The Journal of Infectious Diseases

MAJOR ARTICLE

Defining the Role of Cellular Immune Signatures in Diagnostic Evaluation of Suspected Tuberculosis

Alice Halliday,[1,2] Tereza Masonou,[1] Mica R. Tolosa-Wright,[1,3] Yanping Guo,[4] Long Hoang,[1] Robert Parker,[1] Aime Boakye,[1,3,5] Yemisi Takwoingi,[6] Amarjit Badhan,[1,3] Pooja Jain,[1,3] Ishita Marwah,[1,3] Luis C. Berrocal-Almanza,[1,3] Jonathan Deeks,[6] Peter Beverley,[1,3] Onn Min Kon,[1,3,7] and Ajit Lalvani[1,3]

[1]TB Research Centre, National Heart and Lung Institute, Imperial College London, London, United Kingdom, [2]Bristol Vaccine Centre, Cellular and Molecular Medicine, Life Sciences, University of Bristol, Bristol, United Kingdom, [3]Health Protection Research Unit in Respiratory Infections, Imperial College London, London, United Kingdom, [4]St Mary's Flow Cytometry Facility, Imperial College London, London, United Kingdom, [5]NIHR Imperial Clinical Research Facility, Hammersmith Hospital, London, United Kingdom, [6]Institute of Applied Health Research, University of Birmingham and NIHR Birmingham, Biomedical Research Centre (University Hospital Birmingham NHS Foundation Trust and University of Birmingham), Birmingham, United Kingdom, [7]St Mary's Hospital, Imperial College Healthcare NHS Trust, London, United Kingdom

Background. Diagnosis of paucibacillary tuberculosis (TB) including extrapulmonary TB is a significant challenge, particularly in high-income, low-incidence settings. Measurement of *Mycobacterium tuberculosis* (Mtb)-specific cellular immune signatures by flow cytometry discriminates active TB from latent TB infection (LTBI) in case-control studies; however, their diagnostic accuracy and clinical utility in routine clinical practice is unknown.

Methods. Using a nested case-control study design within a prospective multicenter cohort of patients presenting with suspected TB in England, we assessed diagnostic accuracy of signatures in 134 patients who tested interferon-gamma release assay (IGRA)-positive and had final diagnoses of TB or non-TB diseases with coincident LTBI. Cellular signatures were measured using flow cytometry.

Results. All signatures performed less well than previously reported. Only signatures incorporating measurement of phenotypic markers on functional Mtb-specific CD4 T cells discriminated active TB from non-TB diseases with LTBI. The signatures measuring HLA-DR$^+$IFNγ$^+$ CD4 T cells and CD45RA$^-$CCR7$^-$CD127$^-$ IFNγ$^-$IL-2$^-$TNFα$^+$ CD4 T cells performed best with 95% positive predictive value (95% confidence interval, 90–97) in the clinically challenging subpopulation of IGRA-positive but acid-fast bacillus (AFB) smear-negative TB suspects.

Conclusions. Two cellular immune signatures could improve and accelerate diagnosis in the challenging group of patients who are IGRA-positive, AFB smear-negative, and have paucibacillary TB.

Keywords. diagnostic; flow cytometry; latent tuberculosis infection; T cell; tuberculosis.

In high-income, low-incidence regions, including Europe and North America, the proportion of active tuberculosis (TB) cases that are paucibacillary and/or extra-pulmonary tuberculosis (EPTB) is large and rising [1, 2]. These clinical presentations usually necessitate invasive sampling, typically bronchoalveolar lavage (for paucibacillary pulmonary TB and biopsies for EPTB). Moreover, clinical samples are commonly *Mycobacterium tuberculosis* (Mtb) culture-negative and acid-fast bacillus (AFB) smear-negative, which makes diagnosis clinically challenging, prolongs duration of hospital admission, and delays treatment initiation [3]. Hence, there is an important unmet clinical need for a rapid, noninvasive blood test with sufficient specificity to rule-in paucibacillary and EPTB.

A rational approach to detect TB infection in paucibacillary cases is to use tests measuring the Mtb-specific adaptive immune response, which provides an amplified signal of a low bacillary burden infection. Measuring such responses is possible from blood samples, obviating the need for invasive sampling. Available immune tests for TB include the interferon-gamma release assays (IGRAs), which are commonly used in the diagnostic work-up of active TB; indeed, the sensitivity of currently available and next-generation IGRAs far exceeds that of culture [4, 5]. However, IGRAs lack specificity for diagnosing active TB because they also score positive in patients with non-TB illness who have incidental, concomitant latent TB infection (LTBI). More sophisticated tests measuring additional aspects of cell-mediated immune responses to Mtb may provide improved specificity. Indeed, certain cellular immune signatures, based on detection of Mtb-specific T cells by flow cytometry, are sufficiently different between active TB and LTBI to discriminate between the two [6–11]. This provides important proof-of-principle that such signatures might be used as follow-on tests after a positive IGRA result during diagnostic

Received 4 January 2021; editorial decision 7 June 2021; accepted 14 June 2021; published online July 31, 2021.

Correspondence: Ajit Lalvani, MA, DM, FRCP, FMedSci, FFPH, Tuberculosis Research Centre, National Heart and Lung Institute, Imperial College London, St Mary's Campus, London, W2 1PG, United Kingdom (a.lalvani@imperial.ac.uk).

The Journal of Infectious Diseases® 2022;225:1632–41

https://doi.org/10.1093/infdis/jiab311

work-up to determine whether the Mtb infection is active or not [7, 12]. Although 2 of the signatures have been corroborated in independent study populations [13–15], all studies to date have utilized preselected individuals and case-control study designs in which healthy LTBI individuals served as controls. None of these promising signatures have been evaluated in routine clinical practice where patients present with suspected (but not yet confirmed) active TB and therefore include individuals with non-TB illnesses that clinically mimic TB. Furthermore, the patient group that would benefit from these new tests has not been identified. Therefore, we determined to rigorously evaluate the ability of cellular immune signatures to distinguish patients with active TB from those with non-TB illnesses and concomitant LTBI in a clinically relevant population in real-life clinical practice. Our study population therefore comprised patients who tested IGRA-positive within a large, prospective, multicenter cohort of patients presenting with suspected active TB representing the full, natural clinical spectrum of TB (including a large proportion with EPTB) and non-TB illnesses that clinically mimic TB [4].

METHODS

Recruitment of Patients and Diagnostic Categorization

Individuals were recruited into the "IGRAs in Diagnostic Evaluation of Active TB" (IDEA) study (ethical approval reference 11/H0722/8), a prospective, multicenter, cohort study in routine practice, conducted across 10 inpatient and outpatient hospital sites in England between 2011 and 2013 as previously described [4]. Patients were recruited at the time of clinical suspicion of active TB; therefore, the diagnosis was unknown at enrollment, and treatment decisions were not affected by the study design. Participants gave informed consent for collection and use of their blood samples. All patients were followed up for up to 6 months to determine final diagnosis using a previously validated predefined classification [4, 5]. More importantly, the classification ensures the inclusion of TB patients who are culture-unconfirmed (or "highly probable TB") using a stringent, validated composite reference standard [4, 5]. To ensure a standardized diagnostic approach across different sites, the clinical diagnosis of each patient was verified by a panel of at least 4 expert TB physicians for the purposes of the IDEA study [4]. A clinically indeterminate diagnosis was attributed to individuals in instances in which a final diagnosis of active tuberculosis was deemed neither highly probable nor reliably excluded by the expert panel. The final cohort was representative of cases presenting with symptoms suggestive of active TB and investigated in multiple routine UK TB services.

All patients in the IDEA study who had consented for use of their samples in additional studies were eligible to be included in the "VAlidation of New Technologies for the Diagnostic Evaluation of Tuberculosis" (VANTDET) study, which evaluated various novel technologies (including blood transcriptomics, serum proteomics, and flow cytometric detection of cellular immune signatures) using samples collected during IDEA [16].

Study Design: Nested Case-Control Selection

Blood/peripheral blood mononuclear cell (PBMC) samples from IGRA-negative suspects would not yield sufficient Mtb-response T-cell events in these flow cytometry assays (which all include measurement of interferon [IFN]γ) to allow for a result to be determined. Thus, the objective of this study was to evaluate the ability of cellular immune signatures in patients with suspected active TB and a positive IGRA status, ie, to discriminate IGRA-positive patients presenting with suspected TB who, after full clinical assessment, are diagnosed with TB from those who are diagnosed with non-TB illnesses with concomitant LTBI. Similar to the IDEA study, patients considered clinically indeterminate were excluded because they cannot contribute to the estimation of sensitivity and specificity [4]. Numbers of human immunodeficiency virus (HIV)-infected individuals were so small that we could not reliably ascertain test performance within this subgroup of individuals; thus, HIV-infected participants were excluded. We included all 75 eligible patients diagnosed with non-TB illnesses who tested IGRA-positive and who had PBMCs available, ie, "Other Diseases with LTBI" (OD&LTBI) as controls. We then randomly selected 79 IGRA-positive patients diagnosed with active TB (ie, "cases") for inclusion (Figure 1). This provided a study population derived from routine practice comprising approximately equal numbers of patients who were subsequently diagnosed with TB and patients in whom TB was ruled out.

Antigen Stimulation and Flow Cytometry

The 6 signatures evaluated were as follows: (1) %TNFα-only - the proportion of Mtb-specific $CD4^+$ TNFα-only cells among the cytokine ($IFN\gamma^+$, $IL\text{-}2^+$, $TNF\alpha^+$) positive population [6]; (2) $\%T_{EFF}$ - the proportion of differentiated effector memory ($CD45RA^-CCR7^-CD127^-$) cells within the Mtb-specific $CD4^+$ TNFα-only cells [7]; (3) SCORE - a score combining signature 1 and the presence of a Mtb-specific $CD8^+$ $IFN\gamma^+$ T-cell response [8]; (4) TAM-TB - the ratio of CD27 MFI between the Mtb-specific $CD4^+$ $IFN\gamma^+$ cells and the overall $CD4^+$ population [9]; (5) $\%CD27^-CD45RA^-$ - the proportion of $CD27^-CD45RA^-$ cells within the Mtb-specific $CD4^+$ $IFN\gamma^+$ population [10]; and (6) %HLA-DR - the proportion of HLA-DR^+ cells within the Mtb-specific $CD4^+$ $IFN\gamma^+$ population [11].

Stimulation of baseline PBMCs with purified protein derivative (PPD) and an Mtb-specific peptide pool comprising peptides from antigens ESAT-6, CFP-10, Rv3615c, and Rv3879c was followed by staining with fluorescent-labeled antibodies, and acquisition of cellular profile data using flow cytometry was designed to allow simultaneous detection of all 6 signatures using methodology that resembled the original publications [6–11]; full details are in the Supplementary Material.

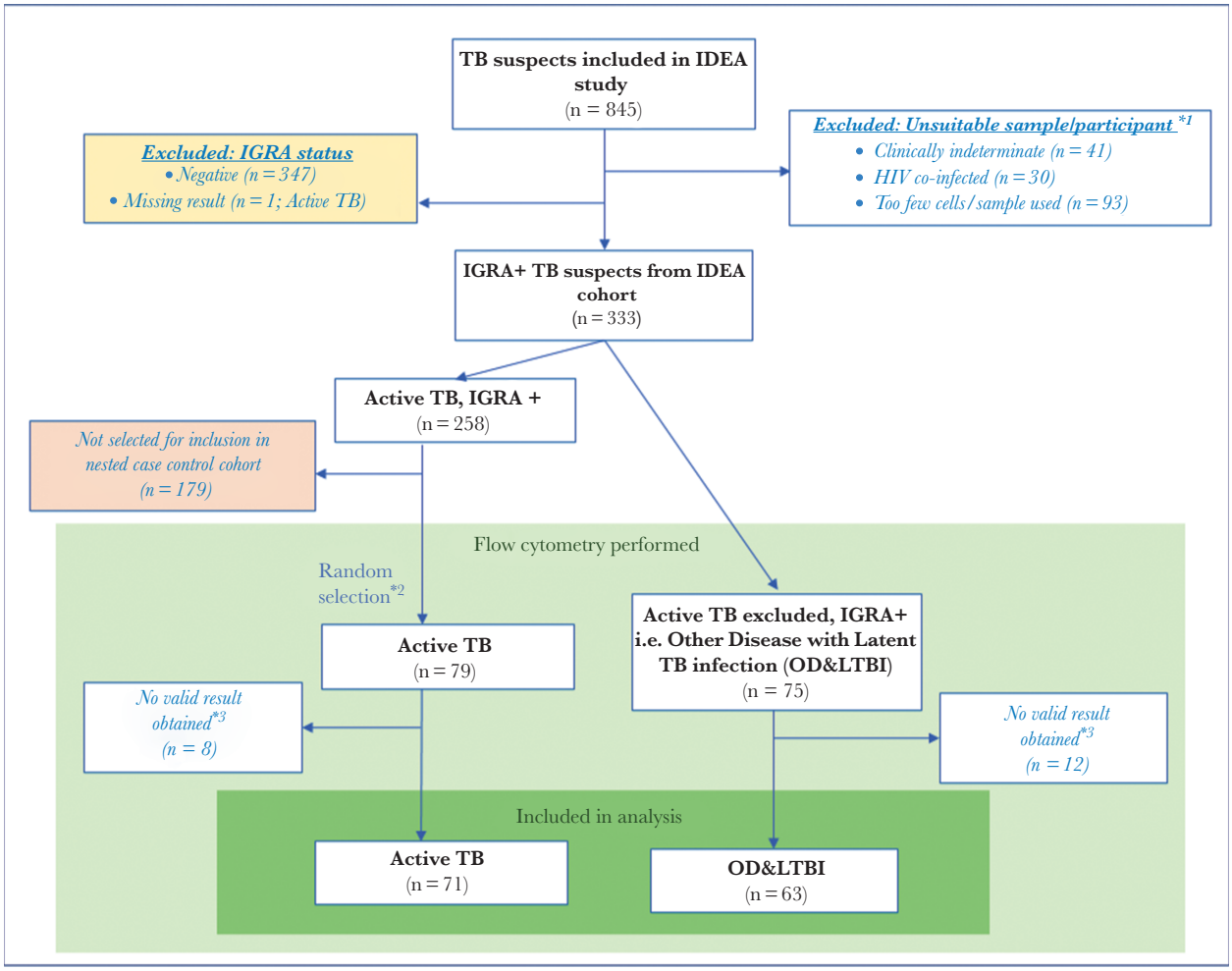

Figure 1. Flow chart showing selection of cohort to achieve a balance of interferon-gamma release assay (IGRA)-positive tuberculosis (TB) and other diseases with latent TB infection (OD&LTBI) subjects. This diagnostic evaluation study uses samples from participants recruited into the IGRAs in the Diagnostic Evaluation of Active TB (IDEA) study, in which n = 845 participants were included in the analysis [4]. From the IDEA final cohort, 512 participants were deemed ineligible for this particular study, due to either unsuitable samples/participant characteristics (n = 164) including indeterminate diagnosis or having HIV coinfection[*1], or having IGRA-negative status (n = 348)[*2]. A total of 79 individuals with active TB were randomly selected from the IGRA-positive TB cases to achieve a cohort with a balanced number of cases/controls, because 75 individuals had OD and concomitant LTBI[*4]. The light green shaded area indicates the samples that were selected to be included in the nested case-control cohort to validate the cellular immune signatures in this study. The dark green area indicates the total number of samples included in the final analysis; those who did not respond above background with all cytokines were excluded because not all signatures were measurable[*4]. [*1]Human immunodeficiency virus (HIV) coinfected and clinically indeterminate individuals were excluded from this study. [*2]To achieve a balanced study design and avoid prioritization of either sensitivity or specificity of the tests, 79 IGRA-positive TB cases were randomly selected for inclusion in the study. [*3]Of the154 individuals included in the study cohort and assayed, 134 responded with sufficient cytokine-producing cells to measure all 6 T-cell signatures (87%); these were included in the final analysis, whereas any with inadequate responses with 1 or more cytokines were excluded (n = 20).

Analysis

Flow cytometry data were collected onto BD FacsDIVA v8.01 (BD Biosciences) software and exported onto FCS files. Gating for all signatures was performed using FlowJo v10 (TreeStar) software according to the gating strategies laid out in the original publications (Supplementary Material). Statistical analysis was performed using GraphPad Prism v7. To compare demographic and clinical characteristics between TB and OD/LTBI groups, either the χ^2, Mann-Whitney *U* test, or unpaired *t* test was used based on the type of data (ie, categorical or Gaussian/non-Gaussian numerical). Flow cytometric signatures were compared between patient groups either using the Mann-Whitney *U* test (for 2 groups) or the Kruskal-Wallis test for multiple comparisons, with Dunn's post hoc test to identify differences between groups. Diagnostic sensitivity and specificity were calculated at different cutoffs and receiver operator characteristic (ROC) curves were plotted. Area under the curve (AUC), likelihood ratios, and predictive values were calculated with 95% confidence intervals (CIs) (Supplementary Material).

RESULTS

Cohort Characteristics

Of the 154 PBMC samples assayed, 134 (87%; 71 of 79 TB cases and 63 of 75 OD&LTBI) gave responses in all the required

cytokine-secreting T-cell populations to allow measurement of the diagnostic performance of all 6 signatures; these 134 individuals thus comprised the final validation cohort (Figure 1). Demographic and clinical characteristics of the cohort are presented in Table 1. One third (34%) of the patients diagnosed with active TB lacked a confirmatory culture result and 81% were lacking a positive smear test result, reflecting the limitations of smear microscopy and culture in routine practice. This proportion of culture-confirmed cases (66%) reflects the overall parent population of TB patients within the parent study [4]. Demographic and clinical features, stratified by culture status, are presented in Supplementary Table 2 , and details of the final diagnoses of patients are presented in Supplementary Tables 3 and 4.

Signature Values in Tuberculosis (TB) and Other Diseases With Latent TB Groups

We evaluated the performance of all 6 signatures for the detection of active TB in our cohort. Four signatures measure the phenotype of cytokine-responsive (functional) $CD4^+$ T cells ($\%T_{EFF}$ [7], TAM-TB [9], $\%CD27^-CD45RA^-$ [10], and %HLA-DR [11]), whereas 2 measure populations defined only by cytokine secretion (ie, functional signatures: %TNFα-only [6], SCORE [8]) (Supplementary Table 1) after stimulation with antigen. Figure 2 depicts the range in values of the 4 phenotypic signatures after PPD stimulation in the patient groups (Figure 2A–D). These 4 signatures gave significantly higher values in TB patients compared with those with OD&LTBI. In contrast, the 2 purely functional signatures that did not include cell-surface phenotypic markers (%TNF-α-only and SCORE) were not significantly different (Supplementary Figure 1A and B) and did not discriminate between the 2 patient groups (Supplementary Figure 1C and D). Some significant differences were observed between functional subsets, ie, TB patients had significantly higher proportions of $IFN\gamma^+IL\text{-}2^+TNF\alpha^-CD4^+$ T cells, but significantly smaller proportions of $IFN\gamma^-IL\text{-}2^+TNF\alpha^+$ and $IFN\gamma^-IL\text{-}2^+TNF\alpha^-$ compared with the OD&LTBI group (Supplementary Figure 1E and F), but none of these differences were sufficient to provide diagnostic discrimination (data not shown). Eighty-five patients (63% of the cohort) had sufficient PBMCs to allow for stimulation with the Mtb-specific antigen peptide pool, and, of these, 71 (84%) were responders for all signatures.

Table 1. Demographic and Clinical Characteristics of the Validation Cohort (n = 134)

Characteristic		TB	OD/LTBI	*P* Value
Total	N	71	63	
Age	Years, median (range)	32 (16–76)	39 (17–80)	.009[a]
Gender	Female, n (%)	20 (28.2)	25 (39.7)	.159[b]
Ethnic Origin	Indian Subcontinent, n (%)	47 (66.2)	26 (41.3)	.044[b]
	Asian (all other), n (%)	3 (4.2)	4 (6.3)	
	Black, n (%)	13 (18.3)	19 (30.2)	
	White, n (%)	7 (9.9)	9 (14.3)	
	All Other[c], n (%)	1 (1.4)	5 (7.9)	
Height (m)	Median (range)	1.68 (1.5–1.89)	1.66 (1.5–1.96)	.736[d]
	Missing, n (%)	28 (39.4)	19 (30.2)	
Weight (kg)	Median (range)	62.0 (41.3–110.4)	70.0 (42–132)	.009[a]
	Missing, n %	0 (0)	2 (3.2)	
BMI	Median (range)	22.0 (15.7–42.2)	24.3 (14.9–47.2)	.009[a]
	Missing, n %	28 (39.4)	19 (30.2)	
BCG Vaccinated	Yes, n (%)	50 (70.4)	39 (61.9)	.297[b]
	No, n (%)	21 (29.6)	24 (38.1)	
Culture Status	Positive, n (%)	47 (66.2)	0 (0)	<.0001[b]
	Negative, n (%)	19 (26.8)	44 (69.8)	
	Indeterminate, n (%)	0 (0)	1 (1.6)	
	Not tested, n (%)	5 (7.0)	18 (28.6)	
Smear Status	Positive, n (%)	13 (18.3)	3[e] (4.8)	.0103[b]
	Negative, n (%)	48 (67.6)	41 (65.1)	
	Not tested, n (%)	10 (14.1)	19 (30.2)	
Clinical Setting	Outpatient, n (%)	53 (74.6)	51 (81.0)	
	Inpatient, n (%)	18 (25.4)	12 (19.0)	

Abbreviations: BCG, bacillus Calmette-Guérin; BMI, body mass index; LTBI, latent tuberculosis infection; OD, other diseases; TB, tuberculosis.

[a]Mann-Whitney *U* test was used to compare medians of non-Gaussian numerical variables.

[b]The χ^2 test was used to compare proportions of categorical variables.

[c]All "other" ethnicity includes the following: mixed white and black African (n = 1); Hispanic (n = 1); all other (n = 3) and unable/unwilling to respond (n = 1).

[d]Unpaired *t* test was used to compare means of normally distributed numerical variables.

[e]The n = 3 smear-positive individuals in the OD/LTBI group were diagnosed with an atypical *Mycobacterium* infection.

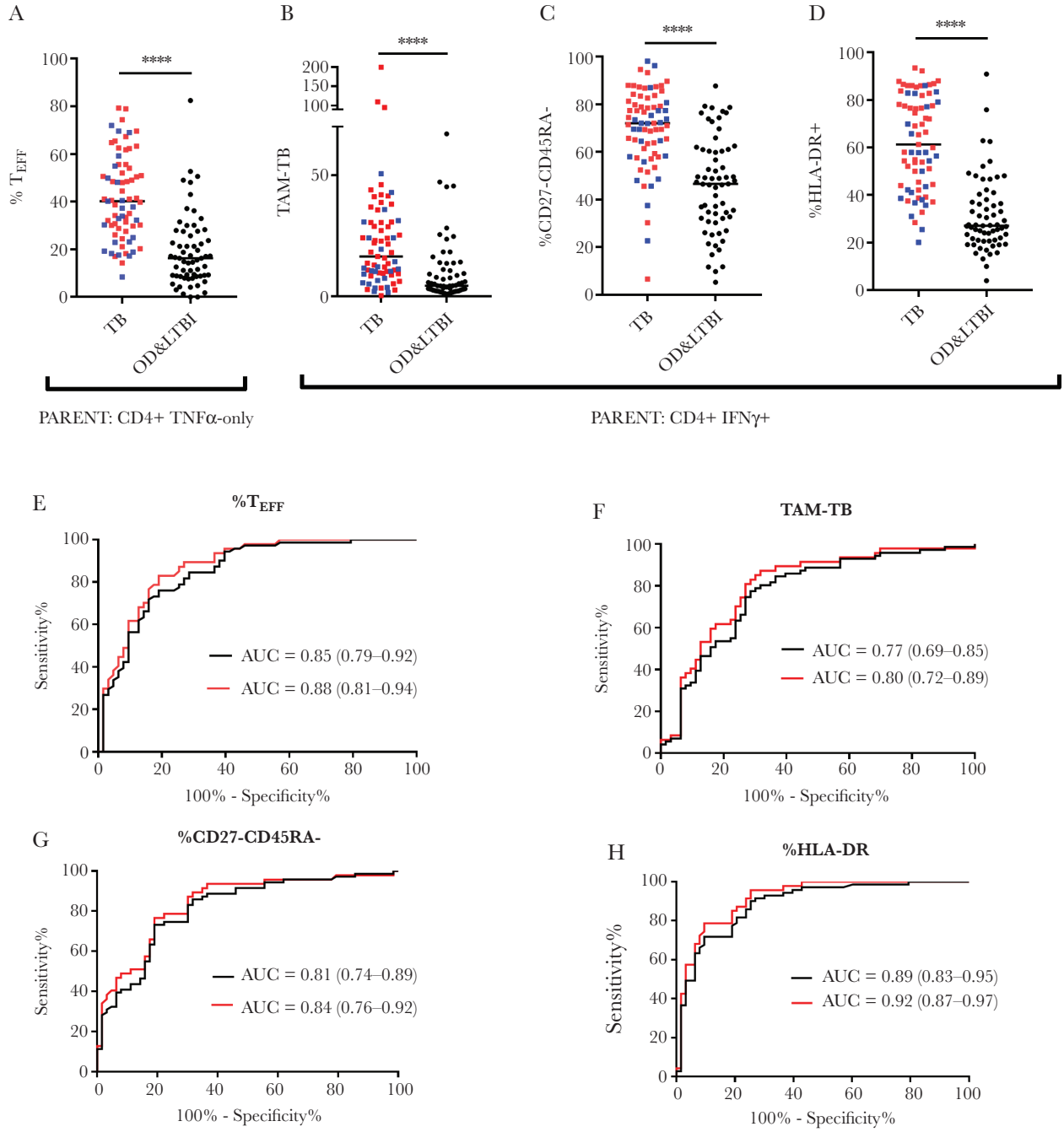

Figure 2. Validation of cellular immune signatures that measure the phenotype of cytokine-secreting *Mycobacterium tuberculosis* (Mtb)-specific CD4$^+$ T cells after purified protein derivative (PPD) stimulation for the diagnosis of active tuberculosis (TB). Dot plots showing the values of signatures in the patient groups are shown in A–D. For each plot, TB patients are represented by squares and colored by culture status (red = positive; blue = negative), and other diseases with latent TB infection (OD&LTBI) patients are represented by black circles. The receiver operator characteristic (ROC) curves for the phenotypic signatures are shown in E–H with the AUC and 95% confidence intervals indicated; black ROC curves show the performance of signatures for detection of all TB, whereas red ROC curves show performance for culture-positive TB only.

By virtue of their positive IGRA status, all individuals in this cohort had already been found to mount Mtb-specific T-cell responses. However, PPD antigen can also detect cross-reactive responses to Bacillus Calmette-Guérin in vaccinated individuals who are not infected with Mtb. Thus, we sought to compare signature performance after stimulation with either PPD or Mtb-specific peptides. The Mtb peptide stimulation was comparable to PPD stimulation for all signatures, with good correlation in results between the 2 stimuli and comparable diagnostic performance for all 6 signatures (Supplementary Figure 2); however, PPD stimulation generally resulted in a larger number of analyzable events (responsive T cells).

Diagnostic Performance of Signatures for Detecting Active Tuberculosis

The ROC curve for each of the 4 phenotypic signatures demonstrated ability to discriminate TB from OD&LTBI, listed in order of performance as quantified by AUC (95% CI) as follows: %HLA-DR 0.89 (0.83–0.95), %T_{EFF} 0.85 (0.79–0.92), %$CD27^{-}CD45RA^{-}$ 0.81 (0.74–0.89), and TAM-TB 0.77 (0.69–0.85). In contrast, the 2 functional signatures discriminated poorly with AUC (95% CI) of 0.57 (0.48–0.67) for %TNF-α-only and 0.60 (0.50–0.69) for SCORE (Table 2, Figure 2E and F, Supplementary Figure 1). For each of the 6 signatures, a balanced cutoff for test positivity was determined by identifying the point at which sensitivity overlapped with specificity, as described previously [11] (Table 2). For the 4 discriminatory phenotypic signatures, sensitivities ranged from 74.6% to 81.7% and specificities from 74.6% to 80.9% with correspondingly high positive predictive values ([PPVs] 93.2% to 95.2%). In our cohort, culture provided 100% specificity (94.3–100.0), but only 66.2% (54.0–77.0) sensitivity for all TB. The 4 phenotypic signatures detected TB in the majority (62.5%–70.8%) of TB patients who had either a negative culture result or where no culture was performed (Supplementary Table 5). However, none of these index tests performed with comparable specificity to that of culture (Supplementary Table 6).

Signature Performance in Tuberculosis Patient Subgroups

We also assessed the performance of signatures for detecting culture-confirmed TB (Figure 2, Supplementary Table 7). The phenotypic signatures all performed better for detecting culture-confirmed TB (AUCs from 0.80 to 0.92) compared to all TB (ie, culture-confirmed and culture-unconfirmed combined), with the same order of accuracy as in the full cohort. The best-performing signatures were %HLA-DR and %T_{EFF}, providing sensitivities of 85.1% (95% CI, 71.7–93.8) and 83.0% (95% CI, 69.2–92.3), respectively, and with both having a specificity of 80.9% (95% CI, 69.1–89.7) for culture-confirmed TB.

The Use of T-Cell Phenotypic Signatures to Detect Paucibacillary, Smear-Negative Tuberculosis

Smear microscopy is often used as a rapid rule-in test to determine whether a patient should be started on anti-TB treatment. However, a significant proportion of TB suspects are smear-negative (eg, 86% in the overall IDEA cohort recruited in routine practice) [17]. Despite the introduction of rapid molecular tests (eg, GeneXpert), which have a sensitivity greater than smear microscopy but less than culture [18], a large proportion of the smear-negative TB cases are not confirmed by culture or molecular tests, amounting to a substantial unmet clinical need. Because the %HLA-DR signature provided the highest sensitivity whilst %T_{EFF} provided highest specificity for all TB cases, we evaluated the use of these signatures as potential rapid tests in smear-negative patients with suspected TB. The diagnostic performance of both signatures in this

Table 2. Performance Characteristics of the 6 T-Cell Signatures for Detecting TB the Full Cohort (n = 71 TB Cases and n = 63 OD&LTBI: The Pretest Probability of TB in the IGRA-Positive Cohort of Patients With Suspected TB was 0.81) After PPD Stimulation of PBMCs

Performance Characteristic	%TNF-α-only (Harari [6])	%T_{EFF} (Pollock [7])	SCORE (Rozot [8])	TAM-TB (Portevin [9])	%$CD27^{-}CD45RA^{-}$ (Petruccioli [10])	%HLA-DR (Adekambi [11])
AUC (95% CI)	0.57 (0.48–0.67)	0.85 (0.79–0.92)	0.60 (0.50–0.69)	0.77 (0.69–0.85)	0.81 (0.74–0.89)	0.89 (0.83–0.95)
Cutoff[a]	<28.85	>28.55	<3.18	>9.46	>61.25	>41.05
Sensitivity (%) (95% CI)	53.5% (41.3 -65.5)	76.1% (64.5–85.4)	52.1% (39.9–64.1)	74.7% (62.9–84.2)	74.7% (62.9–84.2)	81.7% (70.7–89.9)
Specificity (%) (95% CI)	55.6% (42.5–69.1)	81.0% (69.1–89.8)	60.3% (47.2–72.4)	73.0% (60.4–83.4)	77.8% (65.5–87.3)	79.4% (67.3–88.5)
PPV (%) (95% CI)[b]	85.7 (80.8–89.5)	95.2 (92.1–97.1)	86.7 (81.7–90.5)	93.2 (89.9–95.5)	94.3 (91.2–96.4)	95.15 (92.3–97.0)
NPV (%) (95% CI)[b]	19.4 (14.7–25.2)	40.5 (30.7–51.2)	20.3 (15.6–25.8)	36.8 (27.5–47.0)	38.21 (28.9–48.5)	46.6 (34.5–59.2)

Abbreviations: AUC, area under the curve; CI, confidence interval; IGRA, interferon-gamma release assays; LTBI, latent tuberculosis infection; NPV, negative predictive value; OD, other diseases; PBMCs, peripheral blood mononuclear cells; PPD, purified protein derivative; PPV, positive predictive value; TB, tuberculosis; TNF, tumor necrosis factor.

[a]Cutoff values were selected by identifying the point on the receiver operator characteristic curve at which sensitivity and specificity overlap.

[b]Positive predictive value and NPV were calculated as described in the Supplementary Material.

clinically challenging group was high, with an AUC of 0.92 (95% CI, 0.87–0.97) for the %HLA-DR signature and 0.89 (95% CI, 0.82–0.96) for the $\%T_{EFF}$ signature (Table 3). These signatures provided high sensitivities for both overall TB (81.2% [95% CI, 67.4–91.0] and 83.3% [95% CI, 69.8–92.5], respectively) and culture-confirmed TB (86.2% [95% CI, 68.3–96.1] and 82.8% [95% CI, 64.2–94.1], respectively). In this IGRA-positive, smear-negative population with high pretest probability (0.81 for all TB and 0.59 for culture-confirmed TB), both signatures provided a high PPV for both populations (94.5% [95% CI, 90.2–97.0] and 94.7% [95% CI, 90.4–97.1], respectively, for all TB and 86.4% ([95% CI, 77.1–92.3] and 89.1% [95% CI, 79.2–94.6], respectively, for culture-confirmed TB), thus acting as reliable rule-in tests.

DISCUSSION

We aimed to determine the diagnostic accuracy and potential clinical utility of 6 previously described flow cytometric T-cell signatures in a cohort of patients with suspected TB recruited in routine clinical practice. We assessed the role of the signatures at the appropriate point in the diagnostic work-up, ie, to stratify TB suspects with positive IGRA results into those who have active TB versus those who have non-TB diagnoses with concomitant LTBI. This is the first evaluation of these signatures in a routine practice cohort that includes both the full clinical spectrum of active TB cases (including EPTB) and a wide natural spectrum of patients with other diseases that clinically resemble TB and coincidental LTBI (OD&LTBI). Moreover, it is the first study to propose a specific clinical role for these tests in the context of other available diagnostic tools.

We found that only signatures measuring the cell-surface phenotype of functional Mtb-specific T-cell subsets discriminated well between TB and OD&LTBI. The best-performing signatures were the %HLA-DR and the $\%T_{EFF}$ signature, followed by 2 signatures incorporating measurement of CD27 ($\%CD27^{-}CD45RA^{-}$ and TAM-TB). Despite providing reasonable discrimination between active TB and OD&LTBI, all of the phenotypic signatures performed less well than previously reported (with an optimal AUC of 0.89). This likely reflects the rigorous evaluation of the signatures in this real-life cohort in routine clinical practice compared with the preselected study populations used previously.

We further evaluated the diagnostic performance of the 2 best-performing signatures in the subgroup of patients with culture-confirmed TB, the gold-standard diagnosis. We found the phenotypic signatures had, if anything, even higher diagnostic concordance with culture (ie, the signatures predicted culture-confirmed TB better than all TB cases).

Given the availability of rapid microbiological tests to detect a large proportion of culture-positive TB patients with high bacillary load (eg, smear microscopy tests and molecular tests such as GeneXpert), the unmet clinical need in the diagnostic work-up of suspected active TB is in the substantial subgroup of patients who are negative on the currently available rapid tests (ie, smear and GeneXpert), or who require invasive sampling procedures (eg, many cases of EPTB). Therefore, we evaluated the use of the best-performing signatures, %HLA-DR and $\%T_{EFF,}$ in the IGRA-positive smear-negative TB suspects. In this group of patients, TB remains in the differential diagnosis because of the positive IGRA result, but the negative smear result

Table 3. Performance of the %HLA-DR and $\%T_{EFF}$ Signatures for Detecting TB in Smear-Negative TB Suspects, After Stimulation of PBMCs With PPD

Performance Characteristic	%HLA-DR Signature	$\%T_{EFF}$ Signature
Performance for Detecting Smear-Negative TB (All Culture Status Included) in T-SPOT-Positive Individuals, Where the Pretest probability Is 0.81[a]		
AUC (95% CI)	0.92 (0.87–0.97)	0.89 (0.82–0.96)
Cutoff[b]	>42.85	>22.8
Sensitivity (%) (95% CI)	81.3 (67.4–91.1)	83.3 (69.8–92.5)
Specificity (%) (95% CI)	80.5 (65.1–91.2)	80.5 (65.1–91.2)
PPV (%) (95% CI)[c]	94.6 (90.2–97.0)	94.7 (90.4–97.1)
NPV (%) (95% CI)[c]	50.8 (36.0–65.4)	53.7 (37.7–69.0)
Performance for Detecting Smear-Negative Culture-Positive TB (n = 29) Pretest Probability of Culture-Positive TB in All Smear-Negative IGRA-Positive TB Suspects = 0.59[a]		
AUC (95% CI)	0.94 (0.89–0.99)	0.91 (0.85–0.97)
Cutoff[b]	>43.1	>27.8
Sensitivity (%) (95% CI)	86.2 (68.3–96.1)	82.8 (64.2–94.2)
Specificity (%) (95% CI)	80.5 (65.1–91.2)	85.4 (70.8–94.4)
PPV (%) (95% CI)[c]	86.4 (77.1–92.3)	89.1 (79.2–94.6)
NPV (%) (95% CI)[c]	80.3 (61.7–91.1)	77.5 (60.5–88.5)

Abbreviations: AUC, area under the curve; CI, confidence interval; IGRA, interferon-gamma release assays; NPV, negative predictive value; PBMCs, peripheral blood mononuclear cells; PPD, purified protein derivative; PPV, positive predictive value; TB, tuberculosis.

[a]Prevalence of TB within the population indicated was calculated in overall IGRAs in the Diagnostic Evaluation of Active TB (IDEA) study cohort, as described in methods in the Supplementary Material.

[b]Cutoff values were selected by identifying the point at which sensitivity = specificity and both are maximized.

[c]Positive predictive value and NPV were calculated as described in Supplementary Material.

makes active TB less likely. A test that can rapidly rule-in a diagnosis of TB in this setting before culture results are available would advance clinical practice. For this key subgroup of TB patients, the PPV of approximately 95% implies that either of these 2 tests can be used as rule-in tests to accelerate diagnosis of active TB in IGRA-positive patients. Although the pretest probability (prevalence) of TB in this IGRA-positive, smear-negative subgroup is high, a positive cellular immune signature result will enable the clinical decision to commence treatment before culture results become available or where they are unavailable for lack of suitable clinical samples. However, given their poor NPVs, these immune signatures have no role as rule-out tests in this setting. Smear-negative TB and EPTB are associated with treatment delays [3]; positive cellular immune signature results could expedite treatment in these important subgroups; however, our dataset does not permit us to determine whether this would have been the case in our cohort. One potential explanation for the discrepancies in the performance of functional Mtb-specific CD4 T-cell signatures is differences in experimental setup and protocol between studies, because the resting and stimulation times influence the functional profile of T cells [19], as does cryopreservation [20]. However, the relative consistency with which the signatures measuring phenotype of functional subsets perform across studies suggests that measurement of these signatures is less affected by differences in experimental protocols.

Our study has some limitations. This study addressed a specific but clinically challenging subgroup of TB patients. As such, our results are naturally not generalized to all TB suspects including those with a negative IGRA result. Of note, we did not evaluate the utility of these signatures in children, in whom paucibacillary TB and EPTB are common. Therefore, in future studies, researchers should prioritize evaluation of the %HLA-DR and $\%T_{EFF}$ signatures in children. Furthermore, although the parent study included some HIV-positive TB cases, there were insufficient numbers of eligible IGRA-positive patients in this subgroup to assess diagnostic performance in HIV-coinfected TB suspects. Previous studies including HIV-positive patients have demonstrated good performance of both the %HLA-DR signature and the $T_{EFF}\%$ signature, irrespective of HIV status [11, 13, 14], although the %HLA-DR signature required different cutoffs in HIV^+ populations [13, 14]. Some of the PBMC samples we used failed to respond with any of the cytokines being measured (13%), precluding measurement of all signatures in parallel; therefore, these were excluded from analysis. This suggests that if used in a routine diagnostic service setting, these tests might result in a proportion of assay failures. However, given that we used frozen PBMCs that had been stored for approximately 3 years, we anticipate that the rate of nonresponsiveness would be substantially reduced if fresh blood or short-term PBMC storage was used instead, as would be the case in routine diagnostic service laboratories. Finally, the role we have defined for use of these tests is not for all patients presenting with suspected TB but rather for the specific, clinically challenging subgroup who are IGRA-positive and smear-negative.

Based on the evidence provided, the 2 best-performing tests are potentially useful in low-incidence settings, where a significant proportion of patients presenting with TB, particularly those with EPTB, test negative on the available rapid tests. Their high PPV in the IGRA-positive smear-negative population reflects the IGRA-positive rates and TB incidence in our real-life cohort recruited in routine clinical practice and are therefore likely generalizable to other high-income, low-incidence settings. The potential clinical use of these tests in other settings remains unclear and warrants further investigation. Given the technical complexity of using flow cytometry as a diagnostic test, it is unlikely that these tests would be practicable outside major centers in high-incidence, low-income settings in their current format. Therefore, future studies should aim to simplify the protocols to minimize complexity and requirement for technical expertise.

We present the largest study to date to evaluate the diagnostic performance of T-cell signatures for active TB diagnosis. To enable rigorous and clinically relevant assessment of the potential role these new tests could have in clinical practice, we focused specifically on the hard-to-diagnose patients with clear unmet clinical need. We found that 2 signatures have a potential clinical role in the diagnostic work-up of active TB in the important subgroup of patients who are IGRA-positive and smear-negative and typically have paucibacillary or EPTB. Further evaluation in an independent cohort in routine clinical practice, preferably including children, is now warranted to validate our findings and support development of these 2 signatures into regulatory-approved in vitro tests for routine diagnostic services.

CONCLUSIONS

We propose that future biomarker and diagnostic studies use similar approaches, ie, prospective evaluation in clinically relevant cohorts in routine practice, evaluation in patient groups with unmet clinical need, and in the context of other test results during the diagnostic work-up. Such approaches could move the whole field forward by reliably "gating out" or confirming the clinical utility of promising new tests for TB [21].

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

Acknowledgments. We thank the patients for their involvement in the IGRAs in Diagnostic Evaluation of Active TB (IDEA) study and donation of samples for the purpose of future diagnostic research. We thank past and present members of the TB Research Centre and the National Institute for Health Research (NIHR) Health Protection Research Unit in Respiratory Infections for their scientific and advisory support throughout the project, including Dan Solanki, Claire Puddephatt, Gulfer Yakici, Helen Piotrowski, Hilary Whitworth, Ann-Kathrin Reuschl, Hiromi Uzu, Umar Niazi, and Katrina Pollock. We also thank the IDEA study team and the VAlidation of New Technologies for the Diagnostic Evaluation of Tuberculosis (VANTDET) scientific advisory group for their input into the IDEA and VANTDET studies. We thank Tingting Wu for financial and administrative support and Christopher Partlett for assistance in the randomization and selection of patient samples.

Financial support. This work was funded by the Efficacy and Mechanism Evaluation (EME) programme, a Medical Research Council and NIHR partnership (Grant Number 12/65/27) and supported by the NIHR-funded Health Protection Research Unit (HPRU) in Respiratory Infections at Imperial College London (NIHR200927). The views expressed are those of the authors and not necessarily those of the NIHR of the Department of Health and Social Care.

Potential conflicts of interest. A. L. is named inventor on patents pertaining to T-cell-based diagnosis. Some of these patents were assigned by the University of Oxford, Oxford, UK, to Oxford Immunotec plc, resulting in royalty entitlements for the University of Oxford and A. L. A. H. and A. L. have a patent pending around the use of cellular immune signatures for risk stratification of latent tuberculosis infection. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

References

1. Public Health England. Tuberculosis in England: 2019. London: Public Health England, **2020**.
2. Sandgren A, Hollo V, van der Werf MJ. Extrapulmonary tuberculosis in the European Union and European Economic Area, 2002 to 2011. Euro Surveill **2013**; 18:20431.
3. Storla DG, Yimer S, Bjune GA. A systematic review of delay in the diagnosis and treatment of tuberculosis. BMC Public Health **2008**; 8:15.
4. Whitworth HS, Badhan A, Boakye AA, et al; Interferon-γ Release Assays for Diagnostic Evaluation of Active Tuberculosis study group. Clinical utility of existing and second-generation interferon-γ release assays for diagnostic evaluation of tuberculosis: an observational cohort study. Lancet Infect Dis **2019**; 19:193–202.
5. Dosanjh DP, Hinks TS, Innes JA, et al. Improved diagnostic evaluation of suspected tuberculosis. Ann Intern Med **2008**; 148:325–36.
6. Harari A, Rozot V, Bellutti Enders F, et al. Dominant TNF-α+ mycobacterium tuberculosis-specific CD4+ T cell responses discriminate between latent infection and active disease. Nat Med **2011**; 17:372–6.
7. Pollock KM, Whitworth HS, Montamat-Sicotte DJ, et al. T-cell immunophenotyping distinguishes active from latent tuberculosis. J Infect Dis **2013**; 208:952–68.
8. Rozot V, Patrizia A, Vigano S, et al. Combined use of *Mycobacterium tuberculosis*-specific CD4 and CD8 T-cell responses is a powerful diagnostic tool of active tuberculosis. Clin Infect Dis **2015**; 60:432–7.
9. Portevin D, Moukambi F, Clowes P, et al. Assessment of the novel T-cell activation marker-tuberculosis assay for diagnosis of active tuberculosis in children: a prospective proof-of-concept study. Lancet Infect Dis **2014**; 14:931–8.
10. Petruccioli E, Petrone L, Vanini V, et al. Assessment of CD27 expression as a tool for active and latent tuberculosis diagnosis. J Infect **2015**; 71:526–33.
11. Adekambi T, Ibegbu CC, Cagle S, et al. Biomarkers on patient T cells diagnose active tuberculosis and monitor treatment response. J Clin Invest **2015**; 125:1827–38.
12. Halliday A, Masonou T, Tolosa-Wright M, Mandagere V, Lalvani A. Immunodiagnosis of active tuberculosis. Expert Rev Respir Med **2019**; 13:6, 521–32.
13. Wilkinson KA, Oni T, Gideon HP, Goliath R, Wilkinson RJ, Riou C. Activation profile of *Mycobacterium tuberculosis*-specific CD4(+) T cells reflects disease activity irrespective of HIV status. Am J Respir Crit Care Med **2016**; 193:1307–10.
14. Riou C, Berkowitz N, Goliath R, Burgers WA, Wilkinson RJ. Analysis of the phenotype of *Mycobacterium tuberculosis*-specific CD4+ T cells to discriminate latent from active tuberculosis in HIV-uninfected and HIV-infected individuals. Front Immunol **2017**; 8:968.
15. Halliday A, Whitworth H, Kottoor SH, et al. Stratification of latent *Mycobacterium tuberculosis* infection by cellular immune profiling. J Infect Dis **2017**; 215:1480–7.
16. Halliday A, Jain P, Hoang L, et al. New technologies for diagnosing active TB: the VANTDET diagnostic accuracy study. Efficacy and Mechanism Evaluation **2021**; 8:5.
17. Takwoingi Y, Whitworth H, Rees-Roberts M, et al. Interferon gamma release assays for Diagnostic Evaluation of Active tuberculosis (IDEA): test accuracy study and economic evaluation. Health Technology Assessment **2019**; 23:23.
18. Dorman SE, Schumacher SG, Alland D, et al; study team. Xpert MTB/RIF Ultra for detection of *Mycobacterium tuberculosis* and rifampicin resistance: a prospective multicentre diagnostic accuracy study. Lancet Infect Dis **2018**; 18:76–84.

19. Kutscher S, Dembek CJ, Deckert S, et al. Overnight resting of PBMC changes functional signatures of antigen specific T-cell responses: impact for immune monitoring within clinical trials. PLoS One **2013**; 8:e76215.
20. Smith SG, Smits K, Joosten SA, et al; TBVI TB Biomarker Working Group. Intracellular cytokine staining and flow cytometry: considerations for application in clinical trials of novel tuberculosis vaccines. PLoS One **2015**; 10:e0138042.
21. MacLean E, Broger T, Yerliyaka S, Fernandez-Carballo BL, Pai M, Denkinger CM. A systematic review of biomarkers to detect active tuberculosis. Nat Microbiol **2019**; 4:748–58.