Intracellular cytokine flow cytometry (ICCFC) for measurements of TB-specific T cells

Hossam M. Abdel Aziz[a,*], Shahira Morsy El- Shafei[a], Assem Fouad Al- Essawy[b], Nehal Sami Mohamed[c], Fadwa Abdel Reheem Mohammad[a]

[a]Clinical and Chemical Pathology Department, Faculty of Medicine, Fayoum University, [b]Chest Department, Faculty of Medicine, Fayoum University, [c]Clinical and Chemical Pathology Department, Chest Hospital, Fayoum, Egypt

*Correspondence to Hossam Mahmoud Abdel Aziz, MD, Assistant Professor, Clinical and Chemical Pathology Department, Faculty of Medicine, Fayoum University, El Fayoum, Egypt
Tel: +20/1554043143;
e-mail: hma05@fayoum.edu.eg

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Background
Tuberculosis (TB) is a communicable disease that is a major cause of ill health, one of the top 10 causes of death worldwide, and the leading cause of death from a single infectious agent (ranking above HIV/AIDS). Multifunctional T cells, defined by their ability to coexpress two or more cytokines, e.g., (TNF-α and IFN-γ), have shown a good diagnostic yield to detect TB infection.

The aim of the study
Assessment of the diagnostic sensitivity of intracellular cytokine flow cytometry analysis of multifunctional specific CD4+ T cells coexpressing TNF-α and IFN-γ after stimulation with phytohemagglutinin for the diagnosis of active pulmonary TB.

Patients and methods
This study was performed on 30 active pulmonary tuberculosis (APTB) patients who were admitted to Fayoum Chest Hospital and 30 healthy controls. CD4+ T cells were stimulated by phytohemagglutinin (PHA) and then were measured using the intracellular cytokine staining technique by flow cytometry to detect CD4+ T cells expressing TNF-α and IFN-γ on whole-blood samples.

Results
Receiver-operator characteristic (ROC) analysis was done to determine the optimum cutoff value for the studied diagnostic markers, which was the difference between the percentages of $CD4^+$ lymphocytes coexpressing TNF+α and IFN+γ before phytohemagglutinin (PHA) stimulation (inactivated CD4+) and after phytohemagglutinin (PHA) stimulation (activated $CD4^+$) in the cases and controls, which was 4.9 with sensitivity 90% and specificity 50%. Using this cutoff, it scored 27 (90%) as positive from 30 APTB of active TB patients and 15 (50%) as negative from 30 of healthy controls.

Conclusion
Multifunctional flow cytometry analysis of specific CD4+ T-cell response may represent a relatively simple and rapid immune-based approach to distinguish between MTB-infected and -uninfected patients.

Keywords:
CD4+ T cells, cytokine, IFN-γ, intracellular cytokine flow cytometry, TNF-α, tuberculosis

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Introduction

Tuberculosis (TB) remains a major global health problem, responsible for ill health among millions of people each year. With a timely diagnosis and treatment with first-line antibiotics for 6 months, most people who develop TB can be cured and onward transmission of infection curtailed [1]. Multifunctional T cells have been linked to bacterial load and disease activity [2]. Flow cytometry has been suggested to possibly improve the diagnostic accuracy of Mycobacterium tuberculosis (MTB)-specific stimulation assays, because MTB-specific T-cell subsets producing different types of cytokines can be analyzed on a single-cell basis [3,4].

Patients and methods

A total of 60 individuals were enrolled in this study, 30 patients with active pulmonary tuberculosis (APTB) (age: mean ± SD = 34.30 ± 10.27), (12 women and 18 men) and 30 age- and sex-matched healthy controls. Diagnosis of active TB was made on the basis of clinical and radiological findings and was confirmed by identification of M. tuberculosis with microbiological methods.

Inclusion criteria

APTB patients who were diagnosed on the basis of the presence of recent clinical symptoms of tuberculosis, a positive sputum-smear test for acid-fast bacilli

confirmed by a positive culture of M tuberculosis by BACTEC MGIT 960 System and characteristic chest radiograph. Both male and female patients consent to the experimental laboratory procedure.

Exclusion criteria

HIV positivity, immunological, or autoimmune diseases and pregnancy.

The controls were healthy individuals with no previous history of TB, no known TB contacts, and were tested negative for TST and negative AFB.

Heparinized peripheral blood samples from APTB patients and healthy controls were collected.

Sample preparation for flow cytometry

For each sample, two tubes were labeled as follows: positive tube containing 500 μl of heparinized blood and 7 μl of phytohemagglutinin and negative tube containing the same amount of blood without phytohemagglutinin. Both tubes were incubated in the CO_2 incubator for 2 hours at 37°C, then removed from the incubator, and 5 μl of Brefeldin A was added to the positive tubes, both tubes were incubated again in the CO_2 incubator for 8 hours at 37°C.

Sample processing combined membrane and intracytoplasmic staining procedure using conjugated antibodies.

From each of the negative and positive tubes, 50 μl of blood was transferred to polypropylene tubes and then 10 μl of membrane-specific conjugated monoclonal antibody CD4 was added to each tube.

The tubes were vigorously vortexed, then incubated for 15 min at room temperature (18–25°C) in the dark, and then 100 μl of intraPrep fixation reagent (5% formaldehyde) was added to each tube.

Then, they were vigorously vortexed and incubated for 15 min at room temperature in the dark, then 4 ml of sterile phosphate-buffered saline (PBS) was added, the tubes were centrifuged for 5 min at 300 × g, and the supernatant was discarded by aspiration.

Then, 100 μl of intraPrep Permeabilization reagent (PBS-buffered, saponin-based, and lysing medium) was added to each tube and mixed without vortexing, incubated for 15 min at room temperature in the dark, and gently shaken manually for 1–2 s.

Ten μl of intracellular-conjugated specific antibody TNF-α and 10 μl of intracellular-conjugated specific antibody IFN-γ was added to each tube, gently vortexed, and then incubated again for 15 min at room temperature in the dark.

Four ml of PBS was added, the tubes were centrifuged for 5 min at 300 × g at room temperature, and the supernatant was discarded by aspiration.

The pellets were resuspended with 500 μl of PBS and processed by flow cytometry analysis.

For data analysis, a sequential gating strategy was used to detect cytokine-producing TNF-α and IFN-γ repertoires among CD4 lymphocytes.

Plot 1: In this plot, side scatter versus CD4+PECY5 of all events was displayed, region E was set around CD4+ events (Figs. 1, 2).

Plot 2: This plot was designed to display IFN-γ FITC versus TNF-α PE of the gated cells of region E. From this histogram, region F was determined, which showed triple coexpression of CD4+ PECY5, IFN-γ FITC, and TNF-α PE.

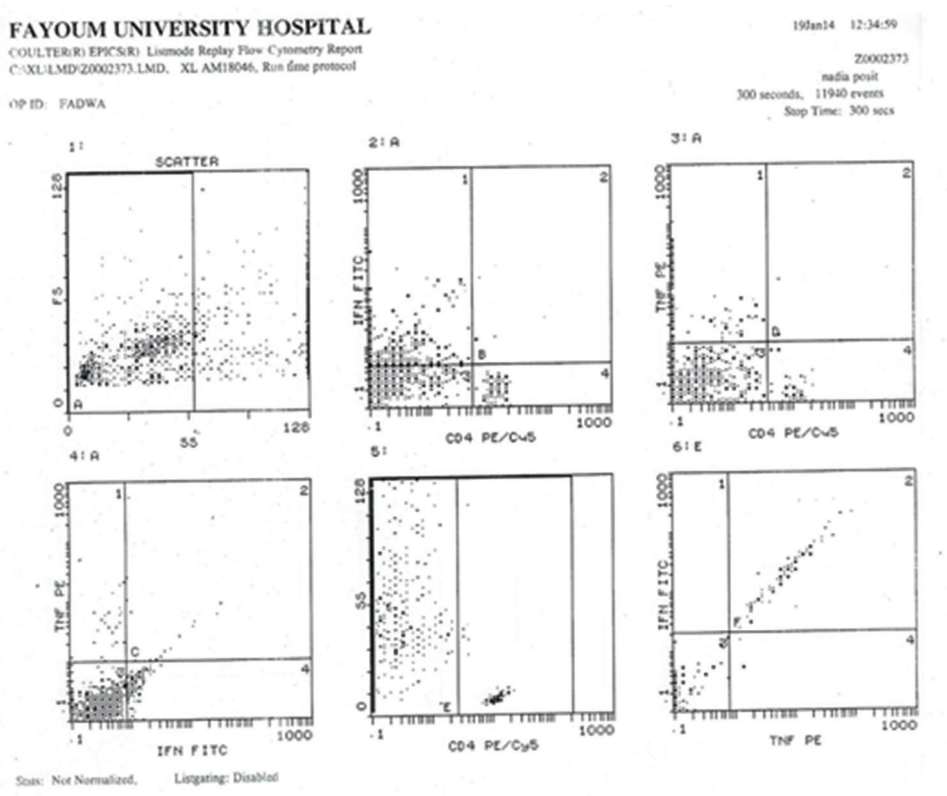
Data were statistically described in terms of mean ± standard deviation (±SD), median and range, or frequencies (number of cases) and percentages. Comparison of numerical variables between the study groups was done using Student *t* test for independent samples when normally distributed and Mann–Whitney *U* test for independent samples when not normally distributed. Accuracy was represented using the terms sensitivity and specificity. Receiver-operator characteristic (ROC) analysis was used to determine the optimum cutoff value for the studied diagnostic markers. *P* values less than 0.05 were considered statistically significant. All statistical calculations were done using computer program SPSS (Statistical Package for the Social Science; SPSS Inc., Chicago, IL, USA) release 15 for Microsoft Windows (2006).

Results

The mean age in years for the active pulmonary tuberculosis group was 35.16 (range of 19–51) and for the healthy-control group, the mean age was 28.83 (range of 19–42). There were no statistically significant differences in age or sex among the patient groups.

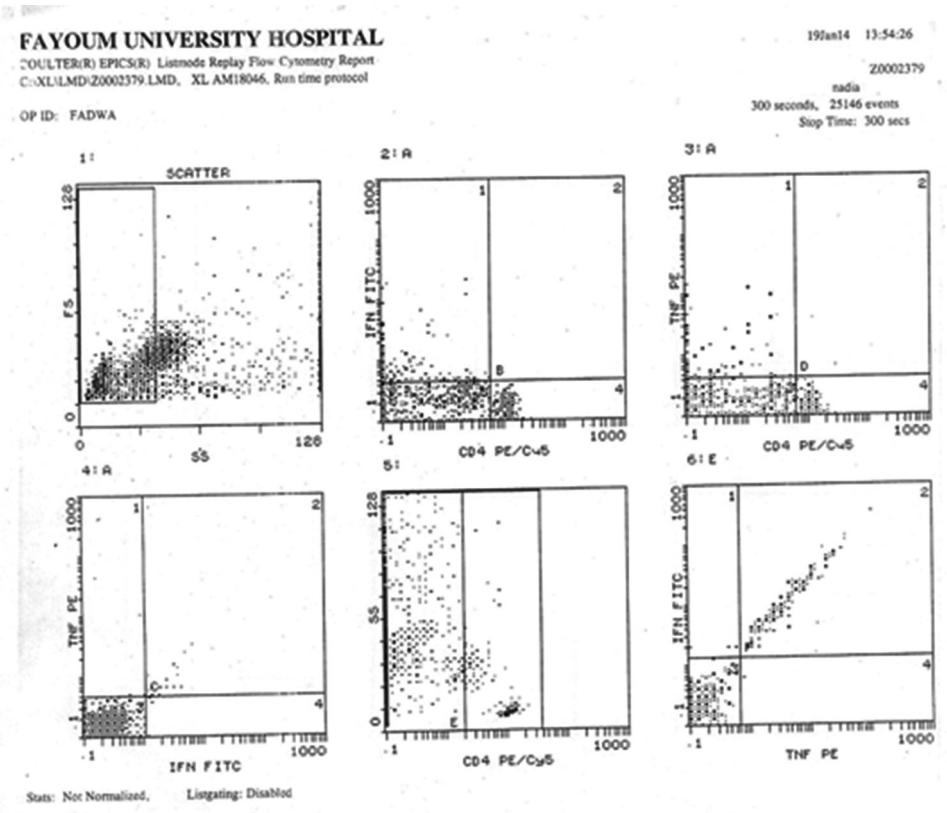
Diagnosis of active TB was made on the basis of clinical and radiological findings and was confirmed by identification of M. tuberculosis with microbiological methods.

Figure 1



Activated CD_4.

Figure 2



Inactivated CD_4.

We identified CD4+ lymphocyte cells coexpressing TNF-α and IFN-γ after being stimulated with phytohemagglutinin (PHA) as activated CD4+ and CD4+ lymphocytes coexpressing TNF-α and

IFN-γ cells, which were not stimulated with phytohemagglutinin (PHA) as inactivated CD4+. The percentage of activated $CD4^+$ cells (38.4 ± 20.3) was higher than inactivated CD4+ cells (18.8 ± 15.5) in the patient group (Figs. 1,2). Also, in the control group, the percentage of activated CD4+ cells was higher (11.6 ± 11.5) than the inactivated cells (9.4 ± 8.95), but the difference between the percentages of the two types of cells was significantly higher in patient the group (Table 1).

Receiver-operator characteristic (ROC) analysis was used (Fig. 3) to determine the optimum cutoff value for the studied diagnostic markers, which was the difference between the percentages of $CD4^+$ lymphocytes coexpressing TNF-α and IFN-γ after phytohemagglutinin (PHA) stimulation (activated CD4+) and without phytohemagglutinin (PHA) stimulation (inactivated $CD4^+$) in the cases and controls, which was 4.9 with sensitivity 90% and specificity 50%.

We suggest that patients showing the difference between the percentages of activated $CD4^+$ lymphocytes inactivated $CD4^+$ of more than 4.9 to be considered as a TB-positive case, while less than 4.9 is probably not a TB case.

Discussion

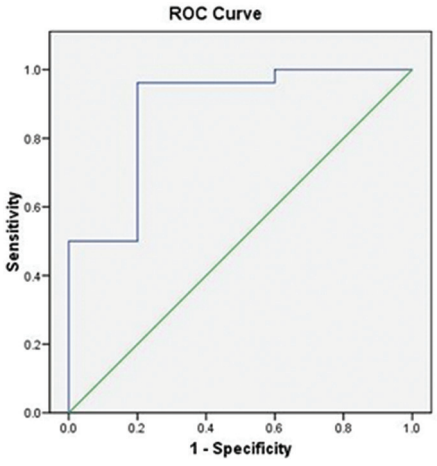
Despite improvements in tuberculosis-control-program performance, the diagnostic sensitivity of active-tuberculosis-case rates in many regions remains at unacceptable levels. Thus, the aim of the current study is to assess the diagnostic sensitivity of intracellular cytokine flow cytometry analysis of multifunctional specific CD4+ T cells coexpressing TNF-α and IFN-γ after stimulation with phytohemagglutinin for the diagnosis of active pulmonary TB.

We hypothesized that a distinct cytokine profile could be useful for the diagnosis of active TB. In our study, the background cytokine production in healthy controls was subtracted from the activated tubes and this difference was statistically significant, *P* value 0.000.

Receiver-operating characteristic (ROC) analysis was performed to calculate the optimal cutoff values for the difference and we found out that a cutoff value more than 4.90 allowed the best combination of sensitivity 90% and specificity 50% to discriminate between active-tuberculosis patients and healthy participants. Using this cutoff, we scored 27 (90%) as positive from 30 APTB of active TB patients and 15 (50%) as negative from 30 of healthy controls. These findings were in agreement with Lichtner *et al.* [5] who suggested that the analysis of activated CD4+ T coexpressing TNF-α and IFN-γ cells allowed the discrimination between MTB-infected and -uninfected patients with a cutoff >0.45%, allowing the best combination of sensitivity 94.44% and specificity 95%. This finding is also in agreement with Lee *et al.* [6], but with a different cutoff value, which was 8.0 with sensitivity 91% and specificity 100%, this discrepancy can be explained by the different size of groups involved.

Despite different detailed outcomes, variable cutoffs, and variable sensitivity and specificity of the results in different centers, it is clear that this procedure can help in solving the worldwide problem of missed undiagnosed cases, especially after multicenter-result accumulation that will help standardization of the technique.

Figure 3



Receiver-operator characteristic (ROC) analysis.

Table 1 Difference between activated and inactivated CD4+ in APTB and HC

	APTB (NO 30)	HC (NO 30)	*P*
Difference	19.5±10.9	2.2±10.8	0.000

Conclusion

Flow cytometry analysis of specific CD4+ T-cell reaction toward MTB may represent a quite simple and rapid immune-based method that could be used in approaching the diagnosis of APTB. In the current study, we performed receiver operating characteristic (ROC) analysis to calculate optimal cutoff values of the difference between activated and inactivated CD4+, TNFα+, and IFNγ+ lymphocytes to diagnose APTB patients. Our study proposed a cutoff value of 4.9 with sensitivity 90% and specificity 50% to diagnose APTB.

Recommendations

It is recommended that a meta-analysis study should be performed for broader utility of multicentric cutoff values to suggest a universal value.

Embedding this diagnostic procedure is suggested in detection of patient outcomes and prognosis.

It is recommended that more researches should investigate more cytokine evolutions, such as IL-2 and IL-10, which have an important role in TB infection.

This immunological approach needs to be validated in a larger and prospective study and to be extended to other forms of tuberculosis as latent, multidrug resistance MRD and TB with immune suppression.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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