Mycobacterium tuberculosis-specific CD4 T-cell scoring discriminates tuberculosis infection from disease

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In a prospective study, a scoring system based on analysis of the activation state of tuberculosis (TB)-specific CD4$^+$ T-cells was developed that allows reliable discrimination of TB infection and TB disease with high sensitivity and specificity https://bit.ly/3EFG4KX

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Abstract

Background Rapid and reliable diagnostic work-up of tuberculosis (TB) remains a major healthcare goal. In particular, discrimination of TB infection from TB disease with currently available diagnostic tools is challenging and time consuming. This study aimed at establishing a standardised blood-based assay that rapidly and reliably discriminates TB infection from TB disease based on multiparameter analysis of TB antigen-reactive CD4$^+$ T-cells acting as sensors for TB stage-specific immune status.

Methods 157 HIV-negative subjects with suspected TB infection or TB disease were recruited from local tertiary care hospitals in Berlin (Germany). Peripheral blood mononuclear cells were analysed for CD4$^+$ T-cells reactive to the *Mycobacterium tuberculosis* antigens purified protein derivative and early secretory antigenic target 6 kDa/culture filtrate protein 10. The activation state of TB antigen-reactive T-cells, identified by surface expression of CD154, was evaluated according to the expression profile of proliferation marker Ki-67 and activation markers CD38 and HLA-DR. Using data from 81 subjects with clinically confirmed TB infection (n=34) or culture-proven pulmonary or extrapulmonary TB disease (n=47), 12 parameters were derived from the expression profile and integrated into a scoring system.

Results Using the scoring system, our assay (TB-Flow Assay) allowed reliable discrimination of TB infection from both pulmonary and extrapulmonary TB disease with high sensitivity (90.9%) and specificity (93.3%) as was confirmed by Monte-Carlo cross-validation.

Conclusion With low time requirement, ease of sample collection, and high sensitivity and specificity both for pulmonary and extrapulmonary TB disease, we believe this novel standardised TB-Flow Assay will improve the work-up of patients with suspected TB disease, supporting rapid TB diagnosis and facilitating treatment decisions.

Introduction

Tuberculosis (TB) results from infection by members of the *Mycobacterium tuberculosis* complex (MTC), which primarily affects the lung but may also target other organs [1]. It is the leading cause of death among infectious diseases worldwide with approximately 1.4 million deaths in 2019 [1]. An estimated quarter of the world's population carries a TB infection with no evidence of clinically manifest TB disease

[2], previously often referred to as latent TB infection. Therefore, reduction of morbidity, mortality and spread of infection through early diagnosis and timely treatment of TB disease, also referred to as active TB, remain important healthcare goals [1, 3, 4]. However, the current reference standard for diagnosis, *i.e.* pathogen detection by culture, often takes several weeks [5]. Since 2021, the World Health Organization (WHO) recommends molecular rapid diagnostic tests for initial TB diagnostics [6]. However, both methods often rely on invasive sampling from the site of infection, especially for the diagnosis of extrapulmonary TB [5, 7].

Indirect diagnostic tests assessing the specific immune response to TB antigens such as the interferon (IFN)-γ release assay (IGRA) may indicate the presence of an infection with members of the MTC, but do not allow differentiation of TB infection and TB disease [5]. Clearly, new diagnostics are needed to discriminate rapidly and reliably between TB infection and TB disease.

Recent studies that attempted to assess the state of infection with *M. tuberculosis* using multiparameter flow cytometric analysis of functional and phenotypic markers on TB-specific T-cells have identified the activation markers CD38 and HLA-DR and the proliferation marker Ki-67 as promising targets for the discrimination of TB infection from TB disease [8–12]. A general challenge of such flow cytometric assays is the identification of antigen-specific T-cells irrespective of their functional status. CD154 (CD40 ligand), a cell surface marker for T-cell receptor-dependent activation of $CD4^+$ T-cells, has been shown to be an excellent marker for identification of antigen-specific $CD4^+$ T-cells after short-term stimulation [13, 14].

In this study, we developed and validated the TB-Flow Assay, a novel multivariable scoring system based on a standardised multiparameter flow cytometry assay, for discrimination of TB disease from TB infection in a routine diagnostic setting.

Methods

Study participants and blood collection

HIV-negative subjects aged ⩾18 years with suspected TB infection or TB disease were recruited at the Dept of Infectious Diseases and Respiratory Medicine, Charité – Universitätsmedizin Berlin and the Dept of Pneumology, Lungenklinik Heckeshorn, Helios Klinikum Emil von Behring (Berlin, Germany). The study was approved by the local ethics committee (ethics approval number EA1/277/15) and informed written consent was obtained from all participants.

Individual participant data were de-identified before analysis. Participant identities remain exclusively known to the study doctors and their staff. For this study, cohorts of subjects with TB infection and subjects with culture-confirmed TB disease were established. Study participants were prospectively recruited from 2017 to 2019 and followed-up for at least 12 months. The study population consisted of subjects evaluated for the presence of TB infection or TB disease based on a positive IGRA and/or symptomatic clinical presentation with suspicion of TB disease as assessed by clinical experts. Criteria for inclusion in the TB infection cohort for establishment and validation of the scoring system were a positive IGRA, negative results for TB disease and the absence of evidence for TB disease (re)activation during a 12-month follow-up. Diagnosis of TB disease was based on a positive culture, positive PCR results, typical histopathological findings (*e.g.* necrotising granuloma with or without acid-fast bacilli) or clinical evidence of TB disease and the clinical decision to administer anti-TB treatment. However, inclusion in the TB disease cohort for establishment and validation of the scoring system required confirmation of TB disease diagnosis by culture. In order to analyse TB-specific $CD154^+CD4^+$ T-cells with a recent history of *in vivo* activation not influenced by TB therapy, patients with TB disease and >5 days of TB therapy at the time of initial sample collection were excluded from the TB disease study cohort used to establish the TB-Flow Assay.

All enrolled subjects donated 50 mL heparinised blood at study entry. Patients with confirmed TB disease were asked to donate an additional 50 mL blood no earlier than 2 weeks after the initial blood withdrawal and at least 6 days after the start of anti-TB treatment.

Additional methods

For information on sample eligibility, isolation and stimulation of peripheral blood mononuclear cells (PBMCs), study population characteristics, assay performance, and flow cytometry, see the supplementary material.

Statistical analysis

Statistical analyses were performed with Prism version 8.0 (GraphPad, La Jolla, CA, USA). The Mann–Whitney U-test was used for comparison of different donor groups. p-values <0.05 were considered statistically significant. Discriminative performance was determined by receiver operating characteristic (ROC) curve analysis. For optimal discriminative performance, cut-offs at the maximum Youden's index were selected. Confidence intervals are given with a 95% confidence level. Background correction of the frequencies of CD38-, Ki-67- and HLA-DR-positive $CD154^+CD4^+$ cells after purified protein derivative (PPD) and early secretory antigenic target 6 kDa/culture filtrate protein 10 (ESAT-6/CFP-10) stimulation was done by subtracting the corresponding cell frequency of the negative control. The median (range) $CD4^+CD154^+$ background in the study cohort was 0.0031% (0.00–0.30%). Two-fold Monte-Carlo cross-validation of the scoring system in 10 000 iterations entailed 10 000 repetitions of 1) randomly dividing the TB disease and TB infection study cohorts into training and test sets of equal size, 2) using the training set to calculate the cut-off points for the 12 parameters of TB-specific T-cell analysis and for the score of the TB-Flow Assay (TB-Flow Score), and 3) determining the sensitivity and specificity for discrimination of TB infection and TB disease in the test set. Frequency analysis of the cumulated sensitivity and specificity values was used for estimation of assay performance. Analysis showed that each iteration produced a unique training and test set.

Results

Subjects included in the study cohorts

In order to establish cohorts of subjects with TB infection and confirmed TB disease, we enrolled 157 HIV-negative subjects with suspected TB infection or TB disease (figure 1). As part of the TB diagnostic work-up at study entry, blood was taken from these patients for phenotypic characterisation of TB-specific T-cells.

TB infection and TB disease were excluded in 29 individuals with negative IGRA, negative mycobacteriological results and lack of evidence of TB disease during a 12-month follow-up. 43 subjects with positive IGRA results but negative mycobacteriological results and absence of evidence for TB disease during a 12-month follow-up were classified as TB infection without (n=40) and with (n=3) a history of TB disease. 85 patients with TB disease at study enrolment were identified by positive culture and/or PCR, typical histopathological findings (*e.g.* necrotising granuloma) and/or clinical evidence of TB disease in combination with a positive TB IGRA, and consecutive anti-TB treatment. Positive cultures were obtained in 68 of these patients. 17 patients with TB disease but without culture confirmation were excluded from the TB disease cohort used for establishment and validation of the TB-Flow Assay. In addition, 12 culture-positive patients with TB disease and >5 days of TB therapy at study enrolment were excluded to ensure that the analysis included only TB-specific $CD154^+CD4^+$ T-cells with a recent history of *in vivo* activation not influenced by TB therapy.

Also eliminated from the TB disease cohort were 15 subjects with TB infection (n=8) or TB disease (n=7) without $CD4^+$ T-cell response to stimulation with ESAT-6/CFP-10 (figure 1), two subjects with high stimulation-independent T-cell activation and autofluorescence, and one subject with insufficient PBMC numbers. The lack of response to ESAT-6/CFP-10 stimulation in the TB-Flow Assay largely corresponded to the IGRA results for those subjects. Among nonresponders, 14 out of 15 subjects had negative (TB disease n=5) or borderline positive IGRA results close to the cut-off point of positivity of 0.35 $IU \cdot mL^{-1}$ (TB disease n=2, TB infection n=7). Only one of the subjects had a strong positive IGRA result at study enrolment (TB infection n=1) (data not shown).

A total of 47 patients with TB disease and 34 subjects with TB infection were included into cohorts for establishment and performance validation of the TB-Flow Assay (figure 1, and supplementary tables E1 and E2). None of the subjects received systemic immunosuppressive or antineoplastic therapy. All subjects included in the TB disease cohort had started anti-TB treatment at the time of study enrolment or at latest 3 weeks after initial blood collection. To investigate the effect of TB therapy on the assay results, a second blood sample was drawn from 36 culture-confirmed patients with TB disease no earlier than 2 weeks after the initial blood withdrawal and at least 6 days after the start of anti-TB treatment.

The age range in the TB infection cohort was 25–70 years (median 42 years) with 23 male and 11 female subjects (supplementary table E1). The TB disease cohort comprised 29 male and 18 female patients aged 19–84 years (median 33 years), and included patients with pulmonary TB (n=30), extrapulmonary TB (n=15) and pulmonary as well as extrapulmonary TB (n=2) (supplementary table E2).

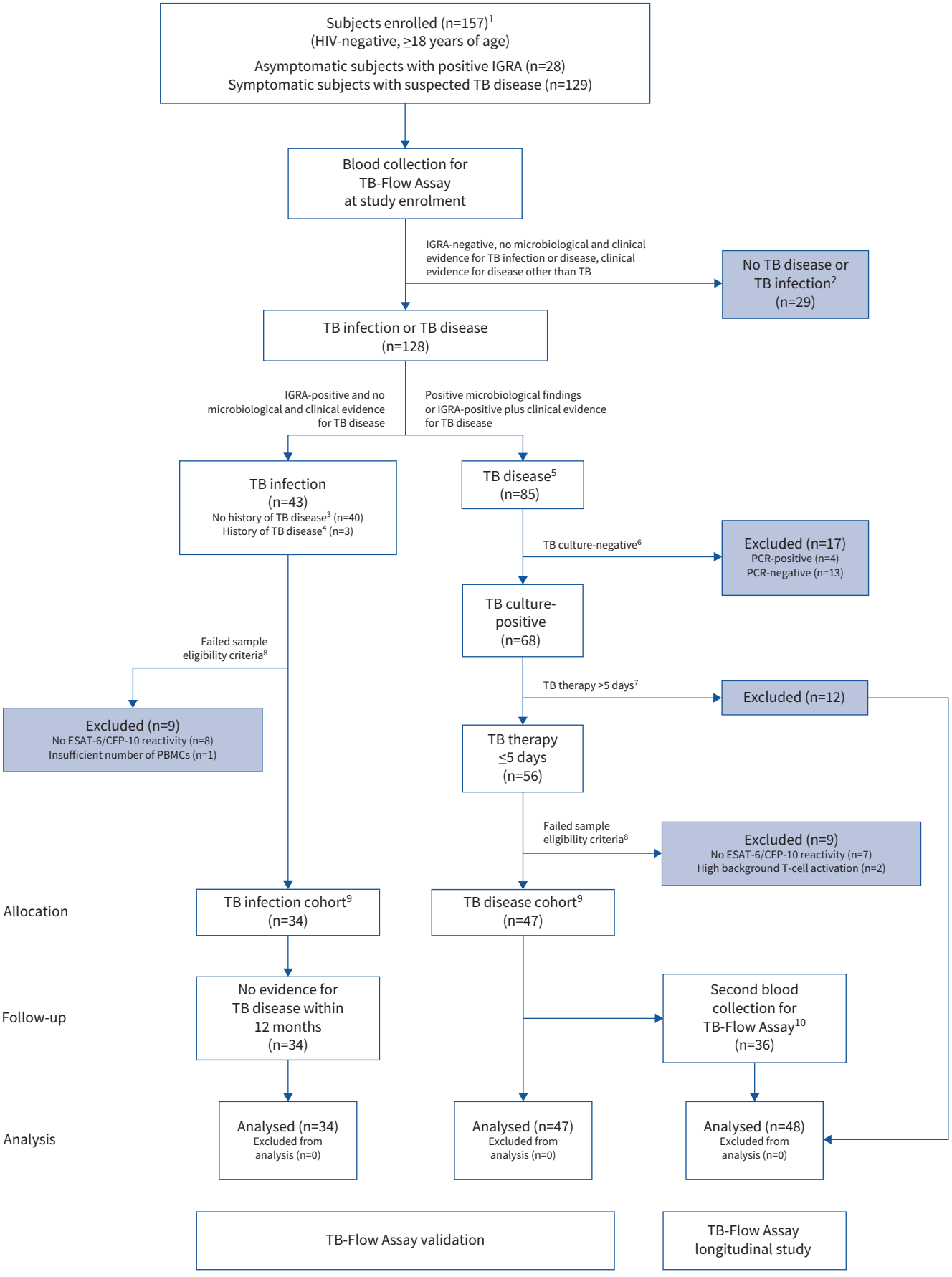
Subjects enrolled (n=157)[1]
(HIV-negative, ≥18 years of age)
Asymptomatic subjects with positive IGRA (n=28)
Symptomatic subjects with suspected TB disease (n=129)
Blood collection for TB-Flow Assay at study enrolment
IGRA-negative, no microbiological and clinical evidence for TB infection or disease, clinical evidence for disease other than TB
No TB disease or TB infection[2] (n=29)
TB infection or TB disease (n=128)
IGRA-positive and no microbiological and clinical evidence for TB disease
Positive microbiological findings or IGRA-positive plus clinical evidence for TB disease
TB infection (n=43)
No history of TB disease[3] (n=40)
History of TB disease[4] (n=3)
TB disease[5] (n=85)
TB culture-negative[6]
Excluded (n=17)
PCR-positive (n=4)
PCR-negative (n=13)
TB culture-positive (n=68)
Failed sample eligibility criteria[8]
Excluded (n=9)
No ESAT-6/CFP-10 reactivity (n=8)
Insufficient number of PBMCs (n=1)
TB therapy >5 days[7]
Excluded (n=12)
TB therapy ≤5 days (n=56)
Failed sample eligibility criteria[8]
Excluded (n=9)
No ESAT-6/CFP-10 reactivity (n=7)
High background T-cell activation (n=2)
Allocation
TB infection cohort[9] (n=34)
TB disease cohort[9] (n=47)
Follow-up
No evidence for TB disease within 12 months (n=34)
Second blood collection for TB-Flow Assay[10] (n=36)
Analysis
Analysed (n=34)
Excluded from analysis (n=0)
Analysed (n=47)
Excluded from analysis (n=0)
Analysed (n=48)
Excluded from analysis (n=0)
TB-Flow Assay validation
TB-Flow Assay longitudinal study

FIGURE 1 Inclusion and exclusion criteria for the tuberculosis (TB) infection (right) and TB disease (left) cohorts used for establishment and validation of the TB-Flow Assay. [1]: subjects were enrolled during diagnostic work-up for confirmation or exclusion of TB disease. [2]: absence of TB infection and TB disease as confirmed by negative mycobacteriological and interferon-γ release assay (IGRA) results, no empirical anti-TB treatment administered, and no evidence of TB disease during a 12-month follow-up. [3]: TB infection without history of TB disease defined by positive IGRA, but no diagnostic evidence of TB disease during a 12-month follow-up. [4]: history of TB disease defined by positive IGRA and successfully completed antimycobacterial therapy, and no diagnostic evidence of TB disease during a 12-month follow-up. [5]: TB disease with or without positive TB culture result (culture-negative TB disease is defined by either a positive TB PCR result, typical histopathological findings (*e.g.* necrotising granuloma) or clinical evidence of TB disease and consecutive anti-TB treatment. [6]: patients with TB disease without a positive TB culture result were excluded. [7]: patients with TB disease that were under anti-TB treatment for >5 days at the time of initial blood collection were excluded. [8]: eligibility criteria included sufficient number of peripheral blood mononuclear cells (PBMCs) available for the test, no or negligible stimulation-independent ("background") T-cell activation and autofluorescence in the unstimulated control, and sufficient early secretory antigenic target 6 kDa/culture filtrate protein 10 (ESAT-6/CFP-10)-reactive $CD4^+$ T-cells (for details, see Methods). [9]: cohorts used for establishing and validation of the TB-Flow Assay. [10]: patients with culture-confirmed TB disease were asked to give a second blood sample no earlier than 2 weeks after the initial blood sample and at least 6 days after the initiation of their anti-TB treatment.

Frequency of PPD- and ESAT-6/CFP-10-specific $CD4^+$ T-cells does not allow discrimination between TB disease and TB infection

PPD- and ESAT-6/CFP-10-reactive $CD4^+$ T-cells of the subjects in the TB infection (n=34) and TB disease (n=47) cohorts were quantified based on CD154 expression after short-term stimulation with PPD or ESAT-6/CFP-10 (figure 2a). We did not observe significant differences between the TB disease and TB infection cohorts regarding the frequency of PPD-reactive $CD154^+CD4^+$ T-cells (area under the curve (AUC) 0.577, 95% CI 0.451–0.702). The frequency of ESAT-6/CFP-10-reactive $CD154^+CD4^+$ T-cells was significantly increased in patients with TB disease compared with subjects with TB infection (figure 2b). However, ROC curve analysis demonstrated only a very limited discriminatory capacity for $CD154^+$ frequency in ESAT-6/CFP-10-reactive $CD4^+$ T-cells (AUC 0.683, 95% CI 0.566–0.800) (figure 2b).

Increased frequency of $CD154^+CD4^+$ T-cells expressing CD38, Ki-67 and HLA-DR in patients with TB disease compared with subjects with TB infection

Through flow cytometry analysis of the well-characterised T-cell activation and proliferation markers CD38, HLA-DR and Ki-67 [8–12], we assessed the activation state of $CD154^+CD4^+$ T-cells in the TB infection and TB disease cohorts after stimulation with PPD and ESAT-6/CFP-10. Six parameters were analysed for each TB antigen: $CD38^+$, $HLA\text{-}DR^+$ and $Ki\text{-}67^+$ cells within $CD154^+CD4^+$ T-cells (figure 3a, left panel) and $CD38^+CD154^+$, $HLA\text{-}DR^+CD154^+$ and $Ki\text{-}67^+CD154^+$ cells within total $CD4^+$ T-cells (figure 3a, right panel). Supplementary figure E2 and figure 3b show the gating strategy and the expression of CD38, HLA-DR and Ki-67 by PPD-reactive $CD154^+CD4^+$ T-cells for representative subjects with TB infection and TB disease. As figure 3c illustrates, the frequencies of $CD38^+$, $HLA\text{-}DR^+$ or $Ki\text{-}67^+$ PPD- or ESAT-6/CFP-10-specific $CD4^+$ T-cells were significantly higher ($p<0.0001$) in the TB disease cohort compared with the TB infection cohort.

To evaluate the discriminatory capacity of these markers, we performed ROC curve analyses and used Youden's index to determine the cut-off points for the best discrimination of TB infection and TB disease (figure 3c and supplementary table E4). Diagnostic sensitivity and specificity at cut-off ranged from 57.5% to 89.4% and 85.3% to 100.0%, respectively (AUC 0.787–0.925) (supplementary figure E3 and supplementary table E4).

To minimise the impact of flow cytometer performance variability and operator bias on the analysis, and to enable analysis on different flow cytometers independently of instrument settings, we established standardisation procedures that employ calibration beads for automated gating of marker-positive cell populations (supplementary figure E2b).

An integrative scoring system increases discriminatory capacity

We created a scoring system that integrates the 12 parameters of the TB-specific T-cell analysis after stimulation with PPD and ESAT-6/CFP-10, comprising the frequencies of $CD38^+CD154^+$, $HLA\text{-}DR^+CD154^+$ and $Ki\text{-}67^+CD154^+$ cells within 1) $CD154^+CD4^+$ T-cells and 2) total $CD4^+$ T-cells (figure 4a). Parameters with marker frequencies that exceeded the corresponding cut-off points (described earlier) were assigned the value of 1, otherwise they were assigned the value of 0. The sum of the 12 assigned values constitutes the TB-Flow Score, which therefore may range from 0 to 12.

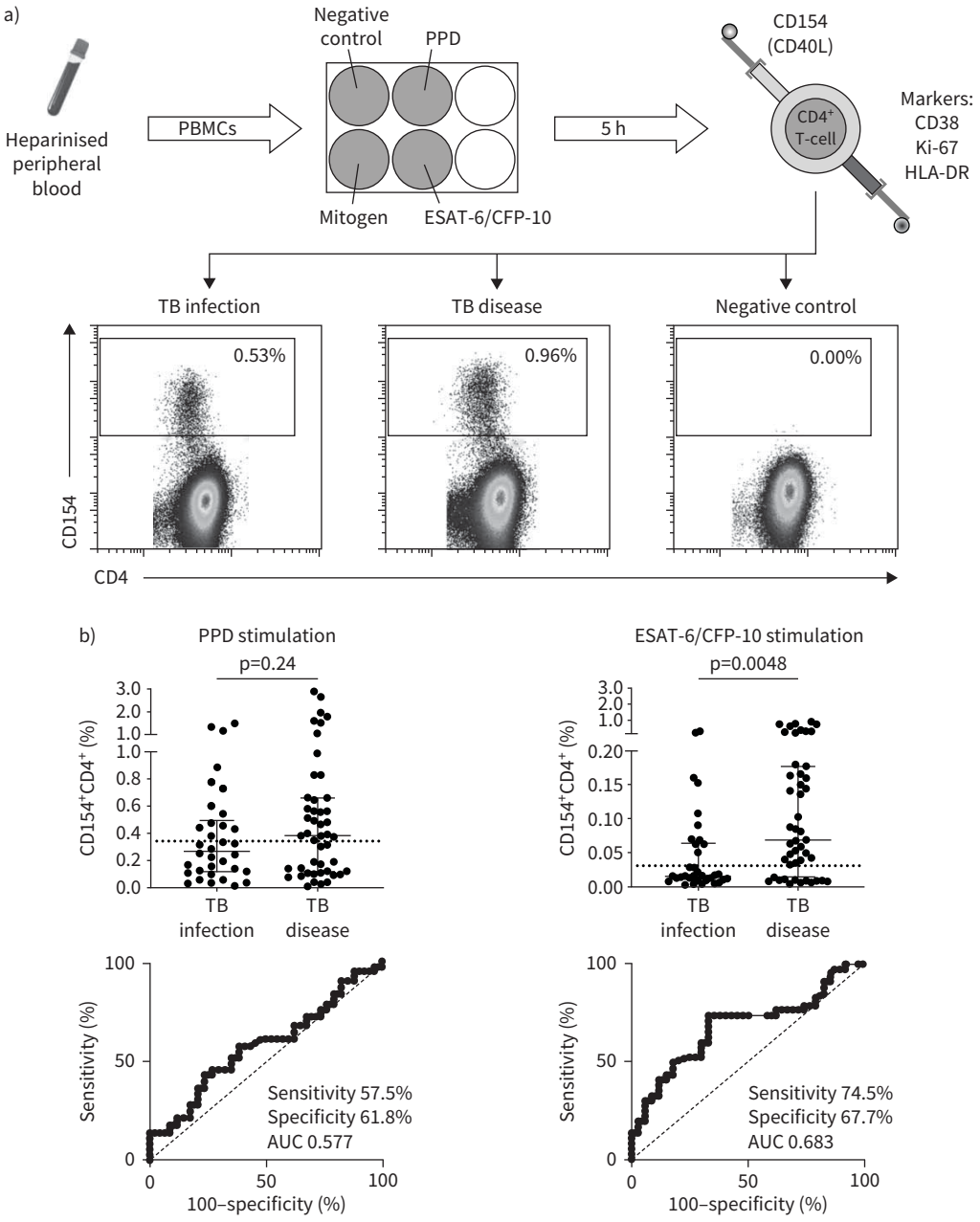

FIGURE 2 General procedure of the TB-Flow Assay and CD154 expression in the tuberculosis (TB) infection and TB disease study cohorts. a) Peripheral blood mononuclear cells (PBMCs) were isolated from heparinised peripheral blood and stimulated for 5 h with the TB antigens purified protein derivative (PPD) and early secretory antigenic target 6 kDa/culture filtrate protein 10 (ESAT-6/CFP-10) peptides. Positive and negative control PBMCs were stimulated with mitogen or unstimulated, respectively. Antigen-specific $CD4^+$ T-cells react to ligation of the T-cell receptor with surface expression of CD154 (CD40 ligand (CD40L)). After stimulation PBMCs were harvested and stained with fluorescence-labelled antibodies specific for CD154, the activation markers CD38 and HLA-DR, and the proliferation marker Ki-67. Marker expression on $CD4^+$ T-cells was analysed by flow cytometry. Dot plots show a representative example of CD154 expression by $CD4^+$ T-cells of subjects with TB infection (top) and TB disease (bottom) after PPD stimulation. b) Plots show the background-corrected frequency of $CD154^+CD4^+$ T-cells after stimulation with PPD (left) or ESAT-6/CFP-10 (right) in the TB infection and TB disease study cohorts. Cut-offs (dotted lines) for discrimination of TB infection and TB disease were calculated using receiver operating characteristic (ROC) curve analysis and Youden's index. Curves below the plots show the ROC curve analysis for discrimination of TB infection and TB disease. AUC: area under the curve.

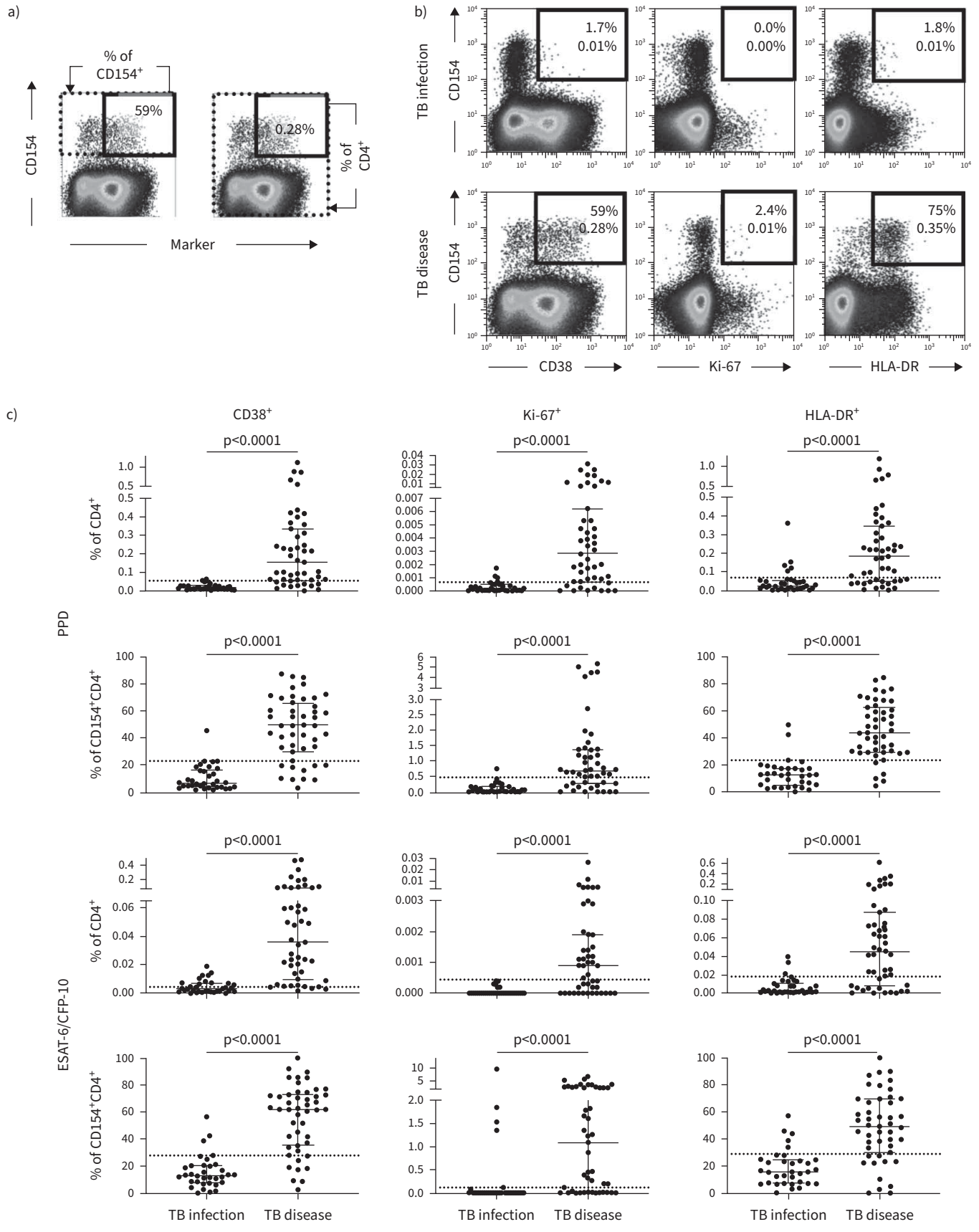
a)
% of CD154+
59%
0.28%
% of CD4+
CD154
Marker
b)
TB infection
TB disease
CD154
1.7%
0.01%
0.0%
0.00%
1.8%
0.01%
59%
0.28%
2.4%
0.01%
75%
0.35%
CD38
Ki-67
HLA-DR
c)
CD38+
Ki-67+
HLA-DR+
PPD
ESAT-6/CFP-10
% of CD4+
% of CD154+CD4+
p<0.0001
TB infection
TB disease

FIGURE 3 Expression of CD38, HLA-DR and Ki-67 by tuberculosis (TB) antigen-specific $CD154^+CD4^+$ T-cells in subjects with TB infection or TB disease. a) For each activation/proliferation marker, frequencies of marker-positive TB-specific $CD154^+CD4^+$ T-cells were determined in relation to the population of TB-specific $CD154^+CD4^+$ T-cells (top) and total $CD4^+$ cells (bottom). b) Representative data for marker expression on $CD4^+$ T-cells from a subject with TB infection and a patient with TB disease after stimulation with the TB antigen purified protein derivative (PPD). Frequencies of marker-positive $CD154^+CD4^+$ T-cells within $CD154^+CD4^+$ T-cells (upper numbers) and within total $CD4^+$ T-cells (bottom numbers) are given. c) Background-corrected frequencies of PPD-specific (top) and early secretory antigenic target 6 kDa/culture filtrate protein 10 (ESAT-6/CFP-10)-specific (bottom) marker-positive $CD4^+$ T-cells expressing the markers CD38, HLA-DR or Ki-67 within total $CD4^+$ T-cells and within $CD154^+CD4^+$ T-cells. Median and interquartile range of the TB infection and TB disease cohorts are shown in the dot plots. Marker-specific cut-offs (dotted lines) for discrimination of TB infection and TB disease were calculated using receiver operating characteristic curve analysis and Youden's index.

The optimal TB-Flow Score cut-off point for discrimination of TB infection and TB disease in the cohorts was 3.5 as determined by ROC curve analysis (AUC 0.978, 95% CI 0.945–1.000) and Youden's index. At this cut-off point only three out of 47 subjects in the TB disease cohort and one out of 34 subjects in the TB infection cohort showed a TB-Flow Score that was not consistent with the clinical diagnosis (supplementary table E5). The resulting diagnostic sensitivity and specificity was 93.6% (95% CI 82.5–98.7%) and 97.1% (95% CI 84.7–99.9%), respectively (figure 4b).

a)

	PPD			ESAT-6/CFP-10		
	$CD38^+$	$Ki\text{-}67^+$	$HLA\text{-}DR^+$	$CD38^+$	$Ki\text{-}67^+$	$HLA\text{-}DR^+$
% of $CD4^+$	0/1	0/1	0/1	0/1	0/1	0/1
% of $CD154^+CD4^+$	0/1	0/1	0/1	0/1	0/1	0/1

TB-Flow Score (0–12)

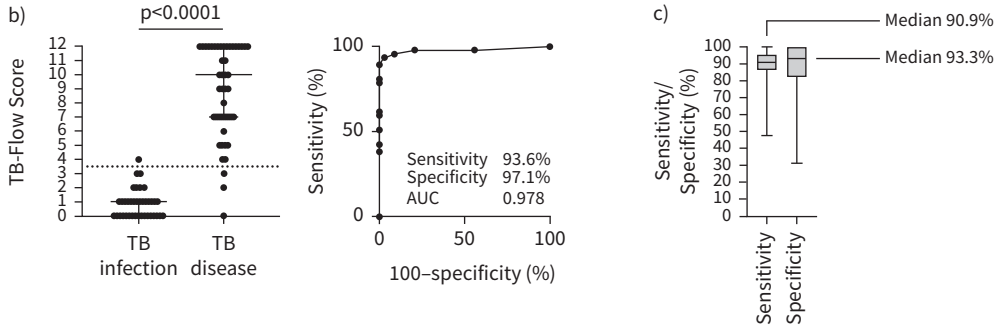

FIGURE 4 Calculation and characteristics of the TB-Flow Score. a) The TB-Flow Score is based on analysis of the frequencies of $CD38^+$, $HLA\text{-}DR^+$ and $Ki\text{-}67^+$ $CD4^+$ T-cells specific for the tuberculosis (TB) antigens purified protein derivative (PPD) and early secretory antigenic target 6 kDa/culture filtrate protein 10 (ESAT-6/CFP-10) (peptide pool). The TB-Flow Score is calculated by comparison of marker frequencies within total $CD4^+$ T-cells and within $CD154^+CD4^+$ T-cells with cut-offs specific for each cell population. It corresponds to the number of cell populations with a frequency of marker-positive cells above the cut-off. 12 combinations of antigen, marker and reference cell population were analysed. Therefore, the TB-Flow Score can assume values from 0 to 12. b) TB-Flow Score for the TB infection and TB disease cohorts (left) and the receiver operating characteristic (ROC) curve for discrimination of the two cohorts (right). Median, interquartile range (IQR) and TB infection/disease cut-off (dotted line) are marked in the plot. The TB-Flow Score cut-off point for discrimination of TB infection and TB disease was also calculated using ROC curve analysis and Youden's index. c) Range of diagnostic sensitivity and specificity values calculated with two-fold Monte-Carlo cross-validation (10 000 iterations). Box plots show the median sensitivity (90.9%) and specificity (93.3%) values calculated in the 10 000 iterations. IQR and minimum–maximum values are also shown.

To estimate the unbiased diagnostic performance of the TB-Flow Assay we validated the generalised statistical performance of the scoring system with 10 000 unique combinations of training and test sets using two-fold Monte-Carlo cross-validation in 10 000 iterations [15]. The median (interquartile range (IQR)) diagnostic sensitivity and specificity over all iterations was 90.9% (86.4–95.5%) and 93.3% (82.4–100.0%), respectively (figure 4c). Cut-off points in the range 2.5–4.5 were calculated for 93.1% of iterations, with the cut-off point 3.5 being most frequent (supplementary figure E1a). Therefore, TB-Flow Score values below or above this range would correspond with a higher confidence to TB infection or TB disease, respectively (supplementary figure E1b).

We found neither sex nor age of the test subjects had an impact on the result of the TB-Flow Assay (figure 5a and supplementary figure E4). Importantly, the test equally recognised pulmonary as well as extrapulmonary TB disease. Stratification of the TB disease cohort by infection site showed that 31 out of 32 subjects with pulmonary TB disease and 13 out of 15 subjects with extrapulmonary TB disease were assigned a score that indicated a TB disease (figure 5b). The median (IQR) TB-Flow Score of subjects with pulmonary and extrapulmonary TB disease was 9.5 (6.0–12.0) and 10.0 (7.0–12.0), respectively.

All 47 subjects of the TB disease cohort were treated for ⩽5 days or had not received TB therapy prior to blood collection. The median (IQR) TB-Flow Score of the TB disease cohort was 10.0 (7.0–12.0). In order to assess the influence of anti-TB therapy on our test system, we analysed 48 patients with culture-confirmed TB disease that had been under therapy for >5 days at the time of sample collection. The patients were stratified into the following groups according to days of completed therapy: 6–30 days (n=30), 31–90 days (n=8) and >90 days (n=10). The median (IQR) TB-Flow Score in these groups was 8.5 (5.8–11.0) for 6–30 days, 6.5 (2.3–8.8) for 31–91 days and 3.0 (2.0–5.8) for >90 days (figure 5c). The median TB-Flow Score clearly declined with progression of anti-TB therapy. This is in accordance with data from other groups who demonstrated a decline of TB antigen-specific T-cells expressing CD38, HLA-DR and Ki-67 under TB therapy [8, 10].

One prerequisite for inclusion of subjects in the TB disease cohort was confirmation by TB culture. Consequently, we excluded 17 subjects without positive TB culture from the TB disease cohort, 13 of whom fulfilled the criteria for sample eligibility (see Methods) and had been receiving TB therapy for ⩽5 days (supplementary table E3). Despite the lack of culture confirmation, these patients had been categorised as TB disease cases and received TB treatment based on clinical presentation and other diagnostic evidence such as PCR (n=4), histological evidence (n=3) or radiography results (n=6) alone. Interestingly, all patients with a positive PCR result were identified as cases with TB disease by the TB-Flow Assay, whereas only six out of nine patients diagnosed with TB disease based on histology or radiography and clinical presentation were classified as subjects with TB disease (figure 5d).

A total of 15 clinically confirmed subjects with TB infection (n=8) and patients with culture-confirmed TB disease (n=7) had been excluded from the TB infection and TB disease cohorts owing to a lack of T-cell response to ESAT-6/CFP-10 stimulation. These ESAT-6/CFP-10 nonresponders were analysed using a different scoring (PPD Score), which exclusively assesses the T-cell response to PPD stimulation (supplementary figure E6a). Like the TB-Flow Score, the PPD Score was established based on the TB infection and TB disease cohorts excluding the ESAT-6/CFP-10 nonresponders. ROC curve analysis of this partial score (AUC 0.967, 95% CI 0.931–1.000) showed a diagnostic sensitivity and specificity of 96.3% (95% CI 87.3–99.5%) and 92.9% (95% CI 80.5–98.5%), respectively, at cut-off point 1.5 (supplementary figure E6b and supplementary table E4). Using the PPD Score, all ESAT-6/CFP-10 nonresponders with TB disease (n=7) and seven out of eight ESAT-6/CFP-10 nonresponders with TB infection were correctly classified regarding the presence or absence of mycobacterial disease (supplementary figure E6c).

Discussion

Here, we introduce the novel TB-Flow Assay that enables fast discrimination of TB infection from TB disease regardless of the site of infection. Based on multiparameter flow cytometry analysis of TB-specific $CD4^+$ T-cells from peripheral blood, the TB-Flow Assay can be performed using easily accessible sample material. Integrating 12 different flow cytometric parameters into one unifying TB-Flow Score allowed for superior sensitivity and specificity, and facilitates interpretation of the results. To the best of our knowledge, the TB-Flow Assay is the first flow cytometric assay allowing discrimination of TB infection and TB disease in a routine diagnostic setting due to standardisation procedures which minimise assay variability. Patient evaluation using the novel TB-Flow Assay appears to be a promising tool for optimising patient management as well as to gain further insight into TB pathogenesis [16]. One crucial point of the WHO End TB Strategy is the exclusion of TB disease before initiating preventive treatment of TB

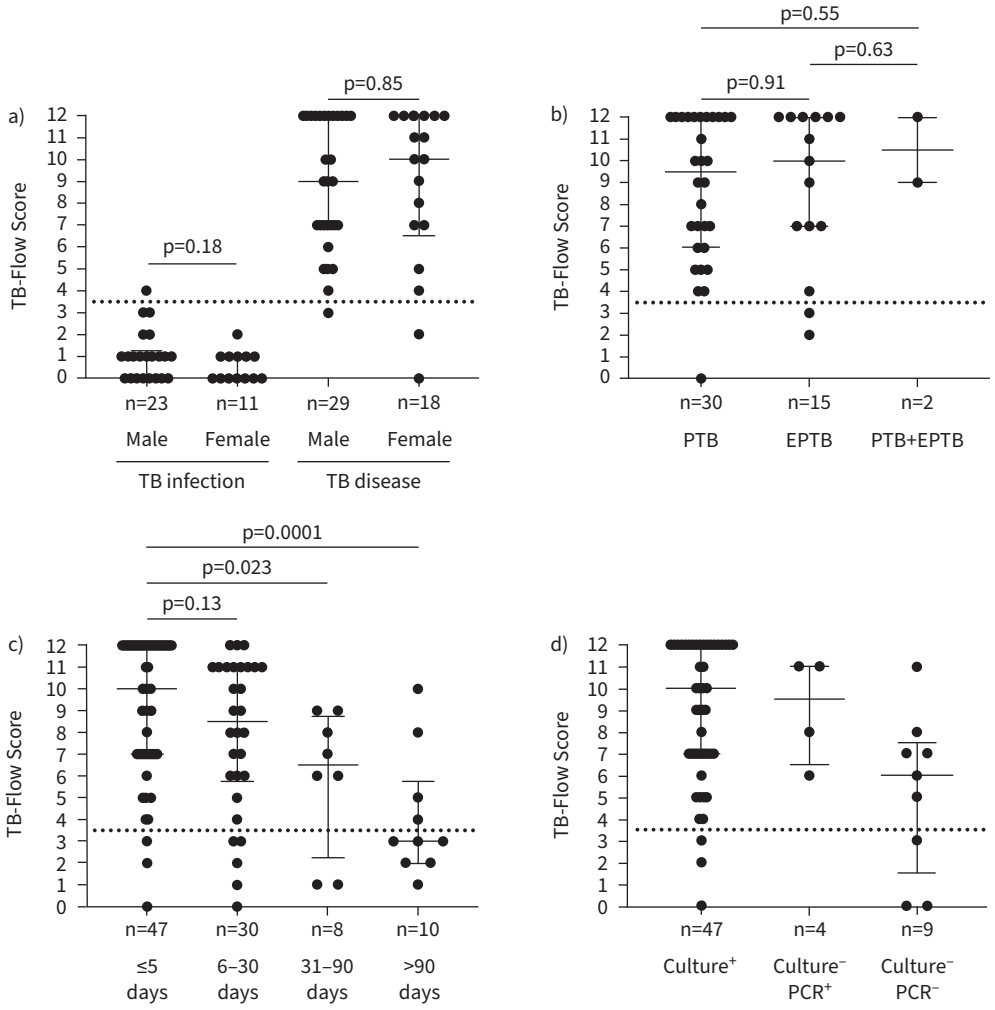

FIGURE 5 TB-Flow Score results with regard to sex, infection site and therapy duration, and for culture-negative patients with tuberculosis (TB) disease. a) Impact of sex on the TB-Flow Score. Subjects with TB infection and TB disease are subdivided into female and male. Median (interquartile range (IQR)) TB-Flow Score: 1.0 (0.0–1.3) for TB infection male, 0.0 (0.0–1.0) for TB infection female, 9.0 (7.0–12.0) for TB disease male and 10.0 (6.5–12.0) for TB disease female. No significant sex-specific differences were observed. b) TB-Flow Score with regard to infection site. Median (IQR) TB-Flow Score: 9.5 (6.0–12.0) with one false-negative result for pulmonary TB disease (PTB), 10.0 (7.0–12.0) with two false-negative results for extrapulmonary TB disease (EPTB), and 10.5 (9.0–12.0) with no false-negative results for pulmonary and extrapulmonary TB disease (PTB+EPTB). No significant differences in the TB-Flow Score were observed. c) TB-Flow Score of patients with culture-confirmed TB disease with regard to therapy duration. Samples subdivided according to the time between therapy start and blood collection. A total of 95 samples were analysed. Group "⩽5 days" corresponds to the TB disease cohort. Median (IQR) therapy duration: 0 (0–3) days for "⩽5 days", 13 (9–20) days for "6–30 days", 45 (41–69) days for "31–90 days" and 143 (110–218) days for ">90 days". The TB-Flow Score declines with duration of therapy, which corresponds to a reduced expression of the markers CD38, HLA-DR and Ki-67. d) TB-Flow Score for patients with culture-negative TB disease. A total number of 13 enrolled patients had been diagnosed with TB disease without a positive TB culture and had undergone ⩽5 days of antimycobacterial therapy at the time of blood collection. Positive PCR results for *Mycobacterium tuberculosis* complex were available for four of these patients ($Culture^{-}PCR^{+}$). Nine patients had been diagnosed based on clinical presentation, pathohistological results and/or radiography/computed tomography results ($Culture^{-}PCR^{-}$) alone. TB-Flow Score values are presented for these two patient groups and the cohort of culture-confirmed TB disease ($Culture^{+}$). Median (IQR) TB-Flow Score: 10.0 (7.0–12.0) for $Culture^{+}$, 9.5 (6.5–11.0) for $Culture^{-}PCR^{+}$ and 6.0 (1.5–7.5) for $Culture^{-}PCR^{-}$. Median and IQR are shown for each group; the dotted line marks the cut-off for optimal differentiation of TB infection and TB disease.

infection [17]. We have shown that the TB-Flow Assay is a reliable method to discriminate TB infection from TB disease. However, its use is currently limited to regions with diagnostic laboratories equipped with six-colour flow cytometry and cell culture facilities. Previously, a pilot study by Musvosvi *et al*. [18] suggested that flow cytometric immunological assays using fewer markers can be applied in TB diagnostics. Nonetheless, future studies with larger cohorts are warranted to elucidate whether simplified T-cell-based assays will retain high sensitivity and specificity over a broad range of disease manifestations and are suitable for implementation in resource-poor regions.

In recent years, research on the diagnostic use of TB-specific T-cells for discrimination of TB infection from TB disease has focused on multidimensional functional [19–24] or phenotypic characterisation of TB-specific $CD4^+$ T-cells [8–12, 18, 25, 26]. Our approach applies multidimensional analysis of the activation state of TB-specific $CD4^+$ T-cells. Identification of $CD4^+$ T-cells reactive to the TB antigens PPD or ESAT-6/CFP-10 by expression of CD154 and analysis of the expression of the activation/proliferation markers CD38, HLA-DR and Ki-67 enabled reliable and consistent quantification of *in vivo* activated TB antigen-specific $CD4^+$ T-cells. The potential use of CD38, HLA-DR and Ki-67 for discrimination of TB infection and TB disease has been recently demonstrated [8, 10–12], but so far has not been established as a routine diagnostic test, most likely due to technical and standardisation issues.

The cell surface marker CD154 has distinct advantages in the enumeration of antigen-specific T-cells compared with the expression of IFN-γ. CD154 is quickly and transiently expressed on the cell surface of all T-cell subsets upon T-cell receptor activation [13, 14], exhibits little background expression, and can be easily and reliably stained on the cell surface after just 4–5 h of stimulation. In contrast, IFN-γ is only expressed by a variable fraction within the T-helper type 1 T-cell subset, and requires intracellular staining and secretion blocking agents [8, 10–12]. Importantly, CD154 is a general marker for antigen-specific $CD4^+$ T-cells [13] and has been shown to identify a greater fraction of TB antigen-specific $CD4^+$ T-cells than IFN-γ [27].

Similar to the results obtained by others [8, 10–12, 18], we found that analysis of the frequency of PPD- or ESAT-6/CFP-10-reactive T-cells expressing CD38, HLA-DR or Ki-67 enabled differentiation of TB infection and TB disease, whereas the overall frequencies of PPD- or ESAT-6/CFP-10-reactive T-cells by themselves were not sufficient for a reliable discrimination.

However, in our hands, single-marker analysis did not allow discrimination of TB infection and TB disease with a diagnostic sensitivity >90% for any of the 12 assessed combinations of markers, antigens and reference populations. Therefore, we integrated these distinct 12 combinations into a scoring system. This increased the diagnostic performance of the TB-Flow Assay and allowed robust discrimination of TB infection from TB disease in our study cohorts with excellent diagnostic sensitivity and specificity. Two-fold Monte-Carlo cross-validation [15] in 10 000 iterations demonstrated a robust performance of the scoring system with a median diagnostic sensitivity and specificity of 90.9% and 93.3%, respectively. Moreover, this approach allowed us to define ranges of high confidence for classification as TB infection and TB disease using the TB-Flow Score. However, this needs to be confirmed by future prospective studies.

Importantly, our test equally distinguished pulmonary and extrapulmonary TB disease from TB infection using ⩽10 mL peripheral blood. This is crucial since the diagnostic work-up of patients with suspected extrapulmonary TB disease frequently requires invasive sample collection, which may delay diagnosis. We did not find significant differences in marker expression between pulmonary and extrapulmonary TB disease (supplementary figure E5). Moreover, we did not observe a significant influence of subject age or sex. However, due to the limited number of elderly patients we cannot with all certainty exclude age-related effects on the outcome of the TB-Flow Assay.

The TB-Flow Assay gave results inconsistent with the clinical diagnosis in four out of 81 subjects in our study cohorts. These cases may highlight limitations of immunological tests. TB pathogenesis is complex. Primary and post-primary TB may result in clearance, latent infection, rapid as well as slow development to overt disease or an oscillation between different disease states [16, 28, 29]. Consequently, both infection activity and immune response vary over time, and may pass states of contained or minimal disease activity manifesting as symptomless latent, incipient or subclinical TB [28]. Therefore, inconsistent results of the TB-Flow Assay with respect to clinical presentation or patient assessment may give reason for further in-depth analysis, including repetitive testing of the same patient, as has been successful for invasive fungal infection [30].

Interestingly, the TB-Flow Assay confirmed the diagnosis in all patients with TB disease that had a positive PCR result but lacked TB culture confirmation. In contrast, only six out of nine patients with TB

disease who had been diagnosed based solely on histology, radiography or clinical presentation were classified as TB disease cases by our assay. Although the reasons for these discrepancies remain speculative, the possibility remains that the empirical diagnoses of TB disease in these cases had not been correct.

Our study has several limitations. First, although the TB-Flow Assay identified cases of pulmonary and extrapulmonary TB disease equally well, larger studies are warranted for confirmation given the relatively low number of patients with extrapulmonary TB disease.

Second, we did not include subjects with HIV infection, patients undergoing immunosuppressive and antineoplastic treatment or subjects aged <18 years. In HIV-infected patients presenting with advanced immunodeficiency, assessment of TB-specific T-cell responses may be compromised. However, it has been demonstrated that CD38, HLA-DR and Ki-67 are appropriate markers for the discrimination of TB infection and TB disease in HIV-infected TB patients with moderate immunodeficiency [12, 31]. Future investigations need to include cohorts of patients with HIV infection and advanced immunodeficiency as well as otherwise immunosuppressed patients. Similarly, TB diagnostics for children is known to be challenging [32]. However, preliminary results indicate that the TB-Flow Assay also allows effective differentiation of TB infection and TB disease in subjects aged 6–17 years (our unpublished data).

In addition, although our TB disease study cohort was representative regarding age, sex, site of infection and ethnicity of the general TB patient population in Western Europe [33], further studies are warranted to demonstrate comparable performance of the TB-Flow Assay across geographically and genetically diverse populations.

Unfortunately, we were unable to obtain information on bacille Calmette–Guérin (BCG) vaccinations for the majority of participants. However, we believe the BCG status is unlikely to affect the result of the TB-Flow Assay: 1) ESAT-6/CFP-10 proteins are not expressed in BCG vaccine strains, hence a vaccination will not induce T-cells reactive to these proteins, and 2) the activation status of T-cells reactive to PPD antigens should not be affected by BCG status except in the case of a very recent BCG vaccination. Nevertheless, the performance of the TB-Flow Assay should be validated in populations where BCG vaccination is routinely performed.

TB patients poorly or not at all responding to stimulation with ESAT-6/CFP-10 peptides pose a problem to immunological assays employing these antigens [34, 35]. As described for IGRA, we also observed a small number of ESAT-6/CFP-10 nonresponders among patients with culture-confirmed TB disease [36, 37]. With only one exception, the lack of response to ESAT-6/CFP-10 stimulation coincided with a negative or borderline positive IGRA result. Importantly, all ESAT-6/CFP-10 nonresponders in our cohorts reacted to stimulation with PPD. Scoring marker expression only for PPD-reactive $CD4^+$ T-cells also discriminated TB infection from TB disease in these subjects. Although such an approach would not distinguish tuberculous and nontuberculous mycobacterial infections, it may nonetheless be utilised for the diagnosis of mycobacterial disease in general.

In accordance with the results of others [8, 10], we found that the frequency of TB-specific $CD4^+$ T-cells expressing CD38, Ki-67 and HLA-DR as well as the TB-Flow Score declined with progression of effective TB therapy. This may enable use of the TB-Flow Assay for early assessment of treatment success. However, larger longitudinal studies directly comparing changes in T-cell phenotype and bacterial burden under therapy in drug-responsive and drug-resistant TB cases are required to evaluate the use of the TB-Flow Assay as a monitoring tool for therapy response.

In summary, our blood-based TB-Flow Assay allows discrimination of pulmonary and extrapulmonary TB disease from TB infection within 24 h with excellent diagnostic sensitivity and specificity, and including ESAT-6/CFP-10 nonresponders. It incorporates the relevant negative and positive controls, thresholds to determine technical validity of measurements, and a bead-based standardisation method for minimisation of error due to day-to-day variability of the flow cytometry equipment. Hence, we present a test appropriate for routine use in TB diagnostics. We believe the TB-Flow Assay will facilitate rapid disease diagnosis and support clinical decision making.

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Author contributions: C. Meisel, A. Scheffold, D. Schürmann, H-D. Volk, T. Meyer and A. Mantei designed the study and supervised manuscript preparation. T. Meyer and A. Mantei performed the research, participated in the collection of patient data and analysed the data. A. Mantei, T. Meyer, D. Schürmann and C. Meisel wrote the manuscript. M. Schürmann, D. Schürmann, D. Krieger, T. Bauer, C. Beßler and H. Bias were responsible for patient recruitment. A. Scheffold and P. Bacher provided substantial assistance in setting up the assay on basis of the identification of TB-specific T-cells. All authors agreed to submit the final manuscript.

Conflict of interest: A. Scheffold and P. Bacher are advisors to Miltenyi Biotec who own IP rights on the use of CD154 for antigen-specific T-cell detection. A. Scheffold, C. Meisel, H-D. Volk, P. Bacher, T. Meyer and A. Mantei are listed as inventors in a patent (10 2018 131 696.8) that has been issued and a patent (PCT/EP2019/084392) that is pending on the discrimination of TB infection and TB disease using the method described herein. The remaining authors declare no competing financial interests.

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