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BRIEF REPORT

IGRA-positive patients and interferon-gamma/interleukin-2 signatures: Can the Fluorospot assay provide further information?

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Abstract A goal of testing for latent tuberculosis (TB) infection is to identify individuals who are at increased risk for the development of active TB. No laboratory tool is currently available to distinguish between individuals in the process of progressing from latent TB infection towards active disease and those who are not. Determination of the interferon-gamma and interleukin-2 T cell signature might provide an additional and rapid tool to evaluate treatment necessity and clinical management of a patient. Here, we present three cases of interferon-gamma release assay-positive patients with differing interferon-gamma and interleukin-2 signatures when analyzed by the Fluorospot assay.

Keywords Fluorospot · Latent tuberculosis infection · T-cell signature · Interleukin-2 · Interferon-gamma

Introduction

The latent state of infection with *Mycobacterium tuberculosis* [latent tuberculosis infection (LTBI)] is probably maintained by an active immune response in the host initiated by *M. tuberculosis* which results in host-controlled persistence of the organism. LTBI can progress to active TB disease a long time after the initial infection [1]. Infection with *M. tuberculosis* manifests as a spectrum of clinical presentations, including those patients who will progress to active disease, those who maintain persistent, lifelong infection but do not progress to active disease, those who temporarily suppress infection but later develop active disease, and those who are able to effectively clear the pathogen.

In countries with a low incidence of TB, targeted testing for LTBI among risk groups is an important approach to prevent active TB disease [2]. In most developed countries, the diagnosis of LTBI increasingly relies on the results of two ex vivo interferon-gamma (IFN-γ) release assays (IGRA), namely, the QuantiFERON®-TB Gold In-Tube assay (QFT; Qiagen AG, Hombrechtikon, Switzerland) and the T-SPOT.*TB* assay (Oxford Immunotec, Oxford, UK) [3]. These routine laboratory tests utilize specific *M. tuberculosis* antigens [T-SPOT.*TB* assay: early secretory antigen target-6 (ESAT-6) and culture filtrate protein-10 (CFP-10); QTF (TB7.7) assay: ESAT-6, CFP-10, and Rv2654c] that are absent from Bacillus Calmette–Guérin (BCG) vaccine and most non-tuberculous mycobacteria to stimulate IFN-γ release. As such, these tests significantly minimize the number of wrongly diagnosed LTBI cases due to unknown vaccination status compared to the tuberculin skin test (TST) [3, 4]. However, the questions of whether IGRAs can differentiate between persons who will develop active TB disease and those who will not, and if so by which mechanism, remain unanswered. There is currently no laboratory tool available to directly detect individuals in the process of progressing from LTBI towards active TB disease. In their recent meta-analysis, Diel and colleagues showed that the pooled positive predictive value (PPV) for progression to active TB disease using

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commercial IGRAs was 2.7 % in the included studies and that the PPV increased to 6.8 % when only high-risk groups were considered [5]. These authors reported that the pooled value of the negative predictive value for progression to active TB disease was very high—99.7 %.

Identifying individuals who are at an elevated risk of reactivation and/or progression to active TB would aid clinical management decisions, as *M. tuberculosis* therapy is lengthy and adverse effects are not uncommon. In this context, cytokine profiling is a promising tool [6]. IFN-γ/interleukin-2 (IL-2) profiling identifies T-cell cytokine signatures which reflect their memory phenotype and defines functional T-cell subsets, namely, effector cells that mainly secrete IFN-γ only and long-lived central-memory T-cells secreting only IL-2. Antigen clearance is typically associated with dominant IL-2 functional T-cell signatures while, in contrast, high antigen loads are associated with dominant IFN-γ functional T-cell signatures. A dynamic relationship has been demonstrated in intracellular infections between antigen load and distinct IFN-γ and IL-2 profiles of antigen-specific CD4+ T-cells [7]. In the context of *M. tuberculosis* infection, a study by Millington and colleagues [8] examined samples from active TB patients both pre- and post-initiation of treatment. These authors showed a significant decline in the frequency of ESAT-6/CFP-10-specific T-cells secreting IFN-γ over 28 months and a significant increase in the number of ESAT-6/CFP-10-specific T-cells secreting IL-2 during the 6 months of treatment [8]. In another study, Casey and colleagues [9] compared patients with active TB and those with LTBI and found that the latter had a significantly lower proportion of ESAT-6/CFP-10-specific IFN-γ-only-secreting T-cells and a significantly higher proportion of ESAT-6/CFP-10-specific dual IFN-γ/IL-2-secreting and IL-2-only-secreting T-cells.

Here we present three cases of persons with positive IGRA results (QFT- and T-SPOT.*TB*-positive) who were putatively infected with *M. tuberculosis*, with markedly different single-cell IFN-γ and IL-2 signatures when measured using the Fluorospot assay described in the following section (Figs. 1, 2). When used in a diagnostic laboratory environment the IFN-γ/IL-2 Fluorospot assay produces low-cost single-cell cytokine profiles for IFN-γ and IL-2. These results provide useful information for optimizing clinical management of potentially infected patients. Compared to the previously described enzyme-linked immunosorbent assay (ELISA) method of determination of IFN-γ and IL-2 using plasma supernatant of QuantiFERON-TB Gold (QFT ®; Qiagen, Hilden, Germany), the Fluorospot assay has the advantage of not being restricted to the linear range of the ELISA readout and the usability for immunosuppressed patients since peripheral blood mononucleated cells (PBMCs) are collected independently. Additionally, cells secreting both IFN-γ and IL-2 simultaneously can be distinguished from mono-secreting cells [9, 10].

Patient	QFT (IU/ml)	T-SPOT.TB (ESAT-6) (SFU)	T-SPOT.TB (CFP-10) (SFU)	Fluorospot IFN-γ (*) (FITC-SFU)	Fluorospot IL-2 (*) (CY3-SFU)	
1	6.38	26	53	112	16	IFN-γ -dominant signature
2	7.72	182	256	212	198	IFN-γ/IL-2 -mixed signature
3	0.52	10	0	4	11	IL-2 -dominant signature

Fig. 1 Three cases of persons with positive interferon-gamma (*IFN-γ*) release assay (IGRA) results. Patient 1: 62-year-old female; strong positive IGRA QFT and T-SPOT.*TB* results; Fluorospot INF-γ dominant signature. Patient 2: 31-year-old female; strong positive IGRA QFT and T-SPOT.*TB* results; Fluorospot IFN-γ/IL-2 mixed signature. Patient 3: 55-year-old male; weak positive IGRA QFT and T-SPOT.*TB* results; Fluorospot IL-2 dominant signature. QFT was considered to be positive at >0.35 IU/ml, the T-SPOT.*TB* was considered to be positive at >7 spot-forming cells (*SFC*). *Asterisks* For stimulation, the pooled antigen mixture mycobact.tub.filtrate1 (Autoimmun Diagnostika GmbH) was used for the Fluorospot assay. The different assays used (IGRA QFT, T-SPOT.*TB*, Fluorospot IL-2) are described in full in the text. *IL-2* Interleukin-2, *FITC* fluorescein isothiocyanate

Methods

The QFT and T-SPOT.*TB* assays were performed according to the respective manufacturer's instructions. Spot-forming cells (SFCs) were counted using an AiD-iSpot reader (Autoimmun Diagnostika GmbH, Strassberg, Germany). PBMCs were collected using Becton–Dickinson Vacutainer Cell Preparation Tubes (BD BioSciences, San Jose, CA) following centrifugation for 20 min at room temperature (RT)/1,650 *g* (Haereus Megafuge 1.0R; Thermo Scientific, Waltham, MA). The plasma supernatant was discarded and the cells were washed twice with 15 ml 10 % phosphate buffered saline and centrifuged for 15 min at RT/300 *g* (Haereus Megafuge 1.0R). The cells were then collected and re-suspended in AIM-V media, a fully-defined serum-free tissue culture media for immunological studies [AIM V® Medium, liquid (research grade); AlbuMAX® Supplement; Life Technologies, Carlsbad, CA], counted using a Cell-Dyn Emerald flow-cytometer (Abbott AG, Liestal, Switzerland), and adjusted to 250,000 PBMC/100 µl.

The IFN-γ/IL-2-Fluorospot assay was performed as follows: 250,000 PBMCs (100 µl), including anti-CD28 monoclonal antibody (mAb) and a co-stimulatory anti-CD28 immunoglobulin G (IgG) antibody to counterbalance

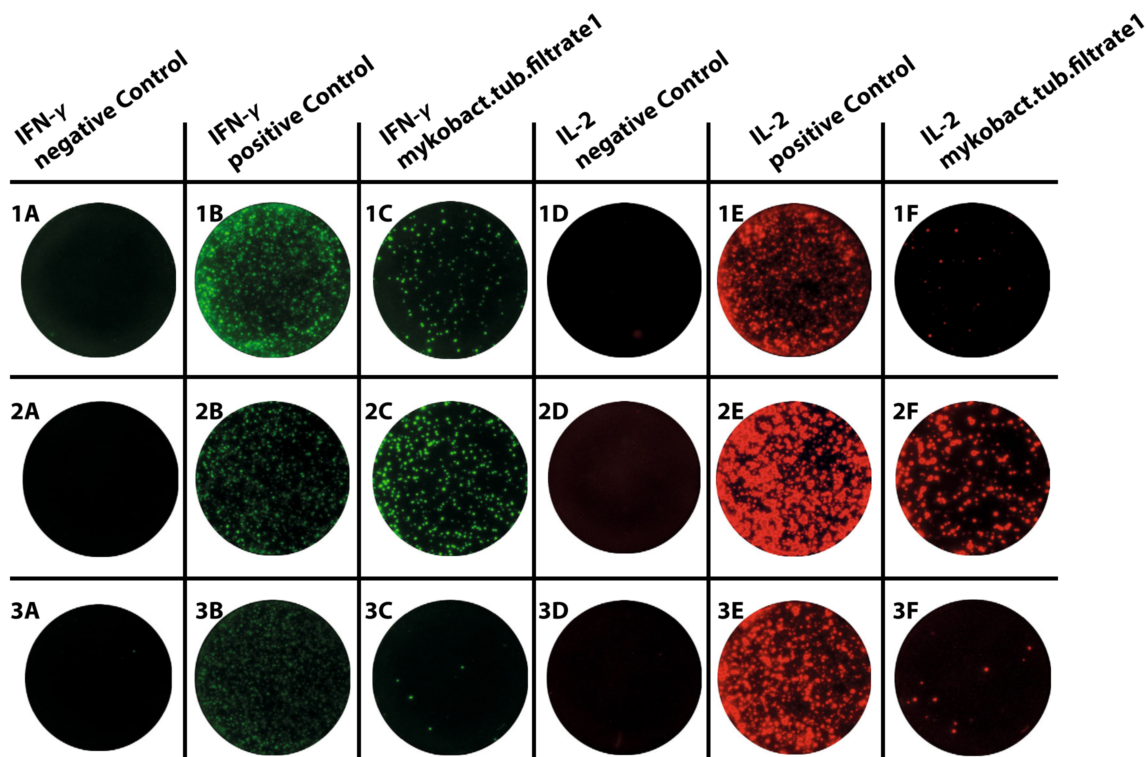

Fig. 2 Three cases of IGRA-positive patients with different IFN-γ and IL-2 signatures by the Fluorospot assay. *1A–1F* Patient 1 (62-year-old female), IFN-γ dominant signature. *2A–F* Patient 2 (31-year-old male), IFN-γ/Il-2 mixed signature. *3A–3F* Patient 3 (55-year-old male), IL-2 dominant signature

sequestering of IL-2 by membrane-bound anti-IL-2 capture antibodies [11] (Anti-Human CD28 mAb CD28-A, 0.1 µg/ml; MABTECH AB, Nacka Strand, Sweden), were seeded to each well of an IFN-γ/IL-2 capture pre-coated PVDF membrane plate (MABTECH AB). For each patient, each well contained plain AIM-V (negative control; Fig. 2, columns A and D), pokeweed mitogen (Autoimmun Diagnostika GmbH, GER) (positive control; Fig. 2, columns B and E), and 100 µl of the *M. tuberculosis* antigen mixture mycobact.tub.filtrate1 (Autoimmun Diagnostika GmbH) (Fig. 2; columns C and F). The plates were incubated at 37 °C with 5 % CO_2 for 20–24 h, then washed and incubated at room temperature for another 2 h with FITC-conjugated anti-IFN-γ (7-B6-1-FS-FITC; MABTECH AB; diluted according to the manufacturer's instructions) and 0.5 µg/ml biotinylated anti-IL-2 (IL2-II-biotin; MABTECH AB) detection mAbs. The plates were then washed again and incubated at room temperature for 1 h with fluorophore-labeled monoclonal anti-FITC-Green antibody and streptavidin-red (MABTECH AB Fluorospot kit; 1 µg/ml/Streptavidin-PF555 and anti-FITC-PF488P, PromoCell GmbH, Heidelberg, Germany). After washing, the plates were incubated at room temperature for 15 min with 100 µl/well of fluorescence enhancer (MABTECH AB) before being emptied and air-dried in the dark. SFCs were counted using an automated AiD-iSpot reader system to detect PF488P and PF555 fluorophores (ERLIFL05; Autoimmun Diagnostika GmbH). The automatic readouts were validated by visual inspection of the spots by at least two different persons.

Results

Patient 1 was a 62-year-old female of Swiss descent, in good general condition, health care worker (HCW). The infection was identified via a hospital active TB contact screening program (no prolonged contact with the infected patient). The clinical history revealed a period of prolonged coughing as a child, a positive TST test (>20 mm) of unknown date, and a link between the coughing and an unknown BCG-vaccination status. No abnormalities were detected on the chest X-ray. Both IGRA tests were clearly positive: the QFT result was 6.38 IU/ml and the T-SPOT.*TB* results of 26 spots for ESAT-6 and 53 spots for CFP-10, respectively. In clinical terms the positive IGRAs were interpreted as indicating a historical contact with *M. tuberculosis* during adolescence. Even though the recent exposure may not have resulted in infection, in this case a previous infection might be progressing to active disease (i.e., re-activation), and this transition stage was only detected by chance. The patient refused preventive

isoniazid (INH) treatment against *M. tuberculosis*. However, later testing with the Fluorospot assay revealed a strongly IFN-γ dominant signature (Fig. 1, Patient 1; Fig. 2, 1A–F). This IFN-γ dominance might indicate an ongoing immunological process directed against *M. tuberculosis* [8] that supports the need for follow-up monitoring, particularly considering the untreated status.

The second patient was a 31-year-old female of Kosovar descent, in good general condition, HCW. The infection was identified through routine hospital entry screening. The status of the BCG vaccination could not be confirmed; however, an inoculation scar was detected (arm). The clinical history revealed confirmed close contact with an active TB patient (within the family) at the age of 16 years. No post-exposure contact screening or treatment against *M. tuberculosis* was recorded, and no TST was performed. No abnormalities were detected on chest X-ray. Both IGRA tests were clearly positive: the QFT result was 7.72 IU/ml and the T-SPOT.*TB* results were 182 spots for ESAT-6 and 256 spots for CFP-10, respectively. The Fluorospot assay revealed a mixed IFN-γ/IL-2 signature (Fig. 1, Patient 2; Fig. 2, 2A–F). The patient received drug treatment against *M. tuberculosis* (rifampicin 600 mg, once daily for 6 months).

The third patient was a 55-year-old male of Swiss descent, in good general condition, HCW. The infection was identified through routine hospital screening. The status of the BCG vaccination was confirmed. The clinical history showed repeated potential exposures during several professional assignments in Eastern Europe over the past years. No abnormalities were detected on chest radiograph, and no TST was performed. Both IGRA tests were weak positive: the QFT result was 0.52 IU/ml and the T-SPOT.*TB* results were 10 spots for ESAT-6. The Fluorospot assay revealed an IL-2 dominant signature (Fig. 1, Patient 3; Fig. 2, 3A–F). The patient did not receive preventive INH therapy against *M. tuberculosis.*

Discussion

We have shown that clearly distinguishable IFN-γ and IL-2 signatures can be obtained in IGRA-positive individuals using the Fluorospot assay. These IFN-γ and IL-2 signatures can be used as an additional criterion by clinicians, allowing for a personalized risk assessment of IGRA-positive subjects for which there are no further clinical, radiological, or anamnestic parameters available. Individuals with IFN-γ dominant or mixed signatures whose immune system might be in the process of actively fighting *M. tuberculosis* could bear a higher risk of developing active disease and therefore be indicated for treatment or clinical follow-up. Determination of the IFN-γ and IL-2 functional signature might also help to further assess the status of individuals showing increased within-subject variability (e.g., multiple conversions and reversions in serial testing), a phenomenon encountered using both the QFT and the T-SPOT.*TB* assays [12, 13], as well as individuals with consistent low positive QFT results (≥0.35 to <1.0 IU/ml), who generally seem to have an overall higher IL-2/IFN-γ ratio when compared to QFT intermediate responders (1.0–5.0 IU/ml) or QFT strong responders (>5.0 IU/ml) [10]. Additional data are needed to obtain cut-off values that allow a clear distinction between IFN-γ, IL-2, and IFN-γ/IL-2 mixed signatures. Further studies to determine not only IFN-γ and IL-2 activity, but also the possible role of TNF-α as an additional Fluorospot marker [14] are warranted.

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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