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Combination of mean spot sizes of ESAT-6 spot-forming cells and modified tuberculosis-specific antigen/phytohemagglutinin ratio of T-SPOT.TB assay in distinguishing between active tuberculosis and latent tuberculosis infection

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SUMMARY

Objectives: Distinguishing between active tuberculosis (ATB) and latent tuberculosis infection (LTBI) remains challenging.
Methods: The modified T-SPOT.TB assay was performed in 499 participants (243 ATB and 256 LTBI) and another 322 participants (162 ATB and 160 LTBI) who were diagnosed in Qiaokou (training) and Caidian (validation) cohort respectively.
Results: The mean spot sizes (MSS) of early secreted antigenic target 6 (ESAT-6) spot-forming cells (SFC) of T-SPOT.TB assay in ATB patients was significantly higher than that in LTBI individuals. 1.0×10^5 was the optimal number of cells added to phytohaemagglutinin (PHA) well for obtaining more accurate TB-specific antigen to phytohaemagglutinin (TBAg/PHA) ratio. The area under the curve of the diagnostic model by combination of ESAT-6 SFC MSS and modified TBAg/PHA ratio in distinguishing ATB from LTBI was 0.959 in training cohort, with a sensitivity of 90.12% and a specificity of 91.02% when a cutoff value of 0.46 was used. This diagnostic model showed similar performance in the validation cohort. The area under the curve, sensitivity, and specificity were 0.962, 93.21%, and 90.00%, respectively. Further flow cytometry analysis showed that ESAT-6 stimulation induced a significantly higher mean fluorescence intensity of IFN-γ^+ cells in lymphocytes compared with culture filtrate protein 10 (CFP-10) stimulation. In contrast, CFP-10 stimulation induced a significantly higher percentage of IFN-γ^+ cells in lymphocytes compared with ESAT-6 stimulation.
Conclusions: The combination of the MSS of ESAT-6 SFC and the modified TBAg/PHA ratio of T-SPOT.TB assay showed great value in discriminating ATB from LTBI.

Introduction

Tuberculosis (TB) is one of the leading causes of death from a single infectious agent worldwide.[1] As reported by the World Health Organization, there are an estimated 10 million new cases of TB, resulting in almost 1.3 million deaths in the year 2017.[2] Besides, about 1.7 billion people, accounting for 23% of the world's population, are infected with *Mycobacterium tuberculosis* (Mtb).[2] Although most Mtb-infected individuals remain latent TB infection (LTBI) and are asymptomatic throughout their lifetimes, approximately 5–10% of them will progress to active tuberculosis (ATB).[2,3] Thus, the development of new and accurate methods for distinguishing ATB from LTBI is very important for TB management and control.

Acid-fast staining, Xpert MTB/RIF, and Mtb culture are most commonly used methods for the diagnosis of ATB. The

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sensitivities of these methods are unsatisfactory under low bacterial loads and the scope of application of them is not included for diagnosing LTBI.[4] Interferon-gamma (IFN-γ) release assays (IGRAs) including QuantiFERON-TB Gold In-Tube (QFT-GIT) and T-SPOT.TB (T-SPOT) can be used to diagnose Mtb infection, but both of them cannot distinguish ATB from LTBI.[5] QuantiFERON-TB Gold Plus (QFT-Plus), the second-generation IGRA which measures $CD8^+$ T cell response, was reported to have potential value in this field.[6] However, this approach still remains to be validated in future studies. Although recent studies have shown that genomics, transcriptomics, and proteomics are of value in distinguishing between ATB and LTBI,[7–16] these methods need either special equipment or complicated procedures, and the clinical feasibility of them also remains to be determined. Besides, our previous studies reported that the ratio of TB-specific antigen to phytohemagglutinin (TBAg/PHA ratio) in T-SPOT assay could be used to discriminate ATB from LTBI,[17,18] but there is a dispute that the number of PHA well cannot be accurately counted in some cases. So far, the differentiation of ATB and LTBI is still a major challenge in clinical practice.

In this study, we first describe a new diagnostic indicator-mean spot sizes (MSS) of early secreted antigenic target 6 (ESAT-6) spot-forming cells (SFC) in T-SPOT assay, and reveal that it has an adjunctive role in distinguishing ATB from LTBI. Moreover, we designed a modified T-SPOT assay with a decreased number of peripheral blood mononuclear cells (PBMCs) added to PHA well. The aim of this step is to achieve accurate counting of PHA SFC, which results in obtaining a more reliable TBAg/PHA ratio. Finally, our diagnostic model further shows that combination of ESAT-6 SFC MSS and modified TBAg/PHA ratio has prominent capability in differential diagnosis between ATB and LTBI.

Methods

Study participants

The present study was carried out from January 2017 to August 2019 at Tongji Hospital (Qiaokou cohort, the largest tertiary hospital in central China with 5000 beds) and Sino-French New City Hospital (Caidian cohort, a branch hospital of Tongji Hospital with 1500 beds). Participants in two cohorts were selected based on positive results for T-SPOT assay. Individuals with ATB were diagnosed as having positive results of Xpert MTB/RIF (Cepheid, Sunnyvale, USA) and/or Mtb culture (Mycobacterial Growth Indicator Tube 960 and Lowenstein-Jensen media) in sputum, bronchoalveolar lavage fluid, or biopsy tissue, with clinical symptoms and radiological characteristics suggestive of TB. Individuals with LTBI were defined as healthy people with positive T-SPOT results but without clinical or radiological evidence of ATB. Participants with the following conditions were excluded: (1) age $>$ 70 or $<$ 18 years, (2) HIV-positive, (3) undergoing anti-TB treatment for more than 2 weeks, and (4) receiving immunosuppressive treatment. Ethics approval was provided by the Ethics Committee of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology. All participants provided written informed consent at enrollment.

The modified T-SPOT assay

Blood samples were collected from all recruited participants for modified T-SPOT assay. The procedures of modified T-SPOT assay were basically same as commercial T-SPOT assay (Oxford Immunotec, Oxford, UK), with a small change (another PHA well in which 1.0×10^5 PBMCs were added). Briefly, PBMCs were isolated and added to 96-well plates precoated with anti-IFN-γ antibody. Five wells were used for each patient. There are four wells in which 2.5×10^5 PBMCs were added and stimulated as T-SPOT assay: a positive control well in which PHA was added, a negative control well that contained the medium and two antigen wells that contained ESAT-6 or culture filtrate protein 10 (CFP-10) peptide pools. The fifth well was a modified PHA well in which 1.0×10^5 PBMCs was added. Plates were incubated for 16 h at 37 °C with 5% carbon dioxide, washed with phosphate buffered saline and developed using an anti-IFN-γ antibody conjugate and substrate to detect the presence of secreted IFN-γ. Spots were counted and the MSS of them were calculated, with an automated ELISpot reader (CTL Analyzers, Cleveland, OH, USA). Positive and negative results were defined the same as T-SPOT assay. Results were considered undetermined if the spot amounts in the positive control were $<$ 20 or $>$ 10 in the negative control.

The modified TBAg/PHA ratio

The ratios of (1) ESAT-6 SFC to modified PHA SFC, and (2) CFP-10 SFC to modified PHA SFC were calculated. The larger of the above two values was defined as the modified TBAg/PHA ratio of one patient.

Cell staining and flow cytometry analysis

PBMCs isolated from ATB patients or LTBI individuals were stimulated with ESAT-6 or CFP-10 peptides for 24 h. After stimulation, PBMCs were collected and monoclonal antibodies against the following antigens were added to the cell suspensions: CD45, CD3, CD4, CD8, and CD56 (BD Pharmingen, San Diego, CA, USA), Isotype controls with irrelevant specificities were included as negative controls. All the cell suspensions were incubated for 30 min at room temperature. For intracellular staining, the cells were collected after surface staining. After washing, the cells were fixed and permeabilized and stained with anti-IFN-γ monoclonal antibodies (eBioscience, San Diego, CA, USA). After washings, the pellets were resuspended in 200 µl PBS buffer and analyzed by FACSCanto flow cytometer (Becton Dickinson, San Jose, CA, USA). The different cell subsets were gated as the following criteria: (1) lymphocytes were characterized as $CD45^+$ subset; (2) $CD4^+$ and $CD8^+$ T cells were characterized as $CD45^+CD3^+CD4^+$ and $CD45^+CD3^+CD8^+$ subsets; (3) NK cells were gated as $CD45^+CD3^-CD56^+$ subsets. Data analysis was performed using FlowJo version 7.6.1 software (TreeStar, Ashland, OR, USA).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 6.0 (GraphPad, La Jolla, CA, USA). The Mann-Whitney *U* test and Wilcoxon test were used to compare groups of continuous variables. The chi-square test was used for comparison of categorical data. The cut-off values of different variables for distinguishing ATB from LTBI were selected using receiver operating characteristic (ROC) curve analysis. Area under the curve (AUC), sensitivity and specificity were reported. Statistical significance was determined as $P < 0.05$.

SPSS 19.0 (SPSS, Chicago, IL, USA) was used to conduct the Lasso logistic regression model analysis. A diagnostic model incorporating ESAT-6 SFC MSS and modified TBAg/PHA ratio was developed based on the regression coefficients of multivariate logistic regression.

Results

Participants characteristics

On the basis of clinical and laboratory findings, 499 (243 ATB and 256 LTBI) and 322 (162 ATB and 160 LTBI) individuals

Table 1
Demographic and clinical characteristics of study participants.

Variables	Qiaokou (training) cohort ($n=499$)		Caidian (validation) cohort ($n=322$)		P^*
	ATB ($n=243$)	LTBI ($n=256$)	ATB ($n=162$)	LTBI ($n=160$)	
Sex, male, %	63.37	66.8	62.96	69.38	0.822
Age (mean ± SD), years	47.58 ± 16.77	46.83 ± 13.95	48.43 ± 13.71	46.42 ± 12.06	0.873
Presence of BCG scar	112 (46.09%)	90 (35.16%)	68 (41.98%)	60 (38.75%)	0.884
TB history	53 (21.81%)	0 (0.00%)	40 (24.69%)	0 (0.00%)	0.432
Positive mycobacterial culture	198 (81.48%)	NA	132 (81.48%)	NA	NA
Positive Xpert MTB/RIF	180 (74.07%)	NA	122 (75.31%)	NA	NA

ATB, active tuberculosis; LTBI, latent tuberculosis infection; SD, standard deviation; BCG, bacille Calmette-Guérin; TB, tuberculosis; NA, not applicable. *Comparisons were performed between Qiaokou and Caidian cohorts using chi-square test or Mann-Whitney *U* test. Data were presented as means ± SD, percentages, or number (percentages).

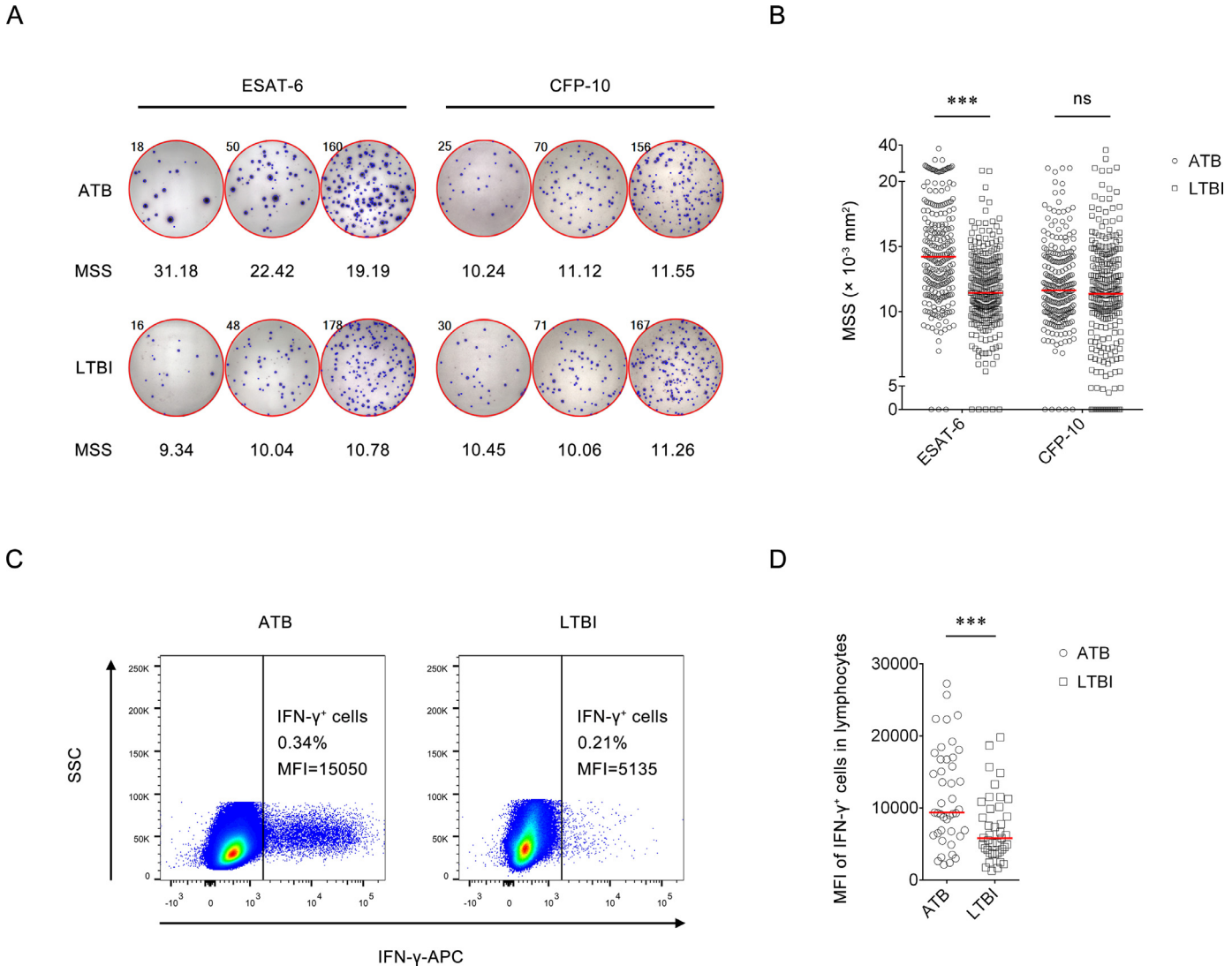

Fig. 1. ESAT-6 and CFP-10 SFC MSS in ATB and LTBI. (A) Representative pictures showing the MSS of ESAT-6 and CFP-10 SFC in ATB patients and LTBI individuals. The number in the upper left corner of each graph indicates the number of SFC in each well. (B) Scatter plots showing ESAT-6 and CFP-10 SFC MSS in ATB patients ($n=243$) and individuals with LTBI ($n=256$) in Qiaokou cohort. Horizontal lines indicate the median. ***$P < 0.001$; ns, no significance (Mann–Whitney *U* test). (C) Representative FACS plots showing the MFI of IFN-γ^+ cells in lymphocyte in ATB and LTBI under ESAT-6 stimulation. (D) Scatter plots showing ESAT-6 SFC MSS in ATB patients ($n=43$) and LTBI individuals ($n=41$). Horizontal lines indicate the median. ***$P < 0.001$ (Mann–Whitney *U* test). ATB, active tuberculosis; LTBI, latent tuberculosis infection; ESAT-6, early secreted antigenic target 6; CFP-10, culture filtrate protein 10; SFC, spot-forming cells; MSS, mean spot sizes; MFI, mean fluorescence intensity.

were recruited in Qiaokou (training) and Caidian (validation) cohort, respectively. The demographic and clinical characteristics of the ATB and LTBI individuals in two cohorts were summarized in Table 1.

The MSS of ESAT-6 and CFP-10 SFC in ATB and LTBI

It was more common to find big spots in ESAT-6 rather than CFP-10 antigen well. Fig. 1A showed that three representative ATB

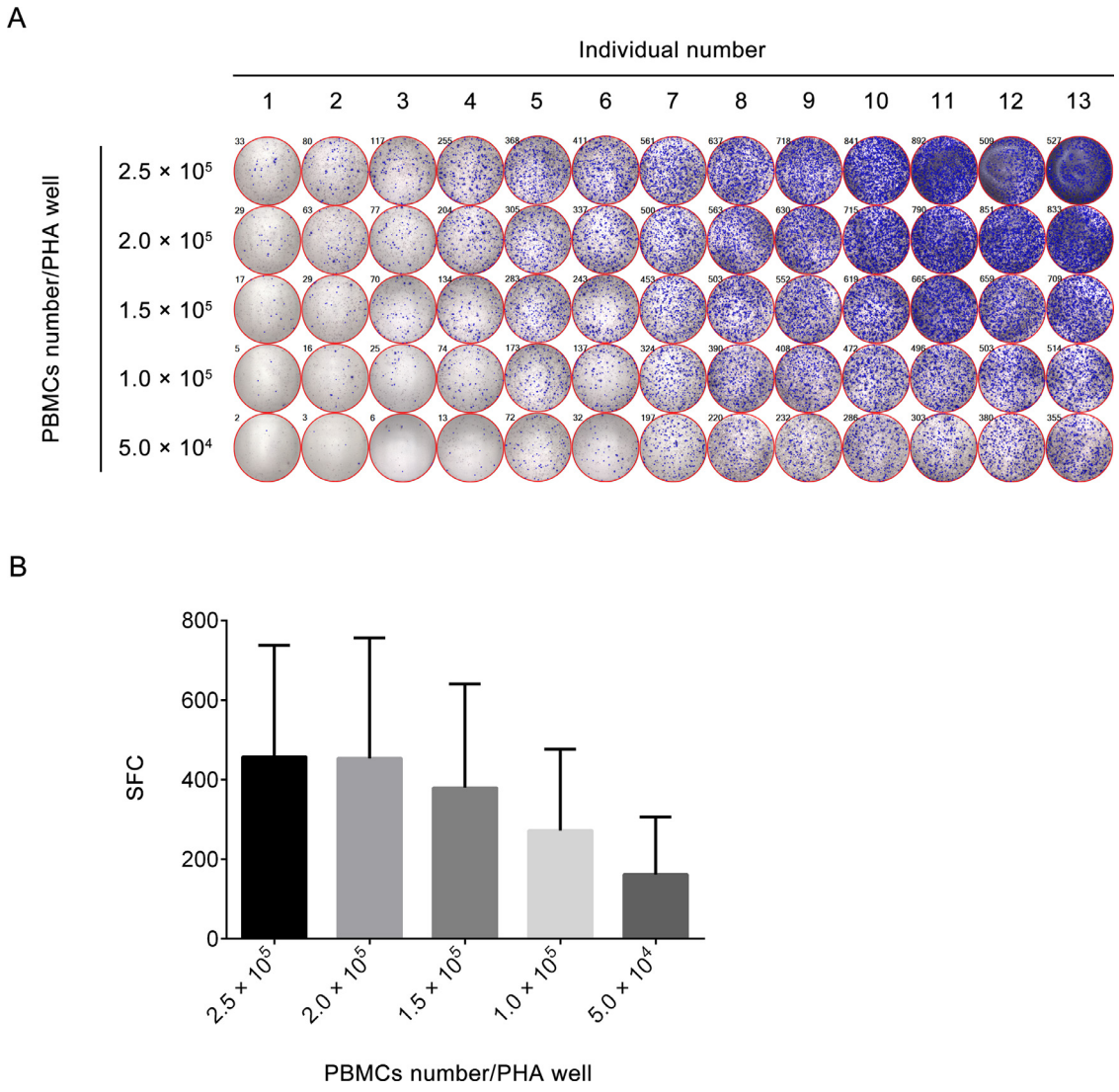

Fig. 2. Determination of the optimal number of PBMCs added into modified PHA well. (A) Representative pictures showing the SFC results in PHA well when different number of PBMCs (2.5×10^5, 2.0×10^5, 1.5×10^5, 1.0×10^5, and 5×10^4) were added into the well. The number in the upper left corner of each graph indicates the number of SFC in each well. (B) Histograms showing the SFC results in PHA well when different number of PBMCs (2.5×10^5, 2.0×10^5, 1.5×10^5, 1.0×10^5, and 5×10^4) were added into the well. Data are shown as the means ± SD. PBMCs, peripheral blood mononuclear cells; SFC, spot-forming cells; PHA, phytohemagglutinin.

patients had obviously bigger ESAT-6 spot than LTBI individuals. The MSS of ESAT-6 SFC in ATB patients was significantly higher than that in LTBI individuals, while no significant difference was found in CFP-10 SFC MSS between these two groups (Fig. 1B). We also used flow cytometry to analyze the production of IFN-γ in lymphocytes after ESAT-6 stimulation. We found the mean fluorescence intensity (MFI) of IFN-γ^+ cells in ATB patients was significantly higher than in LTBI individuals (Fig. 1C, D).

Determination of the optimal cell number in PHA well

The optimal number of PBMCs in PHA well was determined for obtaining accurate TBAg/PHA ratio. Representative figures showed the possible PHA SFC results in most clinical cases (Fig. 2). We found if adding 2.5×10^5 PBMCs to a well, it was difficult for ELISpot reader to count PHA SFC accurately in some cases because too many spots are crowded in a well (individual 11–13). After decreasing the number of cells added to PHA well, the number of PHA SFC was gradually decreased. Ultimately, we used 1.0×10^5 as the optimal number of PBMCs added to PHA well (5×10^4, too low SFC, individual 1–2; 1.5×10^5, 2.0×10^5 and 2.5×10^5, too many SFC, individual 11–13). The modified PHA SFC results were defined as the PHA SFC results under 1.0×10^5 PBMCs added to PHA well.

Establishing diagnostic model based on combination of ESAT-6 SFC MSS and modified TBAg/PHA ratio in Qiaokou cohort

The number of modified PHA SFC in ATB patients was significantly lower than in LTBI individuals (Fig. 3A). The modified ESAT-6/PHA ratio, CFP-10/PHA ratio, and TBAg/PHA ratio in ATB patients were significantly higher than those in LTBI individuals (Fig. 3B). ROC analysis showed that the AUC of the modified ESAT-6/PHA ratio was 0.866 (95% CI, 0.835 to 0.898), with a sensitivity of 62.55% and a specificity of 91.80% when a cutoff value of 0.39 was used to differentiate ATB from LTBI. The AUC of the modified CFP-10/PHA ratio was 0.889 (95% CI, 0.860 to 0.919), with a sensitivity of 67.49% and a specificity of 93.36% when a threshold value of 0.48 was used. Moreover, with a cut-off value of 0.53, the modified TBAg/PHA ratio had AUC of 0.925 (95% CI, 0.903 to 0.948) with a sensitivity of 75.72% and a specificity of 91.41% (Table 2, Fig. 3C).

We also analyzed the diagnostic value of ESAT-6 SFC MSS in ATB patients. ROC analysis showed that the performance of ESAT-6

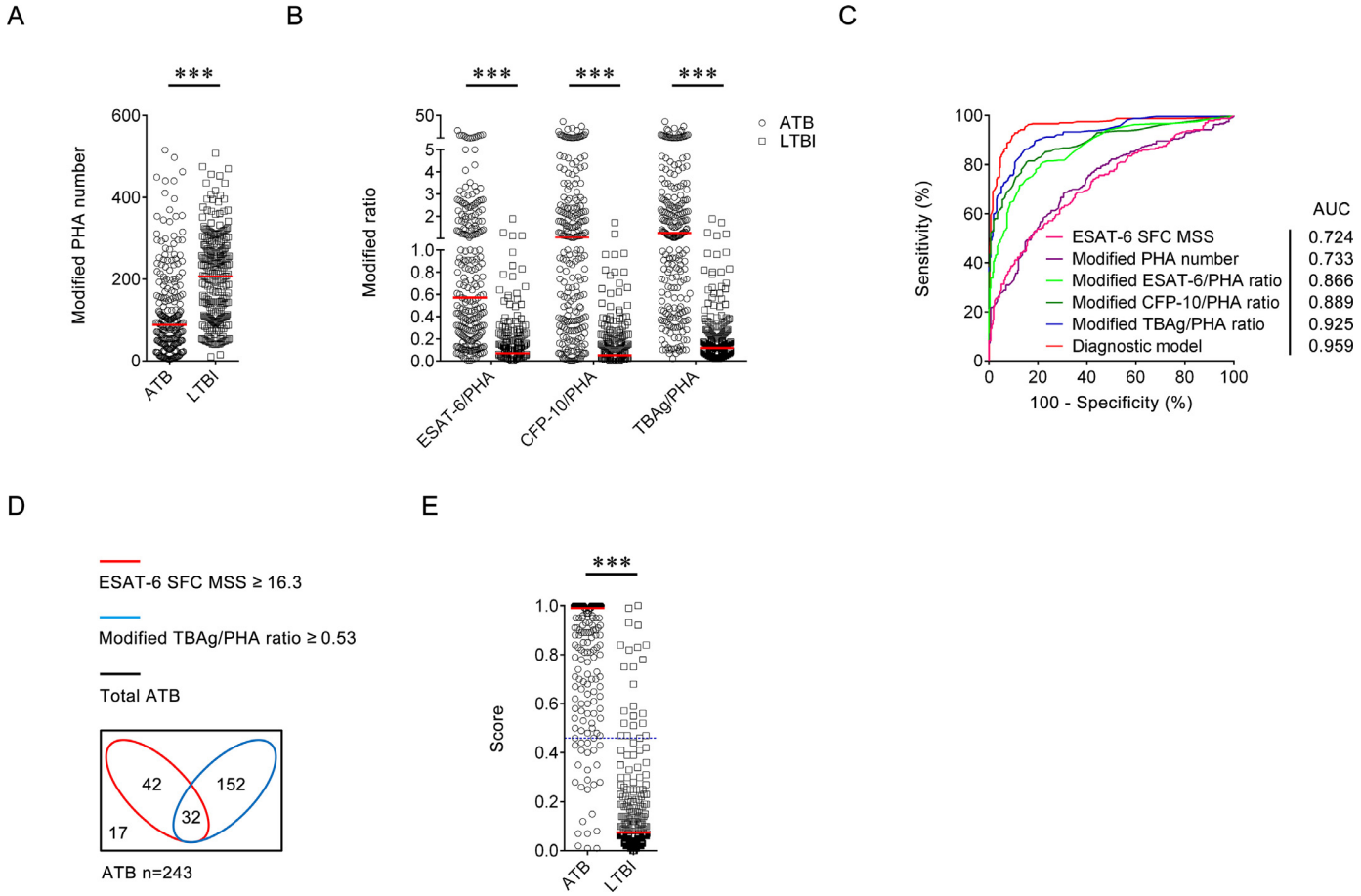

Fig. 3. Establishment of diagnostic model based on combination of ESAT-6 SFC MSS and modified TBAg/PHA ratio in Qiaokou cohort. (A) Scatter plots showing modified PHA number in ATB patients ($n = 243$) and LTBI individuals ($n = 256$) in Qiaokou cohort. Horizontal lines indicate the median. $^{***}P < 0.001$ (Mann–Whitney U test). (B) Scatter plots showing modified ESAT-6/PHA ratio, CFP-10/PHA ratio, and TBAg/PHA ratio in ATB patients ($n = 243$) and LTBI individuals ($n = 256$) in Qiaokou cohort. Horizontal lines indicate the median. $^{***}P < 0.001$ (Mann–Whitney U test). (C) ROC analysis showing the performance of ESAT-6 SFC MSS, modified PHA number, modified ESAT-6/PHA ratio, modified CFP-10/PHA ratio, modified TBAg/PHA ratio, and diagnostic model in distinguishing ATB from LTBI in Qiaokou cohort. (D) Venn diagrams showing the overlap of ESAT-6 SFC MSS and modified TBAg/PHA ratio in ATB patients ($n = 243$) in Qiaokou cohort. (E) Scatter plots showing the score of diagnostic model in ATB patients ($n = 243$) and LTBI individuals ($n = 256$) in Qiaokou cohort. Horizontal lines indicate the median. $^{***}P < 0.001$ (Mann–Whitney U test). Blue dotted lines indicate the cutoff values in distinguishing these two groups. ATB, active tuberculosis; LTBI, latent tuberculosis infection; ESAT-6, early secreted antigenic target 6; CFP-10, culture filtrate protein 10; MSS, mean spot sizes; PHA, phytohemagglutinin; SFC, spot-forming cells; AUC, area under the curve; modified ESAT-6/PHA ratio, the ratio of ESAT-6 to modified PHA; modified CFP-10/PHA ratio, the ratio of CFP-10 to modified PHA; modified TBAg/PHA ratio, the ratio of TB-specific antigen to modified PHA.

Table 2
Diagnostic efficiencies of different methods in distinguishing between ATB and LTBI in Qiaokou cohort.

Methods	Cutoff value	AUC (95% CI)	Sensitivity (%) (95% CI)	Specificity (%) (95% CI)	PPV (%) (95% CI)	NPV (%) (95% CI)	PLR (95% CI)	NLR (95% CI)
ESAT-6 SFC MSS ($\times 10^{-3}$ mm^2)	16.3	0.724 (0.680 to 0.768)	30.45 (24.82 to 36.72)	94.92 (91.27 to 97.15)	85.06 (75.44 to 91.49)	58.98 (54.05 to 63.74)	6.00 (3.42 to 10.53)	0.73 (0.67 to 0.80)
Modified PHA number	53	0.733 (0.689 to 0.777)	31.28 (25.58 to 37.57)	91.41 (87.11 to 94.41)	77.55 (67.79 to 85.11)	58.35 (53.35 to 63.20)	3.64 (2.34 to 5.66)	0.75 (0.69 to 0.82)
Modified ESAT-6/PHA ratio	0.39	0.866 (0.835 to 0.898)	62.55 (56.11 to 68.59)	91.80 (87.56 to 94.73)	87.86 (81.83 to 92.16)	72.09 (66.82 to 76.82)	7.63 (5.00 to 11.62)	0.41 (0.35 to 0.48)
Modified CFP-10/PHA ratio	0.48	0.889 (0.860 to 0.919)	67.49 (61.16 to 73.26)	93.36 (89.39 to 95.97)	90.61 (85.15 to 94.27)	75.16 (69.96 to 79.73)	10.16 (6.37 to 16.22)	0.35 (0.29 to 0.42)
Modified TBAg/PHA ratio	0.53	0.925 (0.903 to 0.948)	75.72 (69.74 to 80.87)	91.41 (87.11 to 94.41)	89.32 (84.08 to 93.04)	79.86 (74.72 to 84.21)	8.81 (5.87 to 13.22)	0.27 (0.21 to 0.33)
Diagnostic model*	0.46	0.959 (0.941 to 0.977)	90.12 (85.49 to 93.44)	91.02 (86.65 to 94.10)	90.50 (85.91 to 93.75)	90.66 (86.26 to 93.80)	10.03 (6.78 to 14.85)	0.11 (0.07 to 0.16)

ATB, active tuberculosis; LTBI, latent tuberculosis infection; ESAT-6, early secreted antigenic target 6; CFP-10, culture filtrate protein 10; SFC, spot-forming cells; MSS, mean spot sizes; PHA, phytohemagglutinin; AUC, area under the curve; CI, confidence interval; PPV, positive predictive value; NPV, negative predictive value; PLR, positive likelihood ratio; NLR, negative likelihood ratio.*Combination of ESAT-6 SFC MSS and modified TBAg/PHA ratio.

SFC MSS was very limited in distinguishing ATB from LTBI. The AUC of ESAT-6 SFC MSS was 0.724 (95% CI, 0.680 to 0.768), with a sensitivity of 30.45% and a specificity of 94.92% when a threshold value of 16.3 was used (Table 2, Fig. 3C). However, the overlap between modified TBAg/PHA ratio and ESAT-6 SFC MSS showed that ESAT-6 SFC MSS had a useful adjunctive role in diagnosing ATB patients (Fig. 3D). Another 42 (17.28%) ATB patients were detected by using ESAT-6 SFC MSS in patients with modified TBAg/PHA ratio < 0.53.

We further established a diagnostic model based on combination of ESAT-6 SFC MSS and modified TBAg/PHA ratio. $P = 1/[1 + e^{-(-8.058 + 4.791 \times \text{modified TBAg/PHA ratio} + 0.406 \times \text{ESAT-6 SFC MSS})}]$ P, predictive value; e, natural logarithm. The AUC of this diagnostic model reached up to 0.959 (95% CI, 0.941 to 0.977), with a sensitivity of 90.12% and a specificity of 91.02% (Table 2, Fig. 3C). When the cutoff value of 0.46 was used, the proportions of false-negative ATB patients and false-positive LTBI individuals were 24/243, 23/256 (Fig. 3E).

The difference between ESAT-6 and CFP-10 stimulation

In T-SPOT assay, the number and spot size were completely different between ESAT-6 and CFP-10 SFC in ATB patients. The number of CFP-10 SFC was found significantly higher than that of ESAT-6 SFC, while the MSS of ESAT-6 SFC was found significantly higher than that of CFP-10 SFC (Fig. 4A, B). However, neither the number nor the spot size showed difference between ESAT-6 and CFP-10 SFC in LTBI individuals (Fig. 4A, B). A further flow cytometry analysis found that CFP-10 stimulation induced a significantly higher percentage of IFN-γ^+ cells in lymphocytes, especially in CD8$^+$ T cells, compared with ESAT-6 stimulation. In contrast, ESAT-6 stimulation induced a significantly increased MFI of IFN-γ^+ cells in lymphocytes compared with CFP-10 stimulation (Fig. 4C–F).

Validation of diagnostic model in Caidian cohort

We performed another blinded validation study in an independent population in Caidian cohort. Similarly, both the MSS of ESAT-6 SFC and modified TBAg/PHA ratio in ATB patients were significantly higher than those in LTBI individuals (Fig. 5A–C). The overlap between ESAT-6 SFC MSS and modified TBAg/PHA ratio in ATB patients was shown in Fig. 5E. The diagnostic model established by combination of these two parameters also showed excellent discrimination between ATB and LTBI. Using the cut-off value of 0.46, the AUC of the diagnostic model was 0.962 (95%CI, 0.940 to 0.983), with a sensitivity of 93.21% and a specificity of 90.00% (Fig. 5D; Table 3). The proportions of false-negative ATB patients and false-positive LTBI individuals were 11/162 and 16/160 (Fig. 5F) in the validation cohort.

Discussion

TB is still the leading infectious disease killer globally.[1] Rapidly distinguishing between ATB and LTBI is a key pillar of TB control. China is a TB-endemic country with a high TB incidence and a large number of LTBI individuals.[19–21] It is essential to find out ATB patients from Mtb-infected populations, which is important for selecting the right patients for initiating anti-TB treatment.[22,23] Such an initiative can help improve patients' prognosis, and it is also required to achieve the WHO End TB Strategy targets.[24] However, existing methods, including traditional tests or emerging immunological or metabolomic technologies, lack sufficient ability to solve this problem.[25–29] The development of sensitive, specific, and effective strategies in the differential diagnosis of ATB in high TB prevalence countries is becoming a global priority.[1,2,4,13,30–32]

As one of two commercially available IGRAs, T-SPOT has been extensively applied worldwide as a diagnostic tool for Mtb

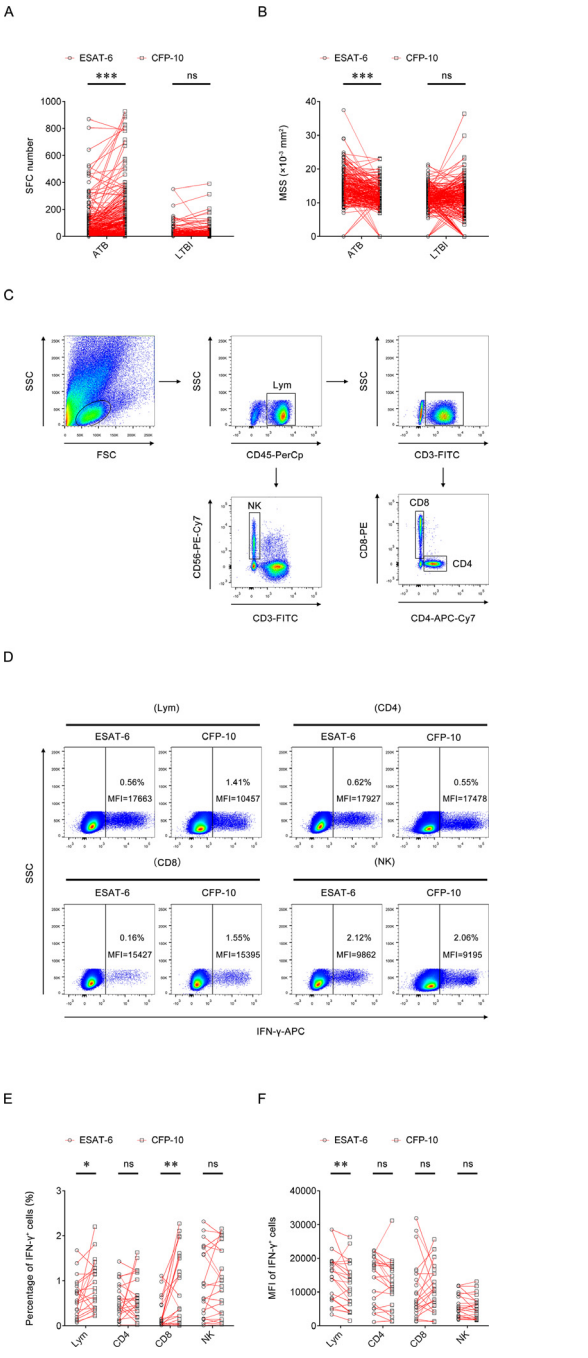

Fig. 4. The difference between ESAT-6 and CFP-10 stimulation. (A) Line graphs showing the results of ESAT-6 and CFP-10 SFC number for each individual in T-SPOT in ATB patients ($n = 243$) and LTBI individuals ($n = 256$) in Qiaokou cohort. One line represents one individual. $^{***}P < 0.001$; ns, no significance (Wilcoxon test). (B) Line graphs showing the results of ESAT-6 and CFP-10 SFC MSS for each individual in T-SPOT in ATB patients ($n = 243$) and LTBI individuals ($n = 256$) in Qiaokou cohort. One line represents one individual. $^{***}P < 0.001$; ns, no significance (Wilcoxon test). (C) Gating strategy used to sort lymphocytes, CD4$^+$ T cells, CD8+ T cells, and NK cells from peripheral blood in ATB patients. (D) Representative FACS plots showing the percentages and MFI of IFN-γ^+ cells in lymphocytes, CD4$^+$ T cells, CD8+ T cells, and NK cells from peripheral blood in ATB patient under ESAT-6 and CFP-10 stimulation. (E) Line graphs showing the percentages of IFN-γ^+ cells in lymphocytes, CD4$^+$ T cells, CD8+ T cells, and NK cells in ATB patients ($n = 22$) under ESAT-6 and CFP-10 stimulation. One line represents one individual. $^{*}P < 0.05$; $^{**}P < 0.01$; ns, no significance (Wilcoxon test). (F) Line graphs showing the MFI of IFN-γ^+ cells in lymphocytes, CD4+ T cells, CD8+ T cells, and NK cells in ATB patients ($n = 22$) under ESAT-6 and CFP-10 stimulation. One line represents one individual. $^{**}P < 0.01$; ns, no significance (Wilcoxon test). ATB, active tuberculosis; LTBI, latent tuberculosis infection; ESAT-6, early secreted antigenic target 6; CFP-10, culture filtrate protein 10; MSS, mean spot sizes; SFC, spot-forming cells; Lym, lymphocyte; MFI, mean fluorescence intensity.

Table 3
Diagnostic efficiencies of different methods in distinguishing between ATB and LTBI in Caidian cohort.

Methods	Cutoff value	AUC (95% CI)	Sensitivity (%) (95% CI)	Specificity (%) (95% CI)	PPV (%) (95% CI)	NPV (%) (95% CI)	PLR (95% CI)	NLR (95% CI)
ESAT-6 SFC MSS ($\times 10^{-3}$ mm^2)	16.3	0.701 (0.644 to 0.758)	33.33 (26.25 to 41.22)	90.00 (84.01 to 93.99)	77.14 (65.28 to 85.99)	57.14 (50.77 to 63.29)	3.33 (2.00 to 5.57)	0.74 (0.66 to 0.83)
Modified PHA number	53	0.772 (0.722 to 0.822)	32.10 (25.11 to 39.95)	93.75 (88.49 to 96.79)	83.87 (71.87 to 91.59)	57.69 (51.42 to 63.73)	5.14 (2.71 to 9.75)	0.73 (0.65 to 0.81)
Modified ESAT-6/PHA ratio	0.39	0.884 (0.847 to 0.923)	70.37 (62.61 to 77.15)	90.00 (84.01 to 93.99)	87.69 (80.50 to 92.58)	75.00 (68.15 to 80.83)	7.04 (4.37 to 11.32)	0.33 (0.26 to 0.42)
Modified CFP-10/PHA ratio	0.48	0.915 (0.883 to 0.946)	76.54 (69.12 to 82.68)	91.25 (85.48 to 94.95)	89.86 (83.26 to 94.14)	79.35 (72.63 to 84.81)	8.75 (5.27 to 14.53)	0.26 (0.19 to 0.34)
Modified TBAg/PHA ratio	0.53	0.947 (0.925 to 0.968)	82.72 (75.81 to 88.02)	90.63 (84.74 to 94.48)	89.93 (83.66 to 94.06)	83.82 (77.28 to 88.80)	8.82 (5.42 to 14.36)	0.19 (0.14 to 0.27)
Diagnostic model*	0.46	0.962 (0.940 to 0.983)	93.21 (87.87 to 96.39)	90.00 (84.01 to 93.99)	90.42 (84.65 to 94.25)	92.90 (87.35 to 96.23)	9.32 (5.85 to 14.86)	0.08 (0.04 to 0.13)

ATB, active tuberculosis; LTBI, latent tuberculosis infection; ESAT-6, early secreted antigenic target 6; CFP-10, culture filtrate protein 10; SFC, spot-forming cells; MSS, mean spot sizes; PHA, phytohemagglutinin; AUC, area under the curve; CI, confidence interval; PPV, positive predictive value; NPV, negative predictive value; PLR, positive likelihood ratio; NLR, negative likelihood ratio.*Combination of ESAT-6 SFC MSS and modified TBAg/PHA ratio.

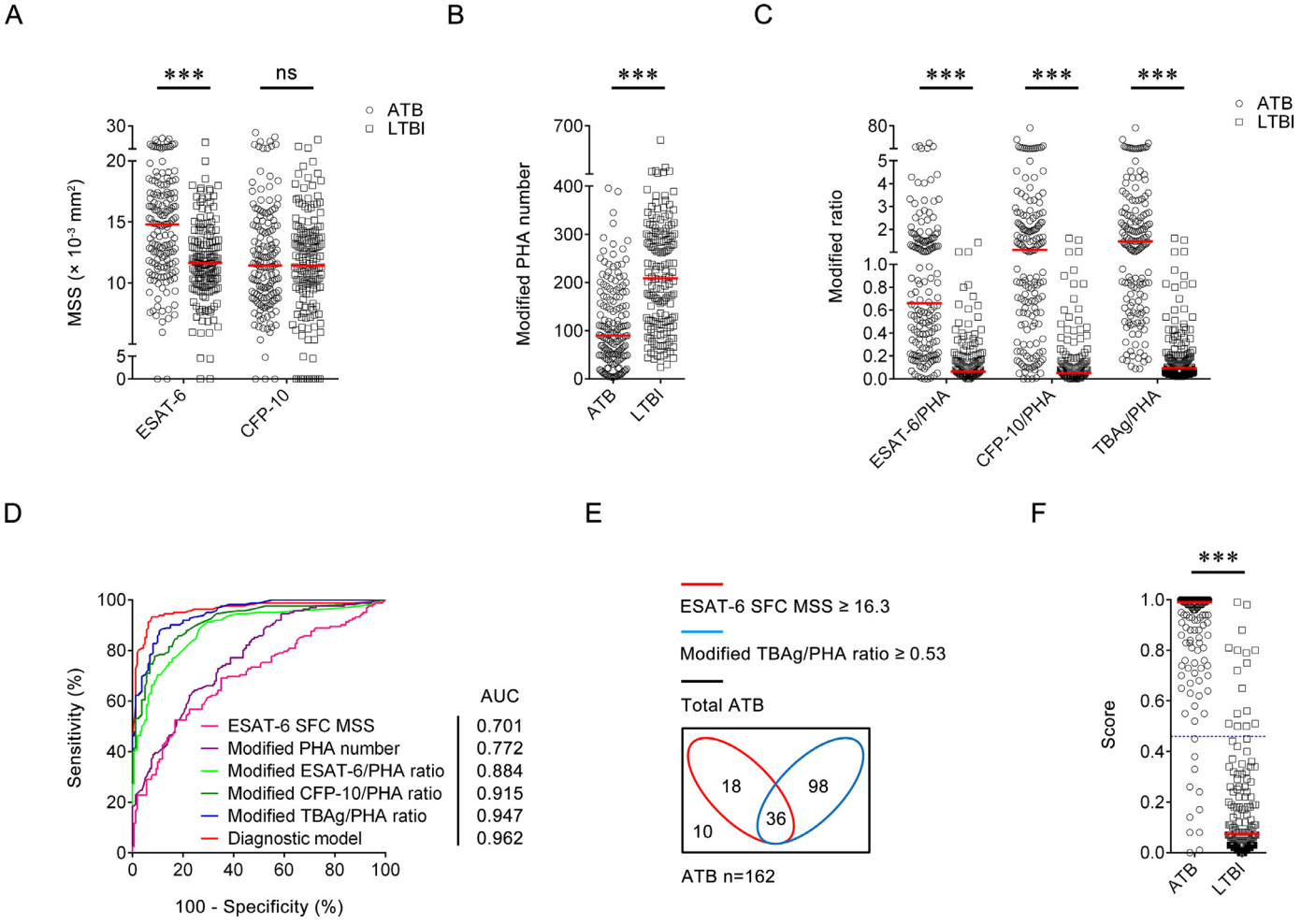

Fig. 5. Validation of diagnostic model based on combination of ESAT-6 SFC MSS and modified TBAg/PHA ratio in Caidian cohort. (A) Scatter plots showing ESAT-6 and CFP-10 SFC MSS in ATB patients ($n = 162$) and individuals with LTBI ($n = 160$) in Caidian cohort. Horizontal lines indicate the median. $^{***}P < 0.001$; ns, no significance (Mann–Whitney U test). (B) Scatter plots showing modified PHA number in ATB patients ($n = 162$) and LTBI individuals ($n = 160$) in Caidian cohort. Horizontal lines indicate the median. $^{***}P < 0.001$ (Mann–Whitney U test). (C) Scatter plots showing modified ESAT-6/PHA ratio, modified CFP-10/PHA ratio, and modified TBAg/PHA ratio in ATB patients ($n = 162$) and LTBI individuals ($n = 160$) in Caidian cohort. Horizontal lines indicate the median. $^{***}P < 0.001$ (Mann–Whitney U test). (D) ROC analysis showing the performance of ESAT-6 SFC MSS, modified PHA number, modified ESAT-6/PHA ratio, modified CFP-10/PHA ratio, modified TBAg/PHA ratio, and diagnostic model in distinguishing ATB from LTBI in Caidian cohort. (E) Venn diagrams showing the overlap of ESAT-6 SFC MSS and modified TBAg/PHA ratio in ATB patients ($n = 162$) in Caidian cohort. (F) Scatter plots showing the score of diagnostic model in ATB patients ($n = 162$) and LTBI individuals ($n = 160$) in Caidian cohort. Horizontal lines indicate the median. $^{***}P < 0.001$ (Mann-Whitney U test). Blue dotted lines indicate the cutoff values in distinguishing these two groups. ATB, active tuberculosis; LTBI, latent tuberculosis infection; ESAT-6, early secreted antigenic target 6; CFP-10, culture filtrate protein 10; MSS, mean spot sizes; PHA, phytohemagglutinin; SFC, spot-forming cells; AUC, area under the curve; modified ESAT-6/PHA ratio, the ratio of ESAT-6 to modified PHA; modified CFP-10/PHA ratio, the ratio of CFP-10 to modified PHA; modified TBAg/PHA ratio, the ratio of TB-specific antigen to modified PHA.

infection. Nearly all studies focused on the application of the number of spots in antigen wells, and few studies pay attention to the size of spots or the use of PHA well. Our team has reported that the TBAg/PHA ratio shows potential value in distinguishing between ATB and LTBI.[18,33] In this study, we first reported that the size of TB-specific antigen ESAT-6 has an adjunctive role in the diagnosis of ATB. Besides, combination of ESAT-6 SFC MSS and modified TBAg/PHA ratio shows a high accuracy in differential diagnosis between ATB and LTBI.

There are two innovations in our study. One is that we use a modified PHA well in which 1.0×10^5 PBMCs were added. This step can ensure the accuracy of PHA counting. This improvement further ensures the reliable use of the modified TBAg/PHA ratio. Another is that we demonstrated that the spot size of ESAT-6 but not CFP-10 could be used to distinguish ATB from LTBI. Although using ESAT-6 SFC MSS alone had a very limited sensitivity in distinguish ATB from LTBI, combining it with other indictors could improve diagnostic accuracy. The sensitivity could be increased by about 10% by combination of ESAT-6 SFC MSS and modified TBAg/PHA ratio (from 75% to 90% in Qiaokou cohort; from 82% to 93% in Caidian cohort). We further determined the scope of application of ESAT-6 SFC MSS and found that its diagnostic performance in low modified TBAg/PHA ratio group was obviously better than that in high modified TBAg/PHA ratio group (Supplementary Table 1, Supplementary Fig. 1). This could be used to explain why ESAT-6 SFC MSS has an adjunctive role in the diagnosis of ATB. Meanwhile, we found that the differential diagnostic performance relies heavily on the differences in modified PHA SFC between patients with ATB and LTBI individuals. Our previous studies have proved that PHA SFC could reflect the immune status of patients.[17,34] Given that patients with ATB are less immune than those with LTBI, the modified PHA SFC was significantly lower in ATB patients compared to LTBI individuals.

There are rare studies concerning the difference between ESAT-6 and CFP-10 stimulation in T-SPOT assay. It is generally believed that combination of ESAT-6 and CFP-10 stimulation can improve the diagnostic sensitivity, but we do not know why this happens. Interestingly, we found an obviously different IFN-γ response by lymphocytes between ESAT-6 and CFP-10 stimulation. ESAT-6 stimulation induced a more powerful lymphocyte response but the number of responsive lymphocytes was relatively low. In contrast, CFP-10 stimulation induced a weaker lymphocyte response but the number of responsive lymphocytes was high. This may be due to differences in their structure, since ESAT-6 has much more helical secondary structure but less stable tertiary structure than CFP-10, which contributes to a superior T cell activation by ESAT-6.[35–37]

The strengths of our study include the following: (1) combination of the number and size of spots was an independent method based on T-SPOT assay alone; (2) the excellent diagnostic performance of the modified TBAg/PHA ratio & ESAT-6 SFC MSS found in Qiaokou cohort was verified in Caidian cohort; and (3) the diagnostic criteria of participants were not ambiguous. ATB patients were diagnosed according to the presence of pathogen, and LTBI individuals were recruited based on a positive T-SPOT assay without any evidence suggestive of TB.

Some limitations of this study should be noted. First, since all participants in this study were recruited based on a positive T-SPOT assay, Mtb-infected patients with negative T-SPOT results were not involved in this study. The proportion of ATB patients with negative T-SPOT results may reach 5–15%,[38–40] and the inclusion of this part of the population would lead to biased diagnostic performance. Second, in view of the fact that the age of the participants recruited ranged from 18 to 70 years, the performance of the diagnostic model in children and the elderly is unclear. Finally, participants who received immunosuppressive therapy and patients with HIV infection were excluded from the study, as immunosuppression may influence the results of T-SPOT assay.

In conclusion, our study has provided evidence to support the use of the diagnostic model established by combination of modified TBAg/PHA ratio and ESAT-6 SFC MSS for discriminating between ATB and LTBI, which could be used as an adjunct tool for diagnosis and exclusion of ATB and provide new strategies for the treatment and prognosis of TB diseases.

Declaration of Competing Interest

None declared.

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Author contributions

YL, FW, and ZS conceived of the research, designed the study, interpreted data and wrote the draft of the manuscript; YL, GT, QL, LM, and YX contributed to the acquisition of clinical data; YL, XY, RO, SW, JY, YZ, WL, and HH recruited the participants, performed experiments and analyzed data. All authors saw and edited the final manuscript.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jinf.2020.04.038.

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