Bifunctional T-Cell-Derived Cytokines for the Diagnosis of Tuberculosis and Treatment Monitoring

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Key Words

Interferon- γ · Interferon- γ release assays · Interleukin-2 · Latent *Mycobacterium tuberculosis* infection · Tuberculosis

Abstract

Background: Diagnosis and treatment monitoring of patients with tuberculosis remain challenging. **Objective:** We have evaluated whether *Mycobacterium*-specific interferon (IFN)- γ and interleukin (IL)-2 bifunctional cytokine immune response assays improve the diagnosis of and correlate to treatment response in pulmonary tuberculosis. **Methods:** Early secretory antigenic target (ESAT)6/culture filtrate protein 10 (CFP10), microsomal triglyceride transfer protein 65 (MTP65) and the purified protein derivative (PPD) tuberculin-specific immune profiles were investigated in peripheral blood mononuclear cells from 19 patients with culture-confirmed tuberculosis and 23 healthy community controls (HCCs; 82.6% with latent *M. tuberculosis* infection) using a novel fluorescence-based dual-colour enzyme-linked im-

munospot (EliSpot) technology (FluoroSpot). **Results:** The frequency of ESAT6/CFP10-induced IFN- γ +IL-2[–] producing cells was elevated ($p < 0.001$), whereas the percentages of specific IFN- γ -IL-2⁺ ($p = 0.002$) and IFN- γ +IL-2⁺ double producing cells ($p = 0.037$) were diminished in tuberculosis patients in comparison to HCCs. A 3-host marker model using a combination of those IFN- γ and IL-2 single-cell responses showed 93.8% sensitivity and 77.8% specificity for tuberculosis. During tuberculosis treatment, the PPD-induced immune responses shifted from an IFN- γ +IL-2[–] dominated profile towards a balance of IFN- γ -IL-2⁺ and IFN- γ +IL-2⁺ double producing cells (all $p \leq 0.05$). **Conclusions:** The addition of antigen-specific IL-2 production to IFN- γ responses by EliSpot in IFN- γ release assays increases diagnostic sensitivity for active tuberculosis.

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Introduction

Tuberculosis is an airborne infectious disease. In the year 2011, approximately 8.7 million people developed tuberculosis and 1.4 million human deaths were attributed to this disease globally [1]. In order to identify individuals with latent *Mycobacterium tuberculosis* infection (LTBI) and to prevent the development of active tuberculosis, targeted immunological testing for acquainted *M. tuberculosis*-specific immune responses is performed in close household contacts of infectious patients, persons with HIV infection and other individuals from groups at risk of developing tuberculosis [2]. In the absence of active tuberculosis, those individuals with an adaptive immune response against *M. tuberculosis*, which was assessed using the tuberculin skin test (TST) or an interferon (IFN)- γ release assay (IGRA), are offered preventive chemotherapy [3].

One of the major limitations of the currently available immunodiagnostic methods for *M. tuberculosis* infection is that neither the TST nor IGRAs are able to distinguish between individuals with LTBI and active tuberculosis [4, 5]. In principle, discrimination between different states of *M. tuberculosis* infection is possible by immunodiagnostic testing. Lately, it has been shown that the proportion of single-positive tumour necrosis factor- α -releasing *M. tuberculosis*-specific CD4⁺ T cells is indicative for active tuberculosis, in contrast to LTBI [6].

One of the most promising cytokine candidates to improve the immunodiagnosis of tuberculosis is the interleukin (IL)-2 [7, 8]. Millington et al. [9] have reported a correlation between mycobacterial load and the frequency of IL-2⁺ and IFN- γ ⁺ secreting T cells. Single IFN- γ ⁺ secreting cells dominate the cellular immune response in untreated active tuberculosis. This immune dominance progressively decreased during tuberculosis treatment while single IL-2⁺ secreting T cell frequency increased.

The preferred technique to evaluate immunophenotypes on unstimulated and stimulated cells for the differentiation of active tuberculosis from LTBI is fluorescence-activated cell sorting (FACS). Enzyme-linked immunospot (EliSpot) analysis is a less expensive, highly sensitive, quantitative and easy-to-use method, yet misses the ability of immunophenotyping by FACS. In order to overcome limitations of the EliSpot analysis, a new dual-colour EliSpot technique has been developed, which allows to distinguish the expression of two cytokines at a single-cell level, which is cheaper and easier to perform than FACS analysis.

This study evaluates the utility of an IFN- γ /IL-2 dual-colour EliSpot to discriminate between tuberculosis and LTBI and to monitor specific cytokine profiles during tuberculosis treatment.

Materials and Methods

Participants

Following written informed consent, adult HIV-seronegative suspects with a first episode of tuberculosis and healthy HIV-seronegative community controls (HCCs) were recruited at local health care centres in the Ravensmead/Uitsig community in Cape Town, South Africa. Pregnancy, chronic cardiovascular or metabolic diseases, immunosuppressive medication and age less than 21 years constituted exclusion criteria. All participants had routinely been vaccinated at birth with *M. bovis* BCG (Danish strain 1331; Statens Serum Institute, Copenhagen, Denmark). Participants with suspected tuberculosis presented with clinical symptoms of cough, fever, night sweats and/or weight loss. Chest X-rays, sputum smear microscopy for the presence of acid-fast bacilli and *M. tuberculosis* culture (BACTEC MGIT 960 system; Becton Dickinson Biosciences, Johannesburg, South Africa) of one sputum sample were performed on all study participants. Tuberculosis was diagnosed by culture positivity. Individuals who were suspected to have tuberculosis with a negative *M. tuberculosis* sputum culture were excluded from further participation in the study. For all culture-confirmed tuberculosis patients, clinical records attested full sensitivity against the first-line drugs. All tuberculosis patients received standard treatment consisting of a 2-month intensive phase with isoniazid, rifampicin, ethambutol and pyrazinamide followed by 4 months of isoniazid and rifampicin.

Volunteers from the same community with identical ethnical and social background who lived in a household with proven active tuberculosis patients within the past 3 months were recruited as HCCs. Their *M. tuberculosis* infection status was investigated either by a TST (2 U tuberculin RT23; Statens Serum Institute) or with a QuantiFERON TB Gold in-tube test (QFT; Cellestis, Carnegie, Vic., Australia). A TST >10 mm in diameter was regarded as positive. QFT positivity was defined according to the recommendations of the manufacturer (the cut-off value for a positive test was >0.35 IU/ml). The study was approved by the Ethics Committee for Human Research of the Stellenbosch University, South Africa (N10/08/274), and the University of Lübeck, Germany (05-096).

Blood Processing

Five millilitre of heparinised peripheral blood were collected by venipuncture and processed within 2 h. Peripheral blood mononuclear cells (PBMCs) were isolated using Histopaque density gradient centrifugation of blood layered on Ficoll-Paque Plus (Sigma Aldrich, Johannesburg, South Africa) [10].

Antigens and Antibodies

Monoclonal anti-CD28 was obtained from AID Autoimmun Diagnostika (AID) GmbH and diluted 1:1,000 with the cell suspension. Recombinant early secretory antigenic target 6 (ESAT6)/culture filtrate protein 10 (CFP10) and microsomal triglyceride

transfer protein 65 (MTP65) from AID were used as *M. tuberculosis*-specific antigens in a ready-to-use format (no specific information concerning ESAT6/CFP10 and MTP65 concentrations was provided by the manufacturer). Purified protein derivative (PPD; Statens Serum Institute) was used at 10 µg/ml. Pokeweed mitogen (5 µg/ml; Biochrom AG, Berlin, Germany) and 10 ng/ml anti-CD3 (clone X35; Beckman Coulter GmbH, Krefeld, Germany) were used as positive controls; 96-well plates had been pre-coated by AID with the two capture antibodies anti-human IL-2 and anti-human IFN-γ. Concentrates of biotin-conjugated anti-human IL-2 and fluorescein isothiocyanate (FITC)-conjugated anti-human IFN-γ secondary antibodies as well as the streptavidin Red-(Cy3) conjugate and anti-FITC green were provided from AID and diluted as recommended by the manufacturer.

Dual-Colour EliSpot for the Detection of Cells Secreting IL-2+ and IFN-γ+

PBMCs (2×10^5) were aliquoted into all designated wells, enriched with 5% foetal bovine serum (Sigma Aldrich) and co-stimulated with anti-CD28. The PBMCs were cultured overnight (16 h) in six different conditions in duplicate: 100 µl RPMI 1640 (Sigma Aldrich; negative control), 100 µl ESAT6/CFP10, 100 µl MTP65, 10 µg/ml PPD, 5 µg/ml pokeweed (positive control) and anti-CD-3 (1 µg/ml/well; positive control). The following day, cells were washed off manually (AID wash buffer). The detection antibodies biotin-conjugated anti-human IL-2 and FITC-conjugated anti-human IFN-γ were diluted (AID dilution buffer), and 100 µl of this secondary antibody suspension were added per well and plates were incubated for 2 h in a humid dark chamber. Unbound detection antibody was washed off. Streptavidin Red-(Cy3) conjugate and anti-FITC green were diluted and plates were incubated (100 µl/well) for 1 h in a humid dark chamber. Plates were washed and 100 µl of the ready-to-use AID enhancer were added and incubated for 15 min; subsequently, the plate was blotted on a stack of towel paper and left to dry overnight in a dark place protected from light. IL-2/streptavidin Red-(Cy3) and IFN-γ/anti-FITC green spots are invisible to the naked eye and can be read by a dual-colour EliSpot Reader system (AID) as two independent signals.

Data Analysis

The AID EliSpot software ELRIFLO5 counted all IFN-γ+ and all IL-2+ producing cells and created an artificial image highlighting the double-stained IFN-γ+IL-2+ spots (fig. 1). Cytokine-producing cells were counted as spot-forming cells (SFCs). Results were analysed and interpreted according to the manufacturer's guidelines. The background response of the negative control was deducted from the stimulated wells. For a correct test interpretation, the positive controls (pokeweed mitogen or anti-CD3) had to induce more than 50 SFCs/ 2×10^5 cells/well of IL-2+ and more than 50 SFCs/ 2×10^5 cells/well IFN-γ+ secreting cells after subtraction of the number of spots in the negative control well and at least twice the number of spots of the negative control well, otherwise the test was indeterminate. Providing that a valid positive control result was found, the net response to ESAT6/CFP10, MTP65 or PPD was considered positive when the test well had at least 10 SFCs and twice the number of SFCs in the negative control well for both cytokines. If PBMCs from a participant showed a specific (>10 SFCs/ 2×10^5 cells/well) immune response with one cytokine, but did secrete less than 10 SFCs/ 2×10^5 cells/well of the

Fig. 1. FluoroSpot analysis of three images from the bottom of the same cell culture well following ESAT6/CFP10-specific stimulation of PBMCs. On the left upper image, streptavidin Red-(Cy3)-bound IL-2+ is detected with a red narrow band fluorescence filter. In green squares, areas on the bottom of the well are highlighted, where no IL-2+ production can be seen, while IFN-γ+ production detected by FITC is seen in the same position on the upper right image obtained through a green narrow band fluorescence filter. Here, red squares show areas of IFN-γ+ production, where no IL-2 production is identified on the left upper image. The picture below is an automated overlay of both images. Double IFN-γ+ and IL-2+ producing cells are highlighted in yellow circles, while single cytokine-producing cells are represented by red and green dots (colors refer to the online version only).

other cytokine towards the same antigen, the one cytokine response below 10 SFCs was assigned 0.1 for analysis. An in vitro response of 0–10 SFCs/ 2×10^5 cells/well of both cytokines to the same antigen was classified as negative response for that specific antigen.

Differences between groups in SFCs for each *M. tuberculosis*-specific antigen were assessed with the Mann-Whitney U test for nonparametric data analysis (GraphPad Prism, version 5.00; GraphPad Software, San Diego, Calif., USA). Sensitivity, specificity and cut-offs were ascertained by receiver-operating characteristic (ROC) analysis using the Statistica program (Statsoft, Tulsa, Okla., USA). The study is reported according to the STARD guidelines.

Fig. 2. Study flow diagram. TB = Active tuberculosis; Ag = specific antigen; Neg = negative control.

Table 1. Characteristics of patients with tuberculosis (TB) and HCCs

	TB	HCCs
Participants, n	19	23
HIV status	negative	negative
Mean age $\pm$ SD, years	33.74 $\pm$ 12.5	33.70 $\pm$ 12.8
Sex: male/female, n	8/11	12/11
QFT: positive/total	n.a.	9/11 (81.82%)
TST: positive/total	n.a.	10/12 (83.33%)

n.a. = Not applicable.

Results

Following written informed consent, 27 individuals with suspected active tuberculosis and 26 healthy HCCs were enrolled in the study between November 2010 and February 2012. One out of 27 individuals suspected to have active tuberculosis (3.7%) was excluded as he did not have a positive *M. tuberculosis* culture from sputum. In 7 tuberculosis patients (25.9%), the required >50 SFCs/ 2×10^5 cells/well in the positive control were not obtained and their tests were classified as indeterminate. Thus, 19 patients with tuberculosis were eligible for the final analysis. Among these 19 tuberculosis patients, the magni-

Table 2. Diagnostic performance of stimulated SFCs using dual-colour EliSpot

<i>M. tuberculosis</i> antigen	Host marker	TB: median SFCs (IQR)	HCC: median SFCs (IQR)	p value	AUC (95% CI)
ESAT6/CFP10	IL-2+	25.25 (0.1–123.1)	32.50 (17.5–50.25)	0.704	0.54 (0.32–0.76)
	IFN- γ +	71.5 (241.3–234.3)	31.5 (0.1–85.38)	0.062	0.69 (0.51–0.87)
	double	15.0 (4.13–55.63)	15.25 (5.13–29.63)	0.704	0.54 (0.34–0.74)
MTP65	IL-2+	0.1 (0.1–12.5)	15.5 (0.1–32.00)	0.032	0.71 (0.54–0.88)
	IFN- γ +	32.5 (16–84)	40.5 (20.5–63.25)	0.748	0.53 (0.33–0.74)
	double	2 (1–5)	6.5 (2–12)	0.054	0.69 (0.52–0.87)
PPD	IL-2+	21.5 (12.25–72.25)	25.5 (10.75–55.5)	0.657	0.55 (0.34–0.75)
	IFN- γ +	51.0 (23.25–97.0)	23.0 (16.0–60.75)	0.103	0.67 (0.49–0.85)
	double	14.5 (3.0–32.25)	9.5 (4.25–22.5)	0.915	0.51 (0.30–0.73)

PBMCs of active tuberculosis patients (TB) and HCCs were isolated and stimulated overnight with *M. tuberculosis*-specific antigens (ESAT6/CFP10 and MTP65) and PPD. The number of SFCs was determined with the EliSpot Reader System ELRIFLO5.

IQR = Interquartile range. AUC and 95% confidence intervals (95% CIs) were calculated with ROC analysis. The italicized value indicates a significant difference.

tude of the immune response differed between the antigens: 16 patients showed detectable T-cell reactivity towards ESAT6/CFP10 (84.2%), 15 patients (78.9%) towards MTP65 and 13 patients (68.4%) towards PPD.

Though all 26 HCCs presented without any symptoms suggestive of tuberculosis, 2 (7.7%) had to be excluded from the study as their sputum culture was positive for *M. tuberculosis* (no further information was available to confirm the clinical status of these 2 participants). In 1 HCC (3.8%), the required >50 SFCs/ 2×10^5 cells/well in the positive control were not achieved and the test was classified as indeterminate. Thus, 23 HCCs were eligible for the final analysis. Of these 23 HCCs, 5 (21.7%), 2 (8.7%) and 2 (8.7%) were low responders towards ESAT6/CFP10, MTP65 and PPD, respectively (fig. 2). Nine of 11 (81.8%) HCCs had a positive TST and 10/12 (83.3%) HCCs had a positive QFT result (table 1).

Diagnostic Utility of the Number of SFCs

After stimulation with ESAT6/CFP10, MTP65 and PPD, the numbers of IFN- γ -IL-2+ and IFN- γ +IL-2+ dual cytokine-producing cells were determined in each participant and compared between tuberculosis patients and HCCs (table 2). Patients with tuberculosis had a trend towards more ESAT6/CFP10-induced IFN- γ + producing cells (median 71.5 SFC) than HCCs (median 31.5 SFC, $p = 0.062$).

The frequency of MTP65-induced IL-2+ producing cells was higher in the HCCs (median 15.5 SFC) than in tuberculosis patients (median 0.1 SFC, $p = 0.032$). Al-

though statistically the median of the MTP65-reactive IL-2+ producing cells differed significantly between the two groups, the overlap in frequencies precluded accurate discrimination between LTBI and tuberculosis on an individual basis (online suppl. fig. 1; for all online suppl. material, see www.karger.com/doi/10.1159/000365816).

No differences in immune responses towards PPD stimulation were found between HCCs and tuberculosis patients.

Diagnostic Utility of the Percentage of Single and Double Producing Cells

The lack of discrimination between tuberculosis and LTBI observed with the different antigens might be due to the high level of interindividual variation of SFCs. To adjust for this variation, each individual cytokine response was evaluated in proportion to the overall specific immune response of the individual. Five different T-cell populations were defined: total IL-2+, total IFN- γ +, IFN- γ +IL-2-, IFN- γ -IL-2+ and IFN- γ +IL-2+ producing cells. The total number of any cytokine-producing cells in response to each stimulus was calculated by adding the SFCs of single IFN- γ - and single IL-2-producing cells and SFCs of double producing cells. This sum was used as common denominator for the calculation of the percentage of each cytokine-producing phenotype, which was compared between the two study groups.

Using this algorithm, the percentage of each cell population in relation to all cytokine-producing cells by ESAT6/CFP10 stimulation discriminated significantly

Table 3. Diagnostic performance of stimulated single and dual IFN- γ /IL-2-producing cells using dual-colour EliSpot

<i>M. tuberculosis</i> antigen	Host marker	TB: median (IQR), %	HCC: median (IQR), %	p value	AUC	Cut-off	Sensitivity, %	Specificity, %
ESAT6/CFP10	IFN- γ -IL-2+	15.09 (0.42–27.36)	34.8 (27.4–46.9)	<i>0.002</i>	0.82	28.25	72	81
	IFN- γ +IL-2–	64.12 (46.2–83.9)	29.7 (1.0–48.0)	<i><0.001</i>	0.85	42.95	67	94
	IFN- γ +IL-2+	18.3 (11.7–27.9)	33.1 (19.1–37.7)	<i>0.037</i>	0.71	20.57	78	63
	total IL-2+	35.9 (16.1–53.8)	70.4 (52.1–99.0)	<i><0.001</i>	0.85	57.05	67	94
	total IFN- γ +	84.9 (72.7–99.6)	65.3 (53.2–72.6)	<i>0.002</i>	0.82	71.75	72	81
MTP65	IFN- γ -IL-2+	0.6 (0.3–11.0)	11.6 (0.6–21.0)	0.067	0.68	11.25	57	80
	IFN- γ +IL-2–	88.4 (77.6–96.6)	70.3 (57.0–89.0)	0.132	0.65	76.23	62	80
	IFN- γ +IL-2+	3.6 (2.0–6.8)	11.0 (4.1–18.5)	<i>0.04</i>	0.71	7.1	71	80
	total IL-2+	9.9 (2.4–22.4)	29.8 (11.1–43.1)	0.124	0.65	21.95	62	80
	total IFN- γ +	99.9 (89.0–99.7)	88.4 (79.0–99.4)	0.067	0.68	88.75	57	80
PPD	IFN- γ -IL-2+	22.1 (15.2–33.0)	27.1 (13.6–44.6)	0.620	0.55	28.81	48	77
	IFN- γ +IL-2–	60.5 (51.6–65.4)	49.0 (29.7–61.3)	0.096	0.67	47.54	48	92
	IFN- γ +IL-2+	19.5 (7.2–25.1)	25.5 (16.5–28.3)	0.103	0.67	24.60	52	77
	total IL-2+	39.1 (34.7–48.5)	51.0 (38.7–70.2)	0.096	0.67	52.46	48	92
	total IFN- γ +	78.0 (67.0–84.8)	72.9 (55.4–86.4)	0.620	0.55	71.19	48	77

PBMCs of tuberculosis patients (TB) and HCCs were isolated and stimulated overnight with *M. tuberculosis*-specific antigens (ESAT6/CFP10 and MTP65) and PPD. The number of SFCs was used to calculate frequencies of IFN- γ -IL-2+, IFN- γ +IL-2–, IFN- γ +IL-2+, total IL-2+ and total IFN- γ + producing cell populations.

IQR = Interquartile range. Significant p values for comparisons between groups are italicized. ROC analysis revealed the AUC and the best cut-off identifying the patients' clinical status with the corresponding sensitivity and specificity.

between tuberculosis and LTBI (table 3). The percentage of ESAT6/CFP10-reactive IFN- γ -IL-2+ producing cells was higher in HCCs than in tuberculosis patients (median 34.74 vs. 15.09%, respectively, $p = 0.002$), as well as the percentage of ESAT6/CFP10-reactive IFN- γ +IL-2+ double producing cells (median 30.11 vs. 18.30%, respectively, $p = 0.037$). The percentage of total IL-2+ producing cells responding towards ESAT6/CFP10 was elevated in HCCs in comparison with tuberculosis patients (median 70.38 vs. 35.88%, respectively, $p < 0.001$). In contrast, the percentage of ESAT6/CFP10-induced IFN- γ +IL-2– producing cells was lower in HCCs than in tuberculosis patients (median 29.63 vs. 64.12%, respectively, $p < 0.001$) as well as the percentage of total IFN- γ + producing cells (median 65.27 vs. 84.91%, respectively, $p = 0.002$). Tuberculosis patients had increased ratios of IFN- γ + producing cells (median 84.91% IFN- γ + producing cells), whereas HCCs displayed a more balanced distribution of 34.74% IFN- γ -IL-2+ producing cells, 30.11% IFN- γ +IL-2+ double producing cells and 29.63% IFN- γ +IL-2– producing cells after ESAT6/CFP10 stimulation.

The percentage of MTP65-induced IFN- γ +IL-2+ double producing cells was higher in HCCs than in tuberculosis patients (median 10.83 vs. 3.57%, $p = 0.040$). Al-

though the number of SFCs of MTP65-induced IFN- γ -IL-2+ producing cells was significantly elevated in HCCs in comparison to tuberculosis patients (table 2), after adjustment for the total cytokine-producing cells, the percentage of IFN- γ -IL-2+ producing cells showed only a trend to be higher (median 11.57 vs. 0.62%, respectively, $p = 0.067$; table 3).

Calculating the percentages of PPD-responsive cytokine populations did not reveal any differences between tuberculosis patients and HCCs.

Using the percentages of antigen-induced cytokine populations, 6 host markers significantly discriminated tuberculosis from LTBI. To calculate the ability of individual markers to assign participants to their correct clinical status, ROC analyses were used to define the best cut-off for each antigen-induced host marker. For example, the optimum cut-off for ESAT6/CFP10-stimulated IFN- γ -IL-2+ producing cells was 28.25% (area under the curve, AUC = 0.82) and active tuberculosis was identified with a sensitivity of 72% and specificity of 81% (table 3). Notably, there was substantial overlap in frequencies between the two cohorts for each marker and therefore none of the antigen-induced cell populations was a promising diagnostic marker on its own.

Fig. 3. Utility of a 3-host marker combination model for diagnosis. Sixteen tuberculosis (TB) patients, defined by *M. tuberculosis* growth in culture, and 18 HCC were analysed. The relative percentage of specific cytokine-producing cells and the best cut-off were determined for each cell population as described in table 3. The cut-off was used to define whether the frequency of cell populations indicated either tuberculosis or LTBI. Each cell population was used as an independent marker. Prediction of TB is shown in dark grey, and prediction of LTBI is shown in light grey, for 3 different phenotypic markers. When ≥ 2 phenotypes supported one of the diagnoses, a final diagnosis of either TB (black) or LTBI (white) was made.

ESAT6/CFP10	% IFN- γ ⁻ IL-2 ⁺ cut off 28.25	% IFN- γ ⁺ IL-2 ⁻ cut off 42.95	% IFN- γ ⁺ IL-2 ⁺ cut off 20.57	Final test result
TB1				TB
TB2				
TB3				
TB4				
TB5				
TB6				
TB7				
TB8				
TB9				
TB10				
TB11				
TB12				
TB13				
TB14				
TB15				
TB16				
HCC1				HCC
HCC2				
HCC3				
HCC4				
HCC5				
HCC6				
HCC7				
HCC8				
HCC9				
HCC10				
HCC11				
HCC12				
HCC13				
HCC14				
HCC15				
HCC16				
HCC17				
HCC18				

Marker indicating TB
Marker indicating NO TB
Test indicating NO TB
Test indicating TB

1 false negative
4 false positive
Sensitivity 93.8%
Specificity 77.8%

Diagnostic Utility of Combination Models

In an attempt to increase specificity, different combination models of 3–6 antigen-induced host markers were used to classify the participants. The cut-off value obtained using the ROC analysis described above was used to classify individuals into two groups. Using 4, 5 or 6 markers in combination did not perform better than a

3-marker model. Participants were allocated to a clinical group according to the results of the majority of the individual marker tests (2/3 or 3/3). Accordingly, the ESAT6/CFP10-induced 3-host marker combination model identified 29 of the 34 participants (85.3%) to the correct clinical group with 93.8% sensitivity and 77.8% specificity. Five (14.7%) participants were misclassified, with 1 false-

Fig. 4. Utility of ESAT6/CFP10 in monitoring treatment. Immune responses of PBMCs towards ESAT6/CFP10 of 11 tuberculosis (TB) patients and 9 HCC were evaluated at recruitment and after 6 months, during which the tuberculosis patients had received 6 months of standard treatment. In a model, the optimal AUC cut-off (determined as described in table 3 and fig. 3) was used to define

TB or LTBI. Each cell population was used as an independent marker. For each phenotype, prediction of TB is shown in dark grey and prediction of LTBI in light grey. When ≥ 2 phenotypes supported one of the diagnoses, a final diagnosis of either TB (black) or LTBI (white) was made.

negative and 4 false-positive classifications. Grouping of individual participants, sensitivity and specificity are shown in figure 3.

Treatment Monitoring

After completion of a 6-month standard tuberculosis treatment, 14 tuberculosis patients and 12 HCCs were re-investigated for changes in their immune response. Percentages of the different cell populations were again calculated. Analysing the ESAT6/CFP10-induced immune response of the complete pairs during successful tuberculosis treatment, the median percentage of IFN-γ- IL-2+ producing cells increased from 9.92% at recruitment to 27.38% after 6 months of treatment, as did the median percentage of IFN-γ+ IL-2+ double producing cells (13.59–23.62%, respectively). In contrast, the median percentage of IFN-γ+ IL-2- producing cells decreased (from 71.71 to 53.19%, respectively). Neverthe-

less, the immune responses of tuberculosis patients towards the antigens ESAT6/CFP10 did not change consistently and were therefore not significant (fig. 4). According to the ESAT6/CFP10-induced 3-host marker combination model, 7 (63.63%) of the 11 tuberculosis patients were still assigned to the clinical status of active tuberculosis, despite having completed 6 months of successful treatment. The median percentages of HCCs changed very little (IFN-γ- IL-2+: from 33.55% at recruitment to 36.73% after 6 months, IFN-γ+ IL-2+: from 37.13 to 25.74% and IFN-γ+ IL-2-: from 30.3 to 36.03%, respectively). One of the HCCs, who had been correctly classified at recruitment, changed his immune response to an IFN-γ+ dominating profile, suggesting active disease, but at the 1-year clinical follow-up he did not develop any symptoms of tuberculosis. The MTP65 immune response showed no significant changes during treatment (data not shown).

Analysing the PPD-induced immune response of the 9 pairs during successful tuberculosis treatment, the median percentage of IFN- γ +IL-2 $^-$ producing cells decreased from 63.24% at recruitment to 40.49% after 6 months of treatment ($p = 0.02$), whereas the median percentage of IFN- γ +IL-2 $^+$ double producing cells and all IL-2 $^+$ producing cells increased: from 19.35 to 29.25% ($p = 0.027$) and from 36.76 to 59.51% ($p = 0.02$), respectively. The significant changes in the frequency of cytokine populations are shown in online supplementary figure 2. PPD-induced immune responses in HCCs did not change significantly.

Discussion

The clinical performance of the new IFN- γ /IL-2 dual-colour EliSpot (FluoroSpot) to discriminate between tuberculosis and LTBI and to monitor specific cytokine profiles during tuberculosis treatment was evaluated in this study. The key findings are: (1) A 3-marker combination model including ESAT6/CFP10-induced IFN- γ -IL-2 $^+$, IFN- γ +IL-2 $^-$ and IFN- γ +IL-2 $^+$ double producing cells improved diagnostic sensitivity in comparison to the EliSpot. (2) ESAT6/CFP10-induced IFN- γ -IL-2 $^+$, IFN- γ +IL-2 $^-$ and IFN- γ +IL-2 $^+$ double producing cells expressed as a percentage of all IL-2 $^-$ and IFN- γ -positive cells had a more promising diagnostic utility than analysis of absolute numbers of SFCs. (3) Analysis of PPD-responsive cell populations hold promise as biomarkers for treatment monitoring.

Unlike the traditional T-SPOT.TB and QFT ELISA, the dual-colour EliSpot allows the assessment of IFN- γ and IL-2-producing cell populations at a single cell level. The lack of diagnostic accuracy of the absolute number of SFCs of cytokine-producing cells may be due to high interindividual variation in immune responses [10], which was decreased by the calculation of cell percentages. These results are in agreement with previous observations using FACS analysis, which showed that LTBI and tuberculosis are associated with distinct T-cell phenotypes [6, 11, 12]: During LTBI, antigen-specific immune responses are dominated by a mixture of central memory cells (mostly IFN- γ -IL-2 $^+$ producing cells) plus effector memory cells (IFN- γ +IL-2 $^+$ double producing cells), whereas active tuberculosis is associated with a predominance of effector memory cells (IFN- γ +IL-2 $^-$) [11].

Furthermore, analysis of the antigen-specific induction of two cytokines allows new insights into immune response capability. It has been generally accepted that a

negative IFN- γ immune response in IGRAs of active tuberculosis patients are false-negative test results [13, 14]. However, our data show that even in the absence of antigen-specific IFN- γ production, other cytokines like IL-2 indicate sensitization to pathogen proteins. As the IFN- γ -IL-2 $^+$ phenotype is more frequent in LTBI, IFN- γ should not be seen as the only proxy for sensitization to *M. tuberculosis*.

Although the immune signature was significantly different between LTBI and tuberculosis regarding 5 different ESAT6/CFP10-induced cytokine production patterns, the overlap between the two groups was substantial for all the markers. None of the markers could correctly classify all participants into their respective groups. However, the 3-marker model allowed correct identification of active tuberculosis with a sensitivity of 93.8% and moderate specificity of 77.8%. This confirms the advantage of combination marker models instead of the use of single markers in tuberculosis diagnostics [15, 16]. A positive 3-marker test may serve as screening or rule-out test to identify people with suspected tuberculosis for subsequent testing with a highly specific test such as GeneXpert MTB/RIF or BACTEC 960 culture to optimise diagnostic workup.

ESAT6/CFP10-induced immune responses as markers for treatment monitoring were not promising due to interindividual variations. There were also fluctuations in immune responses in HCCs. These results are in line with another report [11] describing that neither ESAT6 nor CFP10 could separate untreated tuberculosis patients from another cohort of successfully treated tuberculosis patients. However, the results are in contrast with other studies on serial IGRAs where a decline in IFN- γ production during tuberculosis treatment was observed [17–19]. We presume that the kinetics of the immunological recall during tuberculosis treatment may take longer than observed in this study.

PPD-specific immune responses were superior to the ESAT6/CFP10-specific immune responses to monitor treatment success. The frequencies of IFN- γ +IL-2 $^-$, IFN- γ +IL-2 $^+$ and the total IL-2 $^+$ producing cells changed significantly during treatment of patients with tuberculosis. Our results support previous findings [11] indicating that the PPD-reactive IFN- γ +IL-2 $^+$ double producing cells may be valuable markers for treatment responses.

Our study has several limitations, including the small number of participants. Additional time points and new *M. tuberculosis* antigens and host cytokines need to be incorporated into future studies to evaluate biomarkers for successful treatment responses. Indeed, the heterogeneity in host responses to *M. tuberculosis* antigens ob-

served in this study has been reported previously. Chiapini et al. [20] have evaluated the diagnostic utility of 6 *M. tuberculosis* antigens in IFN- γ EliSpot and IL-2 EliSpot. The AlaDH-IL-2 EliSpot provided the most promising diagnostic results in that setting. Evaluation of the AlaDH-IFN- γ /IL-2 dual-colour EliSpot may improve the diagnostic accuracy observed in the present study. The high number of indeterminate results due to inadequate cytokine responses after stimulation with the positive controls (7/26 tuberculosis patients and 1/24 HHCs) adversely affects test performance. As similar reductions in immune responses in the case of disease have been noted previously, this frequent absence of positive responses in tuberculosis may be due to immune suppression [21, 22] or the systemic predominance of TH2 cytokines over TH1 cytokines during disease [23, 24].

Conclusion

In conclusion, this study has shown that ESAT6/CFP10-reactive IFN- γ + producing cells dominate in active tuberculosis, whereas in LTBI the immune response

is balanced between IFN- γ -IL-2+, IFN- γ +IL-2+ and IFN- γ +IL-2- producing cell populations. A 3-marker model of IL-2- and IFN- γ -producing populations, expressed as percentage of all cells producing these cytokines, has improved the discrimination of LTBI from active tuberculosis patients. However, both diagnostic performance at the time of screening and assessment of immunological changes of such biomarkers during tuberculosis therapy need to be further evaluated before they can be recommended for routine clinical practice [25].

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