RESEARCH ARTICLE

T cells co-producing *Mycobacterium tuberculosis*-specific type 1 cytokines for the diagnosis of latent tuberculosis

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ABSTRACT. Patients treated with tumor necrosis factor (TNF)-α-antagonizing medication are at increased risk of developing active tuberculosis (TB), brought about mainly by reactivation of latent infection. Thus, screening for latent TB infection (LTBI) prior to administration of anti-TNF-α-therapy is required. For a long time, the tuberculin skin test (TST) was the only means of diagnosing LTBI, however, interferon-gamma-release assays (IGRAs), are promising new tools. Fifty two patients with dermatological disorders were included prior to implementation of anti-TNF-α therapy. *Mycobacterium tuberculosis* (MTB)-specific cytokine production, including interferon (IFN)-γ, TNF-α, interleukin (IL)-2 and IL-10, was measured in $CD4^+$ and $CD8^+$ T cells by cytokine flow cytometry following stimulation of peripheral blood mononuclear cells (PBMC) with purified protein derivative (PPD) and early secretion antigenic target (ESAT)-6. Simultaneously, a TST was administered and 11 were TST-positive. Generally, MTB-specific IFN-γ produced by $CD4^+$ T cells correlated well with TST results. $CD4^+$ T cells co-producing specific IFN-γ and TNF-α after ESAT-6 stimulation showed the highest overall agreement with the TST (Kappa [κ] = 0.87). Each single cytokine displayed individual patterns, the expression of IFN-γ, however, showed the highest concordance with the TST (κ = 0.82). This suggests that the enumeration of MTB-specific $CD4^+$ T cells might introduce greater specificity for the diagnosis of latent TB, compared to the TST.

Keywords: diagnosis, latent tuberculosis, *Mycobacterium tuberculosis*, anti-TNF-treatment, IFN-γ, TNF-α

Drugs that antagonize TNF-α are approved for different inflammatory diseases such as rheumatoid arthritis, Crohn's disease and psoriatic arthritis, and provide an outstanding clinical benefit for patients. However, these drugs can reactivate TB in patients who have latent infection [1]. Although one third of the world's population is infected with *Mycobacterium tuberculosis* (MTB), only an estimated 10% of infected individuals will develop active disease during their lifetime [2]. In the remaining 90% of cases, the immune system contains the infection leaving the individual symptom-free. The steady state between host and pathogen is crucially dependent on TNF-α [3]. Inevitably, the delicate balance is disturbed by anti-TNF-α therapy, leading to reactivation of MTB, and progression to active disease. This result, in most cases, in atypical clinical presentations such as extra-pulmonary or disseminated disease [4, 5]. Thus, patient-screening for active and latent TB infection (LTBI), before the administration of anti-TNF-α drugs, is essential.

Currently, screening consists mainly of the 100-year-old tuberculin skin test (TST), which has several limitations. Firstly, the TST has poor specificity, since previous BCG vaccination and environmental mycobacteria exposure can result in false-positive results [6]. False-positive results can lead to unnecessary treatment, introducing a significant risk of the severe side effects associated with isoniazid or rifampin/pyrazinamide therapy [7]. Secondly, and in stark contrast, patients undergoing immunosuppressive therapy more often display negative TST results compared with general population [8]. Thirdly, the TST requires two visits and in addition is subject to variation according to age, sex and latitude [6].

Analysis of the mycobacterial genome has identified the region of difference (RD) 1, which encodes for proteins that are absent in BCG and most environmental mycobacteria [9]. Amongst them, ESAT-6 and culture filtrate protein (CFP)-10 can be used to provoke IFN-γ production in peripheral blood mononuclear cells (PBMC). Recently-introduced, MTB-specific IFN-γ release assays (IGRAs) use these MTB-specific proteins and are expected to yield higher specificity and to replace eventually the TST. To quantify the MTB-specific reactivity in peripheral blood, IFN-γ production of T cells from the peripheral circulation stimulated with respective antigens

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can be measured using commercially available enzyme-linked immunospot (ELISA) or enzyme-linked immunospot assays (ELISPOT) [10]. There is growing evidence that the new IGRAs are highly specific for the diagnosis of LTBI in otherwise healthy individuals [10]. Likewise, the new IGRAs introduce both higher sensitivity and specificity in patients with rheumatoid arthritis, prior to anti-TNF-α therapy [11]. However, evidence is scarce for patients with dermatological disorders [12].

We have established a cytokine flow cytometry assay following stimulation of PBMC with PPD and ESAT-6. PBMCs were obtained from patients with psoriasis, prior to anti-TNF-α treatment. Simultaneously, a TST was administered, and results were compared with the frequency of MTB-specific $CD4^+$ and $CD8^+$ T cells, expressing IFN-γ, TNF-α, IL-2 or IL-10 after stimulation with PPD or ESAT-6, respectively.

PATIENTS AND METHODS

Study population

Fifty two patients were recruited from the Department of Dermatology, Medical University of Vienna, Austria. The study population lived in a region with a low TB incidence (approximately 10.5/100,000) and high rate of BCG coverage, as all Austrian infants were routinely vaccinated until 1989 [13]. Three patients with morbus (M)-Behçet's disease (5.7%) and 49 with psoriasis (92.7%) were included (age: median: 49, range: 23 - 83). Of the 52 patients, 18 were female (34.6%). All patients had a history of immunosuppressive therapy within the previous two years. Systemic immunosuppressive medication was discontinued at least two weeks prior to testing to improve the sensitivity of LTBI screening. According to the American Thoracic Society [14], patients were nevertheless classified as at intermediate increased risk, and a cut-off of 10mm for the TST was applied.

We sought to identify LTBI with the standard procedure, *i.e*. medical history, known exposure to MTB, physical examination and tuberculin skin test (TST). Chest X-ray was performed for all patients to exclude active disease. No known exposure to TB was reported by the study participants. Forty one patients displayed a negative TST (14 female) (age: mean: 48, range: 33-67). Eleven (four female) patients (age: mean: 50; range: 23-79) with a TST ≥ 10mm, were interpreted as potentially latently infected with MTB. Investigators performing the laboratory procedures were blinded to the TST results.

Written informed consent was obtained from all participating individuals. Human experimentation guidelines of the Medical University of Vienna were followed during the clinical research. Ethical clearance was given by the ethics committee of Medical University of Vienna.

Detection of PPD-specific and ESAT-6-specific T-cell cytokine expression by flow cytometry

PBMC were isolated from heparinized blood by ficoll-diatrizoate centrifugation, and plated out into 24-well plates (BD Falcon, Mountain View, CA, USA) at 2 x 10^6 per well. Cells were cultured in 3 mL, ultra-culture medium (UCM) (Bio Whittaker, Walkersville, MD, USA) supplemented with L-glutamine (2 mM/L; Sigma, St. Louis, MI, USA), gentamicin (170mg/l; Sigma) and 2-mercaptoethanol (3.5 µl/L; Merck, Darmstadt, Germany) for 18 h at 37°C in 5% CO_2 and stimulated with purified protein derivate (PPD) (Statens Serum Institute, Copenhagen, Denmark), at a final concentration of 10 µg/mL or with ESAT-6 (Statens Serum Institute, Copenhagen, Denmark, with a final concentration of 5µg/ml. In order to amplify TCR signalling and to facilitate the initial phase of the T-cell activation, the co-stimulatory MAb CD28 (Pharmingen San Diego, CA, USA), was added at a final concentration of 5 µg/mL to those wells that were stimulated with PPD and ESAT-6. Brefeldin A (10 µg/mL final concentration, Sigma) was added after 6h to block protein secretion. After 18 h, cells were harvested on ice, washed twice in phosphate-buffered saline (PBS), and fixed with 2% formaldehyde (1 mL per 2 x 10^6 cells) for 20 minutes. After two additional washes in PBS, the cells were re-suspended in Hank's balanced salt solution (supplemented with 0.3% bovine serum albumin and 0.1% sodium-azide). The cells were washed twice with PBS and made permeable with saponin (0.1%; Sigma), re-suspended with 50 µL of saponin-buffered diluted antibodies and incubated for 25 minutes in the dark. The following monoclonal antibodies were used: MAb IFN-γ [clone: B 27], fluorescein – isothiocyanate [FITC] – labeled; MAb IL-2 [MQ1-17H12], phytoerythrin [PE] – conjugated; IL-10 [JES3-9D7], PE – labeled; TNF-α [MAB -11], PE labeled; Anti - CD4, allophycocyanin [APC] labeled; Anti - CD8, peridinin chlorophyll [PerCP] labeled; [Becton Dickinson, Mountain View, CA, USA].

Four-color staining was performed, and at least 10^5 cells were analysed on a FACS-Calibur (Becton Dickinson) equipped with a two-laser system (488- and 630-nm wavelength, respectively). All cytokine combinations were stained in conjunction with CD4 and CD8. The data were analysed with CELL-Quest software (Becton Dickinson) and the results were expressed as the percentage of cytokine-producing cells in each $CD4^+$ or $CD8^+$ population *(figure 1)*. To assure specificity, spontaneous cytokine production in control wells was subtracted from cytokine production after stimulation with PPD or ESAT-6. Relevant background was not seen in negative controls on staining for IFN-γ, IL-2 or IL-10. Some background (but no more than 0.02%) was restricted to TNF-α *(figure 1)*; 0.02% of $CD4^+$ T cells producing MTB-specific cytokines were interpreted as positive [15].

Statistical methods

Statistical analysis was performed using SPSS 14.0 for Windows, SPSS Inc., Chicago. The Wilcoxon-Mann-Whitney U-test was applied for group differences, bivariate correlations were assessed with Spearman's correlation coefficient. A p-value of < 0.05 (two tailed) was considered significant. Receiver-operating-characteristic curves (ROC) were calculated and expressed as areas under the curve, with an asymptotic 95% confidence interval (CI).

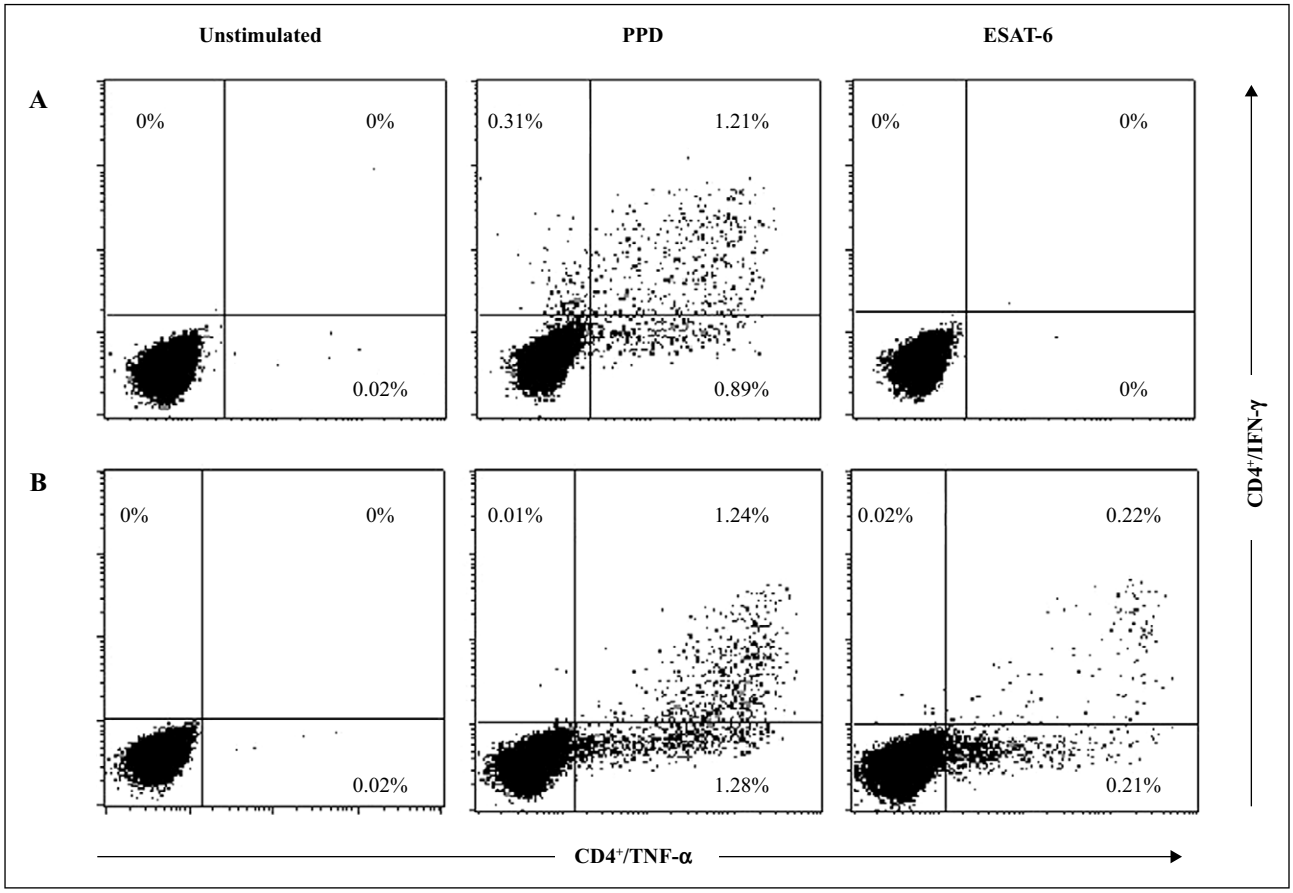

Figure 1

Representative, two-parameter dot plots indicating the frequency of IFN-γ- and TNF-α-expressing $CD4^+$ T cells after PPD or ESAT-6 stimulation of a patient with a negative TST (**A**) and a patient with a positive TST (**B**), prior to anti-TNF-α-therapy. PBMCs were incubated with medium alone (first vertical column), with MAb CD28 and PPD (second vertical column), and with MAb CD28 and ESAT-6 (third vertical column).

RESULTS

The frequency of $CD4^+$ cells expressing IFN-γ, IL-2, IL-10 and TNF-α, as well as co-expressing IFN-γ/TNF-α and IFN-γ/IL-2 after stimulation with PPD: differences between TST positive and TST negative individuals

Significant differences between the TST-positive and the TST-negative group after PPD stimulation were detected *(figure 2)*. However, cytokine expression overlapped remarkably between these two groups.

The frequency of $CD4^+$ cells expressing IFN-γ, IL-2, IL-10 and TNF-α, as well as co-expressing IFN-γ/TNF-α and IFN-γ/IL-2 after stimulation with ESAT-6: agreement between IFN-γ and TNF-α expression and TST results

Differences between TST-positive and TST-negative patients as regards cytokine expression after stimulation were statistically significant and did not overlap *(figure 3)*. ROC analysis comparing MTB-specific IFN-γ and the TST showed an area under the curve (AUC) of 0.840 (CI 95%: 0.675-0.1.005). The degree of agreement, as expressed as Cohen's kappa (κ), was 0.82. TNF-α displayed high reactivity towards antigenic stimulation, showing an AUC of 0.757 (CI 95%: 0.567-0.946), with an agreement of $\kappa = 0.24$. IL-2 expression showed an AUC of 0.623 (CI 95%: 0.41-0.836) and a total agreement of $\kappa = 0.33$.

The best congruence with the TST was reached by $CD4^+$ T cells co-producing IFN-γ and TNF-α after ESAT-6 stimulation, with an AUC of 0.945 (CI 95%: 0.84-0.1.051) and an overall agreement of $\kappa = 0.87$. $CD4^+$ T cells co-producing IFN-γ and IL 2 displayed an AUC of 0.938 (CI 95%: 0.83-1.045), and an overall agreement of $\kappa = 0.80$ *(figure 4)*.

There was a significant correlation between the frequencies of PPD-specific $CD4^+$ T cells and ESAT-6-specific $CD4^+$ T cells detected ($r^2 = 0.2316$, $p < 0.001$). No statistically significant differences were found in $CD8^+$ T cells, either after PPD or after ESAT-6 stimulation.

Measurement of IL-10 yielded no statistically significant differences between the groups.

DISCUSSION

In the current investigation, we compared ESAT-6-specific cytokine production in peripheral $CD4^+$ T cells with TST results in patients with dermatological disorders, prior to anti-TNF-α therapy. We found a close agreement between the two test methods. In contrast, PPD-induced cytokine expression overlapped in TST-positive and TST-negative

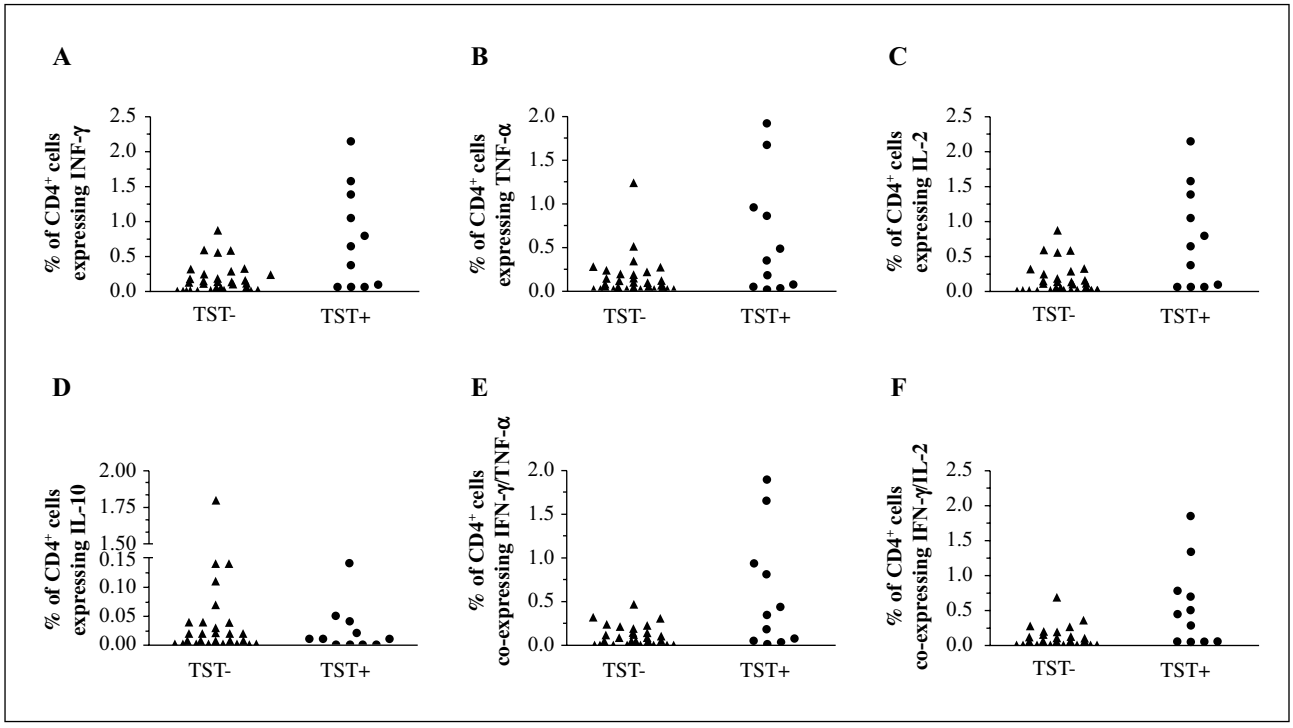

Figure 2

Frequency of cytokine-expressing $CD4^+$ cells after overnight stimulation with PPD in 11 TST-positive and 41 TST-negative patients. **A**) shows IFN-γ expressed by PPD-specific cells. The difference between TST-positive and TST-negative individuals is statistically significant ($p = 0.003$). **B**) and **C**) show TNF-α or IL-2 expressed by PPD-specific cells. The difference between TST-positive and TST-negative individuals is statistically significant ($p = 0.002$ and $p = 0.01$, respectively). IL-10 expression by PPD-specific cells from TST-positive and TST-negative individuals is shown in (**D**). Differences are not statistically significant. The co-expression of IFN-γ/TNF-α and IFN-γ/IL-2 are shown in (**E**) and (**F**). Differences were highly significant for IFN-γ/TNF-α ($p = 0.01$), as well as for IFN-γ/IL-2 ($p < 0.01$). The Mann-Whitney U-test was used to test for significance.

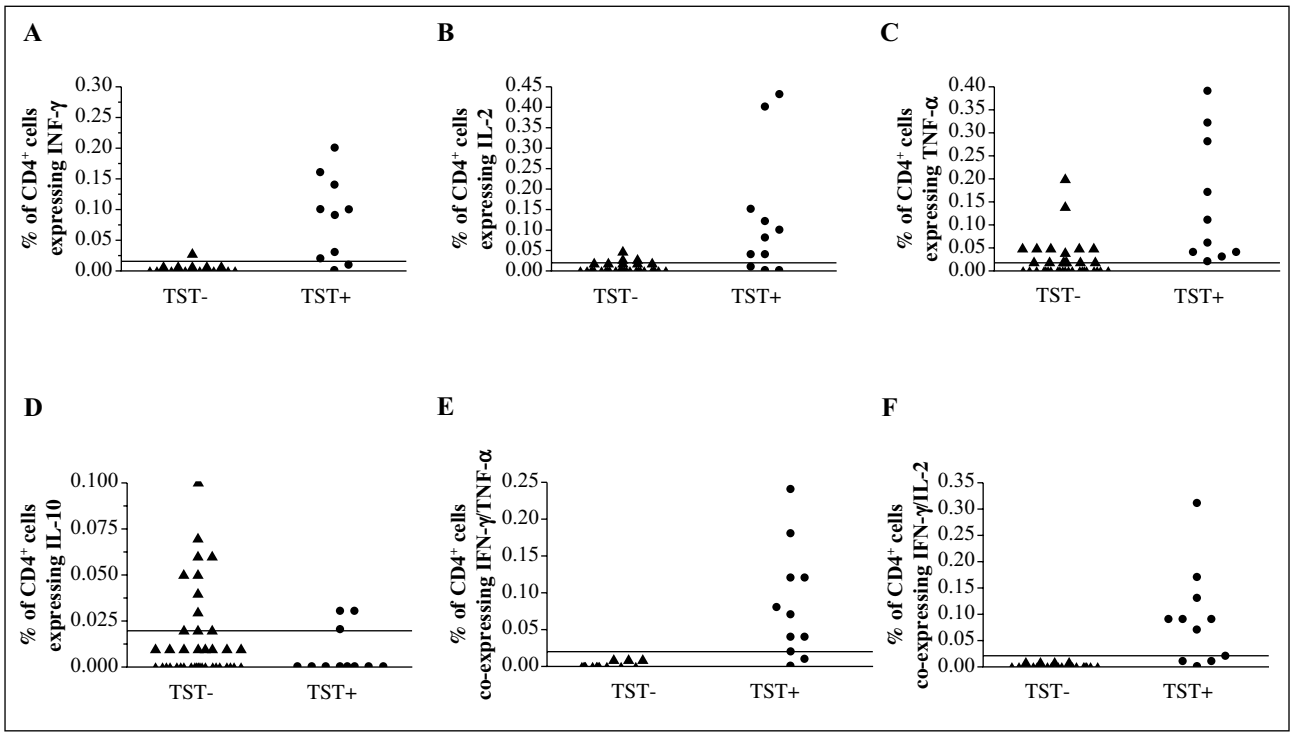

Figure 3

Frequency of cytokine-expressing $CD4^+$ cells after overnight stimulation with ESAT-6 in 11 TST-positive and 41 TST-negative patients. **A**) shows IFN-γ expressed by ESAT-6-specific $CD4^+$ T cells. The difference between TST-positive and TST-negative individuals is statistically significant ($p < 0.001$). **B**) and **C**) show TNF-α or IL-2 expressed by ESAT-6-specific cells. The difference between TST-positive and TST-negative individuals is statistically significant ($p < 0.001$ and $p < 0.01$, respectively). IL-10 expression by ESAT-6-specific cells from TST-positive and TST-negative individuals is shown in (**D**). Differences are not statistically significant. The co-expression of IFN-γ/TNF-α and IFN-γ/IL-2 are displayed in (**E**) and (**F**). Differences were highly significant for IFN-γ/TNF-α ($p < 0.001$), as well as for IFN-γ/IL-2 ($p < 0.001$). The Mann-Whitney U-test was used to test for significance.

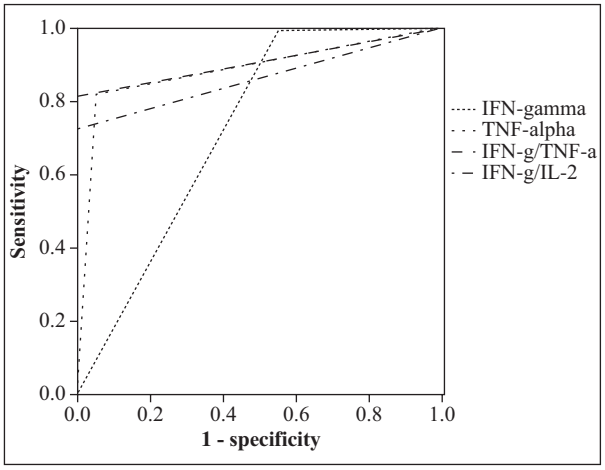

Figure 4
Receiver-operating-characteristic curves for IFN-γ- and TNF-α-expressing, as well as IFN-γ/IL-2- and IFN-γ/TNF-α-co-expressing $CD4^+$ T cells after stimulation with ESAT-6 compared with TST results. A TST ≥ 10mm was interpreted as positive. Areas under the curve for TNF-α and IFN-γ are 0.757 (CI 95%: 0.567-0.946) and 0.840 (CI 95%: 0.675-1.005). For IFN-γ/TNF-α and IFN-γ/IL-2 AUC values are 0.945 (CI 95%: 0.84-1.051) and 0.938 (CI 95%: 0.83-1.045), respectively.

individuals, rendering PPD stimulation unusable for diagnostic purposes, notwithstanding statistically significant differences. The finding that PPD provokes different responses if peripheral blood T cells and intradermal T cells are compared is somewhat counterintuitive, but may be due to the fact that the circulating T cell pool is more cross-reactive to the antigen mixture. Nevertheless, we cannot provide a precise explanation for this discrepancy.

Of note, each cytokine measured displayed a distinct reactivity pattern after ESAT-6 stimulation. Amongst them, IFN-γ expression appeared to be the best marker for LTBI. The highest concordance with the TST was shown for $CD4^+$ T cells co-expressing TNF-α and IFN-γ. Measurement of $CD4^+$ T cells, expressing both IL-2 and IFN-γ, displayed high specificity as well, suggesting that measurement of cytokine co-producing T cells may, overall, be more specific for LTBI than measurement of only one cytokine. This is in line with previous findings in patients with active TB, who had significantly increased frequencies of cytokine-co-producing T cells [16].

IL-10 expression was observed in some patients. However, no significant difference between TST-negative and TST-positive patients was found. Thus, we can only speculate about the role of this particular cytokine in eventually suppressing MTB-specific immune-responsiveness and thereby influencing the results of LTBI testing.

As in all studies on LTBI, the interpretation of our results is hampered by the lack of a gold standard for LTBI. Therefore, we were obliged to link our flow cytometry results to the TST. The reason why the TST is used as the reference is not its convincing performance, but the 100 year-long experience with its shortcomings, as discussed above [6]. Nevertheless, the TST is, to date, the only internationally accepted tool for diagnosis of LTBI [12, 14].

Evaluation of IGRAs in direct comparison with the TST showed greater discordance in populations with BCG vaccination than in those who were not vaccinated, suggesting a higher specificity for IGRAs under such circumstances [10]. In patients with rheumatoid arthritis, IGRAS showed higher sensitivity when compared to the TST [17, 18], which was probably due to diminished skin reactivity in these patients [8, 19, 20].

In patients with psoriasis, recommendations for skin indurations interpreted as positive range from 5 mm as stated by the CDC [21], to 15 mm, which should be applied to any patient with history of BCG vaccination according to the British Thoracic Society [22]. In the current investigation, MTB-specific T cells and TST correlated very well with the intermediate cut-off of 10 mm. This cut off was chosen in the light of an intermediate increased risk of TB reactivation (history of immunosuppressive medication, but none at the time of testing), with a background of full BCG coverage, which might increase the false positive TST rates when using a cut-off of 5 mm.

Comparing $CD4^+$ T cells co-expressing ESAT-6-specific TNF-α and IFN-γ, and TST results, two patients showed discordant results with a positive TST and a negative flow cytometry result. One of these was a patient with M-Behçet's disease, showing no $CD4^+$ T cell reactivity at all after stimulation. Unexpectedly, $CD8^+$ T cells produced high amounts of IFN-γ after both PPD and ESAT-6 stimulation. This unusual cytokine pattern may be attributed to the underlying disease and warrants further study. The second patient, who had a positive TST without MTB-specific $CD4^+$ T cell reactivity, was a woman with a history of treated TB, suggesting that our flow cytometry assay might be able to discriminate between sustained antigenic stimulation by LTBI and previously-treated TB.

It is noteworthy that the high degree of agreement between the new method presented and the TST may be due to the fact that exposure to non-tuberculous mycobacteria in Central Europe is low. Patients included in the recent study were probably all vaccinated in childhood, but the effect of single BCG vaccination in infancy on TST results in adolescence or adult life is still debated [23]. In contrast, in individuals who were vaccinated at two years of age or older, the BCG could be responsible for up to 20% of false-positive TST results [23]. Thus, it is likely that immune-based techniques using the newly discovered antigens are more specific in populations that received BCG vaccination repeatedly, or after infancy. To what extent the TST is influenced by non-tuberculous mycobacteria, remains unclear. It is estimated that only 2% of individuals with exposure to nontuberculous mycobacteria would have false positive results in the TST [23].

Altogether, the quantification of MTB-specific cytokines derived from $CD4^+$ T cells by flow cytometry is a promising new tool for the immune-based diagnosis of LTBI. Whether this immune-based test method is better than the TST in diagnosis of LTBI remains unclear and remains to be evaluated in further investigations. However, it suggests that the flow cytometry assay might yield increased specificity in BCG-vaccinated populations or in populations with high, non-tuberculous mycobacterial exposure. A direct comparison with the commercially-available

assays is not yet possible with the present data. However, analysis of $CD4^+$ cells co-producing cytokines such as TNF-α and IFN-γ should be pursued in order to optimize the performance of immune-based diagnosis of LTBI.

Disclosure. *None of the authors has any conflict of interest to disclose.*

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