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Original article

An IFN-γ and TNF-α dual release fluorospot assay for diagnosing active tuberculosis

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ABSTRACT

Objectives: Currently available interferon (IFN)-γ-release assays (IGRA) cannot discriminate active tuberculosis (TB) from latent TB infection (LTBI), and so have limited clinical utility for diagnosing active TB. Since numbers of tumour necrosis factor (TNF)-α-producing T cells are highly correlated with active TB, we hypothesized that detecting IFN-γ- and/or TNF-α-producing T cells would overcome this limitation of IGRA. This study evaluated the diagnostic performances of the IFN-γ and TNF-α dual release fluorospot assay for active TB.

Methods: Adult patients with suspected TB including recent TB exposers were prospectively enrolled over a 28-month period. In addition to the conventional IGRA test (i.e. QuantiFERON-In-Tube), a fluorospot assay for detecting IFN-γ- and TNF-α-producing T cells was performed. The final diagnoses were classified by clinical category. Patients with confirmed or probable TB were regarded as active TB, and patients with not active TB were further classified as having not active TB with and without LTBI, based on the QuantiFERON-In-Tube results.

Results: A total of 153 patients including 45 with active TB and 108 with not active TB (38 LTBI vs. 70 not LTBI) were finally analysed. The sensitivity and specificity of the QuantiFERON-In-Tube assay for active TB were 84% (95% confidence interval (CI), 70–93) and 70% (95% CI 61–79), respectively. The IFN-γ/TNF-α dual release assay by fluorospot had substantially higher diagnostic specificity (94%) for diagnosing active TB than the IFN-γ single release assay (72%, $p < 0.001$), without compromising sensitivity (84% vs. 89%, p 0.79).

Conclusions: The fluorospot-based IFN-γ/TNF-α dual release assay appears to be a simple and useful test for diagnosing active TB. **J.Y. Kim, Clin Microbiol Infect 2020;26:928**

Introduction

Recently, interferon (IFN)-gamma-releasing assays (IGRAs) have been widely used in clinical practice to diagnose latent tuberculosis infection (LTBI) [1,2]. In some clinical settings such as paucibacillary TB, or TB involving inaccessible sites, IGRAs are used as diagnostic adjuncts for diagnosing active TB [3]. However, they cannot differentiate active tuberculosis (TB) from LTBI. Their clinical utility for diagnosing active TB is limited, especially in intermediate-to-high TB burden countries because the background IGRA positive rate is too high due to the high LTBI rate. A less invasive blood test for diagnosing active TB is urgently needed, because a fast and simple test can be very helpful for early detection of paucibacillary TB, including extrapulmonary TB and pulmonary TB in patients who have difficulty producing sputum.

Recent studies have proposed parallel assessment of the profile of secreted T cell cytokines such as Intracellular Adhesion Molecule-1 (ICAM-1), interleukin (IL)-1ra, IL-2, IL-3, IL-10, IL-12, IL-12p70, IP-10, macrophage colony-stimulating factor (MCSF), vascular endothelial growth factor (VEGF) and tumour necrosis factor (TNF)-α, along with IFN-γ [4–12]. Our previous work indicated that TNF-α-releasing

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assays (TARAs) were particularly promising for diagnosing active TB [13]. Fluorospot is a novel fluorescence-based enzyme-linked immune spot (ELISpot) technology, which is able to simultaneously detect individual cells that secret multiple cytokines, and provides a simple platform that, like the ELISpot assay, can be used in routine clinical practice [14]. We therefore hypothesized that fluorospot-based diagnosis that detected dual IFN-γ- and/or TNF-α-producing T cells might overcome the limitations of IGRAs. This study builds on our earlier publication [13] by developing the clinical application of combined IGRA and TARA; it employs fluorospot-based dual IFN-γ and TNF-α-release assays requiring one-time assay processing, instead of having to process separate IGRAs and TARAs. In this study, we evaluated the diagnostic performances of the IFN-γ and TNF-α dual release assay by fluorospot for active TB.

Methods

Study population and specimen collection

Adult patients suspected active TB and adult subjects with recent TB exposures were prospectively enrolled at two university-affiliated hospitals in Seoul, South Korea, consecutively between November 2016 and February 2019. Microbiological and pathological specimens for diagnosing TB were processed by standard techniques and procedures, as described previously [15]. The IGRA test (i.e. QuantiFERON-In-Tube, Cellestis/Qiagen, USA) was routinely performed in patients with suspected TB. To avoid biases, the results of the fluorospot assay were concealed from the attending physicians, and the researchers were blinded to the diagnoses. The Institutional Review Boards of two hospitals evaluated medical, scientific, and ethical aspects on our study protocol. These two IRBs approved our study protocol. Informed consent was obtained from all participants.

Clinical categories of TB

All patients were classified by a physician investigator (J.H. Park) blinded to the fluorospot results. They were classified based on clinical, histopathological, radiological and microbiological information collected during at least 3 months of follow-up. The various clinical categories of patients with suspected TB have been described previously [13], and are presented in Table S1. Patients with confirmed or probable TB were regarded as reference standards for active TB, and those with not active TB were further classified into not active TB with LTBI and not active TB without LTBI, depending on the results of the IGRA. Patients with possible TB were excluded from the analysis.

Fluorospot assay

Preparation of peripheral blood mononuclear cells (PBMCs) and the detailed procedures of fluorospot assay are described (please see supplementary material).

Results of the fluorospot assay were classified as positive, negative and indeterminate as described elsewhere [13]. Results of the single-colour fluorospot were considered to be positive if the panel test (containing early secretary antigenic target-6 (ESAT-6) and culture-fitrating protein-10 (CFP-10) peptide pools) yielded at least six spot-forming cells (SFCs) more than the negative control when the negative control had five or less SFCs, based on clinical relevance and the manufacturer's recommendation of IGRAs; *or* if the number of spots in the panel test was at least double that of the negative control when the negative control gave more than five SFCs. The results of merged viewing of the dual-colour fluorospots (green (FITC) and red (CY3)) were considered to be positive if the panel test yielded four SFCs more than the negative control. When the positive control gave negative results or the negative control yielded too many spots (>50 SFCs), tests were considered indeterminate.

Statistical analysis

Categorical variables were compared using Fisher's exact test or Pearson chi-square test, as appropriate. Continuous variables were compared using the Mann–Whitney U-test or Student's t-test, or by Kruskal–Wallis one-way ANOVA with Dunn's multiple comparison, as appropriate. Optimal cut-off points were estimated by constructing receiver operating characteristic (ROC) curves. IBM SPSS Statistics for Windows, version 23.0 (IBM Corp., Armonk, NY, USA) was used for these statistical analyses. SFC data analysis and graph plotting were performed with Graphpad Prism 5. Confidence intervals at the 95% level ($p < 0.05$) were considered in all cases.

Results

Study population and clinical characteristics

A total of 175 patients including suspected patients of TB ($n = 109$) and subjects with recent contact ($n = 65$) were initially enrolled. Of these 175 patients, ten with PBMCs of low viability (<70%) were excluded from the study, and fluorospot assays were obtained from 165 patients. An additional 12 patients were excluded from the final analysis; no results of QuantiFERON-In-Tube were available for ten of them, and the two others were finally diagnosed as possible TB (Fig. 1). In the end, 153 patients, including 45 patients classified as active TB (30 confirmed TB and 15 probable TB) and 108 patients with not active TB, were analysed. Of the 108 patients with not active TB, 38 yielded positive QuantiFERON-In-Tube results and were classified as not active TB with LTBI. The remaining 70 patients were classified as not active TB without LTBI (Fig. 1). Detailed clinical characteristics are shown in Table 1.

Diagnostic performances of the fluorospot assay

Typical results of the IFN-γ single release, TNF-α single release, and IFN-γ/TNF-α dual release assays against ESAT-6 or CFP-10 according to clinical classifications are presented in Fig. S1. Of the 153 patients, 69 gave positive IFN-γ single release assay results, 82 gave negative results and two indeterminate results. Similarly, 40 gave positive TNF-α single release assay results, 107 negative results and six indeterminate results, and 43 yielded positive IFN-γ/TNF-α dual release assays, 107 negative results and three indeterminate (Fig. 1).

The diagnostic performances of the QuantiFERON-In-Tube, IFN-γ single release assay, TNF-α single release assay and IFN-γ/TNF-α dual release assay in the 153 patients with suspected TB are shown in Table 2. The overall sensitivity and specificity of the QuantiFERON-In-Tube assays in patients with suspected TB were 84% (95% CI, 70–93) and 70% (95% CI 61–79), respectively; the overall sensitivity and specificity of the IFN-γ single release assay in these patients were 89% (95% CI 75–96) and 72% (95% CI 36–80), respectively and the overall sensitivity and specificity of the TNF-α-single release assay were 78% (95% CI 63–88) and 94% (95% CI 88–98), respectively. The overall sensitivity and specificity of the IFN-γ/TNF-α dual release assay in patients with suspected TB were 84% (95% CI 70–93) and 94% (95% CI 87–97), respectively. The specificities of the TNF-α single release assay (94%) and IFN-γ/TNF-α dual release assay (94%) for active TB were significantly higher than that of the IFN-γ-single release assay (72%) ($p < 0.001$). Additional analyses of the diagnostic performances of the three assays in a subgroup including active TB and not TB with or without LTBI are presented in Table 2.

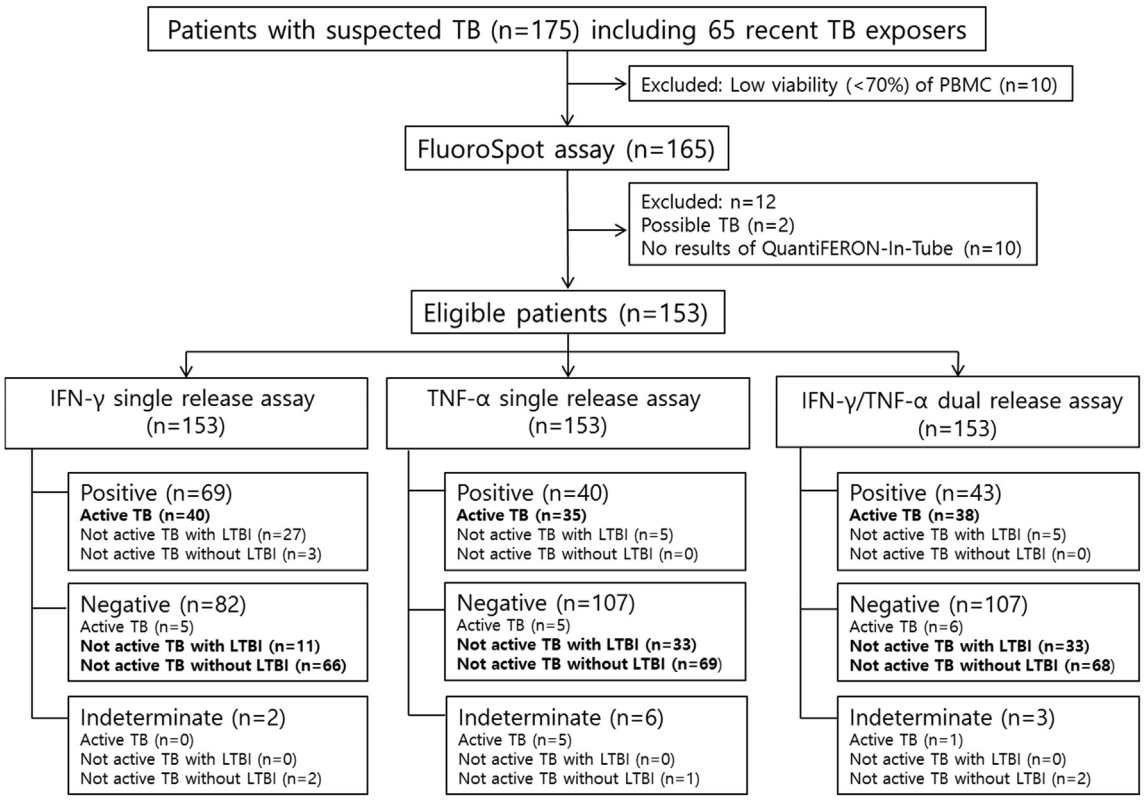

Fig. 1. Study flow diagram.

Table 1
Baseline characteristics of the participants

	Active TB ($n = 45$)	Not TB ($n = 108$)		p[a]
		Not TB with LTBI ($n = 38$)	Not TB without LTBI ($n = 70$)	
Mean age (mean ± SD), years	47.8 ± 16.4	45.6 ± 12.4	42.3 ± 16.8	0.12
Male sex	21 (46.7)	20 (52.6)	31 (44.3)	0.95
TB category				
Confirmed TB	30 (66.7)	NA	NA	NA
Probable TB	15 (33.3)	NA	NA	NA
Underlying condition or illness				
HIV infection	2/42 (4.8)	0/34 (0)	0/65 (0)	0.09
Solid tumour	3 (6.7)	6 (15.8)	5 (7.1)	0.76
Hematologic malignancy	0 (0)	3 (7.9)	5 (7.1)	0.11
Solid organ transplantation	3 (6.7)	1 (2.6)	2 (2.9)	0.36
Bone marrow transplantation	0 (0)	2 (5.3)	2 (2.9)	0.32
Liver cirrhosis	0 (0)	0 (0)	1 (1.4)	>0.99
Haemodialysis	1 (2.2)	0 (0)	1 (1.4)	0.50
Diabetes mellitus	4 (8.9)	2 (5.3)	7 (10.0)	>0.99
Rheumatologic disease	1 (2.2)	2 (5.3)	2 (2.9)	>0.99
Immunosuppressive condition[b]	4 (8.9)	3 (7.9)	2 (2.9)	0.45
Suspected infection site				
Lung	24 (53.3)	29 (76.3)[c]	54 (77.1)[c]	<0.01
Lymph node	14 (31.1)	7 (18.4)	5 (7.1)	<0.01
Skeletal	2 (4.4)	1 (2.6)	6 (8.6)	>0.99
Central nervous system	6 (13.3)	1 (2.6)	6 (8.6)	0.21
Abdominal	3 (6.7)	0 (0)	5 (7.1)	0.69
Pleural	1 (2.2)	0 (0)	1 (1.4)	0.50
Genitourinary	2 (4.4)	2 (5.3)	1 (1.4)	0.63
Miliary	3 (6.7)	NA	NA	NA
Disseminated	9 (20.0)	NA	NA	NA
Others	5 (11.1)	0 (0)	0 (0)	<0.01

Data are presented as number (%) unless otherwise indicated. TB, tuberculosis; LTBI, latent tuberculosis infection; HIV, human immunodeficiency virusData are presented as number (%) unless otherwise indicated.

[a] p value for the comparison between the active TB group and the not TB group.

[b] Patients who underwent solid organ transplantation, bone marrow transplantation, or cytotoxic chemotherapy or took immunosuppressants including corticosteroids within 3 months.

[c] These patients includes 22 and 43 patients with histories of TB exposure in the LTBI group and the not TB without LTBI group, respectively. Of these patients, four patients had abnormal chest images.

Table 2
Comparison of the diagnostic performances of the commercial IFN-γ release assay (QuantiFERON-In-Tube), IFN-γ-single release assay, TNF-α-single release assay, and IFN-γ and TNF-α-dual release assay

	Sensitivity % (n/N[a], 95% CI)	Specificity % (n/N[b], 95% CI)	Positive predictive value % (95% CI)	Negative predictive value % (95% CI)	Positive likelihood ratio (95% CI)	Negative likelihood ratio (95% CI)
Confirmed or probable tuberculosis (n = 45) versus not tuberculosis (n = 108)[c]						
QuantiFERON-In-Tube	84 (38/45, 70–93)	70 (76/108, 61–79)	54 (42–66)	92 (83–96)	2.9 (2.1–3.9)	0.22 (0.11–0.44)
IFN-γ-single release	89 (40/45, 75–96)	72 (78/108, 63–80)	57 (45–69)	94 (96–98)	3.2 (2.3–4.4)	0.15 (0.07–0.35)
TNF-α-single release	78 (35/45, 63–88)	94 (102/108, 88–98)	85 (70–94)	91 (84–95)	14 (6.3–31)	0.24 (0.14–0.41)
IFN-γ/TNF-α dual release	84 (38/45, 70–93)	94 (101/108, 87–97)	84 (70–93)	94 (87–97)	13 (6.3–27)	0.17 (0.08–0.33)
Confirmed or probable tuberculosis (n = 45) versus not tuberculosis with LTBI (n = 38)						
QuantiFERON-In-Tube	84 (38/45, 70–93)	16 (6/38, 7–32)	54 (42–66)	46 (20–74)	1.00 (0.83–1.21)	0.99 (0.38–2.56)
IFN-γ-single release	89 (40/45, 75–96)	29 (11/38, 16–46)	60 (47–71)	69 (41–88)	1.25 (1.00–1.57)	0.38 (0.15–1.00)
TNF-α-single release	78 (35/45, 63–88)	87 (33/38, 71–95)	88 (72–95)	77 (61–88)	5.91 (2.57–13.6)	0.26 (0.15–0.45)
IFN-γ/TNF-α dual release	84 (38/45, 70–93)	87 (33/38, 71–95)	88 (74–96)	83 (67–92)	6.41 (2.81–14.7)	0.18 (0.09–0.36)
Confirmed or probable tuberculosis (n = 45) versus not tuberculosis without LTBI (n = 70)						
QuantiFERON-In-Tube	84 (38/45, 70–93)	100 (70/70, 94–100)	100 (89–100)	91 (92–96)	—	0.16 (0.08–0.31)
IFN-γ-single release	89 (40/45, 75–96)	94 (66/70, 85–98)	91 (77–97)	93 (84–97)	16 (6.0–41)	0.12 (0.05–0.27)
TNF-α-single release	78 (35/45, 63–88)	99 (69/70, 91–100)	97 (84–100)	87 (78–93)	54 (7.7–384)	0.23 (0.13–0.39)
IFN-γ/TNF-α dual release	84 (38/45, 70–93)	97 (68/70, 89–100)	95 (82–99)	91 (81–96)	30 (7.5–117)	0.16 (0.08–0.32)

[a] Number of patients with a positive test result/number of patients tested.
[b] Number of patients with a negative test result/number of patients tested.
[c] Sensitivity and specificity were calculated by comparing active TB and not active TB; Sensitivity: IGRA vs. TARA, p > 0.999; IGRA vs. dual assay, p 0.758; TARA vs. dual assay, p > 0.999; Specificity: IGRA vs. TARA, p < 0.001; IGRA vs. dual assay p < 0.001, TARA vs. dual assay p > 0.999.

M. tuberculosis antigen-specific INF-γ- and TNF-α-producing bifunctional cell responses

While the IFN-γ-producing cell responses to ESAT-6 and CFP-10 were not significantly different in active TB and not TB with LTBI (ESAT-6, 16.38 ± 27.79 vs. 4.29 ± 5.43, p 0.63; CFP-10, 39.78 ± 89.24 vs. 6.40 ± 9.76, p 0.328; Figs. 2a and b), the corresponding TNF-α-producing cell responses (ESAT-6, 15.88 ± 22.41 vs. 3.50 ± 6.51, p < 0.01; CFP-10, 34.35 ± 56.33 vs. 4.53 ± 8.63, p < 0.001; Fig. 2c and d) and IFN-γ and TNF-α producing bifunctional cell responses (ESAT-6, 7.49 ± 13.25 vs. 1.05 ± 2.38, p < 0.001; CFP-10, 13.18 ± 29.81 vs. 1.61 ± 3.23, p < 0.01; Fig. 2e and f) were significantly higher in patients with active TB than in those with LTBI.

In addition, the IFN-γ-producing cell responses to ESAT-6 and CFP-10 were significantly higher in the not TB patients with LTBI than in those without LTBI (ESAT-6, 4.29 ± 5.43 vs. 0.81 ± 1.56, p < 0.01; CFP-10, 6.40 ± 9.76 vs. 0.71 ± 1.19, p < 0.001; Fig. 2a and b). On the other hand, the TNF-α-producing cell responses were not significantly different between the two groups (ESAT-6, 3.50 ± 6.51 vs. 3.72 ± 7.10, p > 0.99; CFP-10, 4.53 ± 8.64 vs. 4.55 ± 9.64, p > 0.99; Fig. 2c and d); the same was true for the IFN-γ- and TNF-α-producing bifunctional cell responses against ESAT-6 (1.05 ± 2.38 vs. 0.26 ± 0.54, p 0.352; Fig. 2e), whereas the bifunctional cell response against CFP-10 was significantly higher in the not TB patients with LTBI than in the those without LTBI (1.61 ± 3.22 vs. 0.26 ± 0.54, p < 0.01; Fig. 2f).

When we selected the optimal cut-off for the IFN-γ/TNF-α dual release assay on the basis of a ROC curve (>3.5 spots in Fig. S2), the sensitivity and specificity of the dual assay were 85% and 95.3%, respectively. The area under the curve of the IFN-γ/TNF-α dual release assay (0.918 ± 0.030, p < 0.001) was higher than that of the IFN-γ single release assay (0.881 ± 0.036, p < 0.001) and that of the TNF-α-single release assay (0.855 ± 0.040, p < 0.001).

Discussion

The commercial IGRA cannot differentiate active TB from LTBI, so the development of an immunodiagnostic test from blood samples to overcome this limitation for diagnosing active TB is urgently needed. We developed a new IFN-γ/TNF-α dual-colour fluorospot assay for active TB that is a simple one-time assay and a diagnostic platform that can be easily applied in real clinical practice. We found that the IFN-γ/TNF-α dual release assay substantially improved diagnostic specificity (94%) in comparison to that of the IFN-γ-single release assay (72%), without compromising diagnostic sensitivity (84% vs. 89%). We thus believe that this new assay will improve the early detection of paucibacillary TB, including extrapulmonary TB and pulmonary TB, in patients who have difficulty producing sputum.

A few studies have assessed *M. tuberculosis*-specific antigen-stimulated bifunctional cell responses, especially IFN-γ and IL-2 cytokine profiles for the differential diagnosis of active TB from LTBI [13,15,16]. Essone et al. reported that ESAT-6- and CFP-10-induced IFN-γ/IL-2 bifunctional cells were less numerous in TB patients than in healthy controls [14]. The authors found that the IFN-γ/IL-2 combined assay increased diagnostic sensitivity (93.8%), but did not improve specificity (77.8%) compared with the IFN-γ single assay [14]. Zhang et al. reported that ESAT-6- and CFP-10-stimulated IFN-γ/IL-2 dual producing T cells were more numerous in active TB than in non-active TB by a fluorospot assay. However, they concluded that the frequency of IFN-γ single producing cells showed highest diagnostic performances, 92.3% specificity and 80.0% sensitivity for active pulmonary TB [16]. Also, Chesov et al. evaluated use of the IFN-γ/IL-2 dual release assay to differentiate active TB from LTBI [17]. While the CPF10-induced IFN-

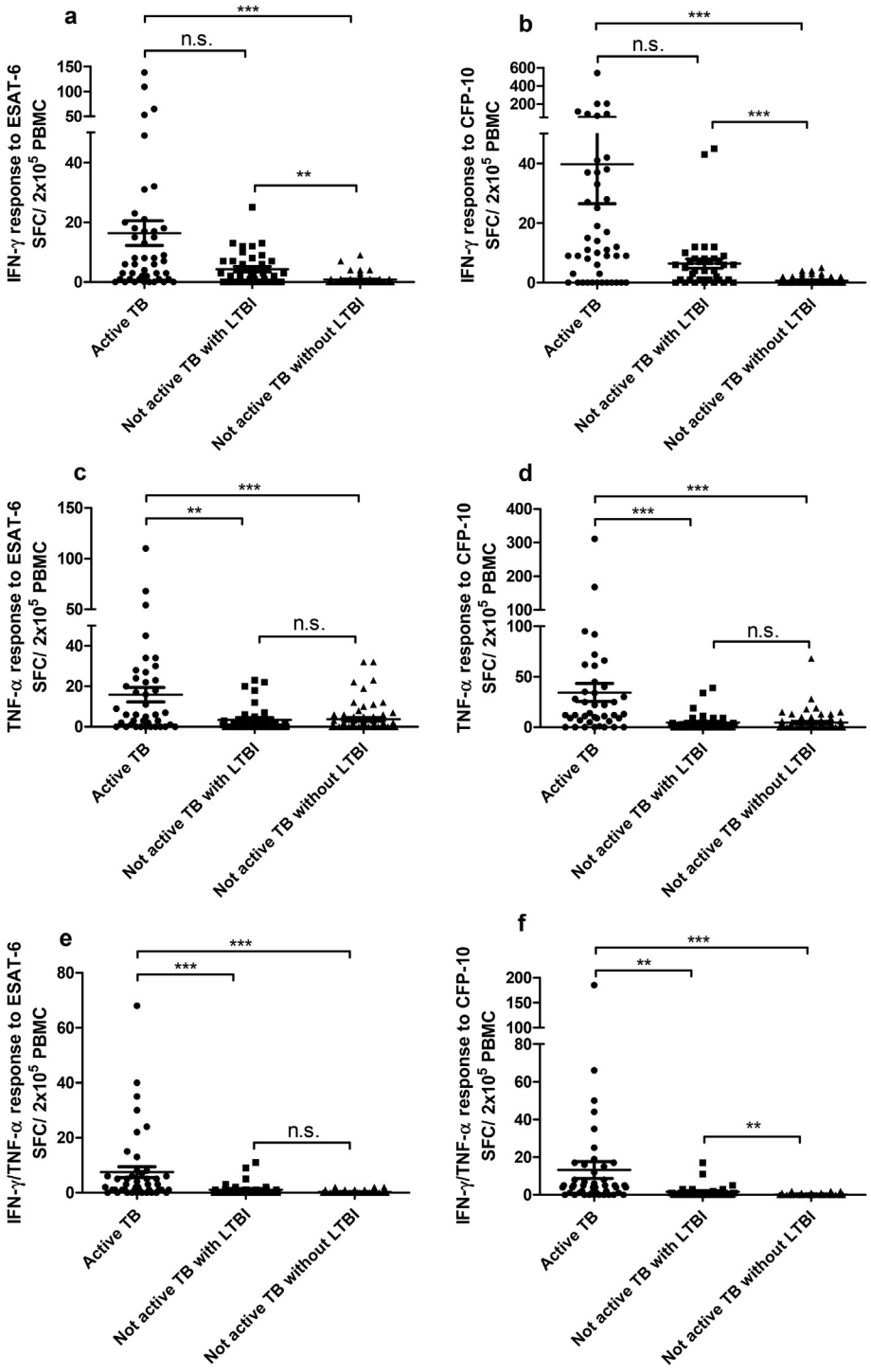

Fig. 2. Mycobacterial antigen-specific interferon (IFN)-γ, tumour necrosis factor (TNF)-α, and dual producing cell responses to ESAT-6 and CFP-10 according to disease category. Spot-forming cells (SFCs) per 200 000 peripheral blood mononuclear cells (PBMCs) stimulated with mycobacterial antigens in the disease categories of active tuberculosis (TB) (circles), not active TB with LTBI (squares), and not active TB without latent TB infection (LTBI) (triangles) are shown. (a,b) IFN-γ producing cell responses against ESAT-6 (a) and CFP-10 (b). (c,d) TNF-α-producing cell responses against ESAT-6 (c) and CFP-10 (d); (e,f) IFN-γ/TNF-α dual-producing cell responses against ESAT-6 (e) and CFP-10 (f). Between-group differences were assessed by Kruskal–Wallis one-way ANOVA with Dunn's multiple comparison test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. n.s., not significant

γ release assay had 89% sensitivity and 76% specificity, ESAT-6- and CFP-10-induced IFN-γ/IL-2 dual release assays yielded 61% and 89% sensitivities and 73% and 59% specificities [17]. These studies suggested that bifunctional IFN-γ/IL-2-producing cells are difficult to differentiate active TB from LTBI.

The diagnostic performances of IFN-γ and TNF-α bifunctional *M. tuberculosis* specific $CD4^+$ T cells have also been evaluated [5–7,12]. Actually, Petruccioli et al. reported that the proportion of any cytokines (IFN-gamma, IL-2 or TNF-alpha) responders to ESAT-6 or CFP-10 was significantly higher in patients with active TB (82%

[9/11]) than in those with LTBI (50% [24/48]) and *M. tuberculosis*-specific IFN-gamma and TNF-alpha producing $CD4^+$ T cells were higher in the active TB group than in the LTBI group [6]. However, they examined $CD4^+$ T cell-derived cytokine profiles by multiparametric flow cytometry, which is too complex for clinical use. In our previous study [13], the TNF-α release assay by ELISPOT had 89% sensitivity and 91% specificity for differential diagnosis of active TB from not active TB including LTBI. However, the ELISPOT-based assay uses separate plates for the IFN-γ single release assay and the TNF-α single release assay. In the present study we adopted a simple fluorospot assay that simultaneously detects IFN-γ- and/or TNF-α single and dual producing T cells, and we demonstrated that this one-time fluorospot assay has acceptable sensitivity and specificity in clinical practice for diagnosing active TB in patients with suspected TB.

According to a systematic meta-analysis, the average sensitivity of T-SPOT.*TB* in human immunodeficiency virus-uninfected patients with active TB was 88% [18,19]. The diagnostic sensitivities in the present study were 89%, 78% and 84% for the IFN-γ single release assay, TNF-α single release assay and IFN-γ/TNF-α dual release assay, respectively. Thus, the present study yielded results consistent with previous IGRA studies. It is worth noting that >60% of the patients with TB in this study had extrapulmonary TB, which indicates that patients with paucibacillary or difficult-to-diagnose TB were represented. In the present study, false negative results of the IFN-γ/TNF-α dual release assay occurred in only six patients, four of whom were extrapulmonary TB patients, namely two TB meningitis patients, one disseminated TB patient and one pulmonary TB patient with stomach and pancreatic cancers. Previous studies have reported that the T-SPOT.*TB* has lower sensitivity in extrapulmonary TB patients than pulmonary TB patients, and that false negative rates differed according to site of infection [15,20]. Our previous study suggested that false negative results of IGRA are associated with TB-specific antigenic load and host immune responses [15]. Hence, further studies are needed of the diagnostic performance of this new assay in patients with high mycobacterial burden TB and in immunocompromised patients with TB.

This study has several limitations. First, some may consider that the indeterminate results in the fluorospot assay limit its clinical use. In this study, we included the patients yielding indeterminate results in the denominator in all analyses, to avoid skewing our findings in favour of greater sensitivity and specificity. Actually, the TNF-α single release assay yielded a relatively large number of indeterminate results (3.9% [6/153]), although we have reported that the commercial IGRA produced more (8.7%) [21]. Of the six patients with indeterminate results, five with active TB gave too many background spots (>50 SFCs) in the TNF-α single release assay, as described in our previous study [13]. We assume that cells such as macrophages and NK cells may secret TNF-α without ESAT-6 and CFP-10 stimulation in patients with active TB, leading to a high background. However, the IFN-γ/TNF-α dual release assay by fluorospot yielded similar numbers of indeterminate results (1.9% [3/153]) to the IFN-γ single release assay (1.3% [2/153]) because background spots that were not double colour spots produced by IFN-γ/TNF-α bifunctional cells were eliminated. Second, we did not assess serial IFN-γ/TNF-α dual release T cell responses in patients who received anti-tuberculous therapy, and future studies of the kinetics of IFN-γ/TNF-α dual release T cell responses after anti-tuberculous therapy may provide further insight into the possibility of using the fluorospot assay as a new biomarker for therapeutic response.

In conclusion, our finding indicate that the diagnostic specificity of the IFN-γ/TNF-α dual release assay by fluorospot is considerably greater than that of the IFN-γ single release assay. Therefore, this dual assay, which is a simple and inexpensive, could be useful for diagnosing active TB in clinical practice.

Transparency declaration

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cmi.2019.11.003.

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