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Antigen-specific CD4- and CD8-positive signatures in different phases of *Mycobacterium tuberculosis* infection☆

Maria Nikolova [a,1], Roumiana Markova [a,1], Rumiana Drenska [a], Maria Muhtarova [a], Yana Todorova [a], Velichko Dimitrov [b], Hristo Taskov [a], Cesare Saltini [c], Massimo Amicosante [c,d,*]

[a] Department of Immunology and Allergology, National Center of Infectious and Parasitic Diseases, 26 Yanko Sakazov, 1504, Sofia, Bulgaria
[b] Regional Tuberculosis Dispensary, Sofia, Bulgaria
[c] Department of Biomedicine and Prevention, University of Rome "Tor Vergata", Via Montpellier 1, 00133, Rome, Italy
[d] ProxAgen Ltd., 35 Sitnyakovo Blvd. 1505, Sofia, Bulgaria

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ABSTRACT

Current diagnostic standards for *Mycobacterium tuberculosis* (MTB) infection do not distinguish between active and latent tuberculosis (TB). To identify specific biomarkers characterizing the different forms of TB infection, we investigated in parallel with the QuantiFERON -TB Gold In-Tube (QFT-IT) the use of flow cytometry measuring CD4 and CD8 MTB-specific immune response in 17 active-TB patients, 21 health care workers (HCW), 14 recent contacts of TB patients (RC-TB), and 10 bacille Calmette Guerin (BCG)–vaccinated healthy controls (BCG-HC).

A correlation ($r = 0.4526$, $P = 0.0002$) was found only between the amount of IFN-γ measured by QFT-IT and the frequency of CD4+/CD69+/IFN-γ+ T cells. The frequency of CD4+/CD69+/IFNγ+ responding T cells was higher in active-TB patients ($0.254 \pm 0.336\%$, $P < 0.01$) compared to the other groups. The response of QFT-IT antigen-specific CD8+/CD69+/IFNγ+ T cells was significantly higher in RC-TB ($0.245 \pm 0.305\%$, $P < 0.05$) compared to the other study groups.

1. Introduction

Tuberculosis (TB) still remains a public health problem of paramount importance (WHO, 2006). It has been estimated that nearly 2 billion people, one third of the world's population, are infected by *Mycobacterium tuberculosis* (MTB), the agent causing the disease, resulting in about 9 million new cases every year together with 2 million deaths (WHO, 2006). The control of TB requires a multifaceted approach by integrating public health interventions with the use of efficient new diagnostic tools, vaccines, and drugs. In particular, the rapid and exact discrimination between subjects with active and latent tuberculosis infection (LTBI) represents one of the main measures of the efficient control of disease diffusion (WHO, 2007).

Different approaches have been described recently in the literature to help in discriminating between LTBI and active-TB status. On one hand, the use of immune response against proteins of MTB associated with different phases of infection, such as dormancy-related proteins (DosR) (Leyten et al., 2006) as well as resuscitating protein factors (Commandeur et al., 2011), gives some insight without a clear-cut solution to the problem. On the other hand, a better discrimination between the different TB stages could be envisaged by a more accurate definition of the immune response to the MTB antigens (Billeskov et al., 2007; Ryan et al., 2009). However, all these approaches are still confined to research laboratories without an immediate transfer to the laboratory of clinical pathology and with no impact on patient management.

Upon infection, MTB stimulates both CD4+ and CD8+ T cells as well other cells of the immune system, eliciting a strong type 1 response dominated by interferon (IFN)-γ secretion (Leyten et al., 2006). The overall response is at the basis of the so-called delayed-type hypersensitivity (DTH) caused by MTB antigens. This phenomenon has been used for more than 1 century to identify MTB-infected subjects by the tuberculin skin test (TST), i.e., the intradermal reaction to tuberculin or PPD (Kaufmann et al., 2005).

In the last decade, extensive studies on subtractive DNA hybridization of pathogenic *M. bovis* and bacille Calmette–Guerin (BCG) together with a comparative genome-wide DNA microarray analysis of MTB H37Rv and BCG allowed the identification of antigens for diagnostic and vaccine development, as the immunodominant early

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* Corresponding author. Tel.: +39-06-7259-6202; fax: +39-06-7259-6207.
E-mail address: amicosan@uniroma2.it (M. Amicosante).

[1] These authors contributed equally to the study.

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secreted antigenic target (ESAT-6) and culture filtrate protein (CFP-10) (Mostowy and Behr 2002). Based on these studies, one of the most significant developments in the diagnostic armamentarium for TB in the last hundred years seems to be the assays based on IFN-γ determination (IGRAs), tests that adapted the T-cell response measure to the need of simplification, and the possibility of automation for clinical laboratories. The evaluation in different clinical settings of the IGRA assays using the *MTB* RD1 antigens has shown many advantages over the tuberculin skin test. Although their sensitivity has not been consistent across the different tests and populations, IGRAs have excellent specificity unaffected by BCG vaccination (Pai et al., 2008). However, IGRAs did not distinguish between LTBI and active TB either (Pai et al., 2008), and contradictory results have been obtained with recently exposed subjects (Mack et al., 2009).

Flow cytometry analysis by intracellular cytokine staining (ICS) following appropriate in vitro stimulation adds to the diagnostic advantages of IGRAs, as it allows the phenotypic differentiation between antigen-specific lymphocyte subsets (Sargentini et al., 2009; Sutherland et al., 2009).

In this study, with the aim of identifying specific biomarkers characterizing the different forms of TB infection, we investigated in parallel with the IGRA test QuantiFERON-TB Gold In-Tube (QFT-IT; Cellestis Ltd., Victoria, Australia) the use of single-cell ICS by flow cytometry for the identification of CD4+- and CD8+-specific immune responses elicited against the same antigens present in the QFT-IT test, by using the cells that are discarded from the QFT-IT test after antigen stimulation and recovery of the plasma supernatant for measuring the IFN-γ.

2. Study population and methods

2.1. Study population

This cross-sectional study was performed from February to December 2009 at the Regional Dispensary for Tuberculosis and Lung Diseases, Sofia (Bulgaria). Table 1 summarizes the demographic and principal clinical characteristics for all the study groups.

Specifically, 17 MTB culture–positive confirmed pulmonary active-TB patients were evaluated before the start of the chemotherapy treatment.

A group of 14 recent family contacts of newly diagnosed acid fast bacilli (AFB)–positive active-TB patients (RC-TB), without a previous documented exposure history of TB, were enrolled in the context of the screening upon contact tracing by IGRAs within 2 weeks from the diagnosis of the index case. At the time of the evaluation, they were presenting no signs of active TB and had negative results on chest X-ray and microbiological screening for MTB. No signs of other active diseases were reported. As far as the clinical screening performed was concerned, they can be considered healthy MTB-exposed subjects.

Furthermore, 21 health care workers (HCW), working in a 50-bed ward with more than 100 admissions of TB patients per year (OSHA annual TB risk due to occupational exposure equivalent to 34.5%, accordingly the QFT-IT results and population data; OSHA, 1997), and 10 BCG-vaccinated healthy controls (BCG-HC) were evaluated.

Blood samples for QFT-IT were obtained from all subjects after obtaining informed consent.

2.2. QuantiFERON-TB Gold In-Tube assay

The QuantiFERON-TB Gold In-Tube assay was performed according to the manufacturer's recommendations.

2.3. Intracellular cytokine detection

The intracellular expression of IFN-γ was evaluated using flow cytometry. To this end, 250 μL of whole blood was recovered from either 1 extra tube of QuantiFERON-TB Gold In-Tube assay with TB antigen (containing the ESAT-6, CFP-10, and TB7.7 peptide cocktail) or with no antigen, after 6 to 8 h of incubation at 37 °C. Thereafter, 12.5 μg/mL of Brefeldin-A (BD Biosciences, San Jose, CA, USA) was added and the blood further incubated for the next 14 h at 37 °C, 5% CO_2 atmosphere. The samples were further incubated with EDTA for 20 min and processed using a BD FastImmune Intracellular Cytokine Detection kit, according to the manufacturer's instructions. One hundred microliters of blood was lysed with 1 mL of FACS™ Lysing solution (BD Biosciences) followed by permeabilization with 0.5 mL of 1 × FACS permeabilizing solution (BD Biosciences) for 10 min. After washing with phosphate buffered saline containing 0.5% bovine serum albumin, simultaneous staining for IFN-γ-FITC/CD69-PE/CD4-PerCP/CD8-APC was performed for 30 min at room temperature in the dark. At least 50,000 CD4+ or $CD8^{high}$+ lymphocytes were collected from each sample using a FACSCanto II flow cytometer and analyzed with the BD FACS Diva 6.1.2 software (BD Bioscience).

The combined use of forward scatter/sideward scatter parameters together with the identification of lymphocytes positive for the CD4 molecule or highly positive for the CD8 molecule allowed the precise identification of CD4+ and CD8+ T cells (Nikolova et al., 2005; Storek et al., 1998), further evaluated for the capability to produce IFN-γ (see also Supplementary Material Figures 1 and 2).

2.4. Statistical analysis

Demographic data are presented as mean ±standard deviation or as median and range, as most appropriate. Comparisons among groups were evaluated by the Mann–Whitney *U* test, Fisher's exact

Table 1
Study population characteristics.

Study groups	*n*	Age, median (range)	Sex (M/F)	Race (country of origin)	BCG status, no. vaccinated (%)	PPD skin test[a], no. positive (%)	Chest X-ray, no. positive (%)	AFB smear, no. positive (%)	QFT-IT, no. positive (%)
Active-TB	17	48 (23–85)	10/7	17 Caucasians (17 Bulgaria)	17 (100%)	11/15 (73.3%)	17 (100%)	11 (64.7%)[b]	13 (76.4%)
Health care workers (HWC)	21	40 (26–59)	1/20	21 Caucasians (21 Bulgaria)	21 (100%)	Not available[d]	Not applicable	Not applicable	10 (47.6%)
Recent contacts of TB patients (RC-TB)	14	44 (20–80)	8/6	14 Caucasians (14 Bulgaria)	14 (100%)	10 (35.7%)	0 (0%)	0 (0%)[c]	7 (50%)
BCG-vaccinated healthy controls	10	47 (41–59)	0/10	10 Caucasians (10 Bulgaria)	10 (100%)	Not available[d]	Not applicable	Not applicable	2 (20%)

[a] Tuberculin skin test positive if the reaction of the Mantoux test with PPD is ≥15 mm as per the Bulgarian Ministry of Health guidelines for the interpretation of the test, in a population with mandatory BCG vaccination at childbirth and a penetration of the BCG vaccine at age 5 higher than 99%.
[b] All subjects were MTB-culture positives.
[c] All subjects were MTB-culture negatives.
[d] Recent tuberculin skin test data for the subjects in the study group not available within the past 10 years.