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Rapid diagnosis of pulmonary tuberculosis by combined molecular and immunological methods

Authors: Claudia Jafari\textsuperscript{1*}, Ioana D. Olaru\textsuperscript{1,2*}, Franziska Daduna\textsuperscript{3}, Martin Ernst\textsuperscript{3}, Jan Heyckendorf\textsuperscript{1,4,5}, Christoph Lange\textsuperscript{1,4,6}, Barbara Kalsdorf\textsuperscript{1,4,5}  
\*shared authorship

Affiliations:
1 Division of Clinical Infectious Diseases, Research Center Borstel, Borstel, Germany  
2 Biomedical Research and Training Institute, Harare, Zimbabwe  
3 Division of Immune Cell-Analytics, Research Center Borstel, Borstel, Germany  
4 International Health / Infectious Diseases, University of Lübeck, Lübeck, Germany  
5 German Center for Infection Research (DZIF), Partner site Hamburg-Lübeck-Borstel, Germany  
6 Department of Medicine, Karolinska Institutet, Stockholm, Sweden

Corresponding author:  
Prof. Dr. Christoph Lange, Division of Clinical Infectious Diseases, Research Center Borstel, Parkallee 35, D-23845 Borstel, Germany. Phone: +49-4537188-3320, email: clange@fz-borstel.de

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Abstract

Diagnosing pulmonary tuberculosis (TB) may be delayed until culture results become available. We ascertained the accuracy of a stepwise diagnostic algorithm for the rapid diagnosis of pulmonary TB by GeneXpert from sputum and/or bronchoalveolar-lavage (BAL) followed by a M. tuberculosis-specific BAL-enzyme-linked immunospot (ELISpot) assay in patients with a suspected diagnosis of pulmonary TB at a clinical referral center in Germany.

Among 166 patients with a presumptive diagnosis of pulmonary TB, 81 cases were confirmed by M. tuberculosis culture from sputum and/or BAL. In 66/81 (81.5%) cases patients had initially M. tuberculosis detected by GeneXpert from sputum. In addition, 6/81 (7.4%) were diagnosed by GeneXpert on BAL-fluid (together 72/81 (88.9%) patients). Out of the remaining 9 patients with negative GeneXpert results from sputum and BAL, BAL-ELISpot identified 8 patients with culture confirmed TB correctly (median time to culture positivity 26 days). At a cut-off of >4000 ESAT-6 or CFP-10-specific Interferon-γ producing lymphocytes per 1,000,000 lymphocytes the specificity of the BAL-ELISpot for active TB was 97%.

In low incidence countries of TB, nearly all patients with active pulmonary TB can be identified within the first few days of clinical presentation using a stepwise strategy with GeneXpert and BAL-Elispot.
Introduction

According to the World Health Organization (WHO), 10.4 million new cases of tuberculosis (TB) occurred worldwide in 2016 [1]. Early diagnosis of pulmonary TB is crucial to avoid transmission of *Mycobacterium tuberculosis*. *M. tuberculosis* culture remains the gold standard for TB diagnosis, but it takes 2-5 weeks on average until culture results become available [2-3]. The detection of acid-fast bacilli (AFB) by sputum smear microscopy is a widely used, inexpensive screening test in patients with presumptive diagnosis of pulmonary TB. However, less than 50% of all notified cases are sputum smear positive [4]. Furthermore, microscopy does not distinguish *M. tuberculosis* from other mycobacterial species. Nucleic acid amplification techniques such as GeneXpert (Xpert MTB/RIF, Cepheid, Sunnyvale, CA; USA) have a short time-to-result of less than two hours and are highly sensitive and specific for the detection of *M. tuberculosis* DNA on AFB-smear-positive samples. However, the sensitivity of GeneXpert for the diagnosis of pulmonary TB decreases to less than 70% for smear negative cases [5]. Thus approximately 15% of adult patients with pulmonary TB remain undetected when initially investigated by GeneXpert.

Following the contact with antigen-presenting cells, *M. tuberculosis*-specific T-cells clonally expand in regional lymph nodes and migrate to the site of the infection where they differentiate into effector T-cells [6-8]. After loss of special phenotypical T-cell surface markers, these cell populations are probably unable to re-enter the blood stream and get enriched at the site of disease [9,10]. In contrast to detection of *M. tuberculosis*-specific effector T-cells in peripheral blood, detection of *M. tuberculosis*-specific effector T-cells by interferon-γ release assay (IGRA) at the site of the infection has a high diagnostic accuracy for active TB even when AFBs and *M. tuberculosis*-DNA are not detectable from local samples like bronchoalveolar-lavage (BAL) [11-15], pleural fluid [16,17], pericardial fluid [18], cerebrospinal fluid [19] or ascites [20]. Enzyme-linked immunospot (ELISpot) test results are available within 24 hours.

In order to ascertain the diagnostic accuracy of the stepwise testing of sputum or BAL by GeneXpert followed *M. tuberculosis*-specific BAL-ELISpot testing for the rapid diagnosis of active pulmonary TB, we prospectively enrolled patients with the presumptive diagnosis of pulmonary TB at a clinical TB referral center in Germany.
Methods

Study participants
We performed a prospective analysis of all patients (age ≥ 17 years) admitted to the Medical Clinic of the Research Center Borstel, Germany, between November 2011 and June 2016 presenting with symptoms and/or chest X-ray suggestive of pulmonary TB. Patients who had received treatment for TB within the last two years prior to admission, and patients with extra-pulmonary TB were excluded from the study. As the initial diagnosis sputum smear microscopy and/or GeneXpert was performed on up to three sputum samples. In the absence of detectable acid-fast bacilli in smear and/or negative *M. tuberculosis*-specific nucleic acid tests on sputum, bronchoscopy with BAL was performed according to national guidelines [21]. Pooled BAL was divided into two aliquots, one each for ELISpot, the other aliquot was sent for BAL microscopy, GeneXpert and culture to the National Reference Center for Mycobacteria in Borstel (Germany). The study flow diagram is shown in figure 1.

The study was approved by the Ethics Committee of the University of Lübeck (14-031A). Reporting follows the STARD criteria [22].

GeneXpert
GeneXpert was performed on sputum and BAL according to manufacturer’s guidelines [23] at the National Reference Center for Mycobacteria in Borstel, Germany.

*M. tuberculosis*-specific ELISpot Test
*M. tuberculosis*-specific ELISpot was performed on PBMC and cells from bronchoalveolar lavage (BALC) as described previously [12]. Results of *M. tuberculosis*-specific ELISpot were considered positive if more than five spot-forming cells (SFCs) were observed in the ESAT-6 or the CFP-10 well, after subtracting the number of SFCs in the negative control well, and if the total number of SFCs in the ESAT-6 or CFP-10 well was at least twice the number of SFCs in the negative control well. The results were considered negative if they did not meet the definition for a positive result and if the number of SFCs in the positive control (anti-CD3, clone X35, 10 ng/ml; Beckman Coulter, Krefeld, Germany) was >20 SFCs after subtracting the number of SFCs in the negative control well and if it had at least twice the number of SFCs of the negative control well. Indeterminate results
were defined as meeting neither the criteria for a positive, nor a negative test result. Due to limited number of cells in BALs, some ELISpot tests were performed with less than 250,000 cells per well, these results were normalized for 250,000 cells per well.

Defining the lymphocyte population by flow cytometry analysis

To adjust for the different percentages of lymphocytes in blood and lavage, 500,000 PBMCs or BALCs were acquired on a FACS calibur (BD Bioscience, Heidelberg, Germany). The respective percentage of lymphocytes out of the population of PBMC or BALC was used to extrapolate SFCs per 250,000 cells per well to 1,000,000 lymphocytes in blood or BAL specimen [12]. Recruitment of *M. tuberculosis*-specific lymphocytes to the lungs was calculated by dividing the number of SFC / 1,000,000 lymphocytes in BALC by the number of SFC / 1,000,000 lymphocytes in the peripheral blood. As previously described, in case the denominator had zero SFC, a value of 0.1 was assigned as published earlier [12,24].

Statistical Analysis

Statistical analysis was performed using STATA version 14 (Stata-Corp., TX, USA) and CATmaker version 1.1 (Center for Evidence-Based Medicine, Oxford, UK). The Mann-Whitney U test was used to compare groups of continuous variables. The cut-offs were selected using receiver operating characteristic analysis. The significance level was set at $\alpha=0.05$.

Results

From 01.11.2011 to 30.06.2016, 412 patients with a presumptive or confirmed diagnosis of TB were admitted to the Medical Clinic of the Research Center Borstel, Germany. Of these, 196 patients had already a confirmed diagnosis of pulmonary TB. Another 50 patients were diagnosed with extrapulmonary TB. These patients were excluded. Of the remaining 166 individuals, who were admitted with presumptive pulmonary TB, 66 patients had a microbiological confirmation of the initial diagnosis made by a positive GeneXpert test result on sputum. The remaining 100 individuals, in whom the diagnosis of TB could not be confirmed by sputum smear microscopy and sputum GeneXpert, but where pulmonary TB was still part of the differential diagnosis, underwent bronchoscopy with BAL.
The median age of these 100 patients was 52.0 years (IQR 35.5-63.0), sixty-one (61%) were male. One patient with culture-confirmed tuberculosis and one patient without tuberculosis were tested HIV-positive.

Fifteen/100 patients had culture-confirmed TB. Six patients with culture-confirmed TB (40%) had a rapid diagnosis of TB by a positive BAL GeneXpert result (of whom two also had detectable acid-fast bacilli) on BAL microscopy, four without detectable AFBs in BAL. Thus, there were 9/100 (9%) patients with negative test results on sputum and BAL by microscopy and GeneXpert that had culture confirmed diagnosis of TB with a median time to culture positivity of 26 days (IQR 16.5-34.5). Patient characteristics are shown in table 1.

Among the 100 patients who underwent bronchoscopy, four were diagnosed with non-tuberculous mycobacterial (NTM) pulmonary infections. Two patients had the evidence of AFBs in BAL and the GeneXpert did not detect *M. tuberculosis*, the mycobacterial species identified from culture in these patients were *M. avium* and *M. simiae*. Two individuals with negative results on BAL microscopy and GeneXpert had a positive culture from BAL for *M. xenopi* and *M. kansasii*. Samples from the remainder 81 patients did not show mycobacterial growth. Patient characteristics and details on the final diagnosis of the patients, who underwent bronchoscopy but were ultimately diagnosed as not-having pulmonary tuberculosis, are shown in the supporting information table 1.

**PBMC- and BALC-ELISpot results**

Among 100 patients with a presumptive diagnosis of pulmonary TB, one had an indeterminate test result in PBMCs. In 1/100 (1%) there was failure of the positive control in the PBMC-ELISpot. In 5/100 (5%) the BALC-ELISpot was indeterminate: two had failure of the negative control and three had failure of the positive control. Using the manufacturer’s definition of a positive test result, among the patients with valid test results PBMC- and BALC-ELISpot were positive in 62/99 (62.6%) and 40/95 (42.1%) individuals, respectively. Active TB could be excluded in 34/62 (54.8%) individuals with a positive PBMC-ELISpot and 25/40 (62.5%) with a positive BALC-ELISpot. Out of 15 patients with culture-confirmed pulmonary TB, PBMC-ELISpot was positive in 14/15 (93.3%) and negative in 1/15 (6.7%). BALC-ELISpot was positive in 15/15 (100%).
For patients with culture-confirmed pulmonary TB, PBMC-ELISpot had a sensitivity of 93% (95%CI 81-100%) and a specificity of 50% (95%CI 38-62%), while the BALC-ELISpot had a sensitivity of 100% and a specificity of 77% (95%CI 67-87%) when the manufacturer’s definition of a positive and negative test result on PBMCs was used.

Table 2 shows all individual test results for the nine culture-confirmed pulmonary TB patients with negative AFB and negative GeneXpert results in BAL. The respective numbers of positive or negative paired PBMC- and BALC-ELISpot results in patients with culture-confirmed pulmonary TB and patients without TB are given in supporting information figure S1.

Concentration of antigen-specific cells at the site of infection

In patients with culture confirmed pulmonary TB, the median antigen-specific SFC was 25 SFC (IQR 6.3-210) per 250,000 PBMC for ESAT-6 and 53.8 (IQR 22.5-177) per 250,000 PBMC for CFP-10. BALC-ELISpot median was elevated to 295 SFC (IQR 52.5-489) per 250,000 BALC for ESAT-6 and 160 SFC (IQR 61-453) per 250,000 BALC for CFP-10. In contrast, patients without TB had a median of 3.8 SFC (IQR 0.1-17.5) per 250,000 PBMC for ESAT-6 and 4.3 SFC (IQR 0.1-16.9) per 250,000 PBMC for CFP-10 and in the BAL the median for both ESAT-6 and CFP-10 was low with 0 SFC per 250,000 BALC. Figure 2 and supporting information figure S1 show the number of antigen specific cells in 250,000 PBMC and BALC in patients with culture-confirmed pulmonary TB but undetectable AFBs on microscopy and negative GeneXpert result compared to patients in whom TB had been excluded.

Concentration of antigen-specific lymphocytes at the site of infection

To account for the different percentages of lymphocytes between individuals, SFC results in PBMC and BAL were normalized for 1,000,000 lymphocytes. In patients with culture-confirmed pulmonary TB, the median of antigen-specific lymphocytes per 1,000,000 in peripheral blood was 121 SFC (IQR 43.9-1228.8) for ESAT-6 and 270 SFC (IQR 109.8-1363.8) for CFP-10, while in the BAL the median was 6370 SFC (IQR 4595.2-14541.7) for ESAT-6 and 6598.5 SFC (IQR 3857.7-13503.7) for CFP-10. By comparison, patients with other diagnosis than TB had a median of 25.5 SFC (IQR 0.8-99.9) ESAT-6 and 27 SFC (IQR 0.7-93.8) CFP-10-specific lymphocytes in peripheral blood, while in the BAL they had a median of 0 SFC (IQR 0-0) of ESAT-6 and 0 SFC (IQR 0-0) of CFP-10 specific lymphocytes per 1,000,000 lymphocytes. Figure 3 and supporting information figure S2 shows
the number of antigen specific lymphocytes per 1,000,000 lymphocytes in PBMCs and BALCs in patients with culture-confirmed pulmonary TB but negative AFB microscopy / negative GeneXpert result compared to patients without TB.

Differentiation of active TB from non-TB

To improve the discrimination between patients without TB and those with culture-confirmed pulmonary TB but undetectable AFB on microscopy and negative GeneXpert result in BAL, different cut-offs were investigated. A cut-off of 50 SFC of ESAT-6 or 50 SFC of CFP-10 specific cells from 250,000 BALCs provides a sensitivity of 100% and a specificity of 91% (IQR 84-98%). Considered the proportion of lymphocytes, a cut-off of at least 4000 antigen-specific lymphocytes for either ESAT-6 or CFP-10 per 1,000,000 BAL-lymphocytes had a sensitivity of 89% (IQR 68-100) and a specificity of 97% (IQR 93-100%). To account for the different percentages of lymphocytes in BAL and peripheral blood a recruitment factor was defined as the ratio of SFC in BALC divided by the SFC in PBMC and multiplied with the lymphocyte factor (% of lymphocytes in PBMC divided by the % lymphocytes in BALC). A recruitment factor of at least 6 for ESAT-6 or CFP-10 had a sensitivity of 67% (IQR 36-97%) and a specificity of 83% (IQR 74-92%).

The area under the ROC curve to differentiate patients without TB from the subgroup of patients with culture-confirmed pulmonary TB but undetectable AFBs on microscopy and negative GeneXpert result was 0.968 for ESAT-6 and 0.985 for CFP-10 specific BALCs, while the area under the ROC curve for PBMC was 0.760 for ESAT-6 and 0.841 for CFP-10. When evaluating the 1,000,000 lymphocytes from the BALC only, the area under the ROC curve was 0.973 for ESAT-6 and 0.978 for CFP-10.

When applying these cut-offs for BAL-lymphocytes (instead of the ones provided by the manufacturer for the use on PBMCs) to the entire group of patients with culture-confirmed pulmonary TB, a number of at least 50 antigen-specific BALCs had a sensitivity of 100% (95% CI: 100 -100%) and a specificity of 91% (95% CI: 84-98%), while a number of at least 4000 antigen-specific BAL-lymphocytes per 1,000,000 lymphocytes had a sensitivity of 89% (95% CI: 68-100) and a specificity of 97% (95% CI: 93-100%). A recruitment factor of at least 6 had a sensitivity of 73% (95% CI: 51-96%) and a specificity of 83% (95% CI: 74-92%).
Table 3 shows the performance of ELISpot on BALCs using these different cut-offs. Supporting information figure S3 gives the graphs of the respective area under ROC curves.

In summary, among 166 patients, who were admitted with a presumptive diagnosis of pulmonary TB, the diagnosis was confirmed by *M. tuberculosis* culture in 81 patients (48.8%). 66/81 (81.5%) had initially a positive GeneXpert result from sputum and in addition 6/81 (7.4%) were diagnosed by BAL GeneXpert (together 72/81; 88.9%). In 8 out of 9/81 (88.8%) patients with negative results on sputum and BAL by microscopy and GeneXpert, BAL-ELISpot identified patients with culture confirmed TB correctly with a specificity of 97%.

With a stepwise analysis of GeneXpert (2h) and BAL-ELISpot only for patients with a negative BAL-GeneXpert test result, the combined sensitivity and specificity for a rapid diagnosis of pulmonary TB was 98.8% and 97.6%, respectively (table 4).

Discussion

We evaluated the accuracy of a stepwise approach consisting of an initial molecular test, the GeneXpert, followed by a *M. tuberculosis*-specific BAL-ELISpot for the diagnosis of pulmonary TB. Using BAL-GeneXpert and BAL-Elispot sequentially, the diagnostic accuracy for sputum-smear and GeneXpert-negative pulmonary TB was 97%. The diagnosis became available within the first days of presentation to the hospital.

GeneXpert has greatly improved the diagnosis of TB. If GeneXpert is performed on two different sputum samples, more than 90% of adult patients with pulmonary TB were being correctly identified by GeneXpert within the first days of presentation to a healthcare facility [25]. However, the sensitivity of GeneXpert is lower in sputum smear negative and in extrapulmonary TB [25,26]. If results from GeneXpert tests are negative, patients could remain undiagnosed for several weeks until culture results of *M. tuberculosis* cultures become available.

*M. tuberculosis*-specific ELISpot performed on cells from the BAL is able to close this diagnostic gap. In the past decade several studies have shown that *M. tuberculosis*-specific ELISpot performed on specimens from the site of the disease e.g. BAL in pulmonary TB, pleural or pericardial effusion in TB pleurisy or TB pericarditis, respectively, ascites in TB peritonitis or
cerebrospinal fluid in TB meningitis [27-30] can improve the case detection rate of TB in paucibacillary disease, but the added value of a stepwise approach of GeneXpert and ELISpot had not been investigated so far.

In active TB, *M. tuberculosis*-specific lymphocytes are recruited from the blood into the disease-affected organ [31,32]. Active disease is more likely with increasing concentration of *M. tuberculosis*-specific cells at the site of infection compared to peripheral blood [24]. Hofland et al. suggested a ratio between SFC in BAL divided by SFC in blood with a cut-off >1 [33]. However, one needs to consider, that lymphocytes predominate in peripheral blood, whereas their proportion in BAL is considerably lower [34]. These differences in percentages of lymphocytes in PBMC and BALC should be acknowledged individually by calculating a lymphocyte-depending recruitment factor of ESAT-6 and CFP 10 specific T-cells between blood and BALC or by normalizing the SFC per 250,000 cells to 1,000,000 lymphocytes [11,24].

Optimal result were obtained in this study with a cut-off of >4000 lymphocytes per 1,000,000 lymphocytes in BAL. When applying this cut-off, patients with paucibacillary pulmonary TB could be identified with a sensitivity of 89% and a specificity of 97%. This cut-off provided an excellent negative predicted value of 98% and a high positive likelihood ratio of 28.9.

Like most studies from low incidence countries, our study is limited by the sample size of patients with culture confirmed active TB and negative GeneXpert results from sputum and BAL. Performing BAL-ELISpot and flow cytometry is also substantially more labour-intensive and requires considerably more technician time than automated *M. tuberculosis*-specific nucleic acid amplification by GeneXpert. However, most patients who are suspected to have pulmonary TB in low-incidence countries of TB with sufficient economical resources will undergo bronchoscopy when results of sputum smear microscopy and sputum GeneXpert analysis are negative, also to exclude alternative diseases such as sarcoidosis, cryptogenic organizing pneumonia, lung cancer, and others. Furthermore, the results cannot be generalized to high burden countries, where repeated exposure to *M. tuberculosis* may alter BAL-ELISpot responses [14] and performance of the ELISpot would be challenging due to limited laboratory capacity.
Due to the high sensitivity for pulmonary TB of GeneXpert performed on respiratory specimen, the performance of *M. tuberculosis* specific BAL-ELISpot can be limited to patients where the GeneXpert result from sputum and BAL are negative and when the differential diagnosis of active pulmonary TB is still to be considered.

In conclusion, in low-incidence counties of TB, nearly all patients with active pulmonary TB can be identified within the first few days of clinical presentation by using a stepwise testing strategy with GeneXpert and BAL-ELISpot.
Acknowledgement

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Christoph Lange and Jan Heyckendorf are supported by the German Center for Infection Research (DZIF).
References

**Tables**

**Table 1**: Individual test results and patient characteristic of the nine patients with culture-confirmed TB with undetectable acid-fast bacilli (AFB) in bronchoalveolar lavage (BAL) and negative GeneXpert results.

MTB: *Mycobacterium tuberculosis*; ITR: indeterminate test result; PBMC: peripheral blood mononuclear cells; TPP: time to culture positivity of the first culture growing MTB, specimen was either sputum* or BAL#.

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Table 2: Individual ELISpot results and recruitment factor for the nine patients with culture-confirmed tuberculosis with acid fast bacilli negative microscopy and negative GeneXpert result in BAL.

PBMC: peripheral blood mononuclear cells, BALC: bronchoalveolar lavage cells, E: early secretory antigenic target (ESAT)-6, C: culture filtrate protein (CFP)-10, Ly: lymphocytes, Rec: recruitment factor = spot forming cells (SFC) in BALC divided by SFC in PBMC multiplied with percentage of lymphocytes in PBMC divided by percentage of lymphocytes in BAL.

PBMC/BAL ESAT-6/ CFP-10 250cells: results are expressed per 250,000 mononuclear cells; PBMC/BAL ESAT-6/ CFP-10 1000Ly: results are expressed per 1,000,000 lymphocytes.

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</tr>
<tr>
<td>9</td>
<td>33</td>
<td>M</td>
<td>5</td>
<td>47</td>
<td>6</td>
<td>61</td>
<td>24</td>
<td>229</td>
<td>107</td>
<td>1085</td>
<td>4.4</td>
</tr>
</tbody>
</table>
**Table 3:** Performance of different cut-offs for *M. tuberculosis* -specific BALC-ELISpot for the diagnosis of active tuberculosis.

1. CFP-10: culture filtrate protein-10, ESAT-6: early secretory antigenic target-6, BALC: bronchoalveolar lavage cells, BAL-Ly: bronchoalveolar lavage lymphocytes, Rec: recruitment factor = spot forming cells (SFC) in BALC divided by SFC in PBMC multiplied with percentage of lymphocytes in PBMC divided by percentage of lymphocytes in BALC, Ratio: ratio of SFC in BALC divided by SFC in PBMC, CI: confidence interval, PPV: positive predictive value, NPV: negative predictive value, CC: correctly classified, LR: likelihood ratio.
2. The test performance was computed for the 100 individuals, who underwent bronchoscopy with BAL.

<table>
<thead>
<tr>
<th>Cut-off</th>
<th>Sensitivity in % (95% CI)</th>
<th>Specificity in % (95% CI)</th>
<th>PPV in % (95% CI)</th>
<th>NPV in % (95% CI)</th>
<th>CC in %</th>
<th>LR+ in % (95% CI)</th>
<th>LR- in % (95% CI)</th>
<th>False positive -&gt; treated unnecessarily</th>
<th>Area under the ROC curve</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFP-10 or ESAT-6 &gt;50 BALC</td>
<td>100 (100-100)</td>
<td>91 (84-98)</td>
<td>60 (35-85)</td>
<td>100 (100-100)</td>
<td>91.9</td>
<td>10.8 (5.1-23.2)</td>
<td>na</td>
<td>6/100 (6%)</td>
<td>ESAT: 0.948 CFP: 0.965</td>
</tr>
<tr>
<td>CFP-10 or ESAT-6 &gt;4000 BAL-Ly</td>
<td>89 (68-100)</td>
<td>97 (93-100)</td>
<td>80 (55-100)</td>
<td>98 (95-100)</td>
<td>95.9</td>
<td>28.9 (7.2-115.3)</td>
<td>0.1 (0.02-0.7)</td>
<td>2/100 (2%)</td>
<td>ESAT: 0.943 CFP: 0.978</td>
</tr>
<tr>
<td>CFP-10 or ESAT-6 Rec &gt;6</td>
<td>67 (36-97)</td>
<td>83 (74-92)</td>
<td>35 (13-58)</td>
<td>95 (89-100)</td>
<td>80.8</td>
<td>3.9 (1.9-7.9)</td>
<td>0.4 (0.2-1)</td>
<td>11/100 (11%)</td>
<td>ESAT: 0.879 CFP: 0.910</td>
</tr>
<tr>
<td>CFP-10 or ESAT-6 Ratio &gt;2</td>
<td>78 (51-100)</td>
<td>84 (75-93)</td>
<td>41 (18-65)</td>
<td>96 (92-100)</td>
<td>83.6</td>
<td>5 (2.6-9.7)</td>
<td>0.26 (0.08-0.9)</td>
<td>10/100 (10%)</td>
<td>ESAT: 0.882 CFP: 0.915</td>
</tr>
</tbody>
</table>
**Table 4:** Performance of a combined approach of GeneXpert and BAL-ELISpot for the diagnosis of active tuberculosis.

BAL-Ly: bronchoalveolar lavage lymphocytes, CI: confidence interval, PPV: positive predictive value, NPV: negative predictive value, CC: correctly classified, LR: likelihood ratio.

Test performance of this stepwise approach of combined GeneXpert and ELISpot refers to 166 individuals (100 BAL, 66 sputum).

<table>
<thead>
<tr>
<th>Cut-off</th>
<th>Sensitivity in % (95% CI)</th>
<th>Specificity in % (95% CI)</th>
<th>PPV in % (95% CI)</th>
<th>NPV in % (95% CI)</th>
<th>CC in %</th>
<th>LR+ in % (95% CI)</th>
<th>LR- in % (95% CI)</th>
<th>False positive -&gt; treated unnecessarily</th>
</tr>
</thead>
<tbody>
<tr>
<td>Combined GeneXpert and ELISpot &gt;4000 BAL-Ly</td>
<td>99 (96-100)</td>
<td>98 (94-100)</td>
<td>98 (94-100)</td>
<td>99 (96-100)</td>
<td>98.2</td>
<td>42 (10.7-165.2)</td>
<td>0.01 (0-0.1)</td>
<td>2/166 (1.2%)</td>
</tr>
</tbody>
</table>
Legends

Figure 1: Study flow diagram. TB: tuberculosis, BAL: bronchoalveolar lavage, GeneXpert: GeneXpert MTB/RIF. MTB Culture: TB diagnosis was confirmed by growth of *Mycobacterium tuberculosis*.

Figure 2: Number of spot forming cells (SFC) of early secretory antigenic target (ESAT)-6- and culture filtrate protein (CFP)-10–specific peripheral blood mononuclear cells (PBMC) and bronchoalveolar-lavage cells (BALC) cells per 250,000 cells in individuals where tuberculosis was excluded (non-TB) and in patients with cultureConfirmed pulmonary tuberculosis (TB) with undetectable acid fast bacilli on microscopy and negative GeneXpert result. A suggested cut-off >50 SFC per 250,000 cells is depicted as dotted line.

Figure 3: Number of early secretory antigenic target (ESAT)-6- and culture filtrate protein (CFP)-10–specific spot forming cells per 1,000,000 lymphocytes in peripheral blood mononuclear cells (PBMC) and bronchoalveolar lavage cells (BALC) in individuals where tuberculosis was excluded (non-TB) and in patients with culture confirmed pulmonary tuberculosis (TB) with undetectable acid-fast bacilli on microscopy and negative GeneXpert. A suggested cut-off >4000 antigen-specific lymphocytes per 1,000,000 lymphocytes is depicted as dotted line.

Supporting information Figure S1
Comparison of spot forming cells (SFC) of early secretory antigenic target (ESAT)-6- and culture filtrate protein (CFP)-10–specific peripheral blood mononuclear cells (PBMC) and bronchoalveolar lavage mononuclear cells (BALC) in patients with culture confirmed pulmonary tuberculosis (TB) with undetectable acid fast bacilli on microscopy and negative GeneXpert result (upper row) and patients with other diagnoses after exclusion of TB (non-TB) (bottom row) by enzyme-linked immunospot (ELISpot).

Supporting information Figure S2: Comparison of spot forming cells (SFC) of early secretory antigenic target (ESAT)-6- and culture filtrate protein (CFP)-10–specific lymphocytes per 1,000,000 lymphocytes from peripheral blood (Blood-Ly) or and bronchoalveolar lavage (BAL-Ly) in patients with culture confirmed pulmonary tuberculosis (TB) and undetectable acid-fast bacilli
on microscopy and negative GeneXpert MT/B/RIF result (upper row) and patients with other
diagnoses after exclusion of TB (non-TB) (bottom row) by enzyme-linked immunospot (ELISpot).

**Supporting information Figure S3**: Area under the ROC curve to differentiate patients without
tuberculosis from patients with culture-confirmed pulmonary tuberculosis for culture filtrate
protein (CFP)-10-specific bronchoalveolar lavage cells (BALC). The recruitment factor (Rec) was
calculated as spot forming cells (SFC) in BALC divided by SFC in peripheral blood mononuclear
cells (PBMC) multiplied with percentage of lymphocytes in PBMC divided by percentage of
lymphocytes in BALC. The ratio (Ratio) was calculated as SFC in BALC divided by SFC in PBMC.
Results are expressed per 250,000 BALC (250 cells) or 1,000,000 BAL-lymphocytes (1000 Ly).