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ORIGINAL INVESTIGATION

Diagnostic performance in active TB of QFT-Plus assay and co-expression of CD25/CD134 in response to new antigens of *Mycobacterium tuberculosis*

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Abstract

The new QuantiFERON-TB Gold Plus employs modified peptides optimized to elicit an IFNγ response from $CD8^+$ cytotoxic T lymphocytes in addition to $CD4^+$ T cells. With a view to improve the difficult identification of TB cases, we assessed the combination of two specific immunological markers comprising IFNγ secretion and T cells co-expression of CD25 and CD134 in response to *Mycobacterium tuberculosis*-specific antigens. A total of 34 subjects with suspected TB and 10 age-matched HD were prospectively enrolled. Assessing the performance of QFT-Plus in terms of the TB1 and TB2 results, we found that in TB patients, the quantitative IFNγ value in TB2 was similar to that in TB1, and we did not find any differences irrespective of the disease (pulmonary or extra-pulmonary). The flow cytometric CD25/CD134 assay, allowed a more accurate differentiation between *M. tuberculosis*-infected and uninfected patients, with a better combination of sensitivity and specificity, especially by evaluation of $CD4^+$ T-cell subset. All individuals with negative QFT-Plus results displayed a positive CD25/CD134 response. Overall, a positive correlation was found between T cells co-expressing CD25/CD134 and IFNγ levels in response to both QFT-Plus TB antigen tubes, as well as between the QFT-Plus TB1 and TB2 tubes. We demonstrated that both TB1 and TB2 induce a higher expression of $CD25^+CD134^+$ markers on $CD4^+$ T cells among infected TB subjects, compared to the lower degree of $CD8^+$ T cells, mainly induced to TB2 stimulation. We suggest that a combined use of classic QFT-Plus and specific CD25/CD134 response may be a useful means in the diagnostic workup for active TB.

Keywords QFT-Plus · Tuberculosis · Flow cytometry · CD25/CD134

Introduction

Tuberculosis (TB) remains a major global health problem with 10.4 million of new TB cases in 2016. Of the nearly 5.4 million of pulmonary TB cases, only the 57% were bacteriologically confirmed and the remaining cases were diagnosed clinically based on symptoms, abnormalities on chest radiography or suggestive histology. Although the number of TB cases has significantly decreased, the proportions of extra-pulmonary TB remained constant, with global estimate ranging from 8 to 24% of total TB cases notified [1].

The bacteriological examination method is the gold standard for TB diagnosis, but the low smear positive rate and the long incubation time to obtain positive cultures, results in a delay in the diagnostic process for pulmonary and extra-pulmonary TB, and consequently, to a delayed optimal treatment and poorer treatment response [2, 3]. Gene Xpert is emerging as a highly specific test for identifying *Mycobacterium tuberculosis*, but has still suboptimal sensitivity in particular in non-respiratory samples [4].

Therefore, immunological methods with high sensitivity and specificity could be useful tools to rapidly identify—or rule out—the presence of TB disease. Blood-based IFNγ release assays (IGRA), including QuantiFERON TB Gold in-tube (QFT-GIT; Qiagen, Hilden, Germany) and T-SPOT. TB (Oxford Immunotec, Abingdon, UK), measure the cell-mediated immune response against some *M. tuberculosis*

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specific antigens [5, 6]. Recently, an updated version of the QFT-GIT has been released. The new QuantiFERON-TB Gold Plus (QFT-Plus) [7] employs two TB specific antigen tubes, called TB1 and TB2, containing peptides from *M. tuberculosis* complex-specific antigens ESAT-6 and CFP-10. In TB1 tube, there are long peptides designed to elicit an IFNγ response from $CD4^+$ helper T lymphocytes, like the original QFT-GIT assay. The TB2 tube, additionally to long peptides, contains a set of newly designed shorter peptides targeted to elicit a response from $CD8^+$ cytotoxic T lymphocytes in addition to those from $CD4^+$ T cells.

Also the flow cytometry has been proposed as a potential tool to help improving TB diagnosis. An interesting blood-based study [8] showed that the stimulation of T cells by antigen or mitogen resulted in the up-regulation of CD25 (interleukin-2 receptor alpha, IL-2Rα) and CD134 (a TNF receptor superfamily member). The co-expression of these two important T-cell surface molecules allows identification of antigen-specific $CD4^+$ T cells.

In the present study, we investigated a cohort of subjects with suspected pulmonary and extra-pulmonary TB using multiple combined immunoassays including IFNγ secretion by means of QFT-Plus assay, and surface co-expression of CD25 and CD134 in response to new antigens of *M. tuberculosis*. Besides, we also evaluated the new tube contains antigens eliciting $CD8^+$ T-cell response.

Materials and methods

Study populations

We prospectively enrolled 34 adult subjects admitted to the Department of Public Health and Infectious Diseases, 'Sapienza' University, Rome, Italy, for a suspected TB. Subjects were classified into the following two groups: (i) 11 subjects with suspected pulmonary TB (6 males and 5 females; median age was 45 years with IQR 25–56 years); (ii) 23 subjects with suspected extra-pulmonary TB (10 males and 13 females; median age was 42 years with IQR 34–55 years). We also recruited 10 healthy control donors (HD) among laboratory staff volunteers as controls (4 males and 6 females; median age was 40.5 years with IQR 34–47 years).

The patients were classified as having active TB if they fulfilled the following criteria: (i) identification of *M. tuberculosis* by microbiologic methods from biological specimens (positive culture for *M. tuberculosis*; or positive PCR using Xpert MTB/RIF, Cepheid, USA) and/or histo-pathological examination of affected tissues consistent with TB and the presence of acid fast bacilli; (ii) or clinical and radiological findings, including appropriate response to a full course of TB specific treatment.

Patients without active TB (no-TB) were defined as those individuals whose culture or histology for *M. tuberculosis* was negative, with resolution of clinical symptoms and radiographic abnormalities following treatment not involving anti-tuberculosis drugs or an eventual alternative diagnosis other than TB.

Measurement of IFNγ levels by QFT-Plus and cytofluorimetric analysis of $CD4^+$ and $CD8^+$ T cells were performed on the same blood samples collected from all patients. The study received approval from the local Ethics Committee of Azienda Policlinico Umberto I, Rome, Italy (Reference number 2669), and informed written consent was obtained from all individual participants included in the study.

QuantiFERON TB Gold- Plus (QFT-Plus) assay

Whole blood from each enrolled subject was tested using the QFT-Plus assay (Cellestis GmbH, QIAGEN Inc. Valencia, CA, USA). QFT-Plus kits were donated by Qiagen and used according to manufacturer's instructions [7]. Levels of IFNγ were quantified by ELISA and the QFT-Plus Analysis Software was used to analyze raw data and to calculate the results in international units per milliliter (IU/ml). The software performs a quality control assessment of the assay, generates a standard curve and provides a test result for each subject. The result was considered positive if IFNγ response after correction for the negative control was $\geqq 0.35$ IU/ml in one of the two TB antigen tubes (TB1 or TB2) or if both tubes (TB1 and TB2) showed $\geqq 0.35$ IU/ml.

CD25/CD134 assay and flow cytometry procedures

Heparinized peripheral blood (0.5 ml) was mixed with an equal volume of RPMI-1640 Medium (Sigma-Aldrich, Germany), and then added to 4 test tubes of QFT-Plus kit, respectively, containing, saline (negative control), peptides derived from ESAT-6 and CFP-10 (tube TB1 and tube TB2) and phytohaemagglutinin (PHA). The tubes were incubated at 37 °C in 5% CO_2 for 44–48 h [8] before antibody staining and analysis by flow cytometry. After 44 h of incubation, 100 µl of whole blood was stained with anti-CD3 PerCP, anti-CD4 PE-Vio770, anti-CD25 APC, anti-CD8 FITC (BD Biosciences, San Josè, USA), anti-CD134 PE (BioLegend, San Diego, CA, USA) for 20 min at 4 °C, followed by treatment with FACS lysing solution (BD Biosciences, according to manufacturer's instructions) and washed twice with FACS buffer (PBS/0.5% BSA, 0.01% sodium azide). Cells were fixed in 1% paraformaldehyde and acquired within 1 h using a MACSQuant Analyzer flow cytometer (MiltenyiBiotec, Germany) after calibration and automatic compensation. From each sample, a minimum of 100.000 events were collected and analyzed using FlowJo software v. 10 (Tree Star, San Carlos, CA, USA). Lymphocytes were identified on the

basis of forward and side-scatter; after gating on $CD3^+CD4^+$ T cells and $CD3^+CD8^+$ T cells, an analysis of CD25 APC and CD134 PE staining events was performed.

Standard gating procedures using fluorescence minus one (FMO) controls (unstimulated) were used to identify positive cells. Background $CD25^+/CD134^+$ expression in negative control was subtracted from stimulated condition. Figure 1 shows the gating strategy used to identify $CD4^+$ and $CD8^+$ T cells co-expressing $CD25^+CD134^+$.

Antigen-stimulated cultures from HD were used to determine the cut-off for a positive response, designed as the mean response (%) + 3 standard deviation (SD) of the TB1 and TB2 values.

The results were considered positive if $CD25^+CD134^+$ responses in either one or in both of the TB antigen tubes (TB1 and TB2) were ≧ of cut-off, after correction for the negative control.

Statistical analysis

Median (interquartile range, IQR) or mean (± SD) of the different parameters were calculated. Non-parametric Mann–Whitney test and non-parametric Kruskal–Wallis ANOVA with Dunn's post-test comparison were used to compare the T cell responses between two or three groups of patients, respectively. Pearson correlation coefficient and Cohen's κ coefficient were used to examine the correlation and the agreement between IFNγ secretion assay and flow cytometric co-expression of $CD25^+/CD134^+$, respectively. All statistical analyses were two-sided, performed using GraphPad Prism Software v. 5 (Software MacKiev), and considered significant at p values < 0.05.

Results

Study subjects

A total of 34 subjects with suspected TB and 10 age-matched HD were prospectively enrolled. According to clinical manifestations, pathological diagnosis, sputum smear, culture, and chest X-ray, 9 (26.5%) subjects were classified as pulmonary TB patients, and 16 (47%) subjects were classified as having extra-pulmonary TB (including pleural, lymphadenitis, abdominal, meningitis, uveitis and bone TB). Finally, in 9 subjects (26.5%) active TB was excluded and another diagnosis was obtained including: lower respiratory tract infections ($n = 1$), non-infectious respiratory diseases ($n = 1$), non-infectious uveitis ($n = 2$), sarcoidosis ($n = 1$), non-infectious lymphadenitis ($n = 3$), spondylodiscitis ($n = 1$); these subjects were classified as no-TB patients. The HD included laboratory staff volunteers (= 10), with no risk factors for *M. tuberculosis* exposure, and tested negative for TST and QFT-Plus.

QFT-Plus assay

Performance of QFT-Plus assay

Among patients with pulmonary TB, the QFT-Plus was positive in 6 (66.7%), negative in 2 (22.2%) and indeterminate in 1 (11.1%), due to a low mitogen value; among patients with extra-pulmonary TB, the test was positive in 13 (81.2%) and negative in 3 (18.8%) of cases. All no-TB patients and HD yielded QFT-Plus negative results. The QFT-Plus detection senstivities of pulmonary and extra-pulmonary TB were 75% (95% CI 34.9–96.8%) and 81.2% (95% CI 54.3–95.9%), respectively. The specificity of QFT-Plus for HD and no-TB group was 100% (95% CI 82.3–100%). Therefore, the overall sensitivity and specificity of QFT-Plus for diagnosis of active TB was 79.1% (95% CI 57.8–92.8%) and 100% (95% CI 82.3–100%), respectively.

Analysis of *M. tuberculosis*-specific responses in QFT-Plus assay

The QFT-Plus results were also evaluated by quantitative means and IFNγ responses following TB1 and TB2 stimulation are summarized in Table 1.

All TB infected patients, including pulmonary and extra-pulmonary, showed similar levels of IFNγ in response to TB1 (median 1.18 IU/mL, IQR 0.21–3.45) and TB2 antigens (1.53 IU/mL, IQR 0.56–5.19; $p = 0.33$) that were significantly higher compared to no-TB subjects and to HD ($p < 0.0001$; Fig. 2a).

Considering only TB-infected patients, the positive QFT-Plus results were stratified according to the ability of subjects to respond to TB1 and TB2. For pulmonary TB patients, the proportion of responders to TB1 and TB2 was 100%. Moreover, for extra-pulmonary TB patients, the 84.6% responded to TB1 stimulation, and 100% responded to TB2. Notably, 2 subjects with extra-pulmonary TB showed a selective response only to the peptides contained in TB2 tubes, suggesting an increase of sensitivity with respect to the old version QFT-GIT.

The quantitative evaluation showed that the IFNγ responses induced by TB1 were in the same range as those induced by TB2, with no differences between pulmonary and extra-pulmonary TB disease ($p > 0.05$ for all comparisons) (Fig. 2b; Table 1).

To have an estimation of effective $CD8^+$ T-cell responses, the ΔIFNγ was calculated by subtracting the IFNγ values obtained in response to TB1, which stimulates $CD4^+$ T cells, from those provided in response to TB2,

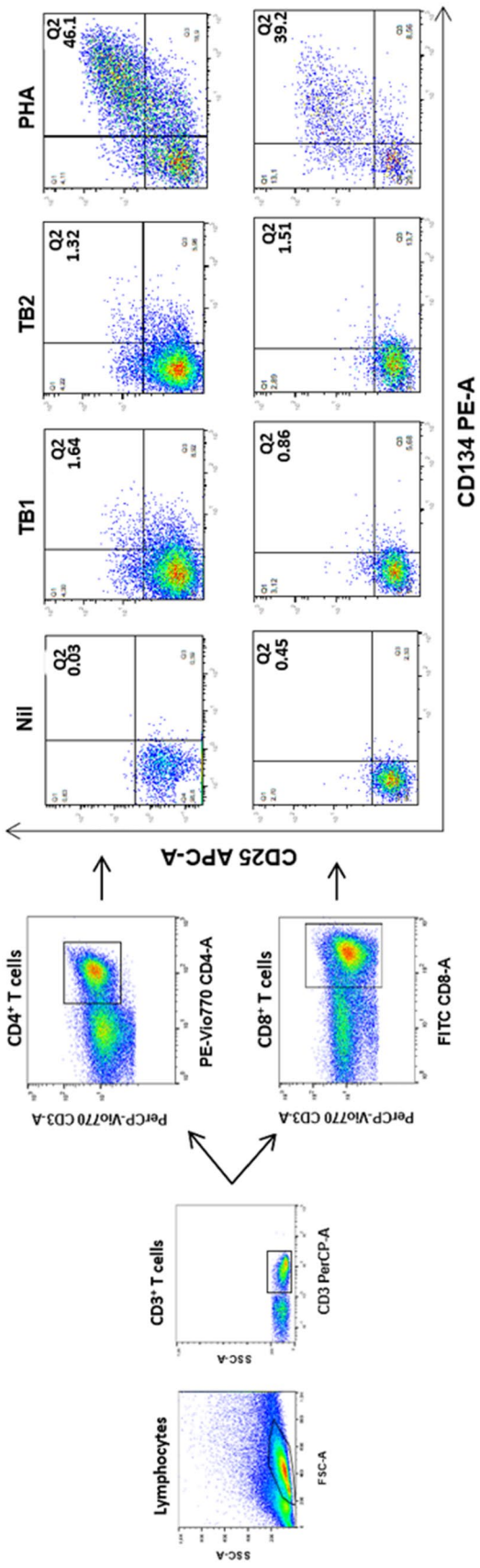

Fig. 1 Representative flow cytometry analysis showing the gating strategy for the analyses of T cells co-expressing $CD25^+/CD134^+$. Flow cytometric gating strategy for detection of T cells co-expressing $CD25^+CD134^+$ markers in response to negative control (Nil), *M. tuberculosis* specific antigens (TB1 and TB2), and phytohemagglutinin (PHA) in a representative subject with active TB. Lymphocytes were gated according to the forward (FSC) and side scatter (SSC) signals. Cells were then gated on $CD3^+$ and the fluorescence intensity for both $CD4^+$ and $CD8^+$ was measured. Gates for $CD25^+$ and $CD134^+$ cells were based on comparison of negative control (Nil) and mitogen (PHA) to include cells highly expressing CD25 plus positive for CD134. The percentages of $CD25^+CD134^+$ T cells are shown in upper right quadrants

Table 1 Quantitative IFNγ responses from QFT-Plus assay in all enrolled subjects

Subjects	*n* (%)	IFNγ (IU/mL)			
		TB 1	TB 2	TB 2–TB 1	*p* value
Pulmonary TB					
QFT-Plus positive	6 (67)	3.33 (1.10–8.54)	4.19 (1.36–7.45)	0.04 (0-0.76)	0.81
QFT-Plus indeterminate	1 (11)	0.05	0.24	0.19	–
QFT-Plus negative	2 (22)	0.15 (0.06–0.24)	0.09 (0.01–0.17)	–	0.66
Extra-pulmonary TB					
QFT-Plus positive	13 (81)	1.93 (0.69–3.45)	2.42 (1.35–5.98)	0.32 (0.005–1.19)	0.23
QFT-Plus negative	3 (19)	0.06 (0-0.18)	0.04 (0.02–0.19)	–	1
No-TB					
QFT-Plus negative	9 (100)	0 (0.0-0.01)	0 (0.0–0.03)	–	0.83
Healthy donors					
QFT-Plus negative	10 (100)	0 (0.0–0.0)	0 (0.0–0.07)	–	0.12

The values expressed as IU/ml are median with interquartile range. TB1 and TB2 are antigen-containing tubes of QFT-Plus

QFT-Plus QuantiFERON-TB Gold Plus

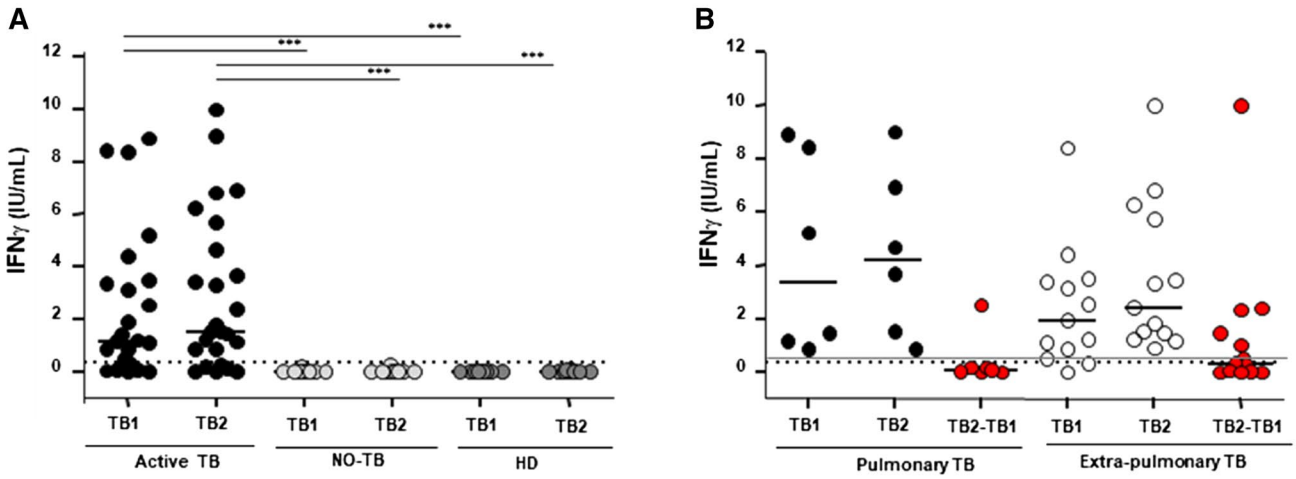

Fig. 2 Antigen-induced IFNγ response from all patients enrolled. The release of IFNγ in response to TB1 and TB2 antigens was evaluated after 18 h of incubation using the QuantiFERON TB Gold-Plus (QFT-Plus; Cellestis GmbH, QIAGEN Inc. Valencia, CA, USA) assay, according to the manufacturer's instructions. The figure shows the antigen-induced IFNγ response. **a** The IFNγ response (IU/mL) was assessed in all TB infected subjects (including 9 with pulmonary TB, and 16 with extra-pulmonary TB), in 9 no-TB subjects and 10 HD. **b** The antigen-induced IFNγ response was assessed only in infected patients with positive QFT-Plus, in according to the type of disease (6 with pulmonary TB, and 13 with extra-pulmonary TB). In addition, the ΔIFNγ (TB2-TB1), subtracting the IFNγ values obtained in response to TB1 from those provided in response to TB2 was calculated. Horizontal bars represent the median values, horizontal dashed lines indicate the cut-off of QFT-Plus positive response corresponding to 0.35 (IU/mL), whereas gray horizontal lines in panel b indicate the cut-off of 0.6 (IU/mL) for positive ΔIFNγ response. Statistical analysis was calculated by non-parametric Mann–Whitney test and non-parametric Kruskal–Wallis ANOVA with Dunn's post-test comparison, to compare the responses between two or three groups of patients, respectively. Significant *p* values are indicated

which stimulates $CD4^+$ and $CD8^+$ T cells. The ΔIFNγ was higher in extra-pulmonary (median 0.32 IU/mL, IQR 0.005–1.91), as compared to pulmonary subjects (0.08 IU/mL, IQR 0–0.19), although the difference did not attain statistical significance ($p = 0.35$; Fig. 2b).

A cut-off > 0.6 IU/mL for ΔIFNγ release was considered as true difference and used as estimation for $CD8^+$ T-cell response [9, 10]. However, only 5 out of 13 (38.5%) extra-pulmonary TB patients and 1 out of 6 (16.6%) pulmonary TB patients had a difference in IFNγ release of > 0.6 IU/mL. Thus, the analysis of the differences TB2-TB1 showed that $CD8^+$ T-cell response was prevalent among the extra-pulmonary TB subjects.

CD25/CD134 flow cytometry assay

The co-expression of CD25 and CD134 by T cells in TB1 and TB2-stimulated whole blood cultures of 44 h duration was assessed in TB patients. In addition, to evaluate the specificity of CD25/CD134 assay, HD and subjects with other diagnosis over TB, were also assessed. The results are summarized in Table 2. Background co-expression of CD25 and CD134 on T cells was extremely low, as shown in a representative active TB subject (Fig. 1).

Analysis of $CD4^+$ T-cell responses to *M. tuberculosis* antigens

Flow cytometric analysis of $CD4^+$ T cells showed significantly higher frequency of $CD25^+CD134^+$ T cells in response to TB1 (median 1.32%, IQR 0.61–2.22%) and TB2 antigens (1.41%, 0.81–2.41%) in TB-infected patients (including pulmonary and extra-pulmonary), as compared to no-TB patients and HD ($p < 0.0001$for TB1, and $p < 0.0001$ for TB2) (Fig. 3a; Table 2). Indeed, based on positive cut-off, corresponding to 0.136% (mean 0.0337, SD 0.0343) for TB1, and 0.119% (mean 0.0338, SD 0.0284) for TB2, we scored as positive all pulmonary and extra-pulmonary and none of no-TB subjects and HD.

Notably, among the active TB group, 3 subjects with extra-pulmonary TB lacked a response to TB1 stimulation, but were considered positive since the TB2 stimulation elicited a selective $CD4^+CD25^+CD134^+$ T-cell response with frequencies of 1.26%, 0.25% and 0.15%, respectively. Thus, the analysis of $CD4^+$ T cells co-expressing CD25/CD134 allowed the discrimination between *M. tuberculosis*-infected and uninfected patients with a sensitivity of 100% (95% CI 86.2–100%) and specificity of 100% (95% CI 82.3–100%) for active TB diagnosis.

Next, to determine whether these markers were differentially expressed in TB infected patients, we compared their expression in according to pulmonary and extra-pulmonary diseases. Among those with pulmonary TB, the proportion of responders to TB1 and TB2 was 100%. Instead, among extra-pulmonary patients, the 81% responded to TB1 and 100% responded to TB2. The co-expression of CD25/CD134 markers induced by TB1 was in the same range as that induced by TB2 in both groups of TB subjects ($p > 0.05$ for all comparisons) (Fig. 3b; Table 2). No differences were observed comparing pulmonary and extra-pulmonary TB patients.

Analysis of $CD8^+$ T-cell responses to *M. tuberculosis* antigens

Analysis of $CD8^+CD25^+CD134^+$ T cells showed different results from those observed in $CD4^+$ T-cell compartment, revealing a lower response among all evaluated subjects. The results of the co-expression of $CD8^+CD25^+CD134^+$ T cells are summarized in Table 2.

Significant higher expression of CD25/CD134 markers on $CD8^+$ T cells, following TB1 (median 0.04%, IQR 0–0.20%) and TB2 stimulation (median 0.18%, IQR 0–0.46%), was observed in TB infected patients (including pulmonary and extra-pulmonary) compared to no-TB subjects and HD ($p = 0.019$ for TB1, and $p = 0.018$ for TB2) (Fig. 3c; Table 2). To note, the majority of uninfected and healthy subjects varied from undetectable to low frequencies (0–0.04%) of activated $CD8^+CD25^+CD134^+$ T in response to both TB1 and TB2 specific antigens. Indeed, based on positive cut-off, corresponding to 0.072% (mean 0.0122, SD 0.0201) for TB1, and 0.074% (mean 0.0193, SD 0.0183) for TB2, we scored as positive 6 out of 9 (66%) pulmonary patients, 11 out of 16 (69%) extra-pulmonary patients, and none of no-TB subjects and HD. The testing information of TB subjects scored as negative by $CD8^+CD25^+CD134^+$ assay are shown in Table 3. Thus, also the analysis of double-positive $CD8^+$ T cells allowed the discrimination between *M. tuberculosis*-infected and uninfected patients, with a sensitivity and specificity for active TB diagnosis of 68% (95% CI 46.5–85%) and 100% (95% CI 82.3–100%), respectively.

Next, the co-expression of $CD25^+CD134^+$ on $CD8^+$ T cells was analyzed by comparing subjects with pulmonary and extra-pulmonary TB. Among those with pulmonary disease, TB1 antigen induced a response in only 33% of subjects and TB2 in 66% of them. Instead, among

Table 2 Analysis of co-expression of $CD25^+CD134^+$ on T cells in all patients enrolled

Subjects	*n* (%)	% $CD4^+CD25^+CD134^+$ T cells		% $CD8^+CD25^+CD134^+$ T cells	
		TB 1	TB 2	TB 1	TB 2
Pulmonary TB	9 (22)	1.23 (0.61–1.19)	1.34 (0.57–1.7)	0.04 (0.005–0.20)	0.18 (0–0.46)
Extra-pulmonary TB	16 (67)	1.52 (0.48–2.45)	1.64 (1.05–3.04)	0.05 (0.0–0.21)	0.15 (0.05–0.43)
No TB	9 (100)	0 (0.0–0.04)	0.02 (0.0–0.06)	0 (0.0–0.02)	0.005 (0–0.027)
Healthy donors	10 (100)	0.02 (0.0–0.07)	0.02 (0.01–0.04)	0 (0.0–0.02)	0 (0.02–0.035)

The results are expressed as median with interquartile range

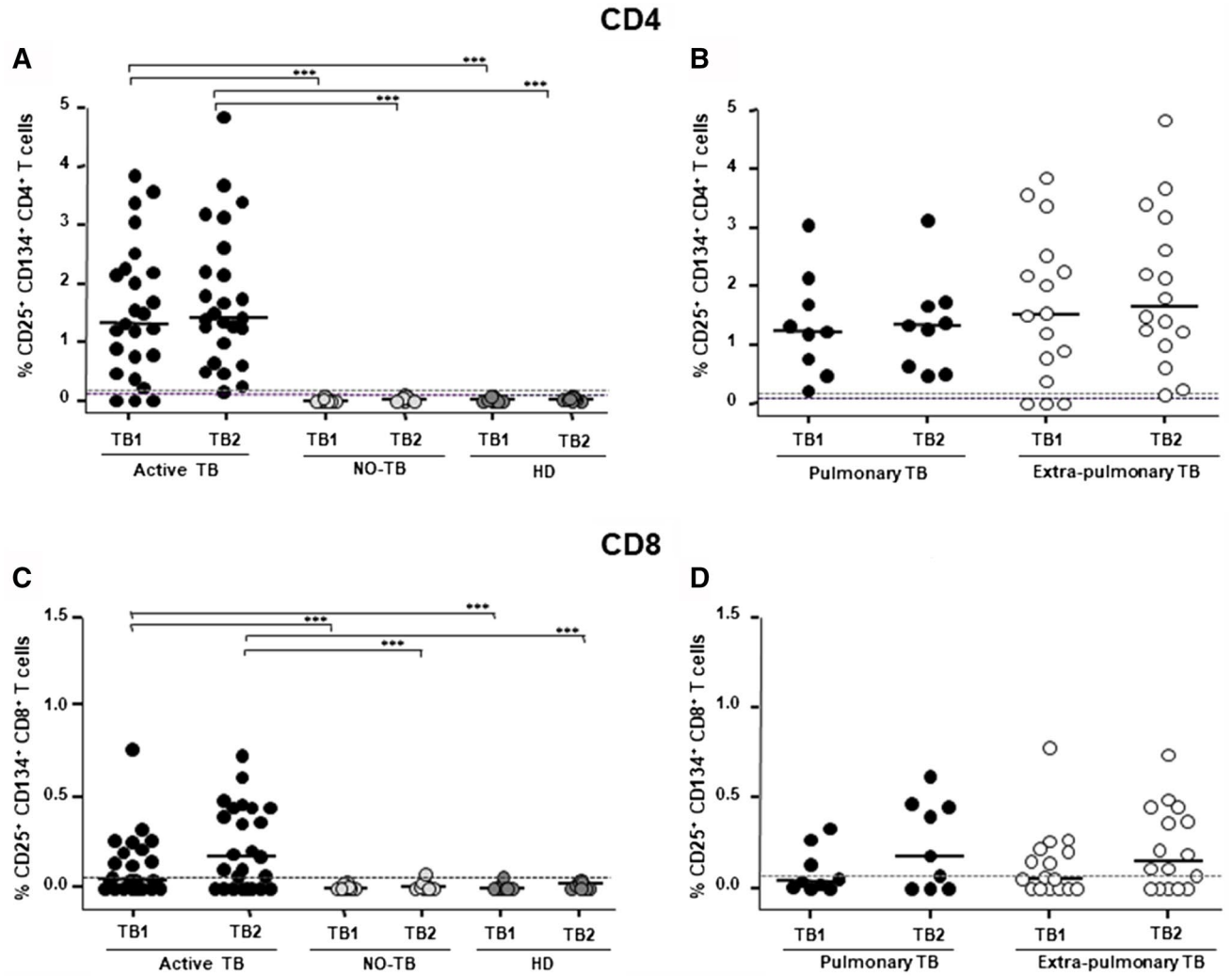

Fig. 3 Characterization of $CD4^+$ and $CD8^+$ T cells co-expressing $CD25^+CD134^+$. The co-expression of CD25 and CD134 by $CD4^+$ and $CD8^+$ T-cell subsets in response to either TB1 or TB2 was evaluated in whole blood cultures of 44 h duration. **a, c** Frequency of $CD4^+$ and $CD8^+$ T cells co-expressing $CD25^+CD134^+$ in response to stimulation with TB1 and TB2, was assessed in TB infected subjects ($n=25$, including 9 with pulmonary and 16 with extra-pulmonary), in subjects without TB ($n=9$) and HD ($n=10$). **b, d** The antigen-induced co-expression of $CD25^+CD134^+$ was assessed in according to the type of disease, in patients with pulmonary TB ($n=9$), and with extra-pulmonary TB ($n=16$). Subjects were defined to be positive or negative to CD25/CD134 assay according to the cut-off defined from HD, as the mean response (%) plus three times the SD of values obtained in response to TB1 and TB2. Horizontal black bars represent the median values, and horizontal gray dashed lines indicate the cut-off of positive CD25/CD134 response. Statistical analysis was calculated by non-parametric Mann–Whitney test and non-parametric Kruskal–Wallis ANOVA with Dunn's post-test comparison, to compare the responses between two and three groups of patients, respectively. Significant *p* values are indicated

extra-pulmonary TB patients, the 44% responded to TB1 and the 69% responded to TB2 stimulation.

Evaluating the frequencies of activated $CD8^+$ T cells, the expression of CD25/CD134 markers induced by TB2 was higher than that induced by TB1 for both pulmonary and extra-pulmonary groups (Fig. 3d; Table 2), but the differences did not attain statistical significance, possibly because of small number of participants ($p>0.05$ for all comparisons). On the other hand, our data indicate that the expression of CD25/CD134 markers on $CD8^+$ T cells was mainly induced by TB2 stimulation with frequencies similar among pulmonary and extra-pulmonary TB subjects ($p=0.88$).

Finally, we compared the co-expression of CD25/CD134 on both $CD4^+$ and $CD8^+$ T cells. Following TB1 stimulation, a significantly higher frequency of activated $CD4^+$ T cells in comparison to $CD8^+$ T cells was observed, both in pulmonary ($p=0.0002$) and extra-pulmonary TB patients ($p=0.0009$), suggesting that TB1 antigen mainly stimulates the $CD4^+$ T cells. Likewise, also following TB2 stimulation, the co-expression of CD25/CD134 was mostly present

Table 3 Testing information for TB patients showing negative $CD8^+CD25^+CD134^+$ T-cell response and discordant QFT-Plus and $CD25^+CD134^+$ T-cell assay results

Subjects	QFT-Plus IFNγ (IU/mL)			% $CD4^+$ $CD25^+CD134^+$ T cells			% $CD8^+$ $CD25^+CD134^+$ T cells		
	TB 1	TB 2	Result	TB 1	TB 2	Result	TB 1	TB 2	Result
1. Pulmonary TB	5.21	4.68	Positive	1.69	1.74	Positive	0.01	0.0	Negative
2. Pulmonary TB	0.87	0.88	Positive	0.76	0.64	Positive	0.04	0.0	Negative
3. Pulmonary TB	0.24	0.17	Negative	0.47	0.50	Positive	0.02	0.0	Negative
4. Pulmonary TB	0.06	0.01	Negative	0.22	0.47	Positive	0.0	0.18	Positive
5. Pulmonary TB	0.05	0.24	Indeterminate	1.19	1.34	Positive	0.33	0.45	Positive
6. Uveitis TB	0.88	0.89	Positive	2.52	0.99	Positive	0.0	0.0	Negative
7. Uveitis TB	0.0	10	Positive	0.0	1.26	Positive	0.0	0.0	Negative
8. Abdominal TB	3.38	5.71	Positive	3.85	4.84	Positive	0.0	0.0	Negative
9. Pleural TB	0.06	0.04	Negative	0.0	0.25	Positive	0.0	0.0	Negative
10. Pleural TB	0.0	0.02	Negative	0.0	0.15	Positive	0.0	0.0	Negative
11. Bone TB	0.18	0.19	Negative	3.38	3.68	Positive	0.05	0.37	Positive

The IFNγ values and the $CD25^+/CD134^+$ frequencies in negative control were subtracted from all stimulated conditions. TB1 and TB2 are antigen-containing tubes of QFT-Plus

QFT-Plus QuantiFERON-TB Gold Plus

on $CD4^+$ T cells than $CD8^+$ T cells both in pulmonary ($p=0.0009$) and extra-pulmonary TB patients ($p<0.0001$) (Fig. 3b, d).

QFT-Plus versus CD25/CD134 flow cytometry assay

After excluding indeterminate results, QFT-Plus assay resulted in an agreement of 88% with a Cohen's kappa of 0.76 with $CD25^+CD134^+$ $CD4^+$ T cells, and in an agreement of 84% with a Cohen's kappa of 0.67 with $CD25^+CD134^+$ $CD8^+$ T cells.

Discordant results were found in 6 subjects (3 pulmonary and 3 extra-pulmonary TB). Among them, five subjects had a negative QFT-Plus and one had indeterminate result. All six cases displayed a positive $CD4^+$ T-cell response, and four subjects a positive $CD8^+$ T-cell response. The characteristics of discordant TB subjects are shown in Table 3.

Next, we compared the expression of CD25/CD134 on $CD4^+$ and $CD8^+$ T cells from TB patients with and without a positive QFT-Plus result. The frequencies of $CD4^+$ and $CD8^+$ T cells co-expressing CD25/CD134 in response to TB1 and TB2 were higher in TB patients with a positive QFT-Plus results (Fig. 4); the difference was statistically significant only for $CD4^+$ T cells in response to TB2 ($p=0.03$).

Correlations among IFNγ secretion and co-expression of CD25/CD134

We examined possible correlations among cytokine production and activation of T cells in TB patients (Fig. 5). IFNγ secretion in response to TB1 was positively correlated with the frequency of activated $CD4^+$ T cells ($r=0.42$, $p=0.03$, $r^2=0.17$; Fig. 5a), but not with the activated $CD8^+$ T cells ($r=0.14$, $p=0.47$, $r^2=0.02$; Fig. 5b).

Following TB2 stimulation, no correlation between IFNγ response and frequencies of activated $CD4^+$ T cells ($r=0.29$, $p=0.15$, $r^2=0.08$; Fig. 5c) and activated $CD8^+$ T cells ($r=-0.00$, $p=0.9$, $r^2=0.00$; Fig. 5d) was observed. This lack of correlation was expected, because in QFT-Plus assay the IFNγ was released simultaneously from $CD4^+$ and $CD8^+$ T cells after TB2 stimulation. Thus, we summed the frequencies of $CD4^+$ and $CD8^+$ T cells co-expressing CD25/CD134 and further examined the possible correlation with IFNγ response. Notably, we found a positive correlation between IFNγ response and the sum of the frequencies of $CD4^+$ and $CD8^+$ T cells co-expressing CD25/CD134 after TB2 stimulation ($r=0.45$, $p=0.020$, $r^2=0.07$; Fig. 5e). Finally, we found a positive correlation between IFNγ in response to TB1 and IFNγ in response to TB2 ($r=0.7$, $p=0.0001$, $r^2=0.49$; Fig. 5f). These results showed a good correlation between two immunologic assays. No significant correlation was observed in HD and subjects with no-TB (data not shown).

Discussion

The impairment of lymphocyte function is commonly observed in active TB patients. Previous studies have shown that depressed proinflammatory cytokine productions, expansion of regulatory T cells and increased production of nitric oxide could suppress T-cell functions, result in impaired immunity of active TB patients [11].

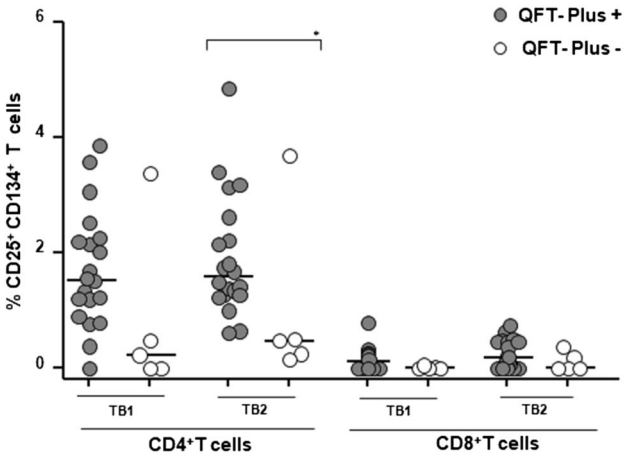

Fig. 4 Co-expression of $CD25^+CD134^+$ on T cells in according to positive and negative QFT-Plus results. The frequencies of $CD4^+$ and $CD8^+$ T cells co-expressing $CD25^+CD134^+$ were assessed in active TB patients with ($n=20$; gray circle) and without ($n=5$; white circle) positive QFT-Plus result. Horizontal black bars represent the median values. Statistical analysis was calculated by non-parametric Mann–Whitney test to compare the responses between two groups of patients. Significant p values are indicated

Also IGRA assays, which depend on the detection of IFNγ in response to TB antigens, and widely used for immunological diagnosis of *M. tuberculosis* infection, sometimes showed negative results in subjects with active TB disease for a relatively low IFNγ secretion [12, 13]. Thus, early detection of *M. tuberculosis* infection remains complicated in particular in the setting of extra-pulmonary forms of TB.

To improve the identification of TB in pulmonary and extra-pulmonary cases, we assessed the combination of two specific immunological markers comprising IFNγ secretion, by means of an IGRA assay, and surface co-expression of CD25 and CD134 in response to *M. tuberculosis*-specific antigens.

First of all, we aimed at evaluating the performance of QFT-Plus in terms of the TB1 and TB2 results, as this assay, new generation of IGRA, included modified peptides that are optimized to activate $CD8^+$ T cells in addition to CD4. Although TB2 elicits an immune response driven by both $CD4^+$ and $CD8^+$ T cells, our results showed that in TB patients, the quantitative IFNγ value in TB2 was similar to that in TB1. The first finding that IFNγ was

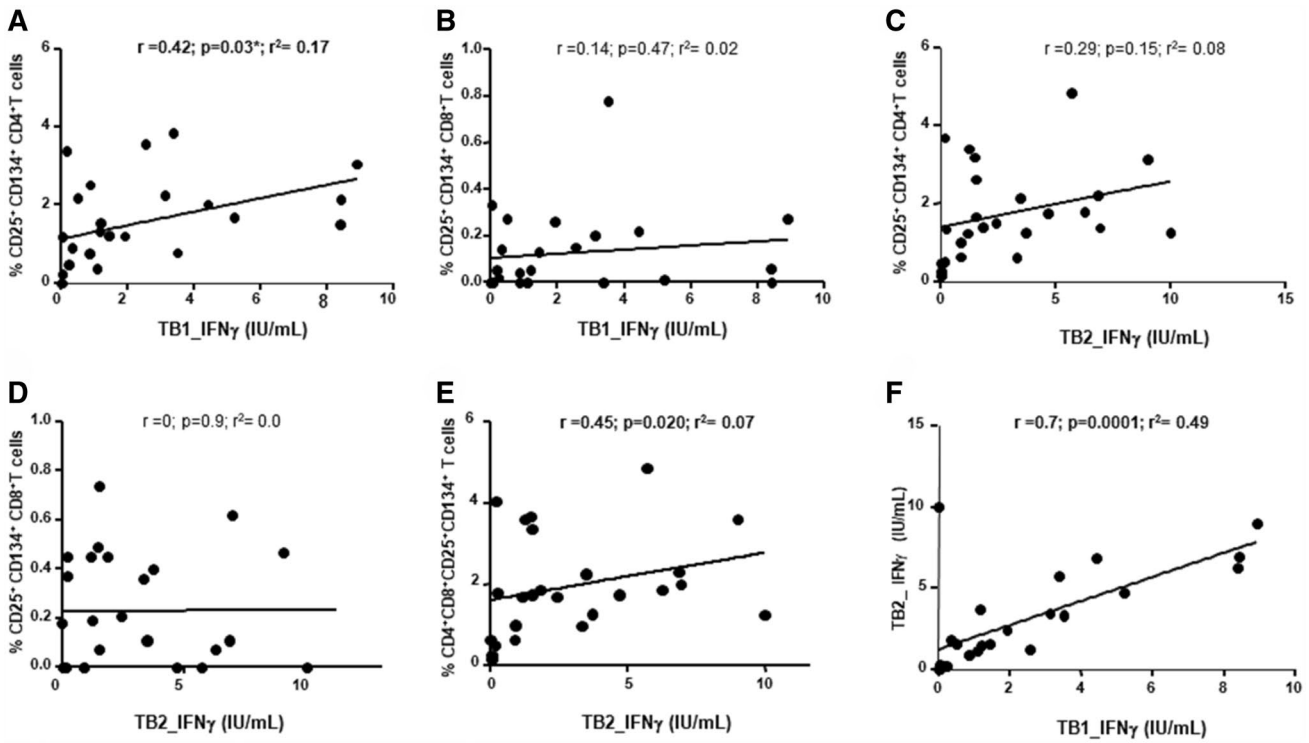

Fig. 5 Correlations between IFNγ secretion, and co-expression of $CD25^+CD134^+$ in response to *M. tuberculosis* specific antigens. The immunologic assays of all TB subjects were correlated using Pearson correlation coefficients. **a** IFNγ response to TB1 stimulation was positively correlated with the frequency of $CD25^+CD134^+$ $CD4^+$ T cells, **b** but not with the activated $CD8^+$ T cells. **c, d** In response to TB2 stimulation there was no correlation between IFNγ response and activated $CD4^+$ T cells and $CD8^+$ T cells. **e** Positive correlation between IFNγ and the sum of the frequencies of $CD4^+$ and $CD8^+$ T cells following TB2 stimulation. **f** Positive correlation between IFNγ in response to TB1 and IFNγ in response to TB2 specific antigen

produced by $CD8^+$ T cells was based on a study of $CD4^+$ T cell-deficient mice [14].

Interestingly, a selective positive response only to peptides contained in TB2 tube, has been observed in 2 individuals with extra-pulmonary TB. The addition of the TB2 antigen tube to QFT-Plus assay could provide a diagnostic advantage over the QFT-GIT version, which stimulates only $CD4^+$ T cells in a single antigen tube (TB1), thus increasing the positivity rates of the new assay. A recent study reported a significant increase in QFT-Plus positivity rates among low-risk workers in health care, compared to QFT-GIT (3.5% vs 2.2%, $p = 0.003$); among the subjects with positive QFT-Plus results the 20% was positive by TB1 only, and 32.6% was positive by TB2 only [15]. However, studies evaluating the QFT-Plus assay, performed in low-TB-incidence setting, have shown high agreement and equivalent performance with QFT-GIT assay, obtaining a sensitivity of 89–90% and a specificity of 84–98% [10, 16–21].

In this study, five patients with active TB displayed a QFT-Plus negative result. This may be correlated with the condition of "*M. tuberculosis*-specific unresponsiveness" as suggested in previous study [22, 23] or due to a higher amount of specific cells at site of infection compared to peripheral blood [24].

We decided to evaluate CD25 (interleukin-2 receptor alpha, IL-2Rα) and CD134 (a TNF receptor superfamily member) surface expression since these markers were recognized to be extremely sensitive and specific tools for the assessment of functional response by individual T-cell subsets to a variety of stimuli [8]. The biological basis of the $CD25^+CD134^+$ phenotype, on which this assay is based, includes the fact that interactions between CD134 and its ligand are critical for memory T cell development and effector T cell survival [25], and the up-regulation of the high-affinity IL-2Rα via CD25 is critical for the proliferation and differentiation of T cells [26].

In the current study, we used the CD25/CD134 assay to measure a broad polyclonal response to TB1 and TB2 antigens, providing a simple assay of antigen-specific T lymphocyte function. The flow cytometric analysis allowed a clear differentiation between *M. tuberculosis*-infected and uninfected patients, with a better combination of sensitivity and specificity that was higher than QFT-Plus, especially by evaluation of $CD4^+$ T-cell subset.

Based on the IGRA format, it is unknown whether the response to TB2 is mediated by the $CD8^+$ or $CD4^+$ T cells, even if can reasonably be assumed that a TB2 selective response could be mediated by $CD8^+$ T cells. As recently shown by Barcellini et al. [10], the difference in IFNγ release between TB2 and TB1 stimulation may provide a surrogate marker of $CD8^+$ T-cell response magnitude. Using this approach, we found that about 40% of extra-pulmonary TB patients showed a $\Delta IFN\gamma > 0.6$ IU/mL, suggesting indirectly that $CD8^+$ T-cell response is indeed associated with extra-pulmonary TB.

By flow cytometry analysis, this result could be more refined, assessing a compartmentalized analysis of T-cell sub-populations (gating the $CD4^+$ and $CD8^+$ T cells separately). Regarding the $CD4^+$ T-cell compartment, the co-expression of $CD25^+CD134^+$ induced by TB1 was in the same range as that induced by TB2 in both groups of TB subjects, but a selected $CD4^+$ T-cell response to TB2 and not to TB1 was observed in 3 subjects with extra-pulmonary TB, including one with uveitis and two with pleural involvement. These findings suggest that the introduction of TB2 tube in the assay has improved the detection of unusual TB disease.

Conversely, in the analysis of $CD8^+$ T cells, the co-expressing $CD25^+CD134^+$ was more frequently detected in response to TB2 antigen, and no distinct differences were observed among both groups of TB subjects. These findings were parallel to those of a recent report, showing a $CD8^+$ T-cell response mainly induced by TB2 and associated to active TB [27].

As regards the $CD8^+$ T-cell compartment, several lines of evidence have pointed out the role of $CD8^+$ T cells in TB [28]. *M. tuberculosis*-specific $CD8^+$ T-cell responses can be detected predominantly in patients with active TB as compared to LTBI subjects [29–31], consistent with the current paradigm associating $CD8^+$ T-cell responses to high antigen burden [29, 32, 33], and declined during anti-TB treatment [29, 34]. Importantly, a recent study performed in young children showed that *M. tuberculosis*-specific $CD8^+$ T cells were detected in active TB disease but not in healthy children recently exposed to *M. tuberculosis*, despite the fact that similar frequencies of $CD4^+$ T cells were present in both groups [35].

We demonstrated that both TB1 and TB2 induce a higher expression of activation markers on $CD4^+$ T cells among infected TB subjects, compared to the lower degree of $CD25^+CD134^+$ up-regulation in $CD8^+$ T cells, mainly due to TB2 stimulation. Thus, the detection of *M. tuberculosis*-specific $CD8^+$ T cells was also a strong predictor of discrimination between infected and uninfected patients, although it was less accurate than $CD4^+$ T cells. The low frequency of antigen-specific $CD8^+$ T cells could be the consequence of the sequestration of these cells at sites of infection, widely observed in TB [36, 37]. Although $CD8^+$ T-cell response is substantially less powerful than the *M. tuberculosis* $CD4^+$ T-cell response, these results highlight the capacity of the cells from active TB patients to respond with both $CD4^+$ and $CD8^+$ T-cell subsets following *M. tuberculosis*-specific stimulation, and further studies targeting children and immunocompromised individuals with low $CD4^+$ T cell counts, may confirm the value of the CD25/CD134 co-expression on

$CD8^+$ T cells in such patients. Hence, it is evident that flow cytometry analysis could have the advantage of detecting $CD8^+$ T-cell response with a range of antigenic conditions besides the short peptides included into new QFT-Plus. Of note, the low degree of $CD8^+$ up-regulation in T cells could be optimized including co-stimulatory antibodies (e.g., anti-CD28 and anti-CD49d) during T-cell stimulation in vitro.

In our study, the CD25/CD134 assay showed an excellent degree of sensitivity for detecting active TB, and a good degree of agreement with QFT-Plus, resembling data reported in two important prospective case control studies, including one performed in low- and high-TB endemic areas with a high proportion of HIV/AIDS subjects [38, 39]. It is very interesting to note that our cut-off values of flow cytometry analysis were similar to that reported by Escalante et al. [39] who used a combinatorial immunoassay approach, comprising IFNγ secretion and co-expression of CD25/CD134 on T cells, as a method to risk stratify patients with LTBI.

The two immunologic assays were qualitatively discordant in 6 individuals with TB (13.6%); all cases with negative or indeterminate QFT-Plus results displayed a positive $CD4^+$ T-cell response, and four subjects showed also a positive $CD8^+$ T-cell response. These findings supported the usefulness of a combinatorial IGRA and flow cytometry analysis for detecting TB disease, especially in cases that are not easy to diagnose. However, the specific comparison of TB patients with and without a positive QFT-Plus results, highlighted an higher frequency of T cells co-expessing $CD25^+CD134^+$ in those with a positive QFT-Plus. The CD25/CD134 assay is not cytokine specific, but measures T-cell activation after stimulation with *M. tuberculosis* specific antigens, and it would also potentially detect *M. tuberculosis* specific TH17 responses and T regulatory, that have been shown to depress IFNγ production by T cells [40]. This could be an explanation for why some participants displayed discordant results (positive CD25/CD134 assay and negative QFT-Plus).

Correlative analysis of frequency of $CD25^+CD134^+$ T cells and amount of IFNγ produced for each specific antigen showed variable results with significant correlations seen following TB1 stimulation, but not following TB2 stimulation. The simplest explanation is that QFT-Plus assay measures the global IFNγ secretion by two functional T-cell subsets, as $CD4^+$ and $CD8^+$, in response to TB2, while flow cytometry detects a $CD4^+$ and $CD8^+$ T-cell responses separately and this should probably be taken into account. This observation seems plausible, because in a further correlative analysis, we showed a positive correlation between IFNγ and co-expression of surface markers evaluated on global $CD4^+$ and $CD8^+$ T cells after TB2 stimulation. These data confirm that $CD25^+CD134^+$ T cells can be considered to be activated antigen-specific T cells responding to the specific antigen stimulus.

The present study has some limitations, such as the relatively small number of patients within each clinical group and the lack of a prospective analysis. Although IGRA assay was developed to detect *M. tuberculosis* latent infection, in the current study, we evaluated active TB patients only.

To our knowledge, in this study, the combination of two specific immunological markers, comprising IFNγ secretion and CD25/CD134 co-expression, was evaluated for the first time utilizing the QFT-Plus platform to generate samples. Our data, though related to a small cohort of patients, revealed that combined use of two immunological analyses has improved the diagnostic accuracy for active TB compared to measuring only IFNγ such as the QFT-Plus.

Furthermore, the cytometry CD25/CD134 analysis, although by itself cannot differentiate active from latent infection, has been used to identify subjects clinically TB infected [13], subjects with LTBI in the setting of advanced HIV co-infection [38], and as an additional diagnostic tool to identify patients on tumor necrosis factor antagonists with latent and active TB, including a severely immunosuppressed case with infliximab-associated disseminated TB [41, 42].

Future studies with larger sample sizes are needed to better understanding of the clinical and biological significance both of differential value between two antigens tubes and new CD25/CD134 assay, and should focus on not only active TB patients but also in a setting of contact tracing, characterized by a high possibility of asymptomatic *M. tuberculosis* latent infection, to obtain data evaluating the performance of these immunologic assay.

In conclusion, the diagnostic delay represents a critical issue in the management of subjects with suspected TB, especially in terms of treatment decisions. It is therefore important to develop new tools and algorithms to increase the diagnostic accuracy of current immunologic assays, including flow cytometric analysis. In this context, we suggest that a combined use of classic QFT-Plus and specific CD25/CD134 response may be a potential additional approach in the diagnostic workup for active TB, particularly in the setting of extra-pulmonary diseases, when the diagnosis it is not easy to establish or it requires invasive procedures.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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