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Factors associated with differential T cell responses to antigens ESAT-6 and CFP-10 in pulmonary tuberculosis patients

Shengsheng Liu, MM[a], Meiying Wu, MM[b], Ertai A, MB[c], Shucai Wu, MB[d], Shujun Geng, MB[d], Zhihui Li, MB[d], Mingwu Li, MB[e], Liang Li, MB[a], Yu Pang, MD[a], Wanli Kang, MM[a], Shenjie Tang, MD[a,*]

Abstract

The T-SPOT.*TB* assay detects cellular immune responses to 2 core *Mycobacterium tuberculosis* antigens, early secreted antigenic target of 6-kDa protein (ESAT-6) and culture filtrate protein-10 (CFP-10). T-SPOT.*TB* has been recently used for auxiliary diagnosis of active pulmonary tuberculosis (PTB). However, testing can produce inconsistent results due to differential PTB patient immune responses to these antigens, prompting us to identify factors underlying inconsistent results.

Data were retrospectively analyzed from 1225 confirmed PTB patients who underwent T-SPOT.*TB* testing at 5 specialized tuberculosis hospitals in China between December 2012 and November 2015. Numbers of spot-forming cells (SFCs) reflecting T cell responses to ESAT-6 and CFP-10 antigens were recorded then analyzed via multivariable logistic regression to reveal factors underlying discordant T cell responses to these antigens.

The agreement rate of 84.98% (82.85%–86.94%) between PTB patient ESAT-6 and CFP-10 responses demonstrated high concordance. Additionally, positivity rates were higher for ESAT-6 than for CFP-10 (84.8% vs 80.7%, $P < .001$), with ESAT-6 and CFP-10 microwell SFC numbers for each single positive group not differing significantly ($P > .99$), while spot numbers of the single positive group were lower than numbers for the double positive group ($P < .001$). Elderly patients (aged ≥66 years) and patients receiving retreatment were most likely to have discordance results.

ESAT-6 promoted significantly more positive T-SPOT.*TB* results than did CFP-10 in PTB patients. Advanced age and retreatment status were correlated with discordant ESAT-6 and CFP-10 results. Assessment of factors underlying discordance may lead to improved PTB diagnosis using T-SPOT.*TB*.

Abbreviations: ATB = active tuberculosis, BCG = Bacillus Calmette-Guérin, BMI = body-mass index, CFP-10 = culture filtrate protein-10, ESAT-6 = early-secreted antigenic target-6kDa protein, IGRA = interferon -γ release assay, IQR = inter quartile range, MTB = *Mycobacterium Tuberculosis*, NTM = non-tuberculous mycobacteria, PTB = pulmonary tuberculosis, SFC = spot forming cell, TB = tuberculosis.

Keywords: culture filtrate protein-10, early-secreted antigenic target-6kDa protein, T-SPOT.*TB*, tuberculosis

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[a] Beijing Chest Hospital, Capital Medical University, Beijing Tuberculosis and Thoracic Tumor Research Institute, Beijing, [b] Souzhou 5th People's Hospital, Souzhou, [c] Chest Hospital of Xinjiang Uygur Autonomous Region, Urumqi, [d] Hebei Chest Hospital, Shijiazhuang, [e] Kunming 3rd People's Hospital, Kunming, China.

[*] Correspondence: Shenjie Tang, Beijing Chest Hospital, Capital Medical University, Beijing Tuberculosis and Thoracic Tumor Research Institute, No.97, Machang, Tongzhou District, Beijing101149, China (e-mail: tangsj1106@hotmail.com).

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1. Introduction

Tuberculosis (TB), a disease caused by *Mycobacterium tuberculosis* (MTB), remains a major global public health concern.[1] Early diagnosis of TB cases is universally important for case detection, successful therapy and control. Bacteriological diagnosis of tuberculosis is the "gold standard" TB test; however, the low sensitivity of sputum smear analysis and the long culture period needed for completion can lead to delayed diagnosis and treatment.[2,3] Meanwhile, molecular diagnostics advances have improved the speed and accuracy of microbiological diagnostic tests over the past decade, but such tests still lack sufficient sensitivity, especially for testing of paucibacillary cases.[4,5] Fortunately, the T-SPOT.*TB* assay, an interferon (IFN)-γ release assay (IGRA), is based on detection of effector T-cells in human whole blood through capture of IFN-γ present in the vicinity of T-cells responding to MTB-specific antigens, including early secreted antigenic target of 6-kDa protein (ESAT-6) and culture filtrate protein-10 (CFP-10).[6–8] This test has been validated for use in detecting MTB infection and does not cross-react with antigens of Bacillus Calmette-Guérin (BCG) and most non-tuberculous mycobacteria (NTM).[7,9,10]

Accumulating evidence has supported the auxiliary diagnostic value of the T-SPOT.*TB* assay for active tuberculosis (ATB) cases in recent years, with sensitivity ranging from 76.7% to 94.7%

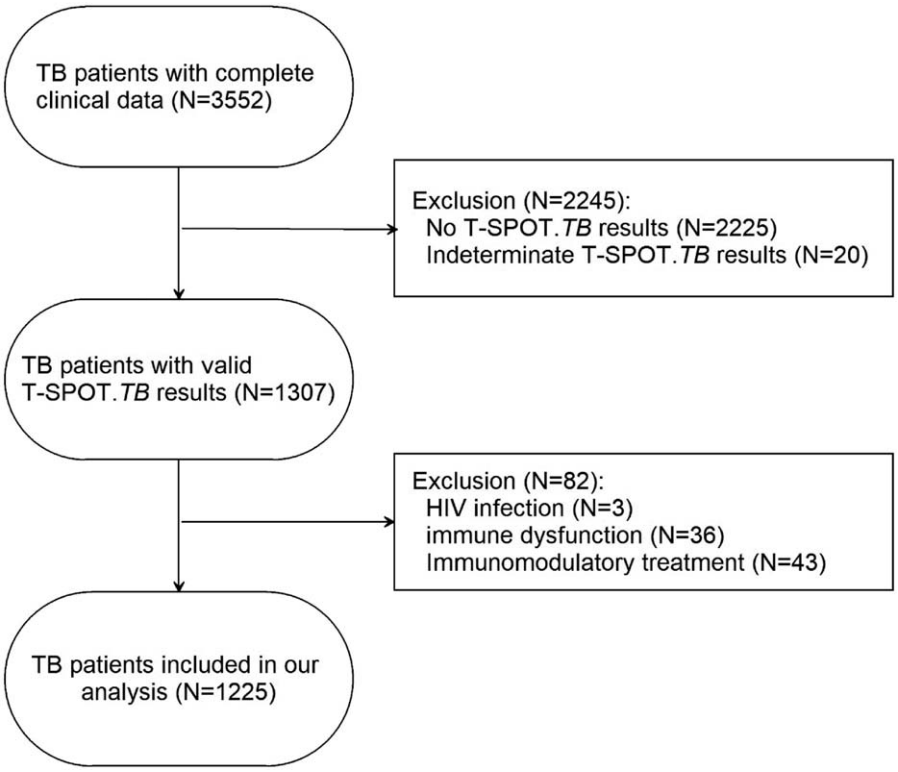

Figure 1. Participant inclusion flowchart. HIV = human immunodeficiency virus, TB = tuberculosis.

and specificity ranging from 63.4% to 91.1%.[11–17] Results are scored as positive if the spot number of either ESAT-6 or CFP-10 wells is positive in clinical practice. However, inconsistent positive responses to these 2 antigens have been obtained due to unknown factors, prompting the present study. Here we retrospectively compared positive rates and spot numbers reflecting T cell responses to ESAT-6 and CFP-10 from active pulmonary tuberculosis (PTB) cases then we identified patient factors associated with inconsistent response results between the 2 antigens. These results should reveal underlying reasons for heterogeneous ESAT-6 and CFP-10 T-SPOT.*TB* assay results for active PTB patients that may be useful for improving clinical decision-making.

2. Methods

2.1. Ethics approval

This was an observational retrospective study and the diagnostic tests were already used in clinical practice. Given that all data were collected anonymously, a waiver of consent was granted by the Ethics Committee of Beijing Chest Hospital, Capital Medical University, which also approved the protocols used in this study.

2.2. Study subjects

We retrospectively recruited ≥15-year-old confirmed PTB in patients with valid T-SPOT.*TB* results who were diagnosed at 5 Specialized Tuberculosis Hospitals in China between December 2012 and November 2015. The 5 hospitals where this multicenter, retrospective comparison study was conducted were located in Beijing Municipality, Hebei Province, Xinjiang Province, Yunnan Province and Jiangsu Province. A total of 1225 PTB patients were included in the study. Figure 1 shows a study flowchart outlining the patient selection process.

2.3. Inclusion and exclusion criteria

Inclusion criteria were as follows:

1. patients with a positive sputum culture for *Mycobacterium tuberculosis*;
2. patients with 2 positive sputum acid-fast smear test results;
3. patients with 1 positive sputum smear and thoracic imaging lesions indicative of active tuberculosis;
4. patients with positive GeneXpert MTB/RIF assay results and thoracic imaging lesions indicative of active tuberculosis;
5. patients with positive pathologic results indicative of pulmonary tuberculosis;
6. patients with typical chest radiograph findings and clinical response to anti-TB treatment consistent with PTB;
7. patients with valid T-SPOT.*TB* tests.

Exclusion criteria were as follows:

1. subjects with HIV infection;
2. patients with immune system disease;
3. patients receiving immunosuppressive or enhancement therapy;
4. patients who did not undergo T-SPOT.*TB* examination or those with indeterminate test results.

2.4. T-SPOT.TB assay

The T-SPOT.*TB* test (Oxford Immunotec Ltd., UK) was performed using peripheral blood mononuclear cells (PBMCs)

isolated from heparinized blood samples according to the manufacturer's instructions. Briefly, PBMCs were isolated and adjusted to a concentration of 2.5×10^6/ml then cells in wells were stimulated with 50 μl each of phytohemagglutinin (positive control), ESAT-6 (panel A), CFP-10 (panel B) or AIMV medium (Invitrogen, USA) (negative control). The procedure was performed in plates pre-coated with anti-interferon-γ antibodies at 37°C for 16 to 20 hours. After addition of alkaline phosphatase-conjugated secondary antibody and chromogenic substrate, the number of spot-forming cells (SFCs) in each well was automatically counted using a CTL ELISPOT system (CTL-ImmunoSpot S5 Versa Analyzer, USA). The results were interpreted as follows:

1. positive: the number of spots in ESAT-6 or CFP-10 wells or both wells was 6 or more;
2. negative: number of spots in ESAT-6 or CFP-10 wells was less than 6;
3. indeterminate: the number of spots in the negative control well was greater than 10 or in the positive control well was less than 20.

All indeterminate results were retested; repeated indeterminate results were dropped from the analysis.

2.5. Data management and statistical analysis

We took measures to guarantee data quality, including use of standardized study protocols and standardized training of research staff. Trained health workers collected medical information using a standardized questionnaire, while results of PTB patient T-SPOT.*TB* tests were obtained from patient medical records. We also collected patient clinical data including demographics, body-mass index (BMI), smoking history, contacts with TB, BCG vaccination status, history of previous TB treatment, bacteriology, imaging, routine examination history, pathology, etc. Categorical variables were summarized as frequencies using proportion values. Concordance between ESAT-6 and CFP-10 results was assessed using Gwet's AC_1 index.[18] Gwet AC_1 = the difference between observed agreement and maximum possible agreement as compared with 1 minus the maximum possible agreement. A Gwet AC1 index value ≥0.75 indicates strong agreement. PTB patient positive results across the 2 antigen tests were compared using the McNemar test. Kruskal–Wallis tests followed by Bonferroni-corrected analysis were used to compare SFCs between positive groups. Multivariable logistic analyses were performed to evaluate factors associated with discordant T cell responses to ESAT-6 and CFP-10 in PTB patients. Odds ratios and 95% confidence intervals for risk were calculated. All data were entered into MS Office Excel (Microsoft, Redmond, WA, USA) datasheets and all analyses were conducted using SPSS software for Windows, version 13.0 (Chicago, IL, USA). A *P* value <.05 was considered statistically significant for all analyses.

3. Results

3.1. Demographic characteristics

A total of 1225 eligible PTB patients were included in the study, of which 67.8% were male with a mean age of 42 years (range: 15–88 years) (Table 1).

3.2. Comparison of T cell responses to ESAT-6 and CFP-10

The overall positivity rate of T-SPOT.*TB* results was 90.3% (95% CI: 88.5%–91.9%) for PTB patients in this study.

Table 1

Demographic characteristics of pulmonary tuberculosis (n = 1225).

Variables	Frequency (%)
gender	
male	831 (67.8)
female	394 (32.2)
age	
15–44	656 (53.6)
45–65	380 (31.0)
≥66	189 (15.4)

Positivity rates of ESAT-6 and CFP-10 were 84.8% (95% CI: 82.7%–86.8%) and 80.7% (95% CI: 78.4%–82.9%), respectively. The positivity rate of ESAT-6 testing was higher than that of CFP-10 testing ($\chi^2 = 13.59$, $P < .001$).

The PTB patient T cell response agreement rate between ESAT-6 and CFP-10 test results was 84.98% (82.85%–86.94%), with strong concordance observed between results (Gwet AC1 = 0.790, 95% CI: 0.758–0.822) ($P < .001$). Of the 184 patients with discordant results, a positive response to ESAT-6 was noted in 117 patients, while 67 had positive responses to CFP-10 (Table 2).

3.3. Interpretation of spot numbers induced by ESAT-6 and CFP-10 responses

Patients with positive TSPOT.*TB* results fell into 3 groups (ESAT-6 single positive: 117 cases; CFP-10 single positive: 67 cases; ESAT-6 and CFP-10 double positive: 922 cases). The median number of spots in each group is shown in Table 3. SFCs for CFP-10 and ESAT-6 microwells in each single positive group were not significantly different ($Z = 69.874$, $P > .99$) (Fig. 2) and SFCs results for double positive patients for the 2 antigens were also not statistically different ($Z = 51.361$, $P = .358$) (Fig. 2). Notably, spot numbers for ESAT-6 in the double positive group were greater than spot numbers for the ESAT-6 single positive group ($Z = 446.456$, $P < .001$) and for the CFP-10 single positive group ($Z = 516.330$, $P < .001$). Meanwhile, spot numbers for CFP-10 in the double positive group were greater than spot numbers for the ESAT-6 single positive group ($Z = 497.817$, $P < .001$) and for the CFP-10 single positive group ($Z = 567.692$, $P < .001$) (Fig. 2).

3.4. Factors associated with PTB patient discordant ESAT-6 and CFP-10 results

Multivariable logistic regression analysis showed that after adjustment for gender, smear, bacterial culture and other factors, factors significantly associated with discordant diagnostic ESAT-

Table 2

Number of positive and negative tests by response to ESAT-6 and CFP-10.

	CFP-10		
Antigen	Negative	Positive	Total
ESAT-6			
Negative	119	67	186
Positive	117	922	1039
Total	236	989	1225

CFP-10 = culture filtrate protein-10, ESAT-6 = early-secreted antigenic target-6kDa protein.

Table 3

The spot numbers in positive T-SPOT.*TB* assay.

Group	N	SFCs (IQR)	
		ESAT-6	CFP-10
ESAT-6 unique positive	117	18 (10,33)	2 (1,4)
CFP-10 unique positive	67	2 (1,2)	14 (8,26)
ESAT-6 and CPF-10 both positive	922	44 (21,82)	50 (20,105)

CFP-10 = culture filtrate protein-10, ESAT-6 = early-secreted antigenic target-6kDa protein, IQR = inter quartile range, SFCs = spot forming cells.

6 and CFP-10 test results included age ≥66 years (OR = 1.593, $P = .034$) and retreatment (OR = 2.166, $P < .001$) (Table 4).

4. Discussion

ESAT-6 and CFP-10, important MTB virulence factors, are the core antigens used in the T-SPOT.*TB* test.[19] These antigens are both encoded by the Region of Difference 1 (RD1) within the MTB genome and form heterodimers in a 1:1 ratio that are secreted by the ESX-1 secretion system.[7,20] In clinical practice, patients with pulmonary tuberculosis mount different T cell responses to antigens ESAT-6 and CFP-10. Therefore, here we investigated factors underlying the observed discordance between ESAT-6 and CFP-10 TSPOT.*TB* results to provide valuable insights for guiding clinical decision-making based on this test.

Our results demonstrated that the agreement rate between ESAT-6 and CFP-10 results was 84.98% (82.85%–86.94%) as evidence of strong results concordance (Gwet AC1 = 0.790, 95% CI: 0.758–0.822), as observed in a previous study.[21] However, we observed 117 ESAT-6 single positive and 67 CFP-10 single positive results overall for all patients, prompting us to consider that responses to ESAT-6 and CFP-10 antigens might exhibit heterogeneity when used for PTB diagnosis even though both antigens are encoded by the same MTB genomic region. Notably, here the observed ESAT-6 and CFP-10 positivity rates (84.8% and 80.7%, respectively) were lower than respective rates reported previously (95.1% and 83.7%).[22] This discrepancy may reflect inconsistencies in sample sizes, inclusion criteria and study populations; our study population originated from 5 provinces and municipalities of China and included a relatively high proportion of patients with hypoproteinemia that may have had false negative IGRA results.[23] Even so, the previous study also demonstrated a higher ESAT-6 positivity rate than that obtained for CFP-10 for active tuberculosis cases, suggesting that ESAT-6 had greater antigenic dominance than CFP-10 and triggered greater IFN-γ release, in accordance with other reported studies.[24–26] For example, 1 study conducted at nine locations assessed T cell reactivity to 59 MTB antigens and found that ESAT-6 showed higher immune-dominance than CFP-10.[24] In another study of ATB cases in the Netherlands, 92% of subjects were positive for T cell reactivity to ESAT-6 peptides, 89% were positive for reactivity to CFP-10 peptides and 86% were positive for both antigens.[21] Similar results were obtained in a Gambian study[25] and in a study by Hesseling et al who obtained higher mean values for T-SPOT.*TB* ESAT-6 responses relative to CFP-10 responses among household contacts of TB patients in South Africa, however, the opposite result was obtained using QFT-GIT,[26] while other studies have also reported results that contradict our findings. For example, in a study of adults with normal chest X-ray findings, more subjects responded to CFP-10 peptides than to ESAT-6 peptides as assessed using an ex vivo

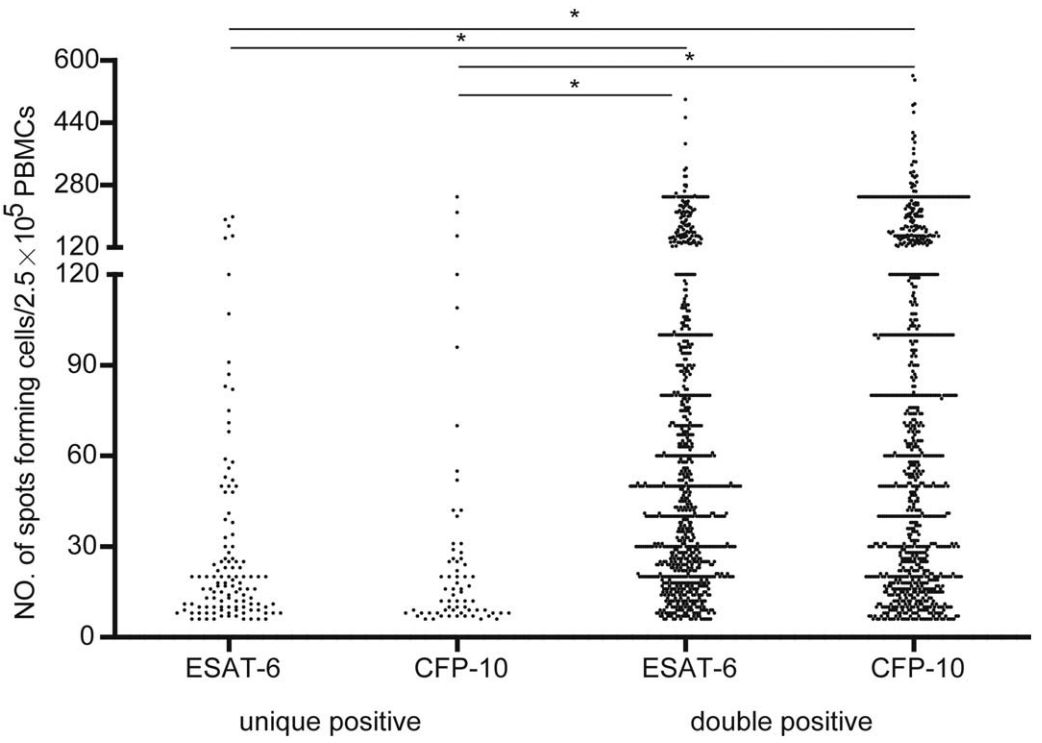

Figure 2. Positive T-SPOT.*TB* assay responses to ESAT-6 and CFP-10. CFP-10 = culture filtrate protein-10, ESAT-6 = early-secreted antigenic target-6kDa protein, PBMC = peripheral blood mononuclear cell. *P value <.01, adjusted by Bonferroni.

Table 4

Factors associated with discordance of ESAT-6 and CFP-10 in PTB patients.

Factors	Total N = 1225	No. (%)with discordance results	Univariate OR (95% CI)	Multivariate adjusted OR (95% CI)	P
Gender					
Male	831	134 (16.1)	Reference		
Female	394	50 (12.7)	0.756 (0.533–1.072)		
Age					
15–44years	656	82 (12.5)	Reference	Reference	
45–65years	380	63 (16.6)	1.391 (0.975–1.986)	1.300 (0.907–1.864)	.153
≥66years	189	39 (20.6)	1.820 (1.194–2.774)	1.593 (1.036–2.450)	.034
Smear					
Negative	536	83 (15.5)	Reference		
Positive	689	101 (14.7)	0.937 (0.684–1.285)		
Culture					
Negative	521	84 (16.1)	Reference		
Positive	704	100 (14.2)	0.861 (0.629–1.180)		
Lung cavity					
No	694	95 (13.7)	Reference		
Yes	531	89 (16.8)	1.270 (0.927–1.738)		
BMI					
<18.5	261	40 (15.3)	Reference		
≥18.5	964	144 (14.9)	0.970 (0.663–1.419)		
Contact of TB					
No	1159	171 (14.8)	Reference	Reference	<.001
Yes	66	13 (19.7)	1.417 (0.756–2.656)	2.166 (1.528–3.070)	
BCG					
No	456	75 (16.4)	Reference		
Yes	769	109 (14.2)	0.839 (0.609–1.155)		
Comorbidity					
No	622	91 (14.6)	Reference		
Yes	603	93 (15.4)	1.064 (0.778–1.456)		
Drinking					
No	1014	147 (14.5)	Reference		
Yes	211	37 (17.5)	1.254 (0.844–1.863)		
Smoking					
No	850	120 (14.1)	Reference		
Yes	375	64 (17.1)	1.252 (0.899–1.743)		
Retreatment*					
No	975	122 (12.5)	Reference		
Yes	250	62 (24.8)	2.306 (1.635–3.253)		
Course					
<1 month	223	26 (11.7)	Reference		
≥1month	1002	158 (15.8)	1.418 (0.911–2.209)		
Diabetes					
No	1068	159 (14.9)	Reference		
Yes	157	25 (15.9)	1.083 (0.684–1.714)		
Albumin					
Normal	914	129 (14.1)	Reference		
Decreased	311	55 (17.7)	1.307 (0.925–1.847)		

* Retreated cases are defined as those anti-TB treated ≥1 month in the past.

BCG = Bacillus Calmette-Guérin, BMI = body-mass index, CFP-10 = culture filtrate protein-10, ESAT-6 = early-secreted antigenic target-6kDa protein, PTB = pulmonary tuberculosis, TB = tuberculosis.

ELISPOT assay of IFN-γ release.[27] In a similar vein, Agarwal et al observed a significantly greater proportion of positive test results for CFP-10 than for ESAT-6 or both antigens at baseline, at 6 months and at 12 months among health care workers.[8] Nevertheless, geographic components and use of different test-specific antigen preparations partly explain these observed discrepancies, while other unknown factors related to specific mechanisms underlying host immune responses to ESAT-6 and CFP-10 or a combination of factors involving different host immune response pathways and response levels may be involved. For instance, it is found that different antigenic epitopes, including those within ESAT-6 and CFP-10, are recognized by specific HLA types.[28,29] Li et al demonstrated that human CD8+ T cells from TB pleurisy patients responded to 4 immunodominant epitopes of MTB CFP-10, with restrictions imposed by HLA-B alleles.[30] In addition, it is possible that different strains of MTB may influence antigen-specific positivity. A study conducted in Madagascar reported that ESAT-6-induced IFN-γ responses were lower in contacts of patients infected with Beijing and Central Asian family MTB strains than in contacts of patients infected with Haarlem, LAM, U, X, and S family MTB strains or ancient MTB strains.[31]

In our study, regression analyses showed that age ≥66 was significantly associated with discordant PTB patient T-SPOT.*TB*

ESAT-6 and CFP-10 responses. Previous studies had determined that the diagnostic efficacy of IGRAs was lower for elderly patients,[32,33] due to their decreased lymphocyte counts and weakened immune function that dampened production of MTB-specific IFN-γ and thus reduced IGRAs sensitivity.[33] Furthermore, elderly patients are often concomitantly afflicted with several diseases that may increase the complexity of their immune responses. Chee et al found that contacts positive for CFP-10 reactivity were significantly younger than those who tested positive for reactivity to ESAT-6 alone or in combination with CFP-10.[34] Meanwhile, Hesseling et al reported significantly different ESAT-6 and CFP-10 spot counts between adults and children as evidence for a possible age-related correlation with antigen response discordance.[26] Although we did not evaluate pediatric TB patients in this work, results of other studies provide support for a potential age-related correlation between antigen responses. At present, it is unclear which of the 2 antigens, ESAT-6 or CFP-10, can better elicit T cells to release interferon and trigger host immune responses in different patient age groups. Nevertheless, age-dependent immune maturation and suppression may be potential underlying mechanisms that partially explain these observations. In line with our hypothesis, when we compared spot numbers between single and double positive groups, our data revealed that spot numbers of the single positive group were lower than those in the double positive group, indicating that patients in the single positive group have poorer immune responses than those in the double positive group. Therefore, elderly patients with attenuated immune responses likely mount different responses to ESAT-6 and CFP-10 antigens.

Another reason for inconsistent results here stemmed from patient retreatment status. Li et al had shown that quantified SFCs numbers to ESAT-6 for previously treated cases were lower than numbers for new cases, while this phenomenon was not observed for CFP-10.[35] Similar results were seen in a study conducted in Korea.[36] Furthermore, several other studies have reported a significant decline in numbers of spots in the ESAT-6 panel during or after anti-TB treatment.[37–39] However, Chee et al demonstrated that treatment had a significant effect on the CFP-10 response but not on the ESAT-6 response.[34] It is thought that during chronic MTB infection, T cells may express an exhausted phenotype whereby the frequency of effector T cells falls as the mycobacterial antigen load declines with treatment, resulting in a progressive decrease of IFN-γ secretion.[34,40] Although the roles that ESAT-6 and CFP-10 proteins play in this process are not yet clear, differential effects of PTB patient treatment on T cell responses to individual antigens ultimately may reflect differences between ESAT-6 and CFP-10 antigenic functions or host immune responses to these antigens, although the specific mechanism warrants further exploration.

To our knowledge, this is the largest multicenter investigation conducted in China to directly compare active PTB case T-SPOT.*TB* positivity rates based on T cell responses to ESAT-6 and CFP-10 antigens and to analyze factors associated with discordant results. This work may help physicians better understand the implications of heterogeneous results for T-SPOT.*TB* antigens as applied to PTB diagnosis, especially in elderly and retreated patients. However, this study also has limitations. First, all patients enrolled in the study were in patients with relatively poor disease status, due to high rates of hypoproteinemia and other complications with possible effects on positivity rates. Second, we did not evaluate T-SPOT.*TB* effectiveness for diagnosing pediatric tuberculosis as only adults were studied; substantial differences have been reported between immune responses to MTB in children versus adults.[41,42] Third, the impact of nontuberculous mycobacterial (NTM) infection was not considered here even though patients infected with *M. kansasii, M. szulgai* or *M. marinum* may also have positive IGRA results, since NTM also harbor genes encoding ESAT-6 and CFP-10 antigens.[7]

In conclusion, ESAT-6 elicited significantly more positive T-SPOT.*TB* results than did CFP-10 in PTB patients. Age ≥66 years and retreatment were independently associated with discordant results for the 2 antigens. Assessment of factors underlying such discordance may improve PTB diagnosis based on T-SPOT.*TB* results.

Author contributions

Conceptualization: Wanli Kang, Shenjie Tang.
Data curation: Shengsheng Liu, Meiying Wu, Ertai A, Shucai Wu, Shujun Geng, Zhihui Li, Mingwu Li, Liang Li.
Formal analysis: Wanli Kang.
Investigation: Shengsheng Liu, Meiying Wu, Ertai A, Shucai Wu, Shujun Geng, Zhihui Li, Mingwu Li, Wanli Kang, Shenjie Tang.
Methodology: Yu Pang, Wanli Kang.
Supervision: Liang Li, Shenjie Tang.
Validation: Shengsheng Liu.
Writing – original draft: Shengsheng Liu.
Writing – review & editing: Yu Pang, Wanli Kang, Shenjie Tang.

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