Online Data Supplement

Mycobacterium tuberculosis-specific CD4 T-cell scoring discriminates tuberculosis infection from disease

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Methods

Isolation and stimulation of peripheral blood mononuclear cells (PBMC): PBMC were purified by density gradient centrifugation within 8 hours after blood sampling.
PBMC were resuspended in RPMI 1640 medium (Biochrom, Berlin, Germany) containing 5% AB serum (Sigma-Aldrich, München, Germany), transferred into flat bottom 96-well plates (Falcon, Berlin, Germany), rested overnight at 37°C and 5% CO_2 and subsequently stimulated for 5 hours at a concentration of 3×10^6 PBMC/mL in a volume of 250 µl with PPD (5 µg/ml; Pharmore, Ibbenbüren, Germany) or ESAT-6/CFP-10 peptide pools (1 µg/ml; JPT, Berlin, Germany). As negative and positive control, PBMC were left unstimulated or were stimulated with SEB (5µg/ml; Sigma-Aldrich), respectively. To prevent internalization of CD154 (CD40L), a monoclonal CD40-blocking antibody (1 µg/mL; Miltenyi Biotec, Bergisch Gladbach, Germany) was added with the stimulants to each well.

Flow cytometry. After stimulation of the PBMC, CD154 and the T-cell activation markers CD38, HLA-DR and Ki67 on $CD4^+$ T cells were analysed by flow cytometry.
Antibodies used for surface staining: CD4-APC-Vio770 (clone M-T321), CD8-VioGreen (clone BW135/80), CD14-VioGreen (clone TÜK4), CD20-VioGreen (clone LT20), CD38-VioBright FITC (clone REA671), CD154-PE (clone 5C8) (all from Miltenyi Biotec GmbH) and HLA-DR-BV421 (clone L243; Biolegend, Koblenz, Germany). The Inside Stain Kit by Miltenyi Biotec was used for PBMC fixation and permeabilization. Ki-67 was intracellularly stained with the Ki-67-APC antibody (clone REA183; Miltenyi Biotec).
Stained cells were acquired on a Navios C10L flow cytometer (Beckman & Coulter, Krefeld, Germany) and analysed using Navios Cytometry List Mode Data Acquisition & Analysis Software (Beckman-Coulter).

Criteria for sample eligibility: Blood samples were considered eligible if the following conditions applied: A) PBMC isolation yielded at least 3.0×10^6 PBMC, B) before background correction, $CD154^+$ frequencies in the $CD4^+$ T cell populations of PPD- and ESAT-6/CFP-10-stimulated culture samples were at least twice the frequency in the negative control and C) the absolute background-corrected number of $CD154^+$ cells in the $CD4^+$ T cell population was at least 10. D) The negative control (unstimulated sample) showed no or negligible stimulation-independent activation and autofluorescence in the $CD4^+$ T cell population.

Supplementary Table E1: Characteristics of subjects of the TB infection study cohort [1]	
	No. of subjects (n=34)
Age (median, range)	42 (25-70)
Sex	
Male	23 (68%)
Female	11 (32%)
Race [2]	
White	25 (74%)
Black	6 (18%)
Asian	3 (9%)
American Indian	0 (0%)
History of earlier TB treatment	3 (9%)
BCG vaccination [3]	
Yes	1 (3%)
No	2 (6%)
Unknown [4]	31 (91%)
Concurrent acute diseases	11 (32%)
Infections	5 (15%)
Strongyloides infection	1 (3%)
Other bacterial infection	4 (12%)
Non-infectious diseases	6 (18%)
Gastritis	1 (3%)
Opthalmologic/rheumatic disease	5 (15%)
Chronic diseases	
Chronical pain syndrome	1 (3%)
Evans syndrome	1 (3%)

[1] Patients were diagnosed with TB infection or had a history of TB treatment completed at least six months before, and showed no evidence of TB disease during further evaluation. All patients had a positive IGRA.

[2] Collection of Race and Ethnicity Data in Clinical Trials. Guidance for Industry and Food and Drug Administration Staff. Document issued on October 26, 2016. October 2016. Clinical Medical. Dockets Management Food and Drug Administration, 5630 Fishers Lane, Rm 1061, Rockville, MD 20852. FDA-2016-D-3561.

[3] Bacille Calmette–Guérin.

[4] Patients' medical history did not show an indication of a former BCG vaccination.

Supplementary Table E2: Characteristics of patients in TB disease study cohort	
	No. of patients (n=47)
Age (median, range)	33 (19-84)
Sex	
Male	29 (62%)
Female	18 (38%)
Race [1]	
White	24 (51%)
Black	10 (21%)
Asian	12 (26%)
American Indian	1 (2%)
Concurrent acute diseases (other than TB)	
Pulmonary NTM infection [2]	2 (4.3%)
Bacterial Pneumonia	1 (2.1%)
History of earlier TB treatment	5 (11%)
BCG vaccination [3]	
Yes	3 (6%)
No	7 (15%)
Unknown [4]	37 (79%)
Clinical TB disease classification [5]	
Pulmonary TB disease	28 (60%)
Extrapulmonary TB disease	17 (36%)
Pulmonary and extrapulmonary TB disease	2 (4%)
Resistance	
Monoresistance	2 (4%)
MDR	1 (2%)
XDR	1 (2%)
Positive culture results for *M. tuberculosis* complex [6]	
Respiratory secretions [7]	30 (64%)
Lymph nodes	10 (21%)
Cervical	5 (10%)
Other [8]	5 (10%)
Bone biopsy	3 (6%)
Lung biopsy	2 (4%)
Ascites	2 (4%)
Mamma fistula secretion	1 (2%)
Chest wall abscess secretion	1 (2%)
Peritoneal infiltrations/abdominal lymph nodes	2 (4%)
Glandular parotis cyst secretion	1 (2%)
Pleural fluid	1 (2%)
Urine	1 (2%)

[1] Collection of Race and Ethnicity Data in Clinical Trials. Guidance for Industry and Food and Drug Administration Staff. Document issued on October 26, 2016. October 2016. Clinical Medical. Dockets Management Food and Drug Administration, 5630 Fishers Lane, Rm 1061, Rockville, MD 20852. FDA-2016-D-3561

[2] *Mycobacterium intracellulare* and *Mycobacterium abscessus* isolated from respiratory secretions were finally determined to be bystanders/colonizers.

[3] Bacille Calmette–Guérin.

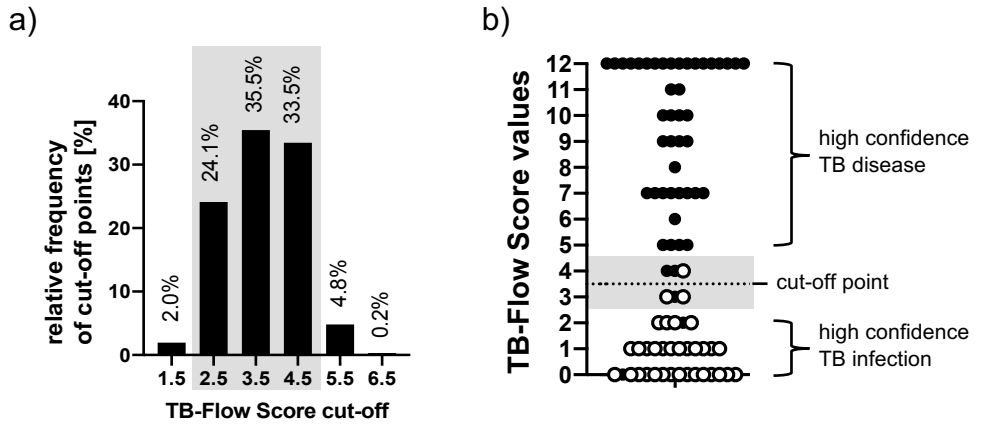
[4] Patients' medical history did not show an indication of a former BCG vaccination.

[5] Defined by culture-positive specimens from respective sites.

[6] Number of culture positive specimens may exceed number of patients as there are patients with more than one positive specimen.

[7] Including sputum, tracheobronchial secretions, and bronchoalveolar lavage (BAL).

[8] Supraclavicular, submandibular, retroperitoneal or mediastinal/hilar lymph nodes.



Supplementary Figure E1: Cut-off distribution in Monte-Carlo cross validation.
a) Histogram of relative frequencies of the cut-off points for TB-Flow Score calculated in 10,000 iterations of Monte Carlo cross validation. A total of 93.1% of calculated cut-off points fall into the range of 2.5 to 4.5 (highlighted in grey). **b)** Proposed ranges of high confidence for TB disease and TB infection classification encompass TB-Flow Score values above 4.5 and below 2.5, respectively. Dotted line: TB infection/disease cut-off point 3.5; Grey area: range of cut-off points 2.5 to 4.5; Full circles: patients with TB disease; Empty circles: subjects with TB infection.

Supplementary Table E3: Characteristics of patients classified as TB disease case without culture confirmation (not included in TB disease study cohort) and eligible for testing with TB-Flow Assay [1,2]	
	No. of patients (n=13)
Age (median, range)	37 (18-73)
Sex	
Male	8 (62%)
Female	5 (38%)
Concurrent acute diseases (other than TB)	0 (0%)
Chronic diseases	
Chronic Hepatitis B infection	2 (15%)
Diabetes type II	1 (8%)
History of earlier TB treatment	3 (23%)
IGRA results	
IGRA-positive	12 (92.3%)
IGRA-negative	0 (0%)
No IGRA results available	1 (8%)
Clinical TB classification [3]	
Pulmonary TB disease	3 (23%)
Extrapulmonary TB disease	10 (77%)
Suggestive results for TB disease	
Chest X-Ray findings only	6 (46%)
Positive examinations for TB disease (other than culture) [4]	7 (54%)
Positive PCR	4 (31%)
Lymph node	2 (15%)
Mastoid process	1 (8%)
Thyroid gland	1 (8%)
Necrotizing granulomas	7 (54%)
Lymph node	3 (23%)
Mastoid process	1 (8%)
Pleural infiltration	1 (8%)
Thyroid gland	1 (8%)
Peritoneal infiltrations	1 (8%)

[1] Diagnosed as TB disease despite negative culture results, based on positive PCR results, demonstration of TB-typical tissue manifestations (e.g., necrotizing granulomas with or without demonstration of acid-fast bacilli), suggestive clinical features or decision for empiric anti-tuberculosis treatment.

[2] Patients that did not fulfil the test criteria or had been under therapy for more than 5 days were excluded.

[3] Defined by clinical evidence of organ involvement based on positive PCR results, demonstration of TB-typical tissue manifestations and/or suggestive clinical features.

[4] Number of TB-positive results may exceed number of patients as there are patients with more than one positive result.

Supplementary Table E4: Cut-off, AUC, sensitivity and specificity of the analysed marker populations *

PPD

	CD38+CD154+ of CD4+	Ki-67+CD154+ of CD4+	HLA-DR+CD154+ of CD4+	CD38+ of CD154+CD4+	Ki-67+ of CD154+CD4+	HLA-DR+ of CD154+CD4+
cut-off	0.056%	0.00065%	0.068%	22.8%	0.45%	23.6%
AUC	0.908	0.861	0.825	0.925	0.851	0.921
95% CI	0.842 to 0.974	0.778 to 0.944	0.735 to 0.915	0.869 to 0.981	0.766 to 0.936	0.858 to 0.984
sensitivity	76.6%	78.7%	68.1%	78.7%%	68.1%	89.4%
95% CI	62.0% to 87.7%	64.3% to 89.3%	52.9% to 80.9%	64.3% to 89.3%	52.9% to 80.9%	76.9% to 96.5%
specificity	97.1%	88.2%	85.3%	97.1%	97.1%	94.1%
95% CI	84.7% to 99.9%	72.6% to 96.7%	68.9% to 95.1%	84.7% to 99.9%	84.7% to 99.9%	80.3% to 99.3%

ESAT-6/CFP-10

	CD38+CD154+ of CD4+	Ki-67+CD154+ of CD4+	HLA-DR+CD154+ of CD4+	CD38+ of CD154+CD4+	Ki-67+ of CD154+CD4+	HLA-DR+ of CD154+CD4+
cut-off	0.019%	0.00045%	0.018%	27.8%	0.11%	29.0%
AUC	0.905	0.823	0.820	0.911	0.787	0.838
95% CI	0.843 to 0.967	0.732 to 0.914	0.726 to 0.913	0.847 to 0.975	0.684 to 0.890	0.747 to 0.929
sensitivity	70.2%	57.5%	70.2%	83.0%	70.2%	76.6%
95% CI	55.1% to 82.7%	42.2% to 71.1%	55.1% to 82.7%	69.2% to 92.4%	55.1% to 82.7%	62% to 87.7%
specificity	100.0%	100.0%	91.2%	91.2%	88.2%	85.3%
95% CI	89.7% to 100.0%	89.7% to 100.0%	76.3% to 98.1%	76.3% to 98.1%	72.5% to 96.7%	68.9% to 95.1%

Scores

	TB-Flow Score	PPD Score
cut-off	3.5	1.5
AUC	0.978	0.975
95% CI	0.945 to 1.000	0.942 to 1.000
sensitivity	93.6%	95.7%
95% CI	82.5% to 98.7%	85.5% to 99.5%
specificity	97.1%	94.1%
95% CI	84.7% to 99.9%	80.3% to 99.3%

* Sensitivity and specificity values refer to sensitivity and specificity at the given cut-offs.

Supplementary Table E5: Characteristics of patients with false-negative or false-positive TB-Flow Assay results.							
	Patient age and sex	**TB-Flow Score (cut-off: 3.5)**	**Clinical background**	**IGRA**	**Signs and symptoms, clinical findings**	**Mycobacteriological results for TB disease**	**Speculative explanation for discrepant TB-Flow Assay results**
Culture-confirmed TB disease with false-negative TB-Flow Assay results falsely indicating TB infection							
1	22 yr, female	2 of 12	Evaluation and removal of glandular parotid cyst without primary suspicion of TB. Recent history of pregnancy.	Negative.	Cyst right salivary gland. No other S/S. Physical and imaging examinations otherwise unremarkable.	Cyst content PCR- and culture-positive without any suggestive histological findings.	Subclinical [E1] TB with insufficient sensitization of lymphocytes. Low frequency of TB-specific T cells caused by impaired cellular T_H1 response due to recent pregnancy.
2	47 yr, male	3 of 12	Suspicion of urogenital infection.	Not done	S/S of urogenital infection. Physical and imaging examinations otherwise unremarkable.	Urine culture-positive. Bladder biopsies showing TB-typical histological findings. Examinations of sputum negative (culture, AFB, PCR).	Urogenital TB disease representing a sanctuary with insufficient sensitization of lymphocytes. Borderline negative response owed to downregulation of activation markers with TB therapy for 5 days.
3	44 yr, female	0 of 12	Routine TB screening because of refugee background.	Positive	No S/S. Thorax CT scan in-conspicuous except for calcified subcarinal LN.	Subcarinal LN needle puncture culture-positive. Negative for all examinations of sputum and bronchial secretion (culture, AFB, PCR).	Long-term asymptomatic, immunologically contained TB disease with desensitization of lymphocytes. Possibly weakened immune response due to refugee status.
TB infection with false-positive TB-Flow Assay results falsely indicating TB disease							
4	41 yr, male	4 of 12	Uveitis primarily considered a result of rheumatic disease. TB screening before immuno-suppressive therapy. TB chemo-prevention administered.	Positive. Two years earlier negative.	No S/S except uveitis.	All examinations of sputum and bronchial secretions negative (culture, AFB, PCR).	Incipient/subclinical [E1] TB leading to detectable lymphocyte sensitization prior to immunological containment or evolution to TB disease.

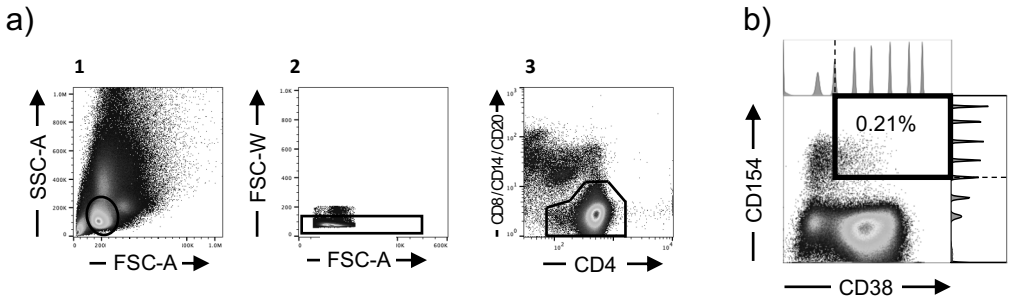
LN = lymph node, S/S = signs and symptoms, TB = tuberculosis, AFB = acid-fast bacilli, IGRA = interferon-gamma-release assay

E1. Drain PK, Bajema KL, Dowdy D, Dheda K, Naidoo K, Schumacher SG, Ma S, Meermaier E, Lewinson DM, Sherman DR. Incipient and subclinical tuberculosis: A clinical review of early stages and progression of infection. *Clin Microbiol Rev* 2018;31(4)

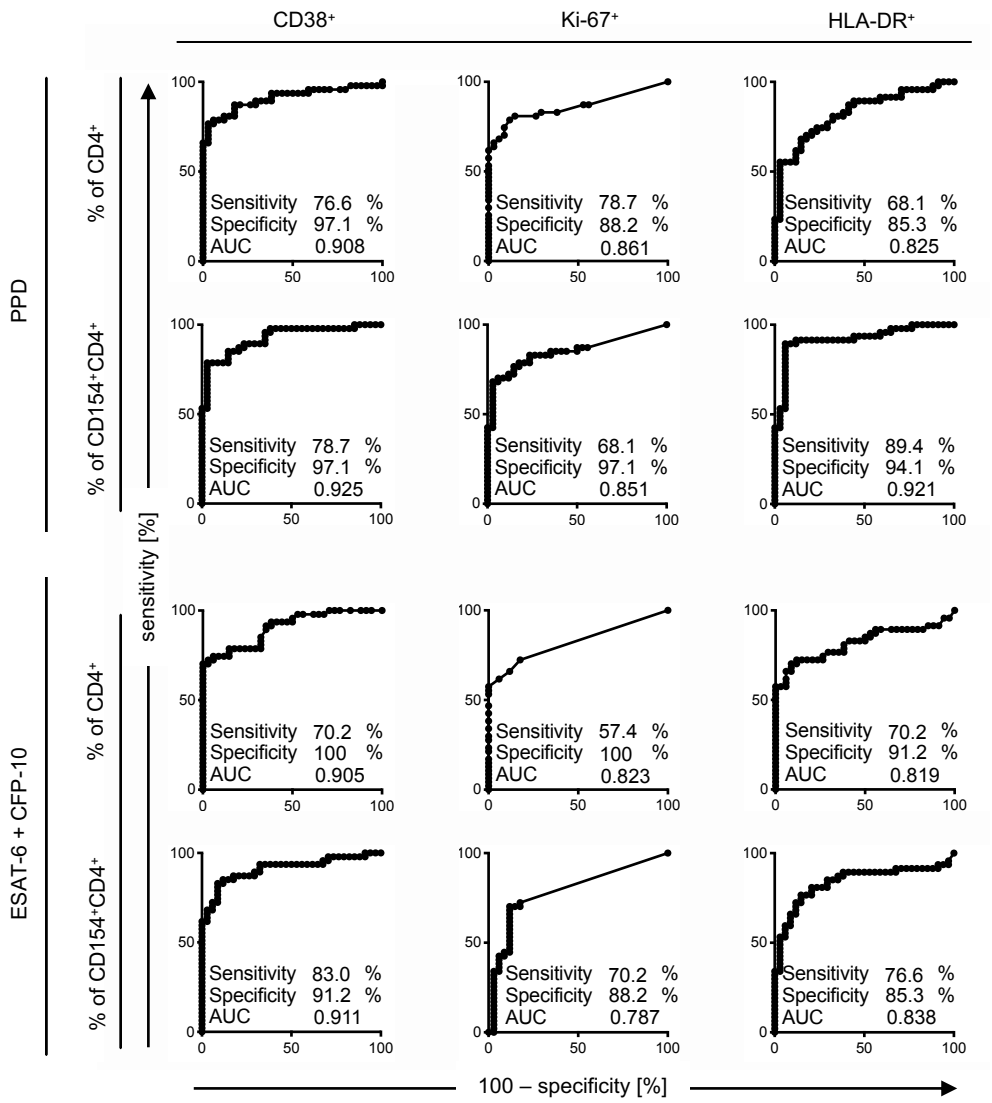
CFP-10	
Peptide-ID	**Sequence**
TB-C10-01	MAEMKTDAATLAQEA
TB-C10-05	KTDAATLAQEAGNFE
TB-C10-09	ATLAQEAGNFERISG
TB-C10-13	QEAGNFERISGDLKT
TB-C10-17	NFERISGDLKTQIDQ
TB-C10-21	ISGDLKTQIDQVEST
TB-C10-25	LKTQIDQVESTAGSL
TB-C10-29	IDQVESTAGSLQGQW
TB-C10-33	ESTAGSLQGQWRGAA
TB-C10-37	GSLQGQWRGAAGTAA
TB-C10-41	GQWRGAAGTAAQAAV
TB-C10-45	GAAGTAAQAAVVRFQ
TB-C10-49	TAAQAAVVRFQEAAN
TB-C10-53	AAVVRFQEAANKQKQ
TB-C10-57	RFQEAANKQKQELDE
TB-C10-61	AANKQKQELDEISTN
TB-C10-65	QKQELDEISTNIRQA
TB-C10-69	LDEISTNIRQAGVQY
TB-C10-73	STNIRQAGVQYSRAD
TB-C10-77	RQAGVQYSRADEEQQ
TB-C10-81	VQYSRADEEQQQALS
TB-C10-85	RADEEQQQALSSQMGF

ESAT-6	
Peptide-ID	**Sequence**
TB-E6-01	MTEQQWNFAGIEAAA
TB-E6-05	QWNFAGIEAAASAIQ
TB-E6-09	AGIEAAASAIQGNVT
TB-E6-13	AAASAIQGNVTSIHS
TB-E6-17	AIQGNVTSIHSLLDE
TB-E6-21	NVTSIHSLLDEGKQS
TB-E6-25	IHSLLDEGKQSLTKL
TB-E6-29	LDEGKQSLTKLAAAW
TB-E6-33	KQSLTKLAAAWGGSG
TB-E6-37	TKLAAAWGGSGSEAY
TB-E6-41	AAWGGSGSEAYQGVQ
TB-E6-45	GSGSEAYQGVQQKWD
TB-E6-49	EAYQGVQQKWDATAT
TB-E6-53	GVQQKWDATATELNN
TB-E6-57	KWDATATELNNALQ

Supplementary Table E6: Sequences of the ESAT-6 and CFP-10 peptides used for stimulation of antigen-specific $CD4^+$ T cells. Peptide length was 14-16 amino acids with 11 amino acids overlap between peptides.

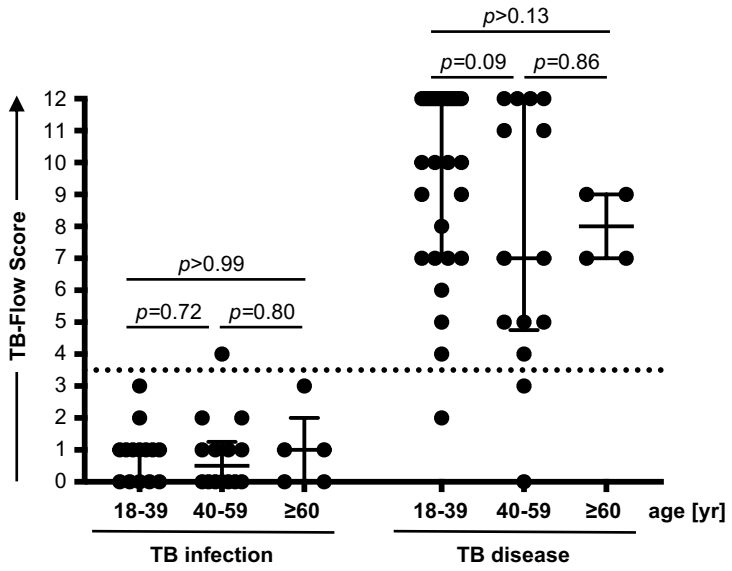


Supplementary Figure E2: Gating strategy and gating using Calibration Beads.
A) Gating strategy: 1. gating of lymphocytes, 2. exclusion of cell aggregates, 3. gating of $CD4^+$ T cells ($CD4^{+/intermediate}CD8^-CD14^-CD20^-$ lymphocytes). **B)** Example for gating marker-positive TB-specific $CD4^+$ T cells using *8 peak Rainbow Calibration Beads*. Each flow cytometry analysis included an acquisition of *8 peak Rainbow Calibration Beads* that exhibit defined fluorescence peaks in all marker channels. The gate boundaries for the populations of antigen-reactive marker-positive cells (i.e., $CD38^+CD154^+$, Ki-67^+CD154^+ and HLA-DR^+CD154^+ cells) were set according to the position of predefined bead peaks (e.g., peak 5 in channel CD154 and peak 6 in channel CD38), minimizing the effects of day-to-day fluctuations and long-term deviation of brightness in the utilized channels on the measurement result.



Supplementary Figure E3: ROC curves for differentiation of TB infection and TB disease by marker expression in TB antigen-specific CD4$^+$ T cells.

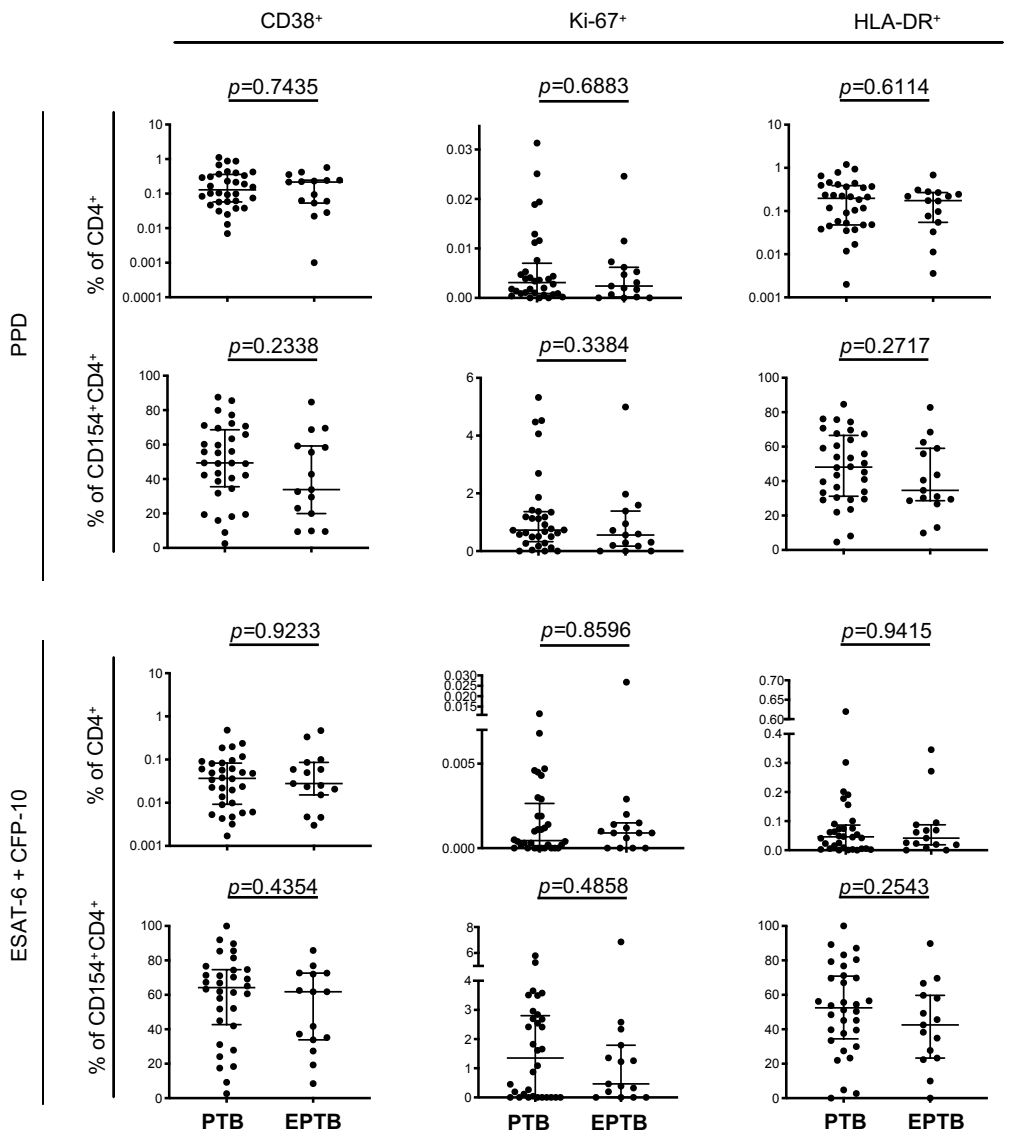
ROC analysis was performed for PPD-specific and ESAT-6/CFP-10-specific CD38$^+$CD154$^+$, HLA-DR$^+$CD154$^+$ and Ki-67$^+$CD154$^+$ T cells within total CD4$^+$ T cells and within CD154$^+$CD4$^+$ T cells. Sensitivity, specificity and AUC (area under curve) for every cell population are given under the ROC curves.



Supplementary Figure E4: TB-Flow Score of subjects with TB infection and TB disease grouped by age.
Subjects with TB infection (left) and TB disease (right) in the age groups 18 to 39 years, 40 to 59 years and 60 years and older are shown with median and IQR.

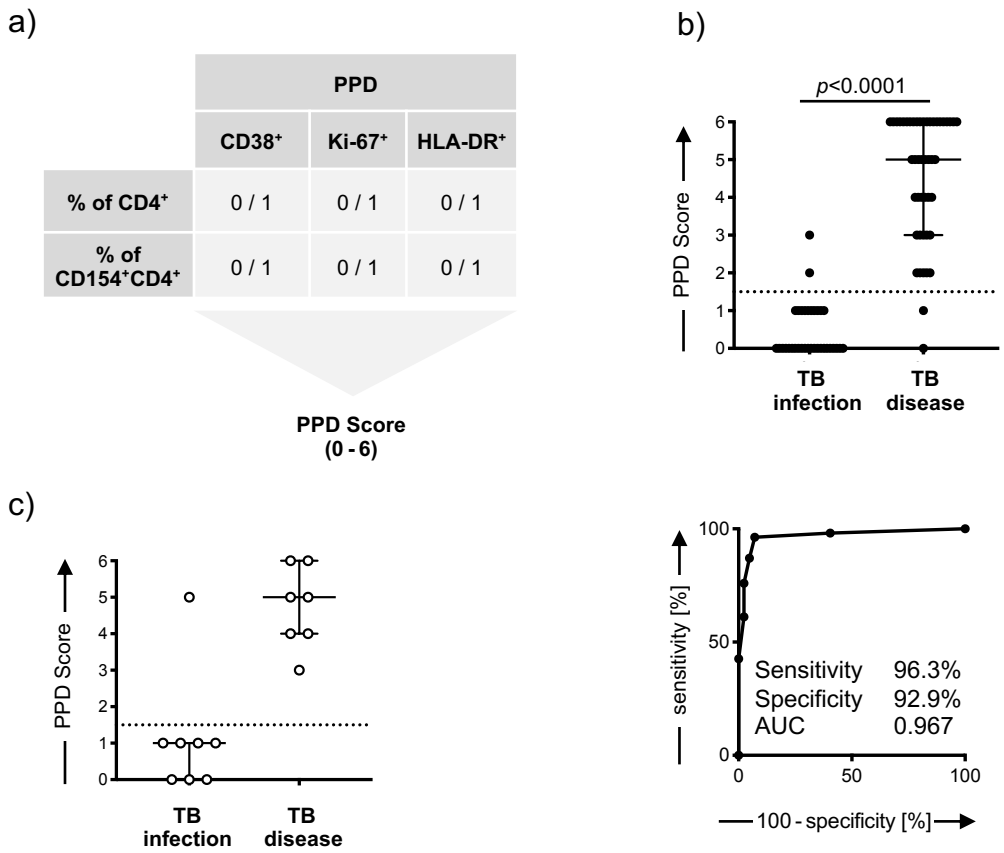
Characteristics of groups:

group	number of subjects	median age (range)	median TB-Flow Score (IQR)
TB infection			
18-39 yr	14	31 yr (25-38 yr)	1 (0.0-1.0)
40-59 yr	15	45 yr (41-59 yr)	0.5 (0.0-1.3)
≥60 yr	5	64 yr (62-70 yr)	1 (0.0-2.0)
TB disease			
18-39 yr	29	29 yr (19-38 yr)	10 (7.0-12.0)
40-59 yr	14	47 yr (40-52 yr)	7 (4.8-12.0)
≥60 yr	4	70 yr (64-84 yr)	8 (7.0-9.0)



Supplementary Figure E5: Frequencies of TB antigen-reactive marker-positive CD4$^+$ T cells in subjects with pulmonary and extrapulmonary TB disease.

Shown are the frequencies of PPD-specific (top) and ESAT-6/CFP-10-specific (bottom) CD4$^+$ T cells expressing the markers CD38, Ki-67 or HLA-DR within total CD4$^+$ T cells or CD154$^+$CD4$^+$ T cells in subjects with pulmonary (PTB) and extrapulmonary (EPTB) TB disease. Median and interquartile range are marked in the dot plots. The populations of patients with PTB and EPTB were compared using the Mann-Whitney test. No statistically significant differences in the frequency of marker-positive CD4$^+$ T cells were observed for patients with PTB and EPTB.



Supplementary Figure E6: PPD score and results of participants excluded for a missing peptide response

A) The PPD score is part of the TB-Flow Score. It is based on analysis of the frequencies of $CD38^+$, $HLA\text{-}DR^+$ and $Ki\text{-}67^+$ $CD4^+$ T cells reactive to PPD. It is calculated by comparison of marker frequencies within total $CD4^+$ T cells and within $CD154^+CD4^+$ T cells with cut-offs specific for each cell population, and corresponds to the number of cell populations with a frequency of marker-positive cells above the cut-off. Six combinations of marker and reference cell population are analysed. The PPD Score can assume values from 0 to 6. Since PPD is known to also elicit T cell responses in TB patients as well as NTM-infected patients the PPD Score is not TB-specific. **B)** Shown are the PPD Score values for the TB infection and TB disease study cohorts (top) and the ROC curve for discrimination of the two cohorts (bottom) based on the PPD Score. Median, IQR and the infection/disease cut-off (dotted line) are marked in the plot. The infection/disease cut-off of the PPD Score was calculated using ROC analysis and Youden's index. **C)** PPD scores of participants with confirmed TB infection or TB disease that were excluded from the validation cohorts due to a missing response to the TB-peptide antigens (ESAT-6 + CFP-10). Using the PPD Score the infection state of ESAT-6/CFP-10 non-responders can be assessed.