ORIGINAL ARTICLE

BCG-specific IgG-secreting peripheral plasmablasts as a potential biomarker of active tuberculosis in HIV negative and HIV positive patients

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ABSTRACT

Background Diagnosis of active tuberculosis (TB) among sputum-negative cases, patients with HIV infection and extra-pulmonary TB is difficult. In this study, assessment of BCG-specific IgG-secreting peripheral plasmablasts, was used to identify active TB in these high-risk groups.

Methods Peripheral blood mononuclear cells were isolated from patients with TB and controls and cultured in vitro using an assay called Antibodies in Lymphocyte Supernatant, which measures spontaneous IgG antibody release from migratory plasmablasts. A BCG-specific ELISA and flow cytometry were used to quantify in vivo activated plasmablasts in blood samples from Ethiopian subjects who were HIV negative or HIV positive. Patients diagnosed with different clinical forms of sputum-negative active TB or other diseases (n=96) were compared with asymptomatic individuals including latent TB and non-TB controls (n=85). Immunodiagnosis of TB also included the tuberculin skin test and the interferon (IFN)-γ release assay, Quantiferon.

Results This study demonstrated that circulating IgG+ plasmablasts and spontaneous secretion of BCG-specific IgG antibodies were significantly higher in patients with active TB compared with latent TB cases and non-TB controls. BCG-specific IgG titres were particularly high among patients coinfected with TB and HIV with low CD4 T-cell counts and an impaired in vitro interferon-γ response.

Conclusions These results suggest that BCG-specific IgG-secreting peripheral plasmablasts could be successfully used as a host-specific biomarker to improve diagnosis of active TB, particularly in people who are HIV positive, and facilitate administration of effective treatment to patients. Elevated IgG responses were associated with impaired peripheral T-cell responses, including reduced T-cell numbers and low M. tuberculosis-specific IFNγ production.

INTRODUCTION

Tuberculosis (TB), caused by Mycobacterium tuberculosis (Mtb), is one of the most important global health problems. Diagnosis of TB is complex as there are different clinical forms with various symptoms of infection and disease, including coinfections with other pathogens such as HIV. To date, methods used for TB diagnosis include assessment of clinical symptoms, pulmonary x-ray, direct microscopy of sputum samples, Mtb culture, cyto-histopathology, PCR and immunological techniques such as the tuberculin skin test (TST) and interferon (IFN)-γ release assays (IGRAs), that is, Quantiferon-TB Gold in-Tube (QFTG) and T-SPOT.TB. However, these methods have important limitations and are often slow, expensive and require advanced equipment or invasive procedures that are difficult to use routinely in resource-poor settings. About 50% of patients with culture-
confirmed pulmonary TB are sputum smear negative and thus microscopy is insufficient to provide accurate diagnosis. Moreover, about 20% of all patients with TB are sputum-negative and culture-negative and must be diagnosed using clinical examination and response to anti-TB treatment. Consequently, TB diagnosis is highly problematic in small children and in patients with suspected extra-pulmonary TB, HIV infection or other immunosuppressive diseases. Furthermore, none of the existing commercial methods clearly separates active TB disease from latent infection, which makes it difficult to select the appropriate chemotherapy.

Antibodies in Lymphocyte Supernatant (ALS) is a non-commercial method that has previously been developed and applied in diagnosis of active pulmonary TB. This method detects antigen-specific antibodies secreted by peripheral blood mononuclear cells (PBMCs) and has also been used to assess mucosal immune responses to oral cholera and typhoid vaccines, and in patients with enterotoxigenic Escherichia coli (ETEC) diarrhoea. In contrast to conventional serology, which involves assessment of stable serum antibodies, the ALS test is based on the spontaneous release of BCG-specific IgG antibodies from peripheral plasmablasts temporarily present in the blood. Our hypothesis is that pathogen-specific antibody secreting cells (ASCs) are only present in blood during active or subclinical disease and not during latent infection or under healthy conditions. To explore whether BCG-specific IgG antibodies secreted by peripheral plasmablasts could be used as a host-specific biomarker to detect different clinical forms of active TB disease among patients who are HIV negative or HIV positive, we assessed the activity of ASCs in blood samples from Ethiopian individuals with sputum smear negative TB.

**METHODS**

**Study subjects**

Participants were recruited at the Chest Unit, Black Lion University Hospital, Addis Ababa, Ethiopia after providing signed informed consent. The study was approved by the national ethical committees in Ethiopia and Sweden. Inclusion criteria were individuals who were HIV negative or HIV positive and sputum smear negative, over 18 years of age with clinical symptoms of suspected TB. Exclusion criteria were patients with a history of previous TB or more than 1 week of antimicrobial chemotherapy, those who used antiretroviral drugs or did not consent to HIV screening. Asymptomatic individuals with no clinical disease were recruited as controls.

Clinical diagnosis was based on typical TB symptoms (persistent cough and general illness including fever, weight and appetite loss, and sweating for >5 months, pleural effusions or chronic non-tender cervical lymphadenopathy > 6 weeks), chest x-ray and positive response to anti-TB treatment (clinical improvement and radiographical resolution of pulmonary TB lesions). Active TB disease was confirmed by a clinical diagnosis of TB and/or positive Mtb culture or cyto-histopathology of clinical specimens. Blood samples obtained from the subjects at the time of diagnosis were used for the QFTG and ALS assays, peripheral CD3/CD4 T-cell counts (FACSCount; BD Biosciences, Franklin Lakes, New Jersey, USA) and to determine HIV status. A detailed description of study subjects, clinical and immunological diagnoses, standard care and HIV testing can be found in the online supplementary material.

**Tuberculin skin test**

The TST measures the presence of delayed-type hypersensitivity in the skin upon intra-dermal injection of 0.1 ml Tuberculin Purified Protein Derivative (PPD) (STU; SSI, Copenhagen, Denmark) in the volar aspect of the forearm. The TST reaction, measured by trained research nurses at 48–72 h after tuberculin injection, was considered positive when the transverse induration was ≥10 mm (≥5 mm for subjects who were HIV positive).

**QuantIFERON-TB Gold in-Tube**

The QFTG assay measures IFNγ production by T cells after in vitro stimulation of whole blood with the Mtb-specific antigens, CFP-10, ESAT-6, TB7.7, according to the manufacturer’s instructions. QFTG values above a cut-off of 0.35 IU/ml were considered positive.

**Antibodies in lymphocyte supernatant**

The ALS assay measures spontaneous release of BCG-specific IgG antibodies from in vivo-derived plasmablasts using in vitro cultures of unstimulated PBMCs and an ELISA as described in the online supplementary material. Briefly, PBMCs were isolated from 3 to 5 ml of blood using cell preparation tubes (CPT; BD Biosciences) and cultured (2.5×10⁶ cells/ml in 48-well plates) in RPMI medium (GIBCO, Invitrogen; Carlsbad, California, USA) for 72 h. Release of IgG antibodies in the culture supernatant was measured using a BCG-specific ELISA. The ASC response is expressed as relative BCG-specific IgG titres and OD values above a cut-off of 0.425 were considered positive.

**Flow cytometry**

Randomly selected PBMC samples (5–10×10⁶ cells) from patients with active TB (n=19), those with latent TB (n=7) and non-TB controls (n=6) were frozen in 1 ml of RPMI with 20% fetal calf serum (FCS) and 10% dimethyl sulfoxide (Sigma-Aldrich, St Louis, Missouri, USA) and stored at −150°C until flow cytometric analysis. After thawing, rested PBMCs were washed with phosphate buffered saline 0.1% FCS and stained for 15 min at 4°C with the following antibodies: CD3 APC, CD20 APC Cy7, CD27 PE , CD38 APC, CD19 PE and IgG PE Cy5 (BD Biosciences). After fixation with 1% paraformaldehyde (Sigma-Aldrich) for 30 min at 4°C, PBMCs were analysed using a Gallios flow cytometer and the Kaluza software (Beckman Coulter, Brea, California, USA; online supplementary material, figure 1).

**Statistical analysis**

Non-parametric analyses were used to calculate p values and included a Kruskal–Wallis test and Dunn’s post test or a Mann–Whitney test. Spearman’s correlation test was used for the correlation analysis. Receiver operating characteristic curves were used to determine the relation between sensitivity and specificity at various cut-off levels of BCG-specific IgG titres (online supplementary material, figure 2). Selection of the best cut-off point (OD 0.425) was based on the level of maximum accuracy. Cohen’s x coefficient and McNemar’s χ² test were used to determine the diagnostic agreement between the ALS assay and clinical diagnosis. Statistical analyses were performed using GraphPad Prism-4 and SPSS V12.

**RESULTS**

**Characterisation of study subjects**

The demographics of the study subjects are outlined in table 1. Among 96 suspected TB cases, clinical and pathological-anatomical diagnoses, and Mtb culture were used to confirm active TB disease in 84 patients (table 1). Patients with active TB
Figure 1: Assessment of BCG-specific IgG antibodies secreted by peripheral plasmablasts in patients with active tuberculosis (TB) (circles) compared with those with latent TB (squares) and non-TB controls (triangles). The graphs show results from (A) the tuberculin skin test (TST) (skin induration), (B) QuantiFERON-TB Gold in-Tube (QFTG) (interferon (IFN)-γ production) and (C) the Antibodies in Lymphocyte Supernatant (ALS) (IgG titres) among the different groups of patients. The dashed lines indicate the positive cut-off level determined for each diagnostic test: PPD ≥10 (mm), IFN-γ ≥0.35 (IU/ml) and IgG titres ≥0.425 (OD). (D) The proportion of IgG+ CD3-CD19-CD20-CD27highCD38high plasmablasts among PBMC samples and the corresponding BCG-specific IgG titres (PBMC culture supernatants) among the study subjects. (E) Correlation analysis between IgG+ plasmablasts (PBMC samples) and the corresponding BCG-specific IgG titres among the study subjects. Graphs are presented as scatter dot plots and the solid bars indicate the median values for each group. The statistical significance of differences in diagnostic performance between the different patients groups was determined using the Kruskal–Wallis test (A–C) or a Mann–Whitney test (D). *p<0.05, **p<0.01 and ***p<0.001. Spearman’s correlation test was used to determine the correlation coefficient $r_s$ (E). A value of $r_s=1$ indicates a perfect positive correlation whereas $r_s=-1$ indicates a perfect negative or inverse correlation.

Figure 2: Assessment of BCG-specific IgG antibodies secreted by peripheral plasmablasts in patients who are HIV negative compared with those who are HIV positive with either active tuberculosis (TB) (circles) or latent TB (squares). Red symbols represents patients with a CD4 T-cell count <200 cells/ml. The graphs show results from (A) the tuberculin skin test (TST) (skin induration), (B) QuantiFERON-TB Gold in-Tube (QFTG) (interferon (IFN)-γ production) and (C) the Antibodies in Lymphocyte Supernatant (ALS) (IgG titres) among the different groups of patients. The dashed lines indicate the positive cut-off level determined for each diagnostic test: PPD ≥10 (mm), IFN-γ ≥0.35 (IU/ml) and IgG titres ≥0.425 (OD). All graphs are presented as scatter dot plots and the solid bars indicate the median values for each group. The statistical significance of differences in diagnostic performance between the different patients groups was determined using the Kruskal–Wallis test (A and B). *p<0.05, **p<0.01 and ***p<0.001.

Tuberculosis

Table 1  Clinical demographics of included study subjects

<table>
<thead>
<tr>
<th>Clinical features, n (%)</th>
<th>Symptomatic TB subjects</th>
<th>Asymptomatic individuals</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total active TB (n=84)</td>
<td>Pulmonary TB (n=35)</td>
</tr>
<tr>
<td>Median age in years (range)</td>
<td>27 (18–72)</td>
<td>27 (18–54)</td>
</tr>
<tr>
<td>Men/women</td>
<td>45/39</td>
<td>20/15</td>
</tr>
<tr>
<td>Abnormal chest x-ray†‡</td>
<td>58 (69%)</td>
<td>35 (100%)</td>
</tr>
<tr>
<td>Positive Mtb culture‡</td>
<td>24 (29%)</td>
<td>24 (69%)</td>
</tr>
<tr>
<td>Positive Mtb</td>
<td>49 (58%)</td>
<td>ND</td>
</tr>
<tr>
<td>Positive cyto-histopathology</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive TST ≥10 mm</td>
<td>75 (89%)</td>
<td>30 (86%)</td>
</tr>
<tr>
<td>Positive QFTG (cut-off 0.35)§</td>
<td>66 (79%)</td>
<td>25 (71%)</td>
</tr>
<tr>
<td>HIV infection</td>
<td>27 (32%)</td>
<td>12 (34%)</td>
</tr>
<tr>
<td>Parasite infections¶</td>
<td>9 (11%)</td>
<td>4 (11%)</td>
</tr>
<tr>
<td>BCG vaccination</td>
<td>17 (20%)</td>
<td>10 (29%)</td>
</tr>
<tr>
<td>Disease outcome (cured)**</td>
<td>62 (74%)</td>
<td>24 (69%)</td>
</tr>
</tbody>
</table>

* Diseases other than TB included patients with confirmed pneumonia (n=3), malignancies (n=4) or non-specific reactive lymphadenitis (n=5).
† Abnormal radiological chest x-ray findings included pulmonary infiltrates, pleural effusions and dense lesions.
‡ Mtb culture was performed on bronchoalveolar lavage obtained from patients with pulmonary TB. Mtb culture-negative patients (n=11) all had clinical symptoms of TB, abnormal chest x-ray findings and responded to standard anti-TB treatment.
§ Indeterminant QFTG responses were found in: pulmonary TB (n=2), pleural TB (n=2), and lymph node TB (n=1). All indeterminant results were from patients with TB/HIV coinfection.
¶ Parasite infections at the time of diagnosis included strongyloides stercoralis, ascaris, trichuris trichiura, amoeba histolitica. n=6/9 patients with TB with parasite infections were HIV positive.
** Disease outcome evaluated 8 months after the start of standard anti-TB therapy included response to treatment as determined by clinical recovery and resolution of lesions evident on the chest x-ray. A few patients (n=11) did not fully recover, and some were lost to follow-up (n=11).

ALs, Antibodies in Lymphocyte Supernatant; Mtb, Mycobacterium tuberculosis; ND, not determined; QFTG, QuantiFERON-TB Gold in-Tube; TB, tuberculosis; TST, tuberculin skin test.

(median age 27 years, men/women: 45/39) were further divided into three groups based on different clinical forms of the disease: pulmonary TB (n=35) or extra-pulmonary pleural TB (n=23) and lymph node TB without pulmonary involvement (n=26) (table 1). According to histopathology and response to conventional antibiotic treatment, diseases other than TB were diagnosed in 12 symptomatic patients (table 1). Three of these patients had latent TB as suggested by a positive TST and QFTG test (table 1). Among 85 asymptomatic individuals (median age: 28.5 years, men/women: 42/43), 45 cases with positive TST and QFTG tests were grouped as latent TB, while 40 individuals who were negative for both these tests were grouped as non-TB controls (table 1). On average, one-third of the study subjects were infected with HIV (table 1).

Elevated levels of circulating BCG-specific IgG-secreting plasmablasts were detected in patients with active TB disease

The majority of patients with active TB had a positive TST (89%) and/or a positive QFTG (79%) test result and consequently these tests could not discriminate active from latent TB (table 1 and figure 1A,B). In contrast, the ALS test revealed that BCG-specific IgG secretion from circulating plasmablasts could be detected in most patients with active TB (91%) but only in a few latent TB cases (16%) (figure 1C). Importantly, median IgG titres were significantly higher in active TB compared with other diseases (p<0.01; data not shown) or latent TB and non-TB controls (p<0.001), which suggests that BCG-specific ASC are only present in the circulation of patients with active TB disease (figure 1C). In line with this finding, multicolour flow cytometric analyses of PBMC samples demonstrated that the proportion of circulating CD3-CD19+CD20-CD27highCD38high plasmablasts expressing cell-surface IgG16 was significantly (p<0.01) higher among PBMCs from patients with active TB (n=19) compared with asymptomatic individuals (n=13), which included those with latent TB and non-TB controls (figure 1D). Importantly, there was a significant correlation (r=0.428, p=0.01) between IgG+ plasmablasts in PBMC samples and BCG-specific IgG antibodies secreted by the PBMCs as determined by the ALS assay (figure 1E).

High BCG-specific IgG titres were associated with low Mtb-specific IFNγ levels in patients with HIV infection and active TB disease

Among subjects who were HIV negative or HIV positive with either active or latent TB, CD4 T-cell counts <200 cells/ml (figure 2, red symbols) were primarily found in those with HIV and active TB (81%). As expected, Mtb-specific IFNγ production in vitro was low in blood samples from patients with TB/HIV coinfection, while IFNγ levels were significantly (p<0.001) higher in samples from subjects who were HIV negative and had latent TB (figure 2B). In contrast, BCG-specific IgG titres were significantly (p<0.001) higher in the TB/HIV coinfected group compared with individuals with who were HIV negative or HIV positive with latent TB (figure 2C). Consequently, the sensitivity (80–100%) and specificity (78–94%) of the ALS test to detect active TB was generally high among patients who were HIV negative or HIV positive, particularly among those with HIV and pulmonary or lymph node TB (table 2). The positive predictive values of the ALS assay were high in most TB groups, but relatively lower among patients who were HIV positive with pleural TB (table 2). The specificity and positive predictive values were also relatively lower comparing active TB with latent TB cases than comparing active TB with non-TB controls (table 2). The negative predictive values were consistently high in all groups (table 2).

Furthermore, we found that the overall agreement between the ALS assay and clinical TB diagnosis (confirmed and clinical
TB cases) was very good (κ>0.742; p>0.625) among all active TB patients, with or without HIV infection (table 3).

**Elevated levels of BCG-specific IgG-secreting plasmablasts correlated with reduced T-cell counts and progression of disease**

Total T-cell counts determined in peripheral blood of the study subjects revealed that the levels of CD3 T cells (figure 3A), and CD4 and CD8 T cells (data not shown), were clearly lower in patients with active TB compared with the other groups, particularly when compared with latent TB cases (p<0.001). We also observed a significant inverse correlation (r=-0.311, p=0.004) between CD3 T-cell counts and BCG-specific IgG titres among patients with active TB (figure 3B) but not among asymptomatic individuals (data not shown). CD4 T-cell numbers were also significantly (p<0.05) lower in active TB patients who were HIV negative or HIV positive compared with individuals with latent TB (figure 3C). Accordingly, there was a significant correlation (r=0.303, p=0.042) between CD4 T-cell counts and Mtb-specific IFNγ production among individuals with latent TB (figure 3D) but not among patients with active TB disease (data not shown).

Comparing different clinical forms of active TB, peripheral CD3 T-cell counts were significantly (p<0.05) higher in patients with local lymph node TB compared with pleural TB (figure 3E). Likewise, Mtb-specific IFNγ production in vitro was significantly higher in patients with TB lymphadenitis compared with those with pulmonary or pleural TB (p<0.001 and p<0.05, respectively) (figure 3F). There was no difference in BCG-specific IgG titres between these groups (data not shown). Patients with TB lymphadenitis also showed a significant correlation (r=0.320, p=0.007) between CD4 T-cell counts and Mtb-specific IFNγ production (figure 3G), but a significant negative correlation (r=-0.429, p=0.03) between CD4 T cells and BCG-specific IgG titres (figure 3H). Of note, HIV-infected patients with TB lymphadenitis (red symbols) who had low CD4 T-cell counts expressed relatively lower levels of IFNγ but higher IgG levels compared with HIV negative patients with TB lymphadenitis (open symbols) who had higher CD4 T-cell counts (figure 3G–H).

**DISCUSSION**

Improved diagnosis and treatment of clinical TB in high-risk groups would have a great impact on preventing the global spread of disease. Here, we explored TB immunodiagnosis using the ALS test, based on assessment of BCG-specific IgG-secreting plasmablasts in peripheral blood samples from patients with sputum-negative TB and asymptomatic individuals. This study provides evidence that circulating IgG+ plasmablasts and spontaneous secretion of BCG-specific IgG antibodies were significantly higher in patients with active TB compared with individuals with latent TB and non-TB controls. BCG-specific IgG titres were particularly high among patients with TB/HIV coinfection and CD4 T-cell counts <200 cells/ml who produced low levels of Mtb-specific IFNγ in vitro. Hence, elevated ASC responses were generally associated with impaired peripheral T-cell responses, including reduced T-cell numbers and low Mtb-specific IFNγ production. These results suggest that detection of BCG-specific IgG-secreting plasmablasts could be successfully used as a diagnostic biomarker to detect different clinical forms of sputum-negative TB and distinguish active TB from latent TB infection in patients who are HIV negative, and particularly, in those with TB/HIV coinfection.

In the steady state, the proportion of migratory and IgG-secreting plasmablasts among PBMCs is very low, but

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**Table 2** Diagnostic performance of the ALS test*

<table>
<thead>
<tr>
<th>Groups</th>
<th>Sensitivity (Latent TB cases n=45)</th>
<th>Specificity (Latent TB cases n=45)</th>
<th>PPV (Latent TB cases n=45)</th>
<th>NPV (Latent TB cases n=45)</th>
<th>Sensitivity (Non-TB controls n=40)</th>
<th>Specificity (Non-TB controls n=40)</th>
<th>PPV (Non-TB controls n=40)</th>
<th>NPV (Non-TB controls n=40)</th>
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<tbody>
<tr>
<td>HIV negative</td>
<td>86 (90.7%)</td>
<td>80 (97%)</td>
<td>89 (86)</td>
<td>86 (86)</td>
<td>86 (90)</td>
<td>86 (96)</td>
<td>94 (94)</td>
<td>74 (98)</td>
</tr>
<tr>
<td>HIV positive</td>
<td>90 (87.2%)</td>
<td>90 (80)</td>
<td>94 (73)</td>
<td>91 (94)</td>
<td>90 (80)</td>
<td>90 (94)</td>
<td>94 (94)</td>
<td>94 (94)</td>
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<tr>
<td>Pulmonary TB</td>
<td>80 (80)</td>
<td>80 (79)</td>
<td>88 (80)</td>
<td>80 (80)</td>
<td>75 (87)</td>
<td>77 (89)</td>
<td>94 (94)</td>
<td>74 (94)</td>
</tr>
<tr>
<td>HIV negative</td>
<td>80 (80)</td>
<td>80 (75)</td>
<td>89 (87)</td>
<td>90 (94)</td>
<td>87 (87)</td>
<td>87 (94)</td>
<td>94 (94)</td>
<td>94 (94)</td>
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<tr>
<td>HIV positive</td>
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<td>80 (67)</td>
<td>89 (80)</td>
<td>80 (80)</td>
<td>67 (89)</td>
<td>67 (89)</td>
<td>94 (94)</td>
<td>100 (94)</td>
</tr>
<tr>
<td>Pleural TB</td>
<td>80 (80)</td>
<td>80 (77)</td>
<td>89 (87)</td>
<td>90 (94)</td>
<td>77 (84)</td>
<td>77 (84)</td>
<td>94 (94)</td>
<td>100 (94)</td>
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<tr>
<td>HIV negative</td>
<td>86 (86)</td>
<td>80 (78)</td>
<td>88 (80)</td>
<td>88 (80)</td>
<td>80 (80)</td>
<td>80 (80)</td>
<td>94 (94)</td>
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<tr>
<td>HIV positive</td>
<td>100 (100)</td>
<td>100 (80)</td>
<td>100 (100)</td>
<td>100 (100)</td>
<td>100 (100)</td>
<td>100 (100)</td>
<td>100 (100)</td>
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</table>

*Patients with active TB were compared with latent TB cases or non-TB controls.
ALS, Antibodies in Lymphocyte Supernatant; NPV, negative predictive value; PPV, positive predictive value; TB, tuberculosis.

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**Table 3** Concordance and agreement between the ALS test and clinical diagnosis among patients with active TB

<table>
<thead>
<tr>
<th>Groups</th>
<th>Concordance</th>
<th>Agreement, Cohen’s κ (SE)</th>
<th>p Value, McNemar</th>
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<tr>
<td>All TB cases</td>
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<tr>
<td>HIV negative</td>
<td>68/75 (90.7%)</td>
<td>0.766 (0.083)</td>
<td>0.725</td>
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<tr>
<td>HIV positive</td>
<td>41/47 (87.2%)</td>
<td>0.742 (0.098)</td>
<td>0.687</td>
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<tr>
<td>Pulmonary TB</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>HIV negative</td>
<td>36/41 (87.8%)</td>
<td>0.804 (0.093)</td>
<td>0.625</td>
</tr>
<tr>
<td>HIV positive</td>
<td>29/34 (85.3%)</td>
<td>0.861 (0.095)</td>
<td>1.00</td>
</tr>
<tr>
<td>Pleural TB</td>
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<tr>
<td>HIV negative</td>
<td>32/34 (94.1%)</td>
<td>0.882 (0.081)</td>
<td>1.00</td>
</tr>
<tr>
<td>HIV positive</td>
<td>22/27 (81.5%)</td>
<td>0.744 (0.171)</td>
<td>1.00</td>
</tr>
<tr>
<td>Lymph node TB</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>HIV negative</td>
<td>34/36 (94.4%)</td>
<td>0.889 (0.067)</td>
<td>1.00</td>
</tr>
<tr>
<td>HIV positive</td>
<td>26/30 (86.7%)</td>
<td>0.913 (0.085)</td>
<td>1.00</td>
</tr>
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</table>

ALS, Antibodies in Lymphocyte Supernatant; TB, tuberculosis.
**Figure 3** Assessment and comparison of peripheral blood T-cell counts, BCG-specific IgG titres and *Mycobacterium tuberculosis* (Mt)-specific interferon (IFN)-γ production in patients with different clinical forms of tuberculosis (TB). Red symbols represent patients with HIV infection. (A) Total peripheral CD3 T-cell counts were determined in cases with active TB (circles), latent TB (squares), and non-TB controls (triangles). (B) Correlation analysis of peripheral blood CD3 T-cell counts and BCG-specific IgG titres among patients with active TB (circles). (C) Peripheral CD4 T-cell counts in patients who were HIV negative or HIV positive with active TB (circles) or latent TB (squares). (D) Correlation analysis of peripheral blood CD4 T-cell counts and Mt-specific IFN-γ production in vitro among patients with latent TB (squares). (E) Total peripheral CD3 T-cell counts and (F) Mt-specific IFN-γ production in vitro in patients with pulmonary TB (PTB), pleural TB or lymph node TB (LNTB) are shown. Correlation analysis of peripheral blood CD4 T-cell counts and (G) Mt-specific IFN-γ production in vitro or (H) BCG-specific IgG titres among patients who were HIV negative (open symbols) or HIV positive (red symbols) with TB significantly elevated upon the continuous antigen exposure resulting after systemic vaccination. Consequently, ASCs should be temporarily present in the peripheral circulation only in patients with active TB disease. By contrast, effector memory T cells persist in the blood of individuals with active or latent TB, and consequently, neither the TST nor the IFNγ release assay can discriminate active from latent TB infection. It is possible that the few latent TB cases with elevated BCG-specific IgG titres may indicate subclinical TB infection associated with an increased risk of developing active TB. It was recently demonstrated that the speed, sensitivity and specificity of the ALS assay can be enhanced by using higher numbers of PBMCs in a concentrated 96-well format (micro-ALS method). This set-up may increase the possibility of detecting borderline cases, with BCG-specific IgG titres close to the cut-off level.

Previous studies have demonstrated that patients with respiratory diseases other than TB possessed significantly lower BCG-specific IgG titres compared with patients with TB. Future studies should also systematically evaluate the ALS test in patients with pulmonary TB compared with patients with respiratory diseases such as bacterial pneumonias, pulmonary malignancies, aspergillosis, bronchiectasis or pleural empyemias, which represents clinical conditions commonly encountered in developing countries that may pose a serious problem to the differential diagnosis. Recently, it was also shown that the BCG vaccine is a superior antigen for detection of ASC in PBMC samples from patients with TB in comparison with a panel of Mt-specific antigens, including LAM, ESAT-6, CFP-10, TB15.3 and TB51A. However, further development of the ALS assay using cocktails of Mt-specific peptide pools may significantly improve the specificity of this test.

To date, most commercial and novel immunological and microbiological assays fail to significantly improve diagnosis of active TB in high-risk groups. Promising results have been obtained using enzyme-linked immunospot or microscopic-observation drug-susceptibility assays. However, these methods require invasive patient sampling techniques or advanced microscopy and time-consuming work with hazardous mycobacterial cultures, and fail to demonstrate efficient diagnostic results in people with TB/HIV coinfection and those who are sputum smear negative and sputum-positive patients groups and, in particular, among those who are HIV positive. High levels of total and Mt-specific serum antibodies have previously been shown in patients with severe forms of TB disease, but the majority of studies reveal highly variable results and suboptimal sensitivity of serology in sputum-negative and sputum-positive patient groups and, in particular, among those who are HIV positive. High levels of total and Mt-specific serum antibodies have previously been shown in patients with severe forms of TB disease, but the majority of studies reveal highly variable results and suboptimal sensitivity of serology in sputum-negative and sputum-positive patient groups and, in particular, among those who are HIV positive.

**Statistical analyses included the Kruskal-Wallis and Spearman’s correlation tests. A value of r=1 for the correlation coefficient r indicates a perfect positive correlation whereas r=−1 indicates a perfect negative or inverse correlation. *p<0.05, **p<0.01 and ***p<0.001.**
Here we provide evidence that the ALS test could be useful to detect active TB among patients who are sputum-negative and those who are immunosuppressed with low CD4 T-cell counts and low QFTG responses. The TST and the QFTG tests depend on the absolute numbers of circulating CD4 T cells, and consequently, demonstrate a significantly reduced sensitivity for the diagnosis of active TB in immunodeficient individuals who often present anergic antigen-specific T-cell responses. Instead, increased CD4 T-cell counts correlated with elevated Mtb-specific IFNγ responses in latent TB (ie, control of TB disease) and in HIV negative patients with local lymph node TB (ie, mild TB disease), while TB/HIV coinfection (ie, advanced TB disease) was associated with reduced CD4 T-cell counts and enhanced secretion of BCG-specific IgG antibodies from peripheral plasmablasts. Importantly, it has been shown that BCG-specific IgG titers gradually decrease after 2 and 6 months of successful anti-TB therapy, but remain high in patients infected with drug-resistant TB, also indicating that high levels of ASCs are maintained during progressive TB.

Interestingly, HIV infection could give rise to different types of B-cell abnormalities, including selective loss of antigen-specific memory B cells but also hypergammaglobulinemia and the production of polyclonal antibodies by activated naïve B cells. Hypergammaglobulinemia has also been observed in patients who are HIV negative and in HIV positive patients with TB or Mycobacterium leprae infection. It is likely that continuous exposure of viral and bacterial antigens in chronic infections with mycobacteria and/or HIV may enhance B-cell activation and antibody-mediated immunity, particularly in patients with impaired T-cell responses. Similarly, in patients with worm infection, reduced CD4 T-cell numbers in blood correlated with enhanced levels of worm-specific IgG antibodies and severity of disease, especially when the release of antigens from pathological lesions was augmented.

Together, our findings suggest that assessment of BCG-specific IgG antibodies secreted by plasmablasts in the peripheral circulation could be exploited as an efficient biomarker to improve diagnosis of sputum smear-negative active TB among patients who are HIV negative or HIV positive. Development and clinical implementation of a rapid and simple point-of-care immunodiagnostic test such as the ALS assay could be very useful for TB control programmes in developing and industrialized countries.

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Competing interests None.

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REFERENCES


SUPPLEMENTARY MATERIAL

METHODS

Study subjects, diagnosis and treatment

The study subjects were screened and recruited from the outpatient clinic and the Chest Unit at the Department of Internal Medicine, Black Lion University Hospital in Addis Ababa, Ethiopia. The responsible clinician informed the subjects about the objectives of the study, where after written consent was obtained and documented. Individuals who agreed to the informed consent were included in the study. The time for enrolment of the study subjects (n=181) was 12 months.

All symptomatic TB suspects (n=96) were sputum-smear negative as determined by acid-fast staining for mycobacteria in induced sputum samples. A confirmed TB diagnosis (n=84) was based on clinical examination, Mtb culture (pulmonary TB cases), histopathology (pleural and lymph node TB cases) or cytology (pleural TB cases), and chest X-ray data as well as response to standard anti-TB therapy. Chest X-ray was used to identify lesions consistent with TB and to distinguish patients with pulmonary infiltrates (n=35) from those with pleural effusions (n=23), i.e. inflammation in the pleural cavity. Patients with local lymph node TB (n=26), characterized by enlarged cervical lymph nodes for more than 1 month, had a normal X-ray.

Patients with confirmed active TB (n=84) received standard anti-TB chemotherapy for 6 months including rifampicin, isoniazid, pyrazinamide and ethambutol and response to treatment was used as retrospective diagnostic criteria for pulmonary TB patients with clinical TB (n=11). Clinical follow-up evaluations were performed at 2, 5, and 8 months and included
clinical and radiologic findings (clinical improvement defined by disappearance of TB symptoms and radiologic resolution of pulmonary TB lesions).

The diagnoses of symptomatic patients with other diseases than TB (n=12) were based on histopathology or response to conventional antibiotic treatment and were confirmed to include pneumonia, malignancies or non-specific reactive lymphadenitis. Patients with pneumonia or non-specific lymphadenitis responded to conventional antibiotic treatment while the cancer patients were remitted to the Oncology Unit at the Black Lion University Hospital, Addis Ababa, Ethiopia.

Latent TB infection (n=45) in HIV-negative or HIV-positive asymptomatic individuals included subjects with a clear chest X-ray as well as a positive TST and QFTG test. Non-TB controls (n=40) included HIV-negative or HIV-positive asymptomatic individuals with a clear chest X-ray as well as a negative TST and QFTG test. Healthy individuals with discordant TST and QFTG test results (n=24) were excluded from this study.

**Mtb culture and cyto-histopathology**

Mtb culture of bronchoalveolar lavage (BAL) fluid was performed at the Armauer Hansen Research Institute, Addis Ababa, Ethiopia, using the established Løwenstein-Jensen (LJ) methodology. After NaOH (4%) treatment, washing and centrifugation of BAL fluids, cell pellets were resuspended in 1,5 ml of 7H9 media from which an aliquot (20-30 µl) were inoculated in LJ-media and incubated at 37°C for 8 weeks with weekly read-out of bacterial growth. Cytology of cell samples from pleural fluid and histology of paraffin-embedded pleural or lymph node tissue involved hematoxylin and eosin staining performed at the Department of Pathology, Black Lion University Hospital, Addis Ababa, Ethiopia.
positive specimens revealed a granulomatous reaction with multinucleated giant cells, epithelioid cells, numerous lymphocytes and necrotic material consistent with TB.

**Clinical samples and HIV screening**

On enrollment, blood samples were obtained from the study subjects for immunodiagnosis using the QFTG and the ALS tests, but also to determine HIV status and peripheral CD3/CD4 T cell counts. After blood sampling, all study subjects were tested for TST reactivity. HIV screening was performed at the Black Lion University Hospital using a national algorithm in three steps: 1) HIV (1+2) Antibody Kit (KHB Shanghai Kehua Bio-engineering; Shanghai, China); 2) HIV 1/2 STAT-PAK Kit (CHEMBIO Diagnostic systems; Medford, NY); 3) Uni-Gold HIV test (Trinity Biotech; Co Wicklow, Ireland). Pre-test counseling was offered to all subjects whereas post-test counseling was provided to HIV-positive subjects by a trained counselor.

**QuantiFERON-TB Gold in-Tube (QFTG)**

For the QFTG assay, 3 ml blood was collected and divided into three aliquots: one test tube coated with Mtb-antigens (CFP-10, ESAT-6, TB7.7), and one negative (Nil) and one positive control tube (Mitogen). The tubes were incubated at 37°C for 16–24 hours before centrifugation and collection of plasma samples. Results of the QFTG ELISA read at 450 nm were determined as positive, negative, or indeterminate using on the QuantiFERON-TB Gold Analysis software.

**Antibodies in Lymphocyte Supernatant (ALS)**

Spontaneous release of BCG-specific IgG antibodies was assessed in PBMC cultures in vitro. PBMCs were isolated from 3–5 ml blood using cell preparation tubes (CPT) pre-loaded with
Ficoll-Hypaque and cultured \((2.5\times10^6\ \text{cells/ml in 48-well plates})\) in RPMI medium supplemented with 10\%FCS, streptomycin, and glutamine (HyClone, Waltham, MA). The supernatant was collected after 72 hours and stored with protease inhibitor cocktail solution (0.2 \(\mu\text{M AEBSF hydrochloride, Calbiochem, Gibbstown, NJ; 1 \(\mu\text{g/ml aprotinin and 10 \(\mu\text{M leupeptin, USB, Lake Placid, NY; 1 mg/ml sodium azide, Sigma, St. Louis, MO})\) at }-70^\circ\text{C. An IgG ELISA was performed in Maxisorb plates (Nunc, Roskilde, Denmark) coated with 1 \(\mu\text{g/well BCG vaccine (Japan BCG Laboratories, Tokyo, Japan) overnight at }+4^\circ\text{C. After blocking with PBS-10\%FCS for 60 min at }37^\circ\text{C, culture supernatants (100 \(\mu\text{l/well}), positive control samples (pooled serum from }n=5\ \text{sputum smear-positive TB patients) and negative control samples (normal rabbit serum and cell culture medium only) were incubated for 2 hours at }37^\circ\text{C before an additional 2 hour incubation with rabbit anti-human IgG-HRP (Jackson Immunoresearch Laboratories, West grove, PA). The plates were extensively washed with PBS-0.05\%Tween-20 (Sigma) between each incubation step. The ELISA was developed at 492 \text{ nm using O-phenylenediamine (OPD) substrate solution (Sigma). Results are presented as BCG-specific IgG titers (OD).}

Receiver-operator characteristic (ROC) curves were used to determine the relation between sensitivity and specificity at varying cut-off levels of BCG-specific IgG titers. The cut-off point for a positive ALS response was determined by constructing a ROC curve from the IgG titers by comparing active TB patients \((n=84)\) with healthy controls \((n=18)\). Selection of the best cut-off point was based on the level at which the accuracy was maximum. The best cut-off point was found to be 0.425, with a sensitivity of 90\% and a specificity of 88\% for the ALS assay (Supplementary material, figure 2).
FIGURES

Figure 1. Gating strategy used in the flow cytometric analyses to detect peripheral plasmablasts. Plasmablasts were identified with a panel including CD3 (UCHT1), CD19 (HIB19), CD20 (L-27), CD27 (L128), CD38 (HIT2) and IgG (G18-145). A representative sample from one patient with active TB is shown.

Figure 2. A receiver-operator characteristic curve was constructed from ASC responses to the BCG vaccine in 84 patients with active TB and in 18 healthy control subjects. The arrow indicate the selected cut-off level used in this study.