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Tuberculosis antigen-specific immune responses can be detected using enzyme-linked immunospot technology in human immunodeficiency virus (HIV)-1 patients with advanced disease

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Summary

There are limited data on the efficacy of T cell-based assays to detect tuberculosis (TB) antigen-specific responses in immune-deficient human immunodeficiency virus (HIV) patients. The aim of this study is to determine whether TB antigen-specific immune responses can be detected in patients with HIV-1 infection, especially in those with advanced disease (CD4 T cell count < 300 cells/μl). An enzyme-linked immunospot (ELISPOT) assay, which detects interferon (IFN)-γ secreted by T cells exposed to TB antigens, was used to assess specific immune responses in a prospective study of 201 HIV-1-infected patients with risk factors for TB infection, attending a single HIV unit. The performance of the ELISPOT assay to detect TB antigen-specific immune responses is independent of CD4 T cell counts in HIV-1 patients. The sensitivity and specificity of this assay for the diagnosis of active tuberculosis does not differ significantly from values obtained in immunocompetent subjects. The negative predictive value of the TB ELISPOT test is 98·2%. A positive predictive value of 86% for the diagnosis of active tuberculosis was found when the combined number of early secretory antigen target-6 (ESAT-6) and culture filtrate protein-10 (CFP-10) IFN-γ spots to CD4 T cell count ratio was > 1·5. TB antigen-specific immune responses can be detected in HIV patients with low CD4 T cell counts using ELISPOT technology in a routine diagnostic laboratory and is a useful test to exclude TB infection in immune-deficient HIV-1 patients. A combination of TB antigen-specific IFN-γ responses and CD4 T cell counts has the potential to distinguish active tuberculosis from latent infection.

Keywords: culture filtrate protein-10 (CFP-10), early secretory antigen target-6 (ESAT-6), HIV-1 infection, interferon gamma (IFN-γ), tuberculosis

Introduction

Tuberculosis is the most common opportunistic infection and the leading cause of death worldwide in human immunodeficiency virus (HIV)-1 infected patients [1]. The clinical and radiological features of tuberculosis (TB) are influenced by the degree of HIV-1 related immune deficiency, especially in patients with CD4 T cell counts < 300 cells/μl [2]. The performance of diagnostic tests such as sputum microscopy and tuberculin skin tests (TST) declines in patients with reduced CD4 T cell counts. There is a pressing need for rapid, sensitive tests to identify both active and latent TB infection to control tuberculosis in HIV-1 infection and other high-risk populations. A number of studies have shown that T cell assays that detect interferon (IFN)-γ secretion in response to TB-specific antigens, early secretory antigen target-6 (ESAT-6) and culture filtrate protein-10 (CFP-10), are significantly better than TST for the diagnosis of active and latent TB infection in immunocompetent individuals [3–8]. There is limited information on the performance of IFN-γ release assays in patients with HIV-1 infection. In this study, we investigate the performance of an enzyme-linked immunospot (ELISPOT) assay (T-SPOT.*TB*; Oxford Immunotec, Oxford, UK) assay to detect TB antigen-specific immune responsiveness in HIV-1 patients.

Material and methods

Study population

HIV-1-positive patients attending a single HIV unit in London were screened for TB infection using the T-SPOT.*TB* ELISPOT assay. The inclusion criteria for this study were individuals with either (1) symptoms, signs and X-ray findings suggestive of active tuberculosis or (2) asymptomatic infection with the following TB risk factors: residence in a TB endemic area for more than 3 months, TB contact, intravenous drug use, diabetes mellitus, chronic renal failure, current immunosuppressive drug therapy. Absolute CD3, CD4 and CD8 T cell counts and percentages were determined using 100 µl of ethylenediamine tetraacetic acid (EDTA) blood samples by four-colour flow cytometric analysis using a Cytomics FC500 flow cytometer (Beckman Coulter Inc., High Wycombe, UK). Plasma viral load was measured using branched DNA amplification technology (Bayer Healthcare, Tarrytown, NY, USA) with a lower limit of detection of 50 copies/ml.

The first 70 patients recruited received tuberculin skin tests (Heaf or Mantoux) using the equivalent of 2 U of tuberculin. However, restrictions on the supply of skin test reagents and authorized personnel resulted in premature discontinuation of this arm of the study. A positive Mantoux test result was defined as skin induration of more than 5 mm in diameter [9] and a positive Heaf test was a grades 2–4 reaction [10]. Clinical information such as age, sex, ethnic background and risk factors for TB infection were recorded. Individuals with clinical features or chest X-ray features suggestive of active tuberculosis had three sputum/bronchial alveolar lavage (BAL) samples requested for acid fast bacilli (AFB) stains and TB cultures. Further investigations, such as TB blood, spinal fluid and tissue cultures, were performed as indicated clinically. Asymptomatic patients with a positive TB ELISPOT test were referred to the TB clinic for further investigations to exclude active TB disease. One individual (P. K.) reviewed the medical notes of all patients studied for at least 3 months after the TB ELISPOT assay to ascertain clinical outcomes. Active tuberculosis was defined as AFB smear-positive or positive culture for *Mycobacterium tuberculosis*, while probable tuberculosis was defined as clinical, radiological features consistent with TB and objective symptomatic or radiological improvement on standard 6-month anti-TB therapy in patients with clinically suspected disease. Asymptomatic HIV patients with no evidence of active TB but who had TB risk factors as defined above and who were not receiving TB chemotherapy were defined as potential latent TB infection. The study was conducted in accordance with local ethical approval (Riverside Research Ethics Committee, REC no. 04/Q0401/108).

The T-SPOT.*TB* ELISPOT assay was performed according to the manufacturer's instructions. Viable peripheral blood mononuclear cells (PBMC) at a concentration of $2{\cdot}5 \times 10^5$ were added to 96-well membrane-bottomed plates precoated with anti-IFN-γ antibodies. Four wells were used for each patient: a positive control well to which phytohaemagglutinin (PHA) was added, a negative control well which contained medium and two wells which contained ESAT-6 or CFP-10 peptide pools. Plates were incubated for 16–20 h at 37°C with 5% CO_2, washed with phosphate-buffered saline (PBS) and developed using an anti-IFN-γ antibody conjugate and substrate to detect the presence of secreted IFN-γ. The number of spots was then counted using an ELISPOT reader. Positive, anergic (less than 80 IFN-γ spots/10^6 cells following PHA stimulation) and indeterminate results were defined according to the manufacturer's instructions.

Table 1. Demographic and clinical characteristics of human immunodeficiency virus-1 infected patients who received a tuberculosis enzyme-linked immunospot and tuberculin skin tests (TST).

Characteristics	Total group ($n = 201$)	TST group ($n = 72$)
Age, median (IQR)	40 (33–46)	40 (33–46)
Male, no. (%)	119 (59)	41 (57)
Ethnic origin		
African, no. (%)	102 (51)	39 (54)
White, no. (%)	69 (34)	25 (35)
Asian/other, no. (%)	30 (15)	8 (11)
CD4 T cell count		
Median (IQR)	213 (77–367)	261 (120–455)
< 300 cells/µl, no. (%)	129 (64)	39 (54)
< 200 cells/µl, no. (%)	96 (48)	25 (35)
< 100 cells/µl, no. (%)	61 (30)	16 (22)
BCG,† no. (%)	n.d.	49 (68)
ART, no. (%)	118 (59)	43 (60)
Viral load		
< 50 copies/ml, no. (%)	87 (43)	35 (49)

†Bacille Calmette–Guérin (BCG) vaccination status determined on the basis of clinical history and the presence of a scar. IQR: interquartile range; n.d. not determined; ART: anti-retroviral therapy.

Statistical analysis

Univariate correlations between T cell counts and PHA/TB antigen-specific induced IFN-γ responses were assessed using Spearman's rank correlation tests. Differences between patients with active/presumptive TB and latent TB were tested for statistical significance using the Mann–Whitney *U*-test. Agreement of the results obtained by TB ELISPOT and TST was determined by calculation of Cohen's κ-coefficient. All statistical calculations were performed with SPSS version 14 (SPSS Inc., Chicago, IL, USA).

Results

We evaluated 201 HIV-1 seropositive subjects for TB infection over a period of 24 months (Table 1): 154 symptomatic patients who presented either with a history of acute respiratory illness or radiological features suggestive of active

Table 2. Performance of tuberculosis (TB) ELISPOT assay for diagnosis of active tuberculosis in HIV-1 infected patients stratified by CD4 T cell count.

Patient group	No.	Sensitivity (%)	Specificity (%)
All HIV patients with active/probable TB	30	90·3	100
CD4 T cell count < 300 cells/μl	22	95·4	100
CD4 T cell count < 200 cells/μl	14	92·9	100
CD4 T cell count < 100 cells/μl	8	87·5	100

tuberculosis and 47 asymptomatic patients who were screened for latent TB infection. Nine (4·5%) TB ELISPOT assays failed according to the manufacturer's criteria. Five patients had anergic PHA-induced IFN-γ responses (four of whom had CD4 < 15 cells/μl) and four individuals had high background counts in the negative control well. Analysis of the effect of low CD4 T cell counts on the performance of the TB ELISPOT showed a weak correlation (Spearman's rho = 0·169, $P = 0·017$) between the CD4 T cell count and the level of PHA-stimulated IFN-γ production in HIV-1 patients. We identified 50 subjects with TB antigen-specific immune responses, of whom 33 (66%) had CD4 T cell counts < 300 cells/μl and nine (18%) had CD4 T cell counts < 100 cells/μl. There was no significant correlation between CD4 T cell count (Spearman's rho = –0·036), CD8 T cell count (Spearman's rho = 0·103) and the magnitude of TB antigen-specific IFN-γ immune responses (Fig. 1). The sensitivity of the TB ELISPOT assay for the diagnosis of active/probable tuberculosis was 90·3% and was not affected by CD4 T cell count (Table 2), although the limited number of cases in HIV-1 patients with a CD4 T cell < 100 cells/μl means that definitive conclusions about the performance of this assay in very advanced HIV-1 associated immune suppression remain to be determined. The specificity of the TB ELISPOT for TB infection in this study was 100%. The assay missed culture-positive tuberculosis in one individual (CD4 T cell count 17 cells/μl). A negative TB ELISPOT result was obtained for one patient who had objective clinical and radiological improvements, which was attributed to TB treatment (CD4 T cell counts 447 cells/μl). No other symptomatic patient with a negative TB ELISPOT assay developed culture-positive tuberculosis or was started on empirical TB treatment within 3 months of a negative test (Fig. 2). The negative predictive value of the TB ELISPOT assay for the diagnosis of TB infection in HIV patients who presented with clinical or radiological features suggestive of this condition was 98·2%. Follow-up of 20 patients with a positive ELISPOT test result who were considered to have latent TB, over a median period of 12 months, showed that two individuals developed active tuberculosis 3 and 10 months later; however, large prospective studies are needed to define the risk and positive predictive value of the TB ELISPOT test for the development of active tuberculosis.

Individuals with active TB had significantly lower CD4 T cells than patients with latent infection (median CD4 T cell count 209 cells/μl *versus* 294 cells/μl, $P = 0·043$). There was no significant difference in CD8 T cell counts between patients with active tuberculosis and latent TB infection (data not shown). The combined number of ESAT-6 and CFP-10 IFN-γ spot-forming cells to CD4 T cell count ratio was significantly higher (median ratio 3·032 *versus* 0·694,

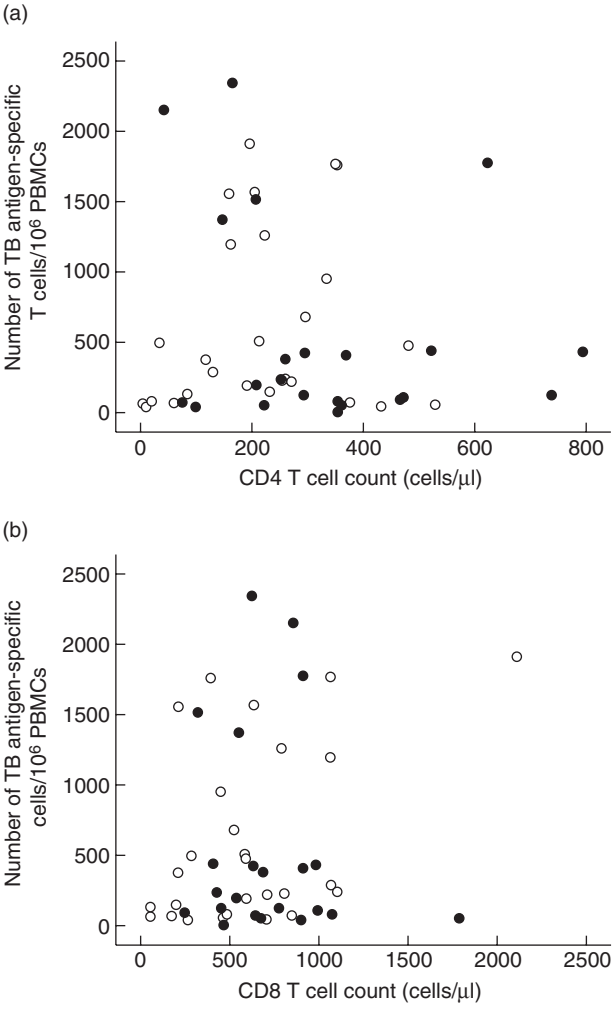

Fig. 1. Scatter plot of the magnitude of tuberculosis (TB) antigen-specific immune responses against CD4 (a) and CD8 (b) T cell counts. The sum of interferon (IFN)-γ spots/10^6 peripheral blood mononuclear cells (PBMC) in response to TB-specific peptide pools early secretory antigen target-6 (ESAT-6) and culture filtrate protein-10 (CFP-10) are plotted against CD4 (a) and CD8 (b) T cell counts observed in each individual who had a positive test result. Open circles represent patients with active/probable tuberculosis, closed circles represent patients with latent TB infection. A positive TB enzyme-linked immunospot assay was defined as follows: the sum of the IFN-γ spots to both peptide pools exceeds that seen in the medium alone well by 25. In this set of experiments the number of IFN-γ spots seen in medium alone well in each individual was < 4 spots/10^6 PBMC.

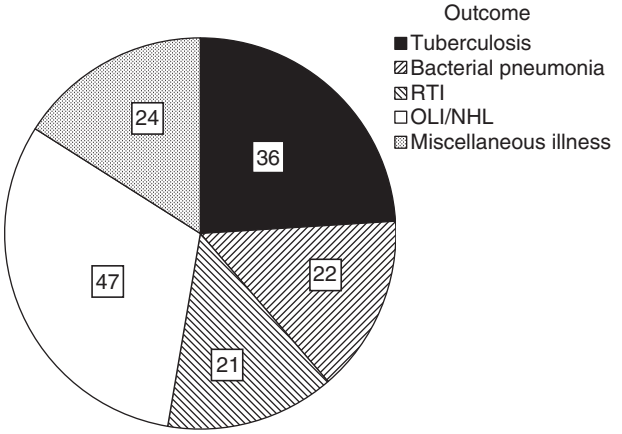

Fig. 2. Clinical outcomes in all symptomatic patients who had a tuberculosis (TB) enzyme-linked immunospot assay test. Clinical outcomes for 154 symptomatic patients in this study were coded using standardized criteria to define pneumonia, lower respiratory tract infection, opportunistic infections and non-Hodgkin's lymphoma. The numbers of patients in each group were as follows: tuberculosis ($n = 36$, 30 patients with active tuberculosis and six individuals with latent TB infection) presumptive bacterial pneumonia ($n = 22$), lower respiratory tract infection ($n = 21$), opportunistic infections involving the lung [*pneumocystis carinii* pneumonia (PCP), *mycobacterium avium* complex (MAC) and non-Hodgkin lymphoma (NHL) ($n = 47$)], miscellaneous illnesses ($n = 24$, including Gram-negative sepsis, pyelonephritis, inflammatory lung disease, lung cancer, cerebrovascular accident, Hodgkin's disease acute illness now resolved). Five patients were lost to follow-up ($n = 3$) or TB cultures are still outstanding ($n = 1$).

$P = 003$) in patients with active tuberculosis compared to latent TB (Fig. 3a). Analysis of the combined number of ESAT-6 and CFP-10 spots to CD8 T cell count ratio also showed a significant difference (median ratio 1·012 *versus* 0·3690, $P = 0·021$) between patients with active and latent TB (Fig. 3b). The positive predictive value, sensitivity and specificity for the diagnosis of active TB was 86%, 68% and 80%, respectively, when the combined number of ESAT-6 and CFP-10 spot count to CD4 T cell count ratio was > 1·5 (Fig. 3c). The positive predictive value was 79% when the combined number of ESAT-6 and CFP-10 spot count to CD8 T cell count ratio was > 1·5; however, the sensitivity and specificity was only 39% and 85%.

Eight patients did not return to have their TST read and in one patient the TB ELISPOT assay did not work. There was good agreement between the results of the TST and TB ELISPOT ($\kappa = 0·74$, $P < 0·001$), with 89·06% ($n = 63$) concordance. Discordant results were seen in seven patients, four of whom had a positive TST and a negative TB ELISPOT result, while three patients had a negative TST and positive TB ELISPOT result. Two of three patients with a positive TB ELISPOT test result and negative skin tuberculin skin test had risk factors that are associated with reduced TST sensitivity for TB infection (active TB disease, CD4 T cell count 42 cells/μl). The reason for the discrepancy between the TB ELISPOT and TST result in the final patient with a history of latent TB is not known.

Discussion

We show that TB antigen-specific immune responses can be detected in HIV-1 patients with reduced CD4 T cell counts and that a combination of TB ELISPOT IFN-γ spots and CD4 T cell counts may distinguish active tuberculosis from latent TB infection. The sensitivity of TST to diagnose active tuberculosis in children and adults with HIV-1 infection is as low as 36–40% [11], and the presence of skin anergy means that false negative TST have been reported in 26–41% of HIV-1 patients who are screened for latent TB infection [12–14]. The sensitivity of the TB ELISPOT assay for the diagnosis of active TB in immune-competent patients is between 83 and 97%, with a specificity of 97–100% [15]. Our data show similar figures for HIV patients, even in those with CD4 T cell counts < 300 cells/μl, in whom the clinical and radiological features of TB are modified by HIV-associated immune deficiency. The sensitivity of the TB ELISPOT assay for the diagnosis of active TB in HIV-1 patients was 90% in a study in Zambia; however, there were no data available on immune status of these patients [16]. Dheda and colleagues showed that PHA-induced IFN-γ secretion was not influenced by low CD4 count in 29 patients with HIV-1 infection; however, they did not assess TB antigen-specific immune responses [17]. The TB ELISPOT and whole blood TB ELISA IFN-γ release assays (IGRA) gave comparable results in 74 HIV-1 infected patients when used to screen for latent TB infection in a high-prevalence country [18]. The median CD4 T cell count for HIV-1 infected patients was 392 cells/μl and only 16 patients had a CD4 T cell count < 200 cells/μl, so it was difficult to ascertain the influence of advanced HIV-1-associated immune suppression on the performance of these tests. Two studies have shown that whole blood TB ELISA worked well in HIV-1 patients who had CD4 T cell counts > 300 cells/μl in low-prevalence TB regions [19,20]; however, a high rate of assay failure due to low PHA responses in HIV patients with CD4 T cell counts < 100 cells/μl was noted. Similar findings have also been noted by other groups assessing the clinical utility of whole blood TB ELISA in other immune-deficient patients [21–23]. The whole blood TB ELISA test was positive in only two of 63 HIV-1 infected patients with a CD4 T cell count < 200 cells/μl in a micronutrient trial that was conducted in a TB endemic area [24], which raises concerns about the performance of this particular test in immune-deficient patients.

A combination of TB ELISPOT IFN-γ spot frequency and CD4 and CD8 T cell counts may have the potential to distinguish between active and latent TB infection in HIV-1-infected patients. This agrees with a recent report from Wilkinson and colleagues, who found that the sum of

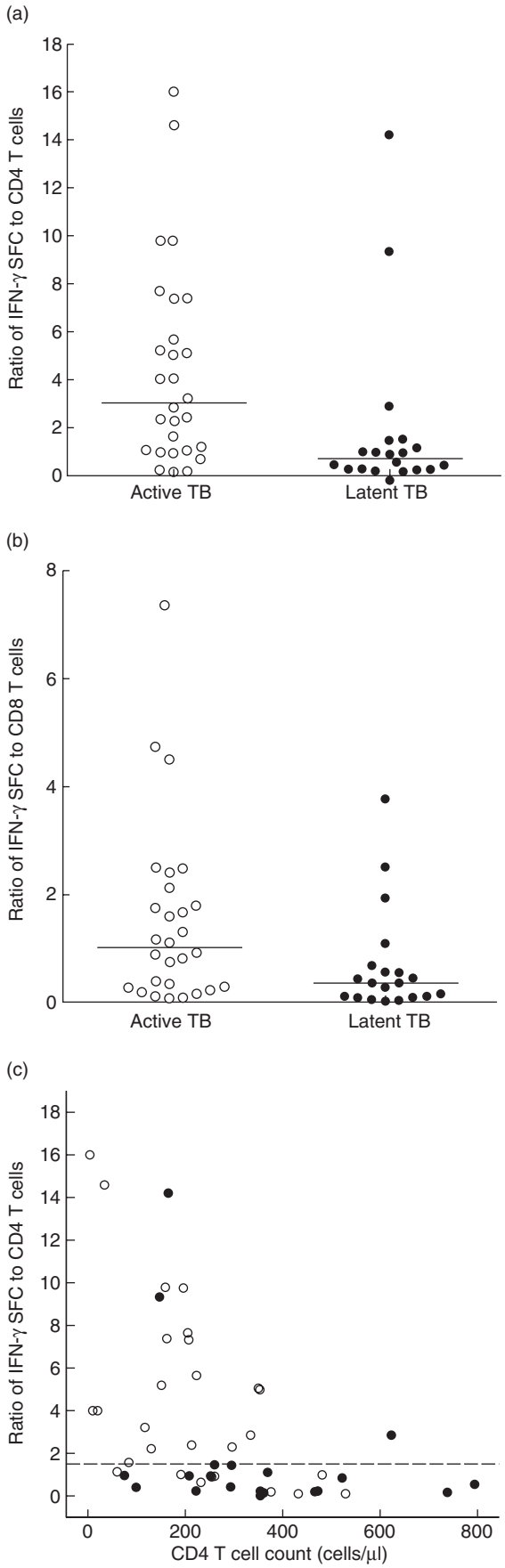

Fig. 3. The ratio of the combined number of early secretory antigen target-6 (ESAT-6) and culture filtrate protein-10 (CFP-10) interferon (IFN)-γ spot-forming cells (SFC) per 10^6 peripheral blood mononuclear cells (PBMC) to CD4 and CD8 T cell count has the potential to distinguish active tuberculosis (TB) from latent TB infection in this patient cohort. (a) Comparison of the combined number of ESAT-6 and CFP-10 IFN-γ SFC to CD4 T cell count ratio in patients with active TB (median ratio 3·032) and latent TB infection (median ratio 0·694) was found to be statistically significant ($P = 0{\cdot}003$). Horizontal lines indicate median values. While the ratio of the number ESAT-6 IFN-γ spots to CD4 T cell counts was significantly higher in patients with active TB compared to latent TB (median ratio 1·189 *versus* 0·177, $P = 0{\cdot}018$), no significant difference was observed for CFP-10 (median ratio 1·095 *versus* 0·319, $P = 0{\cdot}084$). (b) Comparison of the combined number of ESAT-6 and CFP-10 IFN-γ SFC to CD8 T cell count ratio in patients with active TB (median ratio 1·015) and latent TB infection (median ratio 0·369) was found to be statistically significant ($P = 0{\cdot}021$). Horizontal lines indicate median values. No significant difference was observed in patients with active TB compared to latent TB in the number of ESAT-6 or CFP-10 IFN-γ spots to CD8 T cell count ratio. (c) Scatter plot of the combined number of ESAT-6 and CFP-10 IFN-γ SFC to CD4 T cell count ratio plotted against CD4 T cell count. Open circles represent patients with active/probable TB infection, closed circles represent patients with latent TB infection. The dotted line represents a ratio of 1·5.

ESAT-6 and CFP-10 spots divided by CD4 T cell count could distinguish between HIV-1 patients with pulmonary tuberculosis and newly diagnosed HIV-1 patients in South Africa [25]. Further studies are needed to confirm this finding in other HIV-1 cohorts and patient groups at risk of active tuberculosis. In contrast to the African patients studied, 16% of our patients were sputum smear-negative and 60% had extrapulmonary disease, which suggests that this approach may also work in patients whose TB disease is modified by HIV-1-associated immune deficiency. Use of the ELISPOT assay and CD4 T cell count in combination could accelerate the diagnosis of TB infection in routine clinical practice [26], and allow the commencement of anti-TB therapy before the results of TB cultures are available in patients who are believed to have symptoms/radiology consistent with this disease [27]. We acknowledge that our sample size is small and that larger prospective studies are needed to confirm these preliminary findings. Other approaches to distinguish between active disease and latent infection include the identification of purified protein derivative (PPD) [28–30] and TB [31,32] antigen-specific immune responses in BAL or pleural fluid, even when no TB-specific immune responses can be found in blood, and the use of novel TB antigens which are specific for either active disease or latent infection.

Agreement between the results of TST and IGRA range between 53% and 94% in studies of immunocompetent individuals screened for latent TB infection or studies in contact tracing [19,21,23,33]. In this study, discordance

between the results of TST and TB ELISPOT occurred in 11% of patients tested, which agrees with previous reports [34,35]. A history of previous bacille Calmette–Guérin (BCG) vaccination, exposure to environmental mycobacteria, recent exposure to TB or previous TB treatment may account for some TST^+ $IGRA^-$ test results. Reduced sensitivity of IGRA compared with TST has been described [36], and may be a feature of individuals with a history of remote TB exposure [33,37], as was observed in three of four patients in this study. Positive TST responses may persist, whereas TB ELISPOT assays may revert from positive to negative either spontaneously [38–40] or, in some cases, after TB therapy [41–45]. Reversion of IGRA test results may also be influenced by precision of assays used and definition of positive test results [20,35]. Conditions associated with reduced TST sensitivity for TB infection (active TB disease and a CD4 T cell count < 100 cells/μl) may explain the positive TB ELISPOT responses and negative TST observed. The concentration and type of tuberculin preparation may influence the rate of discordant tests results and a criticism of this and other studies [18,25] is that the PPD dose employed may be lower than recommended for clinical diagnosis of TB [9], resulting in potential bias for false negative TST skin test results.

Further studies are required to define the CD4 threshold where the test performance of this assay declines, in particular whether the sensitivity of this assay will decline with CD4 T cell counts < 50 cells/μl. Prospective studies are needed to define what proportion of HIV patients who adhere to TB chemotherapy or prophylaxis will have undetectable TB antigen-specific immune responses in blood at the end of treatment and whether TB immune reconstitution syndrome (IRIS) is associated with persistent ESAT-6 or CFP-10-dependent T cell IFN-γ secretion [46].

In conclusion, we find the *M. tuberculosis*-specific TB ELISPOT test can detect TB antigen-specific immune responses in HIV-1 patients with low CD4 T cell counts. This assay is a useful test to screen for TB infection even in HIV-1 patients with low CD4 T cell counts, and can be used in routine clinical practice. A combination of TB ELISPOT spot number and CD4 T cell count may have the potential to distinguish between active and latent TB infection.

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