Differential expression of activation markers by *Mycobacterium tuberculosis*-specific CD4$^+$ T-cell distinguishes extrapulmonary from pulmonary tuberculosis and latent infection

Paulo S. Silveira-Mattos[1,2,3], Beatriz Barreto-Duarte[1,2,4], Beatriz Vasconcelos[1,2,5], Kiyoshi F. Fukutani[1,2,3], Caian L. Vinhaes[1,2,3], Deivide Oliveira-de-Souza[1,2,3], Chris C. Ibegbu[6,7], Marina C. Figueiredo[8], Timothy R. Sterling[8]*, Jyothi Rengarajan[6,7]* and Bruno B. Andrade[1,2,3,4,5,8,9]*

[1]Instituto Gonçalo Moniz, Fundação Oswaldo Cruz, Salvador, Bahia 40296-710, Brazil
[2]Multinational Organization Network Sponsoring Translational and Epidemiological Research (MONSTER) Initiative, Fundação José Silveira, Salvador, Bahia 40210-320, Brazil
[3]Curso de Medicina, Faculdade de Tecnologia e Ciências (FTC), Salvador, Bahia 40290-150, Brazil
[4]Universidade Salvador (UNIFACS), Laureate Universities, Salvador, Bahia 41720-200, Brazil
[5]Escola Bahiana de Medicina e Saúde Pública (EBMSP), Salvador, Bahia 40290-000, Brazil
[6]Emory Vaccine Center, Emory University School of Medicine, Atlanta, Georgia30322, USA
[7]Division of Infectious Disease, Department of Medicine, Emory University School of Medicine, Atlanta, Georgia, 30322, USA
[8]Division of Infectious Diseases, Department of Medicine, Vanderbilt University School of Medicine, Nashville, TN 37232, USA
[9]Wellcome Centre for Infectious Diseases Research in Africa (CIDRI-Africa), Institute of Infectious Disease and Molecular Medicine, University of Cape Town, Cape Town, South Africa
*T.R.S., J.R. and B.B.A. equally contributed to this work.

Corresponding author: Bruno B. Andrade, MD, PhD. Laboratório de Inflamação e Biomarcadores, Instituto Gonçalo Moniz, Fundação Oswaldo Cruz, Rua Waldemar Falcão, 121, Candeal, Salvador, Bahia 40296-710, Brazil. E-mail: bruno.andrade@fiocruz.br

Summary: Activation markers on *Mycobacterium tuberculosis*-specific CD4$^+$ T cells distinguished active extrapulmonary and pulmonary tuberculosis from latent infection, regardless of HIV status in a large case-control study, serving as an attractive approach for blood-based diagnostic tests for tuberculosis.

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ABSTRACT

Background: Diagnosis of active tuberculosis (ATB) currently relies on detection of *M. tuberculosis* (Mtb). Identifying patients with extrapulmonary TB (EPTB) remains challenging because microbiological confirmation is often not possible. Highly accurate blood-based tests could improve diagnosis of both EPTB and pulmonary TB (PTB), and timely initiation of anti-TB therapy.

Methods: A case-control study was performed using discriminant analyses to validate an approach using Mtb-specific $CD4^+$T-cell activation markers in blood to discriminate PTB and EPTB from latent TB infection (LTBI) as well as EPTB from PTB in 270 Brazilian individuals. We further tested the effect of HIV co-infection on diagnostic performance. Frequencies of $IFN\gamma^+CD4^+$T-cells expressing CD38, HLADR, and/or Ki67 were assessed by flow cytometry.

Results: EPTB and PTB were associated with higher frequencies of $CD4^+$T-cells expressing CD38, HLADR or Ki67 compared to LTBI (all p-values < .001). Moreover, frequencies of $HLADR^+$ (p= .03) or $Ki67^+$ (p< .001) cells accurately distinguished EPTB from PTB. HIV infection did not affect the capacity of these markers to distinguish ATB from LTBI or EPTB from PTB.

Conclusion: Cell activation markers in Mtb-specific $CD4^+$T-cells distinguished ATB from LTBI, and EPTB from PTB, regardless of HIV infection status. These parameters provide an attractive approach for developing blood-based diagnostic tests for both active and latent TB.

Key words: tuberculosis, biomarker, extrapulmonary TB, T-cells, immune activation.

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INTRODUCTION

Diagnosis of active tuberculosis (ATB) disease currently relies on microbiologic tests such as acid-fast smear and culture, and molecular PCR-based assays such as GeneXpert [1] that detect *M. tuberculosis* (Mtb) in patients' sputum. However, the sensitivity of these tests can be low, particularly in extrapulmonary TB (EPTB), in which the bacillary burden is low [2]. Thus, better methods are needed to identify EPTB as well as to discriminate between EPTB, pulmonary TB (PTB) and asymptomatic latent TB infection (LTBI).

EPTB involves organs other than the lungs, such as the lymph nodes (LN), pleura and meninges, and occurs with increased frequency in immunocompromised persons, including those infected with HIV [3]. Diagnosis of EPTB is often more difficult than PTB because patients are more likely to have negative sputum-based tests. Indeed, radiographic-based diagnosis and empirical data on response to anti-TB therapy are commonly used to guide diagnosis of EPTB [4]. Thus, more sensitive and specific diagnostic assays for EPTB that are faster and less-invasive would be a great advance for the field.

Current diagnostic tests for LTBI (e.g, the tuberculin skin test and interferon gamma release assays) are unable to distinguish between LTBI and ATB, and have decreased sensitivity in persons with HIV. A diagnostic test that could accurately distinguish between latent and active TB, including among persons with HIV, would substantially improve our ability to accurately diagnoses and treat these two disease states.

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We have previously identified a blood-based assay in which Mtb-specific $CD4^+$T cells are examined for activation and proliferation markers for diagnosis of active TB [5]. In this assay, the frequency of Mtb-specific $CD4^+$ T cells expressing the immune activation markers CD38 and HLADR, as well as the intracellular proliferation marker Ki67, can accurately identify ATB and successfully distinguish ATB from LTBI in persons from Georgia, U.S.A [5] and the Western Cape, South Africa [6]. Subsequently, it was demonstrated that these markers can also identify ATB in HIV-positive individuals [6]. However, the performance of these diagnostic assays for identifying EPTB has not been previously explored. In the present study, we sought to extend our previously published findings on PTB to additional populations by evaluating cryopreserved peripheral blood mononuclear cells (PBMC) samples from Brazilian patients with EPTB, PTB and LTBI. We report that $CD38^+IFN\text{-}\gamma^+$, $HLADR^+IFN\text{-}\gamma^+$, and $Ki\text{-}67^+IFN\text{-}\gamma^+$ $CD4^+$ T-cells successfully distinguished ATB from LTBI, and EPTB from PTB, regardless of HIV status.

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MATERIALS AND METHODS

Ethics Statement

This study was conducted according to the principles expressed in the Declaration of Helsinki and approved by the Ethics Committee of the Maternidade Climério de Oliveira, Federal University of Bahia (CAAE: 0115.0.054.000–09). Written informed consent was obtained from all participants.

Clinical Study Design

A case-control study was performed using cryopreserved PBMC samples and corresponding clinical and epidemiological data obtained from participants enrolled in a translational study performed at the Instituto Brasileiro para Investigação da Tuberculose (IBIT) and at the Hospital Especializado Octavio Mangabeira (HEOM), Salvador, Bahia, Northeast Brazil, between December 2015 and January 2018. The parent study was focused on characterization of inflammatory markers in different clinical forms of TB, and recruited 1,792 individuals with presumptive TB at the referral primary care clinic at IBIT. These patients underwent clinical assessments and radiological (chest X-ray) examination. In addition, acid-fast bacilli (AFB) screening in sputum smears (by microscopy) and sputum cultures (Lowenstein–Jensen solid cultures) were performed in all patients. At this stage, 235 (13%) individuals were diagnosed with culture-confirmed pulmonary TB (PTB), and 215 (12%) had PTB excluded and were suspected to have extrapulmonary TB (EPTB). Further investigation to confirm EPTB was conducted at a TB referral site at HEOM by performing lymph node (LN) fine needle aspirates (with AFB screening and culture) for TB lymphadenitis, and by pleural fluid drainage with lung biopsy for pleuropulmonary TB. Among the confirmed EPTB cases (n=211, 7%), there were 102

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with TB lymphadenitis, 105 with pleuropulmonary TB, 1 case of TB meningitis, 2 had spinal TB and 1 had abdominal tuberculomas. All individuals were tested for HIV; those who were positive had $CD4^{+}$ T cell counts and HIV viral loads (RNA copies/μL) assessed. All patients screened positive for HIV were diagnosed at the time of study enrollment and had not been treated with antiretroviral therapy previously. The parent study also included participants who were asymptomatic contacts of TB index cases. At the time of study enrollment, HIV-negative individuals who tested positive for QuantiFERON TB Gold-in-Tube (QFT) enzyme-linked immunosorbent assay (ELISA) (Qiagen) were considered to have LTBI, and individuals who were QFT-negative were considered uninfected healthy controls (HC).

At the time of study enrollment and prior to initiation of anti-TB treatment, 10mL of venous blood was collected in sodium heparin tubes for isolation of peripheral blood mononuclear cells (PBMCs) from a subset of participants who consented to blood collection. Cells were cryopreserved in liquid nitrogen at the biorepository of the Laboratory of Inflammation and Biomarkers, Fundação Oswaldo Cruz (FIOCRUZ), Salvador, Brazil. For the immunological assays performed in the present study, selected samples from individuals with confirmed PTB and EPTB were matched on age (±5 years) and sex, with subgroups of HIV-negative and HIV-positive patients, as well as within HC and those with LTBI. For this study, only patients with TB lymphadenitis without pulmonary involvement were included in the EPTB group. Samples used for flow cytometry studies and characteristics of the corresponding study subjects are shown in Table 1. Sample sizes were determined based on calculations of study power of 80% (alpha error: 5%) to detect differences in median frequencies of T cell subsets >2% between active and latent TB, based on a previous study from our group [5].

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Flow Cytometry

Cryopreserved PBMC were thawed and resuspended in RPMI 1640 supplemented with 10% FBS at 10^6 cells per well in 96-well plates and rested for 2 hours at 37°C in 5% CO_2. Cells were washed and resuspended in complete media with Brefeldin-A (Biolegend, San Diego, California, USA) and Monensin (Biolegend, San Diego, California, USA) to block cytokine secretion, and stimulated with ESAT-6 and CFP-10 peptide pools (10µg/ml) overnight at 37°C in 5% CO_2. Cells were then stained for cell surface markers with the following panel of antibodies: CD3 APC-CY7 (clone SK7), CD4 PerCp-Cy5.5 (clone L200), HLADR PE-Cy7 (clone L243), CD38 PE (clone HB7), all from BD Biosciences. Cells were then fixed and permeabilized using the Foxp3 Fixation and Permeabilization Buffer (eBioscience). Intracellular staining to detect IFN-γ Alexa Fluor 700 (clone B27) and Ki67 FITC (clone B56), all from BD Bioscience. Acquisition of stained cells was performed using a BD LSRFortessa cell analyzer (BD Bioscience, San Jose, CA, USA) and analyzed using FlowJo software (BD Bioscience, San Jose, CA, USA). Overall gating strategies together with representative plots are shown in Supplemental Figure 1.

Statistical Analysis

Median values with interquartile ranges (IQR) were compared using the Mann-Whitney *U* test. Receiver Operator Characteristics (ROC) curve analysis was used to test the ability of frequencies of $CD38^+$, $HLADR^+$, and $Ki67^+$ $CD4^+$ T cells to distinguish ATB from LTBI and PTB from EPTB in HIV-negative individuals. The overall accuracy of the biomarkers was examined by comparing the area under the curves (AUCs) with C-statistics. Fisher's exact test was used to compare frequencies of virologically-suppressed HIV-positive subjects between PTB and EPTB groups. Spearman correlation rank analysis was performed to test correlations

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between $CD4^+$T cell counts and frequency of IFNγ^+ $CD4^+$ T cells expressing CD38, HLADR or Ki67 in HIV-positive patients. A p value of < 0.05 was considered statistically significant after adjustment for multiple comparisons using the Holm-Bonferroni method. The statistical analyses were performed using GraphPad Prism 7.0 (GraphPad Software Inc., USA), and JMP 14.0 software.

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RESULTS

Characteristics of the study population

The study groups were similar with regard to age and sex and most of the clinical and epidemiological characteristics. The highest frequency of reported illicit drug use was observed in EPTB patients living with HIV (n=18, 36%, P <0.001, Table 1). Furthermore, additional analyses revealed that patients with active TB (PTB or EPTB) who had HIV coinfection exhibited lower smear grade values more frequently compared to those without HIV (Table 1).

Higher frequencies of Mtb-specific $CD4^+$ T cells expressing CD38, HLADR or Ki67 in Brazilian subjects with ATB compared with LTBI

Frequencies of IFN-γ producing Mtb-specific $CD4^+$ T-cells expressing CD38, HLADR, and Ki67 were compared between ATB and LTBI patients. We found higher frequencies of ESAT-6/CFP-10-specific IFN-γ^+CD4 T-cells expressing the immune activation markers CD38 and HLADR, as well as the intracellular proliferation marker Ki67 in ATB patients (Figure 1A). We next employed cutoff values for these markers which were established in a previous study from our group, [5] and re-examined the discriminatory power in our study sample. Importantly, ROC curve analysis confirmed that each biomarker had the potential to identify ATB cases, with high overall accuracy (Figure 1B). These results validated the use of the $CD4^+$ T-cell activation markers as potential diagnostic biomarkers for ATB in this study population.

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Evaluating the predictive value of $CD38^+IFN\text{-}\gamma^+$, $HLADR^+IFN\text{-}\gamma^+$, and $Ki67^+IFN\text{-}\gamma^+CD4^+$ T-cells in distinguishing EPTB from LTBI and PTB.

We next investigated whether expression of CD38, HLA-DR, and Ki-67 on antigen-specific $IFN\text{-}\gamma^+$ CD4 T cell could identify patients with EPTB. We stimulated PBMCs from HIV-negative individuals with EPTB and compared their activation profiles to those with PTB, LTBI and healthy controls (HC). $CD4^+$ T-cells from EPTB, PTB and LTBI groups exhibited similar frequencies of IFN-γ-producing $CD4^+$ T cells in response to *in vitro* stimulation with ESAT-6/CFP-10 peptides (Figure 2A). However, when the activation and proliferation markers CD38, HLADR and Ki67 were assessed within the gate of $IFN\text{-}\gamma^+$ $CD4^+$ T-cells in the stimulated conditions, major differences were observed between the groups, with both disease groups displaying significantly higher frequencies of cells expressing these markers compared to the LTBI group (Figure 2A). Frequencies of $CD4^+$ T cells expressing any of the three biomarkers tested were significantly higher in EPTB patients compared to those with LTBI (Figure 2A). Interestingly, the frequencies of cells expressing HLADR or Ki67, but not CD38, were higher in patients with EPTB compared to those with PTB (Figure 2A). ROC analysis confirmed that the frequency of cells expressing these markers was able to distinguish EPTB from PTB with high accuracy. The highest performance was achieved when the discriminant model was composed of data on simultaneous expression of the CD38, HLADR and Ki67, simultaneously (Figure 2B).

Frequencies of activated Mtb-specific $IFN\text{-}\gamma^+CD4^+$ T cells expressing CD38, HLADR and Ki67 in PTB and EPTB are comparable in HIV-negative and HIV-positive individuals.

We next sought to determine whether the ability of Mtb-specific T-cells expressing CD38, HLADR and Ki67 to identify PTB and EPTB is altered by HIV status. We stratified the PTB and

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EPTB groups according to HIV status and determined the frequencies of $CD38^+$ IFN-γ^+, $HLADR^+$ IFN-γ^+ and $Ki67^+$IFN-γ^+ $CD4^+$ T-cells in each group. As shown in Figure 3A, HIV status did not substantially alter the frequencies of cells expressing CD38, HLADR or Ki67. Previous studies have suggested that progression of HIV disease gradually impairs capacity to restrain Mtb growth, thus favoring bacterial dissemination and extrapulmonary manifestations of TB [7, 8]. In our study, the frequency of virologically-suppressed patients was just slightly lower in EPTB patients compared to those with PTB, without reaching statistical significance (Figure 3B). Nevertheless, median total $CD4^+$ T-cell counts was lower in EPTB vs. PTB ($p = .039$; Figure 3C), although all HIV patients included in the study had total $CD4^+$ T-cell counts above 350 cells/μL, and were thus not highly immunosuppressed. We further tested correlations between the total $CD4^+$ T-cell count values and the frequencies of Mtb-specific $CD4^+$ T-cells expressing CD38, HLADR or Ki67 in the subgroup of patients with HIV. We found that frequency of $CD38^+$ Mtb-specific $CD4^+$ T-cells was positively correlated with total $CD4^+$ T-cell counts only in EPTB patients, with all other associations not reaching statistical significance (Figure 3D). Figure 3E shows the Spearman correlation plot of such significant association ($r=.42$, $p=.039$).

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DISCUSSION

In the present study, we explored quantification of Mtb-specific $CD4^+$ T-cells expressing activation markers CD38 and HLA-DR and the intracellular proliferation marker Ki67, to diagnose ATB in a Brazilian population. Our primary results extend our previous studies in patients from Georgia, USA and the Western Cape, South Africa [5] to Brazilian subjects. We demonstrate that ATB patients from Brazil express higher frequencies of Mtb-specific $CD4^+$ T-cells expressing CD38, HLADR and Ki67 when compared to individuals with LTBI. To reliably validate results from our earlier studies in our Brazilian population, we used cutoff values previously established for each marker. Using these cutoffs, we observed that quantification of cells expressing these activation markers could reliably distinguish active from latent TB in this study population, with high specificity and sensitivity, as reported previously [9]. Interestingly, similar to interferon gamma release assays, IFN-γ^+ $CD4^+$ T-cells did not distinguish ATB from LTBI. Our findings represent an important step towards validating these three blood-based diagnostic biomarkers and demonstrate their high reliability when used in patient populations from different geographical locations.

Importantly, our studies also show that these biomarkers are also useful for diagnosing EPTB. Extrapulmonary disease accounts for about 20 to 50% of reported TB cases [10]. The most frequent locations are pleural, lymph nodes, bones and joints, central nervous system and gastrointestinal or genitourinary areas. Such anatomical sites represent a challenge for direct visualization of Mtb and therefore hinder accurate diagnosis. According to the World Health Organization (WHO), diagnosis of EPTB is currently based on a positive culture from sputum or extrapulmonary sites, positive histology or strong clinical evidence consistent with active EPTB.

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Notably, two of these three criteria require invasive techniques that can delay the diagnosis of TB [11, 12]. Thus, blood-based markers are attractive for the diagnosis of EPTB, and represent a desirable alternative tool for diagnosing PTB and EPTB. Several recent studies have explored non-sputum based approaches for diagnosing ATB, including transcriptome [13], metabolome [14], proteome [15] and cellular assays [16]. We have recently described that a combination of three plasma markers can distinguish EPTB from PTB and healthy controls in children [17]. In adults, plasma markers have also been shown to differ between PTB and EPTB [9]. However, these studies were exploratory and lacked specificity for Mtb infection. The present study significantly advances the field because it not only validates previously reported cellular biomarkers for identifying ATB in a Brazilian population [5], but also expands its potential use by accurately identifying EPTB cases. Thus, our results demonstrate that ATB patients can be identified by higher frequencies of $CD38^+$ IFN-γ^+, $HLADR^+$ IFN-γ^+ and $Ki67^+$ IFN-γ^+ $CD4^+$, and that persons with EPTB individuals are distinguished by even higher values of such markers compared to those with either LTBI or pulmonary TB disease alone.

Diagnosis of TB in HIV-positive individuals remains challenging. HIV-induced immunosuppression leads to reduced frequency of cavitation, further dampening the sensitivity of sputum-based [18] or radiographic-based assessments. Although clinical/empiric diagnoses are often used, these approaches can also be problematic since clinical manifestations of TB (pulmonary and extrapulmonary) are usually atypical. Moreover, the delayed time to diagnosis in HIV-positive individuals via microbiological-based techniques directly affects clinical prognosis, with increased odds of death and treatment failure [19, 20]. Our findings demonstrating that the higher frequencies of Mtb-specific $CD4^+$ T-cells expressing CD38, HLADR and Ki67 present in

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ATB patients with PTB and EPTB disease compared to those with LTBI, were not significantly influenced by HIV infection status are therefore of great interest. Our studies in HIV co-infected patients corroborate previously reported observations on PTB from South Africa [21, 22] and extend the utility of these biomarkers to diagnosis of EPTB in both HIV-positive and HIV-negative populations in Brazil. Thus, the Mtb-specific T cell-based assay described here can be used as a blood-based diagnostic tool for both PTB and EPTB for all individuals with presumptive TB, independent of their HIV co-infection status. Although flow-cytometry based TB diagnostic tests may not be feasible in some programmatic settings, efforts are underway to develop reasonably-priced commercial assays that could be used in clinical reference laboratories.

Our study has some limitations. We performed a cross-sectional investigation and examined samples obtained from a single timepoint, which preclude us from making conclusions about the utility of using these biomarkers for assessing response to treatment. However, we have previously reported on this in other populations [5]. The number of individuals investigated in this study was relatively small, but was determined by the study power calculations, and the groups were carefully matched to reduce the influence of potential confounding factors. In addition, because we did not have drug susceptibility test results, we were unable to investigate whether drug-susceptible vs. drug-resistant Mtb strains affected immune responses; this is an important avenue for future investigation. Among the persons living with HIV who were investigated here, $CD4^+$ T cell counts were ≥350 cells/μL, so no highly-immunosuppressed patients were examined. Future studies are necessary to test whether the performance of the biomarkers evaluated here are similar at lower $CD4^+$ T cell counts. All patients with pulmonary

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TB in our study exhibited positive AFB in sputum smears. A potential area of interest for future studies is testing the performance of these biomarkers in persons with lower mycobacterial loads (e.g. AFB-negative). Despite these limitations, our study presents robust data demonstrating that biomarkers on $CD4^+$ T cells can distinguish EPTB from LTBI as well as from PTB cases.

In summary, we have validated a reliable, fast, non-invasive blood-based approach that accurately identified patients with active TB compared to LTBI, and also distinguished PTB from EPTB—and both independent of HIV status. These observations are relevant to guide further development of point-of-care diagnosis for both active and latent TB.

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Figure 1. Expression of CD38, HLADR, and Ki67 on IFNγ$^+$CD4$^+$ T cells distinguishes active from latent TB.

(A) Frequencies of CD38$^+$, HLADR$^+$ and Ki67$^+$cells within IFNγ$^+$ CD4$^+$ T cells were examined in PBMCs stimulated with ESAT6-CFP10 peptide pools (10μg/mL) from individuals with latent TB infection (LTBI; n=50) or active TB (ATB; n=100). Lines represent median values and interquartile ranges. Data were analyzed using the Mann-Whitney *U* test. (B) Receiver Operator Characteristics (ROC) curve analyses of the frequency of CD38$^+$, HLADR$^+$ or Ki67$^+$IFNγ$^+$ CD4$^+$ T cells were used to test accuracy to distinguish ATB from LTBI. In (A), dashed lines represent the discrimination thresholds obtained in ROC curve analysis. (C) Graphs in (A) were merged. The red, dashed lines represent the discrimination threshold for each marker and show cutoff values of 18%, 60%, and 5% for CD38$^+$IFNγ$^+$, HLADR$^+$IFNγ$^+$, and Ki67$^+$IFNγ$^+$, respectively. Such thresholds were published previously [5]. AUC, the area under the curve. CI, confidence interval. Sens, sensitivity. Spec, specificity.

Figure 2. In HIV unexposed individuals, EPTB can be distinguished from PTB based on frequencies of IFNγ$^+$CD4$^+$ T cells lymphocytes expressing HLADR$^+$ and Ki67$^+$.

(A) Frequencies of total IFN-γ$^+$CD4$^+$ T cells as well as of CD38$^+$, HLADR$^+$, Ki67$^+$ cells within IFNγ$^+$ CD4$^+$ T-lymphocytes from PBMCs stimulated with ESAT6-CFP10 peptide pools (10μg/mL) obtained from HIV- unexposed healthy controls (HC; n=20), LTBI (n=50), EPTB (n=50), or PTB patients (n=50). Lines represent median values and interquartile ranges. Data from EPTB and PTB were compared using the Mann-Whitney test *U* test. (B) Receiver Operator Characteristics (ROC) curve analysis of frequencies of CD38$^+$, HLADR$^+$, and Ki67$^+$ and when all parameters were considered simultaneously to distinguish EPTB and PTB patients. AUC, an area under the curve. CI, confidence interval. Sens, sensitivity. Spec, specificity.

Figure 3. Frequencies of IFNγ$^+$ CD4$^+$ T cells expressing CD38, HLADR or Ki67 are not substantially altered in active TB patients living with HIV.

(A) Frequencies of IFNγ$^+$ CD4$^+$ T cells expressing CD38, HLADR or Ki67 from PBMCs stimulated with ESAT6-CFP10 peptide pools (10μg/mL) obtained from with PTB and or EPTB stratified by HIV infection status (50 HIV+ and 50 HIV- for each disease group). Lines represent median values and interquartile ranges. Data were compared between HIV+ and HIV- patients using the Mann-Whitney test *U* test. (B) The proportion of HIV+ patients presenting with virological suppression (HIV viral load < 80 RNA copies/μL) was compared between PTB and EPTB groups using Fisher's exact test. (C) CD4$^+$T cell counts were compared between PTB and EPTB patients with HIV using the Mann-Whitney test U test. (D) Spearman correlations between CD4$^+$ T cell counts and frequency of IFNγ+ CD4+ T lymphocytes expressing CD38, HLADR or Ki67 in HIV+ patients. Bars represent the strength of correlation (r values). Black bar indicates a statistically significant correlation while grey bars were nonsignificant. (E) The representative plot of correlation between CD4$^+$T cell counts and frequency of IFNγ$^+$ CD4$^+$ T cells expressing CD38 in EPTB-HIV+ patients lines represent linear regression with a 95% confidence interval.

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Tables, Figures and Legends

Table 1. Characteristics of the study participants.

Characteristic	HC	LTBI	PTB/HIV-	PTB/HIV+	EPTB/HIV-	EPTB/HIV+	P-value
N	20	50	50	50	50	50	
Age – median (IQR)	25 (20-32)	27 (20-33)	29 (19-34)	27 (19-31)	25 (21-29)	26 (20-32)	>.999
Male – no. (%)	10 (50)	25 (50)	25 (50)	25 (50)	25 (50)	25 (50)	>.999
Non-white race – no. (%)	17 (85)	45 (90)	45 (90)	48 (96)	43 (86)	49 (98)	.198
IDU – no. (%)	2 (10)	1 (2)	6 (12)	12 (14)	3 (6)	18 (36)	**<.001**
Smoking – no. (%)	1 (5)	3 (6)	5 (10)	10 (20)	3 (6)	7 (14)	.152
Alcohol abuse – no. (%)	5 (25)	10 (20)	12 (14)	22 (44)	21 (42)	18 (36)	.050
Prior tuberculosis – no. (%)	0 (0)	0 (0)	0 (0)	1 (2)	1 (2)	1 (2)	NA
AFB smear grade – no. (%)							
0	20 (100)	50	0 (0)	0 (0)	0 (0)	0 (0)	<.001
1+/scanty	0 (0)	0 (0)	1 (2)	30 (60)	15 (30)	35 (70)	
2+	0 (0)	0 (0)	24 (48)	15 (30)	30 (60)	13 (26)	
≥3+	0 (0)	0 (0)	25 (50)	5 (10)	5 (10)	2 (4)	

The Kruskal-Wallis test used to compare continuous variables between the groups and the distributions of age while the Pearson's chi-square test was used to compare frequencies. All TB patients had a positive culture for *M. tuberculosis*. P-values in bold font are statistically significant. AFB smear grade was compared between PTB and EPTB groups with or without HIV infection using the Pearson's chi-square test (the HC and LTBI groups were excluded from this analysis). AFB, acid-fast bacilli; EPTB, extrapulmonary tuberculosis; IDU, illicit drug use; IQR, interquartile range; HC, healthy controls; LTBI, latent tuberculosis infection; NA, non-applicable; PTB, pulmonary tuberculosis; TB, tuberculosis; TST, tuberculin skin test. Smear grade from sputum samples for PTB patients or lymph node aspirates for EPTB.

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Figure 1

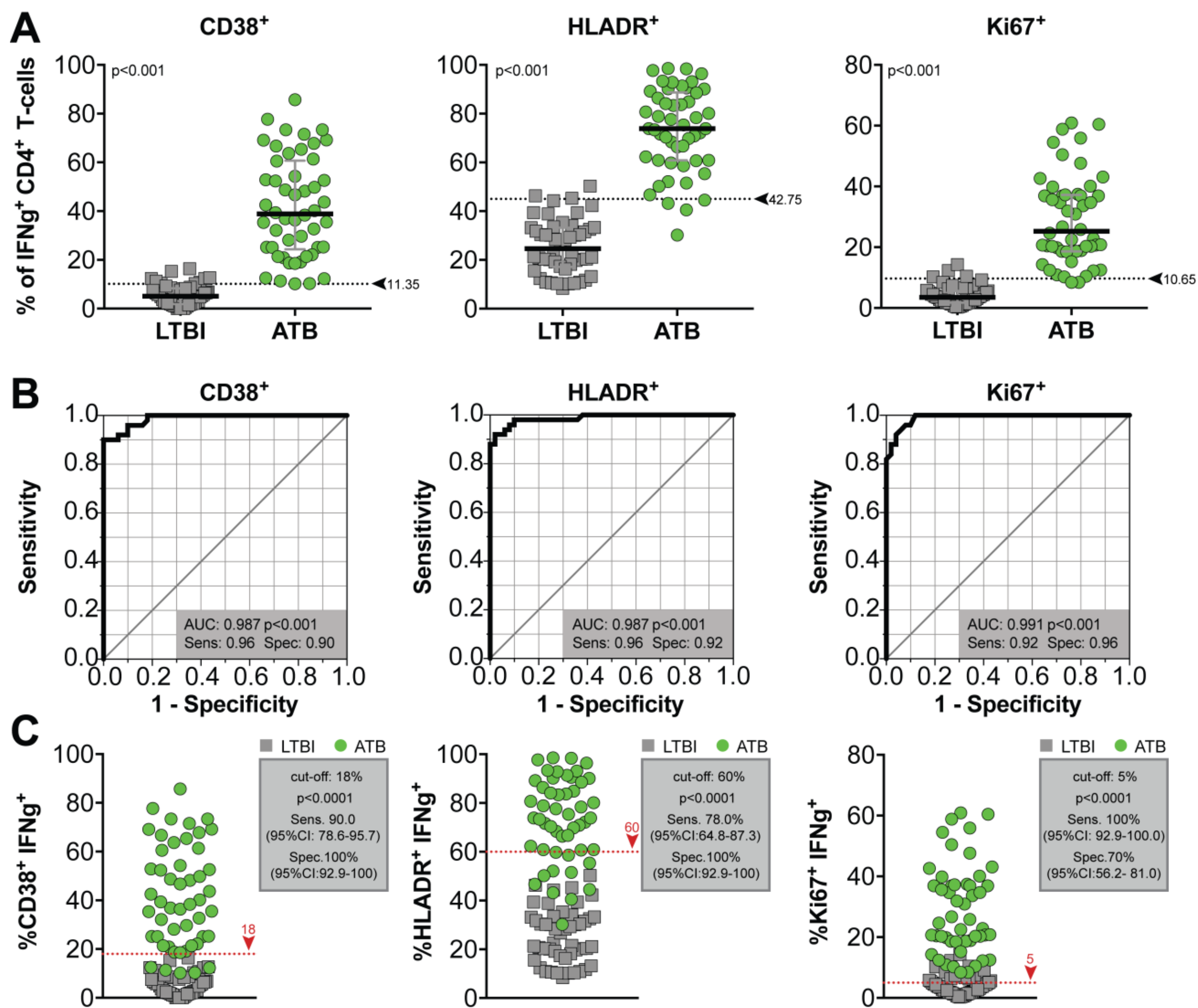

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Figure 2

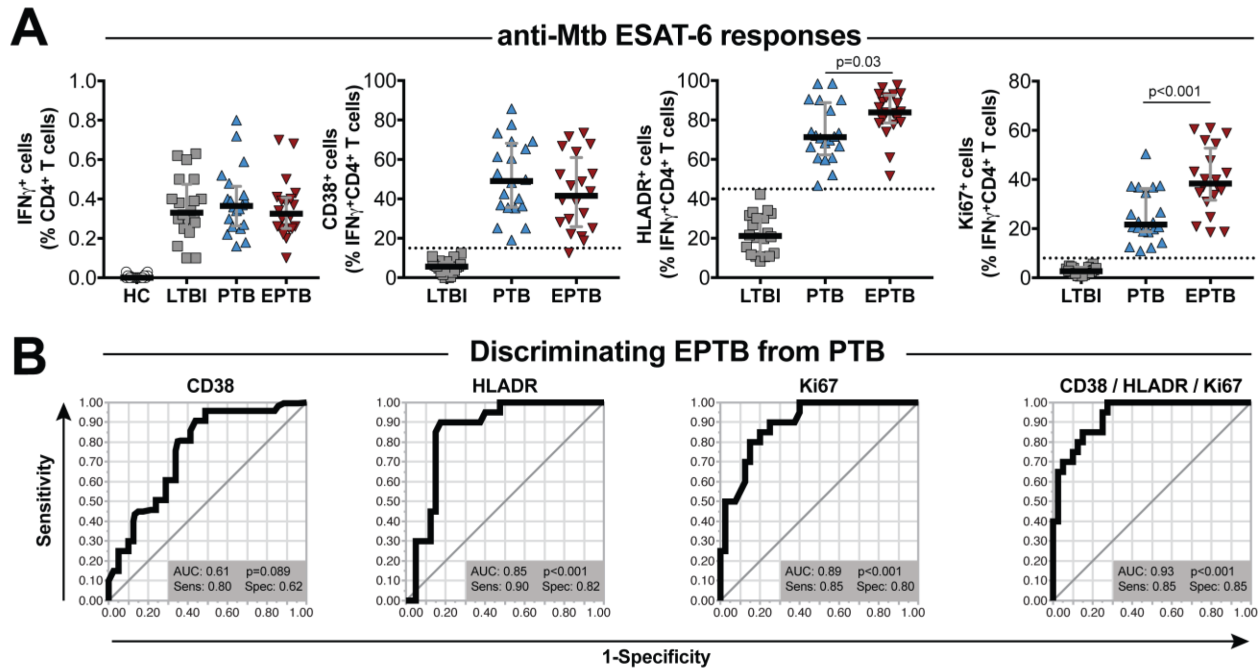

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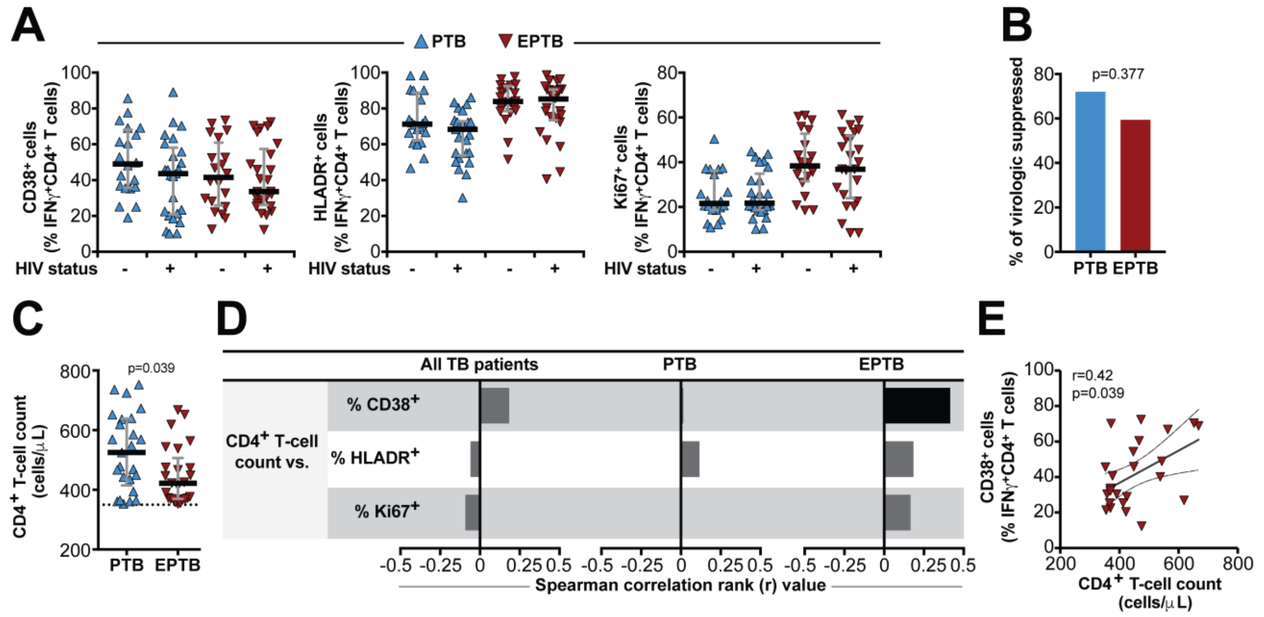
Figure 3



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