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Interferon-gamma (IFN-γ) and Interleukin-2 (IL2) as immunological markers in pulmonary tuberculosis

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Early diagnosis and treatment are important in prevention of tuberculosis (TB) infection. World commonly diagnose pulmonary TB using ZN stain, culture and TST. These tests are either low sensitive or required a long time. Recently intracellular interferon gamma flow cytometry assay has been available for diagnosis of pulmonary TB. We assess this diagnostic method in the diagnosis of pulmonary TB with special concern on its role and interleukin-2 (IL2) as tools to observe the effectiveness of anti-tuberculosis therapy. In our study we aimed to evaluate the diagnostic potential of flow cytometry assay for diagnosis of active pulmonary TB, assess the levels of intracellular Interferon-gamma (IFN-γ) and IL2 in patients with pulmonary TB as tools to observe the effectiveness of anti-tuberculosis therapy and correlate between the levels of IFN-γ and IL2 in the patients with the clinical and radiological findings. In our study intracellular interferon gamma flow cytometry assay and IL2 release were evaluated for pulmonary TB patients, LTBI persons and healthy control persons. Flow cytometry were done twice for the pulmonary TB patients, first at the start of treatment and second after 3 months of treatment. We confirmed that the intracellular interferon gamma flow cytometry assay after *M. tuberculosis*-specific stimulation is sufficient to recognize active TB. IL-2 were more frequently observed in latent TB infected individuals in contrast to active TB patients, and declined with advanced stage of TB. Intracellular interferon gamma flow cytometry assay could function as a powerful immunodiagnostic test to explore pulmonary TB and to predict the efficacy of antituberculosis treatment after 3 months of treatment in cases of pulmonary TB while, IL2 cannot be used to monitor the efficacy of antituberculosis treatment but it declined with advanced stage (stage III) of pulmonary TB in compare to stages I and II.

Key words: Intracellular Interferon-gamma (IFN-γ), flow cytometry, pulmonary tuberculosis (TB), interleukin-2 (IL2).

INTRODUCTION

Tuberculosis (TB) is one of the most common bacterial infections of humans in the world. In 2013, there were 9 million cases and 1.5 million deaths (World Health Organization, 2013). In Egypt, TB is considered the third most important public health problem after schistosomiasis and hepatitis C (WHO, 2010). One of the major problems in the war against TB is the rapid and accurate diagnosis of TB. Tuberculin skin test (TST) is the most used screening method for the diagnosis of TB. However, a past vaccination with Bacillus Calmette Guérin (BCG) can

lead to false positive results using TST. Also, this test has false negative tests due to immune deficiency or anergy and it cannot differentiate patients with active TB from latent tuberculosis infection (LTBI). Moreover, the site of the skin test should be examined by a professional person within 48 to 72 h to evaluate the result of the test (Goletti et al., 2006b; Keeler et al., 2006).

Newer assays setup on the release of interferon (IFN)-γ upon specific *Mycobacterium tuberculosis (M. tuberculosis)* antigen stimulation, named interferon gamma release assays (IGRAs) has recently become available (Pai et al., 2004).

These tests are characterized by the use of early secretory antigenic target-6 (ESAT-6) and culture filtrate protein-10 kDa (CFP-10). These proteins are encoded within the region of difference 1 (RD1) of *M. tuberculosis* but not BCG genome. So, not synthesized by BCG strains and most of environmental mycobacteria (Pathan et al., 2001).

Some of these tests are commercially obtainable and approved for the diagnosis of TB infection. Although, these tests can differentiate vaccinated individuals from *M. tuberculosis*-infected individuals, but it cannot differentiate active TB patients from individuals with latent TB infection (Goletti et al., 2006 a).

In the IGRA procedure, the second stage of measuring IFN-γ is performed by enzyme linked immunospot (ELISpot) assay or enzyme-linked immunosorbent assay (ELISA). The commercial kit for the latter is Quantiferon-TB Gold (QFT) (Cellestis Ltd, VIC, Australia) (Wallis et al., 2010).

Flow cytometry play a promising role in rapid diagnosis of TB, using markers for cell viability and death (Hendon-Dunn et al., 2016). One of the most interesting is intracellular interferon gamma flow cytometry assay (Janossy et al., 2008).

However, these methods cannot differentiate active tuberculosis from LTBI (Herrera et al., 2011). In this study we investigate intracellular interferon gamma flow cytometry assay and IL2 as differential markers between TB patients and LTBI and as prognostic markers of improvement.

METHODOLOGY

This study protocol was approved by the ethical committee in faculty of medicine, Mansoura University.

Study population

Patients admitted to Mansoura chest hospital between January 2015 and December 2015 were evaluated. Enrolled individuals were classified as active TB when the diagnosis was confirmed by a positive *M. tuberculosis* culture from sputum specimens. LTBI was defined by a positive response to the TST and to QFT without any signs of active disease.

Also, healthy persons were enrolled in this study. The healthy subjects were laboratory personnel with negative QFT and without any signs of active disease.

Exclusion criteria were haematological malignancy, HIV, other immunosuppressive disease, current or previous cytotoxic and immunosuppressive treatments.

Clinical samples

Two venous blood samples were collected by venipuncture from each pulmonary patient. The first sample was taken at the time diagnosis of pulmonary TB and the second was taken after 3 months of starting antituberculosis drugs. Blood were tested for the concentration of intracellular IFN-γ by flow cytometry assay and IL-2 release by ELISA. Also, one blood sample was taken from each healthy subject and LTBI persons and tested by the same methods used in the patients.

Methods

All TB patients, LTBI and healthy control persons in our study were evaluated by the following methods.

In vitro stimulation of peripheral blood

In vitro stimulation of peripheral blood was performed using QFT tubes. After 18 h of incubation, part of blood was assayed for CD4/ IFN-γ T cells by flow cytometry and then assayed again after 72 h of incubation.

CD4/ IFN-γ T cells measured by flow cytometry

Blood samples were analyzed for CD4/IFN-γ T cells by flow cytometry using a Fluorescence Activated Cell Sorter (FACS)-calibur flow cytometry (Epics XL Flow cytometry, Coulter company) with specific monoclonal antibodies: Anti-CD4 PE/Cytochrome 5 (PE/Cy5) (clone SK3, mouse IgG1) and anti-IFN-γ Fluorescein isothiocyanate (FITC) (clone 4S.B3, mouse IgG1) .

All antibodies were obtained from Becton Dickinson (BD) Immunocytometry Systems (San Jose, CA, USA).

One hundred micro liters of whole blood was mixed with 20 µL of monoclonal antibodies recognizing CD4 and incubated for 15 min. Haemolysis was performed using 2 mL of a 1:10 diluted 10X concentrate solution for haemolysis (BD company). After 10 min of incubation, the tube was centrifuged at 2000 rpm for 5 min. Then, the supernatant was discarded to remove lysed red blood cells and the remaining pellet was divided into two parts the first part washed twice with 1 mL of PBS and then redissolved in 0.5 mL of PBS.

Nine hundred microliters of working BD FACS permeabilizing solution (BD Biosciences, San Jose, CA) was added to the second part, which was then incubated for 10 min at room temperature, washed, redissolved in 0.5 mL of PBS and then 20 µL of anti-IFNγ Fluorescein isothiocyanate (FITC) was added. Then, the CD4/

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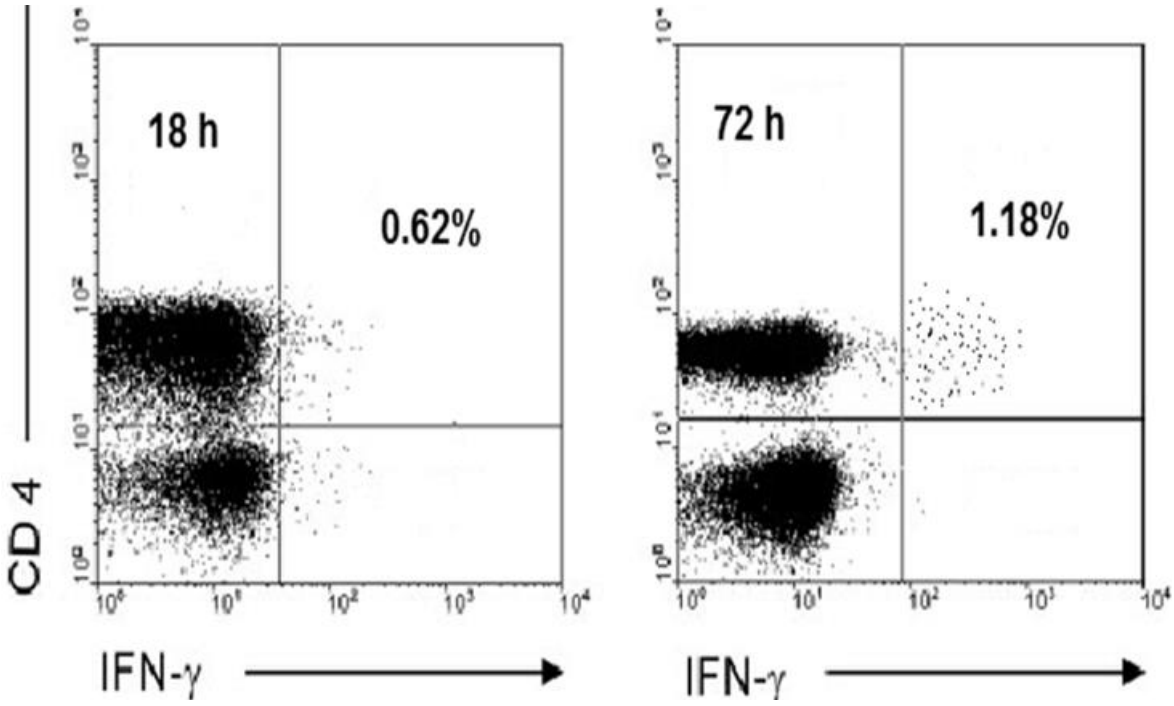

Figure 1. Comparison between CD4/ IFN-γ T cells % measured in active TB patients after 18 h and 72 h of incubation in QFT tubes.

IFN-γ T cells% were calculated and expressed as a percentages from the lymphocytes area noticed in flow cytometry.

Flow cytometer used is Epics XL Flow cytometry PN: 42372238, Coulter company, Miami, Florida 33196, USA.

IL-2 measurement

IL-2 release was evaluated with an ELISA assay (DRG GmbH, Germany), according to the manufacturer's instructions.

Statistical analysis

Mean, standard deviation, frequency, were used to describe data. P value was considered significant if less than 0.05 and highly significant, if less than 0.001. These tests were done using the Statistical Package for Social scientists (SPSS) (SPSS Inc., Chicago, IL, USA).

RESULTS

Patients were categorized according to the size and X-ray findings into three stages following the classification of Dlugovitzky et al. (1999): Stage I, mild cases (5 patients), patients with a single lobe involvement and without apparent cavities; stage II, moderate cases (14 patients), patients display unilateral involvement of two or more lobes with cavities, if present, reaching a total diameter less than 4 cm; and stage III, advanced cases (6 patients), bilateral disease with huge involvement and multiple cavities.

From Table 2 and 3, intracellular INF-γ discriminates active *M tuberculosis*-infected patients from healthy controls but does not discriminates active patients from LTBI persons.

While, IL-2 release differentiate individuals with LTBI from active TB patients. It is believed that the responder cells were T effector memory (TEM) due to the reduced time of *in vitro* stimulation. The cytokine secreting T cells increase when the assay was performed after 72 h of incubation in quantiferon tubes. Also, Table 5 shows that CD4/ IFN-γ T cells% show statistically highly significant P-value for the patients after 3 months of treatment in correlation to improvement (<0.001) while, IL2 values in pulmonary TB patients show no statistically significant P-value in correlation to improvement after 3 months of treatment.

DISCUSSION

Among the 25 active TB patients presented in Table 1; 18 patients were males and 7 patients were females and among the 25 LTBI persons; 19 persons were males and 6 were females. The lower rates of pulmonary tuberculosis in females may be due to the under diagnosis or under reporting of tuberculosis in females as a result of different social and cultural factors. It might also be due to true differences in the rates of infection with *MTB*, reflecting public, cultural, and biological factors that influence the chances for the transmission of *MTB*.

Table 1. Clinical characteristic of enrolled individuals.

Parameter	Healthy control	Latent TB patients	Active TB patients
Number	25	25	25
Age (median) years	32	33.3	30.3
Positive TST	22	25	19
BCG vaccine scar	8	8	9

Table 2. CD4/ IFN-γ T cells % and IL2 measured after 18 h incubation in QFT tubes.

Cytokines	Healthy control	Latent TB	Active TB patients	P value
CD4/IFN-γ%	0.006 ± 0.002%	0.59 ± 0.19%	0.62 ± 0.2%	0.01 (healthy control- active patients) 0.39 (latent TB -active TB)
IL-2 (U/ml)	0.07 ± 0.03	0.66 ± 0.25	0.22 ± 0.07	0.31 (healthy control- active patients) 0.17 (latent TB -active TB)

Table 3. CD4/ IFN-γ T cells % and IL2 measured after 72 h incubation in QFT tubes.

Cytokines	Healthy control	Latent TB patients	Active TB patients	P value
CD4/ IFN-γ %	0.008% ±0.003	0.73% ± 0.19	1.18% ± 0.42	0.01 (healthy control- active patients) 0.22 (latent TB -active TB)
IL-2 (U/ml)	0.13 ± 0.05	11.2 ± 4.1	0.54 ±0.16	P value < 0.01 between LTBI and other groups

These findings are in concurrence with Rajesh et al. (2011) study which found the male-female ratio was 1.5: 1.

Currently, the most widely available test for the diagnosis of pulmonary TB is the direct sputum smear for acid fast bacilli (AFB) by ZN stain and the TST. Because of the low sensitivity of TST and sputum smear by ZN, recent advances have led to a promising generation of alternative tests such as *in vitro* intracellular interferon gamma flow cytometry assay.

Intracellular interferon gamma flow cytometry/ CD4 measured in response to ESAT6 and CFP10 show higher results in subjects with LTBI and patients with active TB than healthy controls (Figure 1) after 18 h of incubation (median 0.59 and 0.62% respectively, vs. 0.006%) as well as after 72 h of incubation (median 0.73 and 1.18%) for LTBI and TB patients, respectively, and 0.008% for the control group.

Intracellular interferon gamma flow cytometry/CD4 T cells measured were not significantly different between the active TB and LTBI groups. In this study neither at 18 h nor at 72 h intracellular IFN-γ allow the differentiation between LTBI individuals and active TB patients (P value: 0.39 and 0.22 after 18 and 72 h of QFT incubation, respectively).

In our study, using healthy controls we determine a positive cut-off response for patients with TB or LTBI > 0.01% of CD4+ IFN-γ+ T cells %. This cut-off percentage agrees with Leung et al. (2009). Moreover, Lichtner et al. (2015) determined that the cut-off response for patients with TB using IFN-γ+ IL-2+ TNF-α+ CD4+ T cells was more than 0.182%.

M. tuberculosis-specific antigens induced the production of IL-2 in LTBI individuals after 18 h of incubation but without significant P value as regard active TB patient and healthy individuals. When the incubation was extended to 72 h, the amounts of IL-2 secreted by LTBI subjects (median: 11.2 U/mL) were significantly higher than those released by active TB patients (median: 0.54 U/mL) and healthy controls (median: 0.13 U/mL) ($p < 0.001$).

So, the amounts of IL-2 measured at 18 h of incubation are inappropriate for determination of active TB patients. However, after 72 h of incubation, IL-2 secretion increases only in individuals with LTBI, in harmony with preliminary data obtained by Sargentini et al. (2009).

These data reflect the increased number of IL-2-secreting and IL-2/IFNγ-secreting central memory T-cells (Millington et al., 2011) and the decreased number of effector memory T-cells which secret IFN-gamma in

Table 4. Correlation between intracellular interferon gamma flow cytometry assay and IL-2 and the stage of pulmonary TB patients after 72h incubation.

Cytokines	Stage I	Stage II	Stage III
CD4/ IFN-γ %	1.26	1.22	1.11
IL-2 (U/ml)	0.44	0.63	0.19

Table 5. Improvement in pulmonary TB patients in relation to IL-2 and CD4/ IFN-γ T cells% before and after 3 months of treatment.

Pulmonary TB patients		IL2 before treatment (72 h QFT incubation)	IL2 3 months after treatment (72 h QFT incubation)	CD4/ IFN-γ at the start of treatment	CD4/ IFN-γ after 3 treatment
No improvement	Frequency	7	7	7	7
	Mean	0.55	0.35	1.42%	1.65%
	Standard deviation	0.28	0.15	0.48	0.51
Improvement	Frequency	18	18	18	18
	Mean	0.26	0.19	0.91%	0.21%
	Standard deviation	0.18	0.09	0.32	0.05
P-value		0.28	0.25	<0.001	<0.001

patients with latent TB infection in comparison with individuals with active TB, owing to the absence of replication of *M. tuberculosis* and, consequently, antigen load. Our results agree with those of Millington et al. (2007) which show IFN γ secreting T cells predominate during active TB. Also the increased number of IL-2-secreting cells in individuals after antituberculous treatment may be considered to be a result of the central memory T-cells expansion, caused by the decreased *M. tuberculosis* antigen load, that are likely to predominate also in LTBI.

On the other hand, some studies found that specific TB antigens induced IL-2^+ IFN-γ^+ secreting cells cannot discriminate between active tuberculosis, LTBI persons and control subjects (Fousse et al., 2011). Conflicting data may be due to different test assays (flow cytometry, QFT), as well as different antigens preparations (Chiacchio et al., 2014) and differences in protocols, e.g. the time of incubation (Marin et al., 2013).

Out of 25 pulmonary TB patients in our study, 18 were found to improve clinically in the laboratory after 3 months of treatment, as revealed in appearance of negative sputum smears by ZN, absence of symptoms and signs of TB and regression of radiographic abnormalities after 3 months of treatment. On the other side, 7 pulmonary TB patients were still without improvement.

Among the pulmonary TB patients without improvement, the mean intracellular interferon gamma/ CD4 measured by flow cytometry at the start of treatment was 1.42% and after 3 months of treatment 1.65% while, in patients with improvements was 0.91% and declined to 0.21% after 3 months of treatment. Intracellular interferon gamma/ CD4 flow cytometry values showed statistically significant P-value in correlation to improvement (<0.001) after 3 months of treatment and this means that the progressive decline of IFN-γ response measured could be used to monitor the efficacy of treatment.

Previous studies on the effect of therapy on intracellular interferon gamma assay by flow cytometry results have generated contradictory data, with some studies as Kim et al. (2014) suggesting that responses to TB-specific antigens might decrease sufficiently with treatment and interferon gamma assay by flow cytometry might use as a tool to monitor the efficacy of treatment, other studies as Mensah et al. (2014) suggesting that responses might not change sufficiently for intracellular interferon gamma assay by flow cytometry to be used for this purpose.

Finally, our study agree with Bertholet et al. (2011) and Feruglio et al. (2014) which show that IL2 alone cannot be used to monitor the efficacy of antituberculosis treatment but it declined with advanced stage (stage III) of pulmonary TB in compare to stage I and II (Table 4).

Conflict of Interests

The authors have not declared any conflict of interests.

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