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. Mtb-
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 (2.5x10^6 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 µM AEBSF hydrochloride, Calbiochem, Gibbstown, NJ; 1 µg/ml aprotinin and 10 µM leupeptin, USB, Lake Placid, NY; 1 mg/ml sodium azide, Sigma, St. Louis, MO) at -70°C. An IgG ELISA was performed in Maxisorb plates (Nunc, Roskilde, Denmark) coated with 1 µg/well BCG vaccine (Japan BCG Laboratories, Tokyo, Japan) overnight at +4°C. After blocking with PBS-10%FCS for 60 min at 37°C, culture supernatants (100 µl/well), positive control samples (pooled serum from n=5 sputum smear-positive TB patients) and negative control samples (normal rabbit serum and cell culture medium only) were incubated for 2 hours at 37°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 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.