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Application of IFN-γ/IL-2 FluoroSpot assay for distinguishing active tuberculosis from non-active tuberculosis: A cohort study

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ABSTRACT

Currently available Interferon-γ release assay cannot reliably differentiate active TB (ATB) from non-active TB (non-ATB). This study aimed to evaluate the diagnostic accuracy of the IFN-γ/IL-2 FluoroSpot assay, which can simultaneously detect IFN-γ and IL-2 secretion, for differentiating ATB from non-ATB. 191 suspected ATB patients with positive T-SPOT.*TB* results were consecutively recruited. 64 (33.5%) participants had ATB, including 22 (34.4%) microbiologically or histologically confirmed TB and 42 (65.6%) clinically diagnosed TB. 119 (62.3%) cases were non-ATB and 8 (4.2%) were clinically indeterminate. After being stimulated with ESAT-6 and CFP-10 antigens, the median frequency and proportion of IFN-γ^+IL-2^- T cells were significantly higher in the ATB group than the non-ATB group ($P < .001$). The areas under the ROC curves of IFN-γ^+IL-2^- T cells were larger than those of total IFN-γ^+ T cells (0.788 vs. 0.739, $p = .323$). With a cutoff value of 25 SFCs/250,000 PBMCs for frequency, sensitivity and specificity of this assay were 73.4% and 69.8% respectively. When combining the frequency and proportions of IFN-γ^+IL-2^- T cells, the sensitivity and specificity were increased to 95.3% in parallel testing and 83.2% in serial testing respectively. In conclusion, IFN-γ/IL-2 FluoroSpot assay is conducive for the diagnosis of ATB in patients with positive T-SPOT.*TB* results.

1. Introduction

Tuberculosis (TB) is one of the top lethal infectious diseases worldwide. The World Health Organization (WHO) has estimated that there were approximately 10.0 million new TB cases and 1.3 million deaths from TB among HIV-negative people in 2017. China is still a high-burden TB country with approximately 889,000 new TB cases identified in 2017 and an incidence of up to 63/100,000 [1]. To achieve the goal of eliminating TB by 2050, we need to detect cases quickly and accurately. However, traditional methods for TB diagnosis are time-consuming, not sensitive enough and sometimes invasive. Moreover, the specificity of tuberculin purified protein derivative (PPD) skin test is low in areas where Bacillus Calmette-Guérin (BCG) vaccination is widely implemented [2].

In recent years, interferon-γ release assays (IGRAs) have been established as routine tests for diagnosing TB infection. However, IGRAs fail to distinguish active TB (ATB) from latent TB infection (LTBI), especially in TB epidemic areas [3]. As reported by a multicenter LTBI epidemiological study in China, nearly 20% of people have LTBI, reducing the specificity of IGRA in diagnosing ATB [4].

In addition to IFN-γ, interleukin-2 (IL-2), another cytokine secreted by activated T cells, is also involved in the Th1 cell-mediated immune response against MTB. It has been reported that IFN-γ^+IL-2^- T cells (T cells that only secrete IFN-γ without IL-2) play an essential role in the cellular immune response against untreated ATB. Compared to ATB patients, LTBI patients have significantly higher IL-2 levels, suggesting a potential role of IL-2 as a biomarker in distinguishing different TB statuses [5–8].

As an adaption of the traditional Enzyme-Linked Immunospot (ELiSpot) Assay, the FluoroSpot assay is capable of detecting multiple

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cytokines simultaneously by utilizing fluorochrome-conjugated antibodies [9]. In the IFN-γ/IL-2 FluoroSpot test system, single IFN-γ-secreting T cells (IFN-γ^+IL-2^-), single IL-2-secreting T cells (IFN-γ^-IL-2^+), as well as dual IFN-γ/IL-2-secreting T cells (IFN-γ^+IL-2^+), can be detected simultaneously, making this test a promising strategy to distinguish ATB from LTBI.

Our team has found the value of IFN-γ/IL-2 FluoroSpot in differentiating ATB from non-ATB in a case-control study before [10]. However, the case-control design might overestimate the differential accuracy of this assay and further studies with cohort designs and large sample sizes are needed. In this prospective cohort study, the diagnostic value of the IFN-γ/IL-2 FluoroSpot assay for distinguishing ATB from non-ATB was evaluated.

2. Materials and methods

2.1. Ethical approval

The study was conducted in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of PUMCH (No: S-715). Informed written consent was obtained from all patients prior to their enrollment in this study.

2.2. Study population and diagnosis of ATB

All patients with suspected ATB from the clinic and ward of infectious diseases and general medicine in Peking Union Medical College Hospital (PUMCH, a 2000-bed tertiary general hospital) between January 2016 and September 2017 were consecutively enrolled. The inclusion criteria included age ≥ 16 years old, HIV-negative, positive T-SPOT.*TB* (Oxford Immunotec, Abingdon, UK) results. ATB was suspected when (1) a patient presented with clinical manifestations consistent with pulmonary or extrapulmonary TB (including fever, cough, chest pain, night sweats, and weight loss), and (2) routine laboratory and radiological findings were in accordance with TB infection.

Clinical information was extracted from electronic medical records. Based on patients' clinical manifestations, imaging findings, microbiological or histopathological results and patient's response to the treatment of TB, diagnosis was independently made by two physicians blinded to the results of IFN-γ/IL-2 FluoroSpot assay. If a patient did not have a confirmed diagnosis at the beginning, an active follow-up at 4 weeks, 8 weeks, and 24 weeks was conducted to obtain the final diagnosis. When disagreement appeared, a chief physician was referred. The diagnostic classification was predefined (Table 1).

2.3. IFN-γ/IL-2 FluoroSpot assay

Eight milliliters of peripheral blood was collected from each patient. IFN-γ/IL-2 FluoroSpot (AID, Straßberg, Germany) assays were performed within 6 h from sample collection according to the manufacturer. PBMCs were firstly isolated by Ficoll-Hypaque gradient centrifugation. 96-well plates pre-coated with monoclonal antibodies against IFN-γ and IL-2 were seeded with 2.5×10^5 PBMCs and anti-CD28 (0.5 μg/ml, AID, Straßberg, Germany) and contained: AIM-V (Gibco™ AIM V Medium liquid, Invitrogen, USA) as a negative control, 5 μg/ml PHA as a positive control, and ESAT-6 and CFP-10 (final concentration of each peptide was 10 μg/ml) as specific antigens. Plates were incubated for 18–20 h at 37 °C in 5% carbon dioxide and then washed with washing buffer. After incubation, the detection antibodies (anti-IL-2 biotin and anti-IFN-γ-FITC) were added to each well and incubated for 2 h at room temperature. Then streptavidine red-conjugate and anti-FITC green were added and another 1 h of incubation was needed. Finally, fluorescence enhancer was loaded to make spots visible under the automated ELISPOT reader (AID iSpot, Strassberg, Germany).

Specific T cells secreting single IFN-γ, single IL-2 and dual IFN-γ/IL-2 were counted and then the frequency and proportion of each T cell subset were calculated and recorded manually. When calculating the proportion, the total number of T cells secreting single IFN-γ, single IL-2 and dual IFN-γ/IL-2 was utilized as the denominator.

The background spot-forming cells (SFCs) in negative control and PHA positive control wells should be ≤10 spots and ≥20 spots respectively. Otherwise, it would be considered as an indeterminate result.

Laboratory personnel who conducted the assays and researchers who interpreted the results were all blind to patients' clinical data, especially final diagnoses.

2.4. Statistical analysis

Receiver operating characteristic (ROC) curves of the frequency and proportion of each group of T cells were developed and the areas under the ROC curves (AUROC) were calculated and compared. The subset of T cells with the largest AUROC was chosen as the potential diagnostic marker for distinguishing ATB from non-ATB. The optimal cutoffs of the frequency and proportion was determined according to ROC curve analysis and then sensitivity, specificity, predictive value (PV) and likelihood ratio (LR) were determined. Parallel and serial testing algorithms combining the frequency and proportion were also utilized to improve the diagnostic sensitivity and specificity [11]. In the parallel testing, diagnosis of ATB was made when either the frequency or the proportion exceeded the cutoff values. In the serial testing, both the frequency and proportion were required to exceed the cutoff values to make the diagnosis.

The Kolmogorov-Smirnov test was adopted to examine whether the variable data showed a normal distribution. Variables that are normally distributed are denoted as the mean ± standard deviation (SD), and variables with an abnormal distribution are denoted as the median and IQR. Enumeration data are presented as percentages and 95% confidence intervals (CIs). Significance was defined as $P < .05$ (two-sided). Statistical analyses were performed using SPSS 24.0 (SPSS Inc., Chicago, IL, USA) and MedCalc version 11 software program (MedCalc Software bvba, Mariakerke, Belgium).

Table 1
Predefined diagnostic criteria of TB infection.

Categories	Criteria
ATB	
Confirmed TB	*MTB* culture (+) or acid-fast staining (+) or PCR (+) of sputum or other samples, OR Typical histologic changes (caseous necrosis, epithelioid granuloma, etc).
Clinically diagnosed TB	Clinical manifestations (including fever, cough, chest pain, night sweats, and weight loss), laboratory results and radiologic features considerably suggesting ATB, AND Appropriate response to anti-TB therapy.
Clinically indeterminate	Patients who still had an unclear diagnosis at 24 weeks follow-up after discharge.
Non-ATB	All microbiological samples were negative in smear acid-fast staining, culture and PCR assays AND A definite alternative diagnosis was identified AND Treatment of the primary disease was effective.

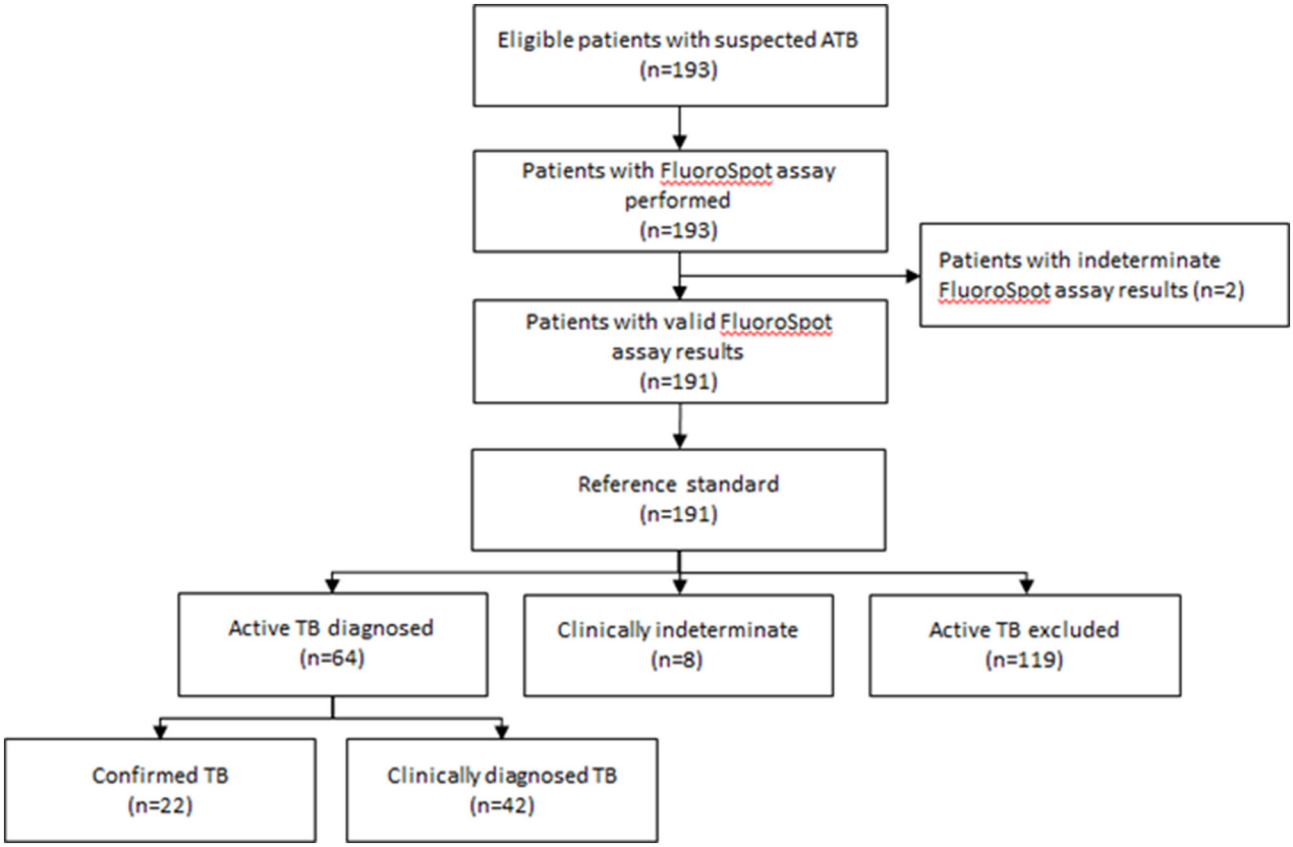

Fig. 1. Flowchart of enrollment of patients with suspected tuberculosis.

3. Results

3.1. Demographic and clinical features of participants

193 participants were enrolled in the study, and 2 patients were excluded due to indeterminate results in the IFN-γ/IL-2 FluoroSpot assay. Thus, a total of 191 subjects were included in analysis, with 86 males (45.0%) and 105 females (55.0%). The 64 ATB patients (33.5%) comprised 9 bacteriologically confirmed cases (14.1%), 13 histologically confirmed cases (20.3%), and 42 clinically diagnosed cases (65.6%). Among them, 19 patients (29.7%) were pulmonary TB, and 45 (70.3%) were extrapulmonary TB. Additionally, 119 patients (62.3%) were diagnosed with non-ATB, and 8 patients (4.2%) were clinically indeterminate (Fig. 1). Age, gender, serous effusion, application of immunosuppressive agents or glucocorticoids, history of TB infection and lymphocyte count were not significantly different between the ATB and non-ATB groups (Table 2).

3.2. Frequencies and proportions of MTB-specific IFN-γ/IL-2-secreting T cells in different TB groups

Compared with the non-ATB group, the median frequency and proportion of IFN-γ^+IL-2^- T cells in IFN-γ/IL-2 FluoroSpot assays were significantly higher for the ATB group ($P < .001$). On the contrary, the proportions of IFN-γ^-IL-2^+ and IFN-γ^+IL-2^+ T cells were both significantly lower for the ATB group (Table 3).

3.3. Diagnostic value of the IFN-γ/IL-2 FluoroSpot assay for distinguishing ATB from non-ATB

Based on ROC curve analysis, AUROC of the IFN-γ^+IL-2^- T cells was larger than that of the total IFN-γ^+ T cells (0.788, 95%CI: 0.723–0.853 vs. 0.739, 95%CI: 0.666–0.811), although the difference was not significant ($p = .323$). Therefore, the group of IFN-γ^+IL-2^- T cells was chosen as the diagnostic marker in our test system and the optimal cutoff value for the frequency and proportion of this subset were 25 SFCs/250,000 PBMCs and 43.6% respectively (Fig. 2).

With a combination of the frequency and proportion of IFN-γ^+IL-2^- T cells, the parallel testing increased the sensitivity of this assay from

Table 2
Demographic, clinical and laboratory characteristics of all participants.

	Confirmed TB	Clinically diagnosed TB	Clinically indeterminate	Non-active TB	Total
Total, n (%)	22 (11.5)	42 (22.0)	8 (4.2)	119 (62.3)	191
Age, Mean ± SD (y)	44 ± 17	52 ± 17	45 ± 14	50 ± 15	50 ± 16
Male, n (%)	13 (59.1)	20 (47.6)	4 (50.0)	49 (41.2)	86 (45.0)
serositis effusion, n (%)	4 (18.2)	13 (31.0)	0	21 (17.6)	38 (19.9)
Use of corticosteroids or immunosuppressive agents, n (%)	5 (22.7)	18 (42.9)	0	43 (36.1)	66 (34.6)
Evidences of previous TB, n (%)	5 (22.7)	9 (21.4)	5 (62.5)	40 (33.6)	59 (30.9)
Median Lymphocyte count [IQR], cells/mm^3	1550 [1190–2080]	1325 [855–1853]	1180 [1083–1605]	1450 [1110–1975]	1420 [1060–1960]

Table 3
Comparisons of frequencies and proportions of IFN-γ- and IL-2-secreting T cells when stimulated by both ESAT-6 and CFP-10.

	ATB group	Non-ATB group	*P* value
	Median [IQR]	Median [IQR]	
Frequency of IFN-γ- and IL-2-secreting T cells (SFCs/250,000 PBMCs)			
IFN-γ^+IL-2^-	64 [18–148]	13 [4–33]	< 0.001
IFN-γ^-IL-2^+	9 [3–43]	6 [2 − 20]	0.091
IFN-γ^+IL-2^+	34 [7–78]	11 [4–30]	0.002
Proportion of IFN-γ- and IL-2-secreting T cells (%)			
IFN-γ^+IL-2^-	56.1 [44.8–73.6]	36.8 [25.0–53.3]	< 0.001
IFN-γ^-IL-2^+	10.6 [3.7–21.0]	17.3 [7.1–33.3]	0.005
IFN-γ^+IL-2^+	28.6 [18.9–34.0]	36.2 [20.2–50.0]	0.006

79.7% to 95.3%, and the serial testing increased the specificity from 69.8% to 83.2% (Table 4).

Frequencies and proportions of IFN-γ^+IL-2^- T cells in patients with ATB and non-ATB and the corresponding 1ikelihood ratios (LRs) were shown in Table 5.

When changing the cutoffs of the frequency and proportion of IFN-γ^+IL-2^- T cells, FluoroSpot assays performed different accuracy in ATB diagnosis. When the number of single IFN-γ-secreting T cells exceeded 250, the PLR of this assay to predict ATB reached 11.16 (95%CI: 1.37–90.67) (Table 5).

4. Discussion

With the advantage of a prospective cohort design and a relatively large sample size, this study evaluated the diagnostic performance of the IFN-γ/IL-2 FluoroSpot assay in distinguishing ATB from non-ATB in T-SPOT.*TB* positive population.

Compared to the T-SPOT.*TB*, the FluoroSpot assay can simultaneously measure multiple cytokines produced by a single cell. IFN-γ^-IL-2^+ T cells, IFN-γ^+IL-2^+ T cells and IFN-γ^+IL-2^- T cells, also known as central memory T cells, effector memory T cells and terminally differentiated T cells, respectively, are considered to represent different stages of T cell differentiation and may play different roles in different TB statuses [12–14]. Therefore, detection of IFN-γ and IL-2 profile by the FluoroSpot assay is expected as a supplementary method to distinguish ATB from non-ATB.

In line with results from a case-control study conducted by Chesov's group, the frequency of IFN-γ^+IL-2^- T cells was significantly higher in the ATB group in our study [15]. As to the proportion, the ATB group possessed significantly different distribution of various T cell subsets compared with the non-ATB group, also consistent with the findings in previous studies [16,17].

It should be noted that the sensitivity and specificity of the FluoroSpot assay were only 73.4% and 69.8% in this study, lower than the sensitivity (92.3% of the pulmonary TB, 91.3% of the extrapulmonary TB) and specificity (80% of the pulmonary TB, 76.9% of the extrapulmonary TB) reported in our previous case-control study [10]. There might be two reasons. Firstly, in our previous study, all ATB patients were bacteriologically or histologically diagnosed. However, in this study, only 22 patients (34.4%) had bacteriologically or histologically confirmed TB, while the remaining 42 ATB patients (65.6%) were clinically diagnosed. Different spectrum of patients may account for the decreased sensitivity and specificity compared to those of previous studies. Secondly, some patients had received diagnostic anti-TB treatment when enrolled, which may alter the cytokine secretion spectrum, potentially reducing the differential diagnostic accuracy [16].

This study showed that the AUROC of the single IFN-γ-secreting T cells was only slightly larger than that of the total IFN-γ^+ T cells and the difference was not significant. This suggested that the FluoroSpot assay was not superior to T-SPOT.*TB* in the differential diagnosis of ATB and non-ATB when only taking the frequency into consideration, which was similar to the findings of Lange et al. [15]. However, it was important to note that in addition to the frequency, FluoroSpot assays also provided information on proportions of different T cell subsets. By combining the frequency and proportion, the parallel and serial testing could raise the sensitivity to 95.3% and specificity to 83.2%, which was of certain value for clinical practice.

Clinical diagnosis is a dynamic process. New information could increase or decrease the probability of the diagnosis. Likelihood ratios capture the extent to which new pieces of information revise probabilities [18]. In order to assist clinicians to make full use of the FluoroSpot results for differential diagnosis, the frequency and proportion of IFN-γ^+IL-2^- T cells were stratified to calculate likelihood ratios, enabling doctors to better interpret results from FluoroSpot

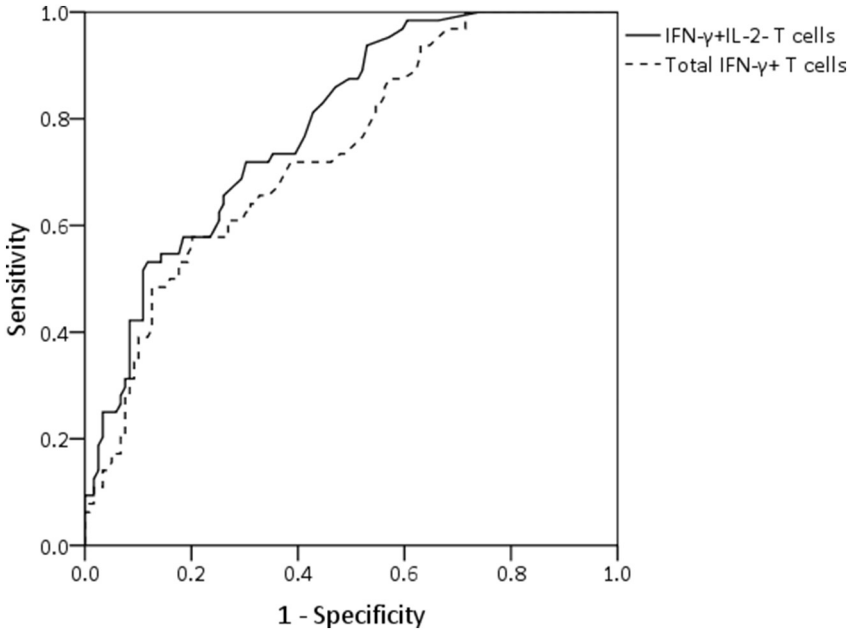

Fig. 2. ROC curve of IFN-γ/IL-2 FluoroSpot assay for differentiating ATB from non-ATB.

Table 4
Diagnostic accuracy of FluoroSpot assay for differentiating ATB from non-ATB.

Parameters	Sensitivity %	Specificity %	PPV %	NPV %	PLR	NLR
	(95%CI)	(95%CI)	(95%CI)	(95%CI)	(95%CI)	(95%CI)
Frequency of IFN-γ^+IL-2$^-$ T cells	73.4(60.9–83.7)	69.8(60.7–77.8)	56.6(45.3–67.5)	83.0(74.2–89.8)	2.43(1.78–3.31)	0.38(0.25–0.58
Proportion of IFN-γ^+IL-2$^-$ T cells	79.7(67.8–88.7)	59.7(50.3–68.6)	51.5(41.3–61.7)	84.5(75.0–91.5)	1.98(1.54–2.54)	0.34(0.21–0.57)
Parallel test	95.3(86.9–99.0)	46.2(37.0–55.6)	48.8(39.8–57.9)	94.8(85.6–98.9)	1.77(1.49–2.11)	0.10(0.03–0.31)
Serial test	73.4(60.9–83.7)	83.2(75.2–89.4)	70.2(57.7–80.7)	85.3(77.6–91.2)	4.37(2.85–6.69)	0.32(0.21–0.48)

PPV: Positive predictive value; NPV: Negative predictive value; PLR: Positive likelihood ratio; NLR: Negative likelihood ratio.

Table 5
PLRs of frequency and proportion of IFN-γ^+/IL-2$^-$ T cells to predict ATB.

	ATB group	Non-ATB group	PLR (95%CI)
	n (%)	n (%)	
Frequency of IFN-γ + /IL-2- T cells, SFCs/250,000 PBMC			
< 10	2 (3.1)	51 (42.9)	0.07 (0.02–0.29)
10–49	26 (40.6)	49 (41.2)	0.99 (0.68–1.42)
50–99	9 (14.1)	9 (7.6)	1.86 (0.78–4.45)
100–249	21 (32.8)	9 (7.6)	4.34 (2.11–8.91)
≥250	6 (9.4)	1 (0.8)	11.16 (1.37–90.67)
Proportion of IFN-γ + /IL-2- T cells			
< 30%	4 (6.3)	43 (36.1)	0.17 (0.07–0.46)
30–49%	19 (29.7)	40 (33.6)	0.88 (0.56–1.39)
50–69%	24 (37.5)	20 (16.8)	2.23 (1.34–3.72)
70–99%	14 (21.9)	7 (5.9)	3.72 (1.58–8.74)
100%	3 (4.7)	9 (7.6)	0.62 (0.17–2.21)
Total	64 (100)	119 (100)	

assays and estimate the posttest probabilities.

To improve this method, we should consider the incubation time for cytokine secretion. Previous studies have confirmed that multifunctional Th1 cell cytokine responses were initiated asynchronously; that is, cells predominantly release one type of cytokine at a time rather than simultaneously secrete multiple cytokines [19–22]. Although the overall integrated response of IFN-γ/IL-2 cell spots could be detected, IFN-γ/IL-2 FluoroSpot plates in our study were only read at a particular point in time, 18–20 hours post-incubation, and thus, we could not perform real-time tracking of the fluorescent signals. Further studies on the time-course of cytokine expression are needed to optimize the incubation time.

There are some limitations in this study. First of all, about two-thirds of the patients with ATB were clinically diagnosed without culture confirmation, which possibly lead to the problem of misdiagnosis. To address this problem, we have followed up for a long time to make the final diagnosis as accurate as possible. Secondly, some patients had received diagnostic anti-TB treatment when enrolled, which potentially affected the secretion profile of IFN-γand IL-2, and possibly affected the diagnostic accuracy of the FluoroSpot assay. Thirdly, this is a single center study. As the study site is the national referral center dealing with complex and rare disorders, the spectrum of disease is expected to be different from TB specific hospitals, and primary hospitals. Selection bias may exist and the results should be extrapolated carefully.

In summary, we conclude that in patients with positive T-SPOT.*TB* results, the combination of frequencies and proportions of IFN-γ^+IL-2$^-$ T cells in the IFN-γ/IL-2 FluoroSpot assay could be used as a supplementary index for differential diagnosis of active TB in countries with a high burden of TB.

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Declaration of Competing Interest

The authors declared there are no conflicts of interest.

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