3 (36/39)      | 84.6 (11/13)      | 94.7 (36/38) | 78.6 (11/14) |
| T-SPOT.TB and TST1TU PPD (parallel) | 94.9 (37/39) | 61.5 (8/13)       | 88.1 (37/42) | 80.0 (8/10)  |
| T-SPOT.TB and TST1TU PPD (serial) | 53.8 (21/39) | 84.6 (11/13)      | 91.3 (21/23) | 37.9 (11/29) |

1, two tests were combined in a ‘parallel’ way that took a positive result when either test was positive and a negative result when both negative.

2, two tests were combined in a ‘serial’ way that took a positive result when both test was positive and a negative result when either negative. The two tests were performed simultaneously and the word ‘serial’ only indicated the combination fashion usually done. PPV, positive predictive value; NPV, negative predictive value.

We further investigated whether the performance of T-SPOT.TB can be improved by combining with either TST1TU-PPD or TST5TU-PPD. A parallel combination test is usually expected to increase sensitivity, but we found that even at the expense of a big reduction in specificity, the sensitivity of T-SPOT.TB did not increase by parallel combining with TST1TU-PPD (figure 2A and 2C), because the false-negative number upon combination failed to decline (Table 6). Obviously, TST1TU-PPD could not help increase true-positives by parallel testing due to its low sensitivity. On figure 2B and 2D, parallel and serial testing both displayed a ‘fatter’ feature than T-SPOT.TB with only growth in false-positives. However, a greater decrement in true-positives than in false-positives after combination suggested that the limitation of TST to identify the true-positives compromised the
combinative PPV and made the improved specificity less helpful to determine true active tuberculosis.

There were some noteworthy limitations of this study. Despite no difference in the sensitivity of T-SPOT.TB between the culture/biopsy-confirmed (n = 45) and clinical probable ATB (n = 30) groups, inclusion of those probable cases in the true ATB group may bias the performance for both T-SPOT.TB and TST and their comparisons. Besides, unbalanced factors of age and TB history between comparative groups may also cause study bias. The more subjects were expected to involve into such investigations.

In conclusion, the TST played a prominent part in the detection of tuberculosis, but its contribution has been on the wane as other immunoassays came into use with superior diagnostic performance over it. This study demonstrated that T-SPOT.TB is a promising tool for diagnosing tuberculosis with pulmonary and extrapulmonary involvement. Moreover, not only cannot TST take major part in immunodiagnosis of ATB, but it was not supported by our study that combining TST with small or regular dose of PPD would become a routine optimized immunodiagnostic strategy in the population with high TB-prevalence and massive BCG-vaccination. We highly recommend that IGRA should be tested as first priority to diagnose LTBI and ATB for those populations, if applicable.

**Author Contributions**

Conceived and designed the experiments: WZ LS XW. Performed the experiments: YF JW SZ JJ FW. Analyzed the data: ND. Wrote the paper: ND YZ WZ.