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Late diagnosis and TCD8 immune response profile of cutaneous tuberculosis: A case report

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ABSTRACT

Introduction: *Cutaneous tuberculosis* (CTB) is a rare form of extra-pulmonary tuberculosis that, when associated with late diagnosis, worsen the quality of life of the sick individuals. This report presents a case of late diagnosis of CTB. Unusual clinical manifestations retarded the correct tuberculosis diagnosis for more than a year. The immune response elicited by this type of tuberculosis as well as the factors that might contribute to the delay in diagnosis was evaluated and discussed. Methodology: Clinical evaluation and flow cytometric analyses of PBMC were realized for a case of CTB and a healthy individual as a control. Results: *M. tuberculosis* specific TCD8+ cell response was analyzed by flow cytometry and revealed positive cells for IL-17, IL-10, TGF-*β* and IDO. The CTB patient presented higher percentage of those cells when compared to a healthy donor. However, TCD8 cells positive for the important protective cytokine, IFN-*γ* was decreased in the CTB patient. Conclusion. The assessment of the *M. tuberculosis* specific TCD8+ immune response showed a regulatory/modulatory phenotype with a reduced IFN-*γ* response when compared to the healthy control that could have contributed to the CTB infection.

Keywords: *Cutaneous tuberculosis*; Late Diagnosis; CD8+ T Cells

1. INTRODUCTION

Tuberculosis is a chronic disease that affects millions of people in the world. It is a disease that can affect every organ of the body and consequently have equally diverse clinical manifestations, but the pulmonary form is the most frequent. Cutaneous tuberculosis (CTB) is a rare form of extra-pulmonary tuberculosis that can be caused by *Mycobacterium tuberculosis*, *M. bovis* and in certain conditions by the attenuated *M. bovis-BCG* [1]. Despite its rare frequency, CTB corect diagnosis and management are fundamental, both for the patient as well as for public health. Additionally, long lasting, misdiagnosed and/or untreated CTB can lead to the development of a variety of types of cancer [2].

The CTB clinical manifestations depend on many factors: the route of infection, the host immune status, and drug resistance and/or bacteria pathogenicity [3-6]. When infection occurs by laboratory accident caused by clinical samples or *M. tuberculosis* cultures injected directly into the skin, the CTB clinical morphological variants are verrucosa cutis (it manifests as a painless, solitary, purplish or brownish-red warty plaque that may extend peripherally causing central atrophy or form fissures that exude pus or keratinous material), lupus vulgaris (plaque type begins as discrete, redbrown papules that coalesce and form plaques with a slightly elevated verrucas border and central atrophy) or skin ulcers (chancres). A lymphatic or hematogenous disseminated miliary TB may cause CTB variants such as escrofuloderma (firm, painless, subcutaneous, red-brown nodules overlying an infected focus, which gradually enlarge and suppurate forming ulcers and sinus tracts that drain watery, purulent, or caseous material), lupus vulgaris (multiple, discrete, smooth 1 - 3 mm brown/red or brown-to-yellowish dome-shaped papules usually located on the face), and tuberculous abscesses (an inflammatory papule develops at the inoculation site and evolves into a firm, shallow, non-tender, nonhealing, undermined ulcer with a granulomatous base) as described before [2,7].

We report a case of cutaneous tuberculosis in a 50-year-old male patient with unusual clinical symptoms that consequently delayed the correct diagnosis for two years. The clinical evolutionary aspects of the disease and the cellular immune response against *M. tuberculosis*

antigen were evaluated. The difficulty in diagnosing extrapulmonary tuberculosis and the immune response associated to its development was reviewed and discussed.

2. CASE REPORT

A 50-year-old HIV-negative male reported the appearance of two papules of gelatinous consistency on the neck with pus secretion in a 12 months interval. The clinical suspicion of a tumor led to a cervical ultrasound analysis that accused two solid cystic firm lesions, with enlarged lymph nodes. CT scan also revealed enlarged lymph nodes in the neck. Removal of the lumps showed necrosis redirecting the diagnosis to an infection, thus an empirical treatment was prescribed: amoxicillin (250 mg) for 10 days, but resulted in no health improvement/ resolution. A thoracic plain chest X-ray film showed normal results.

After eight months, the patient returned to the hospital and complained the appearance of other papules with similar morphology and size on the chest. Again, 10 days of amoxicillin (500 mg) was prescribed. Due to the unaltered clinical signs, two months later, a chest CTS was performed, and revealed the presence of cutaneous abscesses without lung alterations. A series of tests was conducted, in order to identify the etiology of the illness, but all of them showed negative results (**Table 1**). At this time, abscesses and lymph nodes were biopsied and the samples from the abscesses showed a chronic granulomatous inflammation with caseous necrosis. These lesions were positive for acid-fast bacilli.

Two years after the first clinical symptoms, and one year after the first heath assistance, a clinical diagnosis of cutaneous tuberculosis was made. Therefore, TSPOT.TB and tuberculin skin tests (TST) were performed and resulted positive. Culturing and performing biochemical tests of the lymph node samples confirmed *Mycobacterium tuberculosis*. The patient underwent standard treatment for TB with rifampicin, isoniazid, pyrazinamide and ethambutol for 2 months, followed by rifampicin and isoniazid for 4 months.

3. MATERIALS AND METHODS

In order to understand the immune factors that could have contributed for the CTB development and the unusual clinical findings, a 20 mL of blood sample with heparin was collected at the time of the CTB diagnostic. As control, blood from a healthy donor (TST-) matched by sex and age was recruited.

3.1. Peripheral Blood Mononuclear Cells and Cell Culture

Peripheral blood mononuclear cells (PBMCs) were obtained from CTB patient and TST negative control by Ficoll density gradient centrifugation (Ficoll-Paque Plus, GE Healthcare Bio-Sciences AB). The cells were washed twice in saline and distributed in 96-well plates at 2×10^5 cells/mL in RPMI 1640 medium (GIBCO, Invitrogen Corporation) supplemented with 2 mM glutamine, 10 Nm pyruvate, 2 mM amino acids, 50 µg/mL penicillin, 50 µg/mL streptomycin and 10% heat-inactivated bovine serum. They were then incubated with recombinant *Mycobacterium tuberculosis* antigen (MPT-51, 1 µg/mL)

Table 1. CTB patient's laboratorial tests.

Laboratorial analysis		Test	Normal Value Range	Results
X-ray of the spine		Normal	Normal	Normal
Cytomegalovirus	IgG	13.8 U/mL	To 8 U/mL	Reagent
	IgM	2 U/mL	To 15 U/mL	Not Reagent
Toxoplasmosis	IgG	12.6 U/mL	To 10.5U/mL	Reagent
	IgM	Not Reagent	Not Reagent	Not Reagent
Amylase		30 U/L	22 - 80 U/L	Normal
Bilirrubin	Direct	0.11 mg/mL	0 - 0.20 mg/mL	Normal
	Indirect	0.36 mg/mL	0.20 - 0.80 mg/mL	Normal
	Total	0.49 mg/mL	0.20 - 1.00 mg/mL	Normal
TGO		28 U/mL	To 37 U/mL	Normal
TGP		31 U/mL	To 41 U/mL	Normal
Leucogram		5700 mm^3	4000 - 10,000 mm^3	Normal
Prothrombin test		11.9 s	11.1 s - 13.2 s	Normal
VDRL		Not Reagent	Not Reagent	Not Reagent

previously shown to be immunogenic for pulmonary TB patients [8] or with PHA (1 μg/mL) as positive control and cultivated at 37˚C with 5% CO_2 for 96 hours in the presence of anti-CD3 (eBioscience). Cells stimulated with medium alone or anti-IgG1 and anti-CD3 were used as control.

3.2. Flow Cytometry

The following antibodies (Abs) were used for surface and intracellular staining for flow cytometry: IFN-γ-FITC (BD Bioscience Pharmingen), IL-10-PE, IL-17-PE, CD8-APC (eBioscience), TGF-β-PE (IQ Products) and IDO-FITC (Santa Cruz Biotechnology). The cells were also stained with PE-conjugated mouse IgG1 for isotype control. For flow cytometry analysis, medium alone, PHA or TB antigen stimulated cells were treated with Golgi Stop Solution (containing monensin-BD Biosciences Pharmingen), and after 4 h of further incubation, they were harvested for analysis. To perform surface and intracellular staining, the cells were treated with PBS containing 0.05% azide for 20 min. After centrifugation (3000 rpm for 10 min), the cells were stained at 4˚C for 18 minutes with surface marker Abs (CD8-APC). Subsequently, the plates were washed twice with PBS containing 0.05% sodium azide and treated with PermFix (BD Pharmingen, San Jose, USA) for 18 min. For intracellular staining, the cells were permeabilized with Perm Wash buffer (BD Biosciences Pharmingen) and incubated at 4˚C for 18 min with the following specific antibodies: IFN-γ-FITC (BD Biosciences Pharmingen), IL-10-PE, IL-17-PE (eBioscience), TGF-β-PE (IQ Products), and IDO-FITC (Santa Cruz Biotechnology). After washing, the samples were immediately analyzed on a FACSCanto II apparatus (Becton Dickinson, San Jose, USA) at the Farmatec/UFG (Goiás, Brazil). At least 50.000 events were acquired per sample. Data analysis was performed using FACSDiva software (BD Biosciences Becton Dickinson). All studies were approved by the Hospital of the Federal University of Goiás Ethics Committee (Goiás, Brazil), and informed consent was obtained from all participating subjects.

4. RESULTS

To address if the unusual development of cutaneous TB, in this patient, was due to a defective TCD8+ immune response, specific TCD8+ subsets responses were analyzed by flow cytometry. TCD8+ cells were analyzed for specific expression of IFN-γ, IL-17, IL-10, TGF-β and indoleamine 2,3-dioxygenase (IDO) upon stimulation with *M. tuberculosis* antigens. The patient presented low percentage of *M. tuberculosis* antigen specific TCD8+ cells producing IFN-γ (0.7%) compared to healthy control (2.0%) (**Figure 1**). However, the patient presented higher percentage of *M. tuberculosis* antigen specific TCD8+ cells positive for IL-17 (10.9%), IL-10 (3.1%), TGF-β (11.7%) and IDO (6.9%) when compared with TCD8+ cells from the healthy donor (IL-17 = 2.3%; IL-10 = 1.2%; TGF-β = 0.7%; IDO = 0.9%) (**Figures 1** and **2**).

5. DISCUSSION

Cutaneous tuberculosis is re-emerging in countries with high incidence of multidrug resistance pulmonary tuberculosis and HIV, as Brazil [3]. Manifestation of disseminated milliary tuberculosis and CTB is predominant in immunosuppressed individuals [7]. Those individuals need rapid diagnosis and prompt treatment. The first described case of CTB in Brazil was reported around the year 1950 [9]. Since then, there were rare cases reported in the literature [10-12]. Here we report a case of a patient that had a delayed diagnosis of CTB due to unusual clinical symptoms. Although rare cases of CTB have been reported worldwide, to our knowledge this is the first reported case in the Midwestern region of Brazil.

In the case reported here, the patient did not present a previous history of pulmonary TB (X ray had no TB scars), and the patient did not report any previous hospitalization or contact with TB patients, discarding also a direct skin contamination. His lesions were compatible to disseminated TB, and during the disease evolution, the patient always reported pain in the affected regions.

Diagnosis of CTB is a challenge, and requires the association between clinical findings and diagnostic laboratory tests. The usual laboratory tests are biopsies and *Mycobacterium sp* culture of the lesions, polymerase chain reaction (PCR) and acid-fast bacilli test (AFB) to identify the causal agent in the lesions [2,13]. Serological tests and TST (tuberculin skin test) has been used as complementary tests [2,14,15].

The delay in the diagnosis of CTB occurred mainly because of the unusual signs and symptoms presented by the patient. Initially papules or lesions similar to a skin cancer may have misled the physician interpretation, avoiding a suspicion of *M. tuberculosis* infection [2].

Although the pulmonary disease is the most common form of TB, extrapulmonary TB affects 10% - 20% of individuals [15]. Host defense against *M. tuberculosis* is mediated by combination of innate and adaptive immune responses and these responses consist in the activation of macrophages, CD4+ and CD8+ T lymphocytes and B cells in both pulmonary and extra-pulmonary form [14,16].

The protective immune response to TB depends mainly on the activation of T cells with a Th1 phenotype and macrophages, the latter being the primarily effector cells. Th1 cells are a subset of TCD4+ cells that are responsible for producing a series of cytokines such as IFN-γ and lymphotoxin-α that are essential for other cell activation [8,15]. IFN-γ is the main cytokine involved in macro-

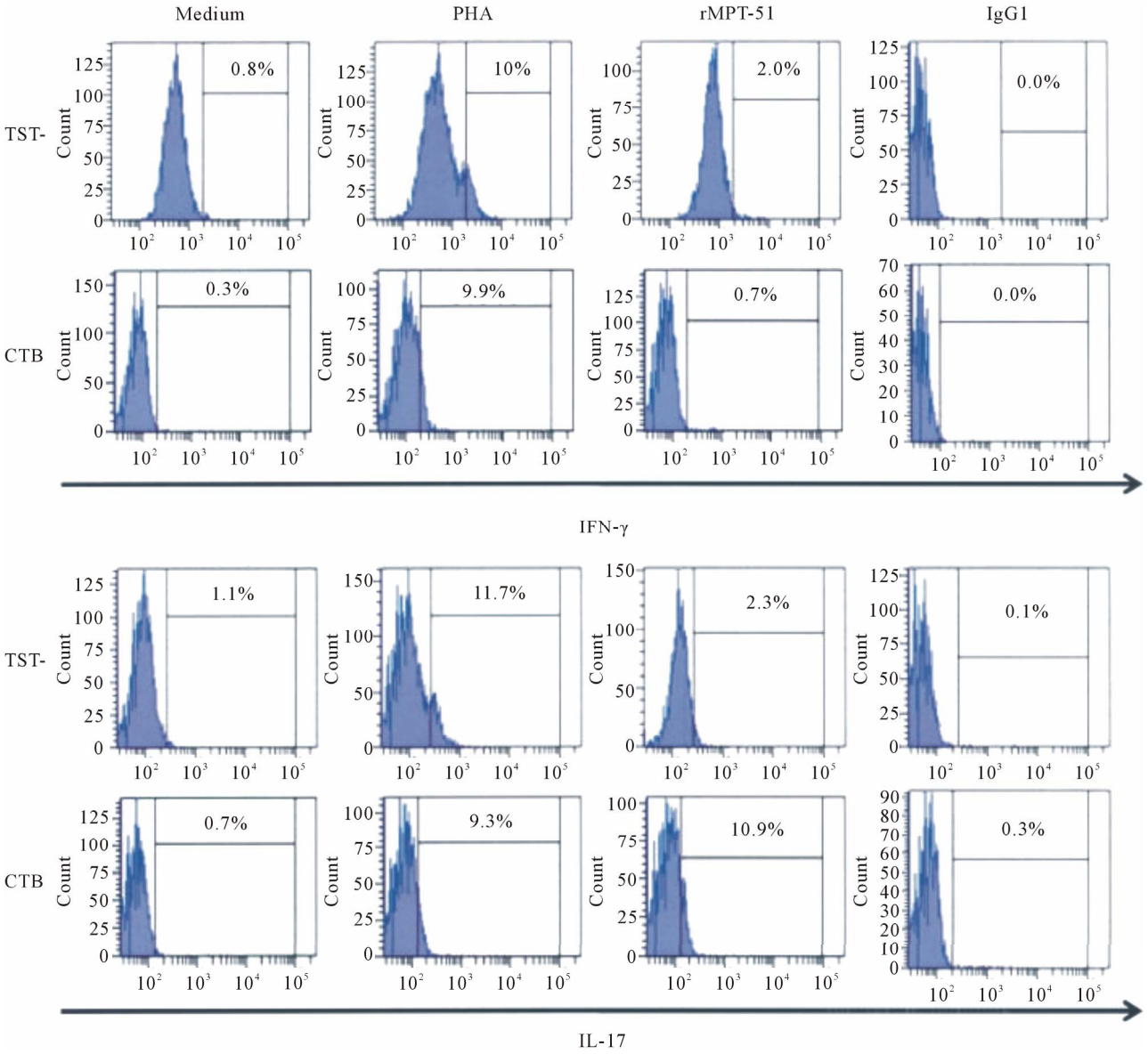

Figure 1. Histograms showing flow cytometry results of the percentage of specific TCD8+ cells positive for IFN-γ and IL-17 from a TST-healthy control control and the CTB patient. Pheripheral blood mononuclear cells (PBMCs) were obtained by Ficoll density gradient centrifugattion (Ficoll-Paque Plyus, GE Healthcare Bio-Science AB). The cells were washed twice in saline and distributed in 96-well plates a 2×10^5 cells/mL in RPMI 1640 medium (GIBCO, Invitrogen Corporation) supplemented. They were then incubated with recombinant *Mycobacterium tuberculosis* antigen (MPT-51, 1 μg/mL) or with PHA (1 μg/mL) as positive control and cultivated at 37˚C with 5% CO_2 for 96 hours in the presence of anti-CD3 (eBioscience). After the culture, the cells were treated with monoclonal antibodies anti-CD8 APC, anti-IFN-γ FITC, anti-IL-17 PE and anti-IgG2 PE as a isotype control and acquired in a FACS-Canto and analyzed using FACS Diva software.

phage activation. Infected macrophages produce cytokines and chemokines that recruit other cells to the site of infection and, when previously activated by IFN-γ, macrophages up regulate membrane MHC proteins and lysosome enzymes, among other molecules to increase bacterial killing [15]. As shown previously, pulmonary and extra pulmonary tuberculosis patients present specific TCD4+ IFN-γ producing cells in response to PPD among other *M. tuberculosis* antigens [16], however, activation of TCD4+ cells does not directly correlates with disease protection or the clinical TB forms, creating the hypothesis that other cell population might be important in the establishment of the several TB clinical forms. TCD8+ cells are known by its cytotoxic function, however it has been shown that TCD8+ cells also produce IFN-γ contributing to TB protection. Although some studies emphasize the TCD8+ role in the murine model of infection, little is known in the human disease [18].

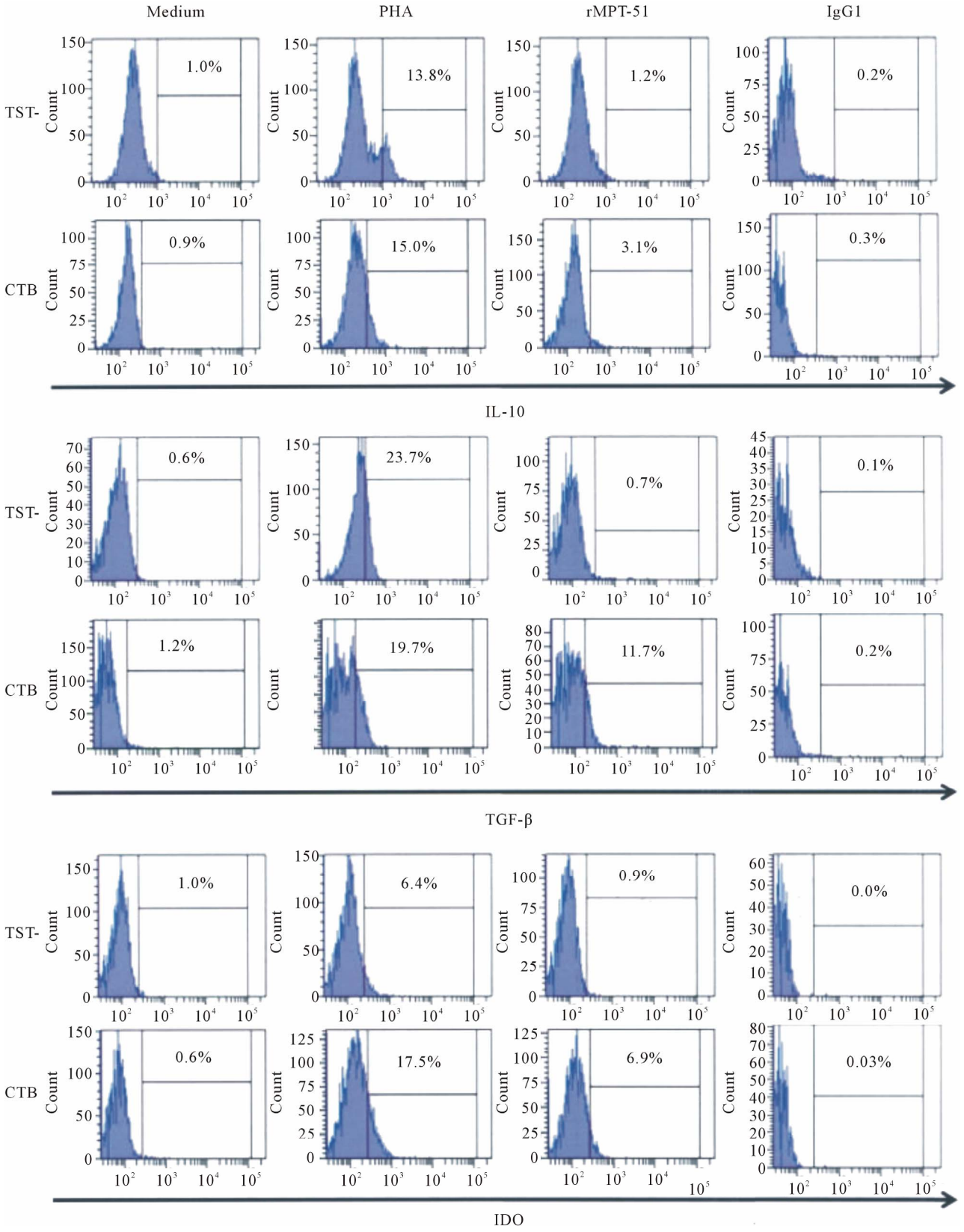

Figure 2. Histograms showing flow cytometry results of the percentage of specific TCD8+ cells positive for IL-10, TGF-*β* and IDO from TST-healthy control and CTB patient. The PBMC cells cultured ad described in **Figure 1** treated with monoclonal antibodies andi-CD8 APC, anti-IL-10 PE, anti-TGF-*β* PE, anti-IDO FITC and anti-IgG1 PE.

In the skin, site of *M. tuberculosis* infection for CTB, dendritic cells and resident macrophages could initiate the immune response and induce the activation of either intraepithelial or peripheral T cells. The intraepithelial lymphocytes are mainly CD8+ and have direct cytotoxic action on infected cells [17]. Many subsets of peripheral TCD8+ cells have been characterized in several diseases [19,20], but in TB their functions are yet unclear. CTB immune response characterization has been done by identification of the cells present in CTB lesions [2,13]. In the present case, macrophages, lymphocytes and giant cells were observed in the histopathological findings (data not shown) only one year after the patient sought health assistance.

Sehgal *et al.* (1992) described the presence of T CD4 and CD8 cells in the peripheral blood of CTB patients by immunohistochemistry and observed that the clinical manifestations affect the percentage of these cells in the blood [15]. Nevertheless, to date, no published data studied the role of TCD8 cells subtypes in CTB. TCD8+ cells contribute by destroying cells infected with the bacilli by the release of granules containing perforin and granzyme and also by activation of other cells through the production of various cytokines. Studies have demonstrated the participation of TCD4+ regulatory cells and TCD8+ cells positive for IDO (an enzyme that promotes tryptophan depletion) in the inhibition and/or down regulation of the immune response [21].

The results presented here revealed a specific TCD8 immune response to *M. tuberculosis* antigens by the CTB patient. The presence of TCD8 cells positive for IL-10 and TGF-γ, which characterize a regulatory phenotype could account for the unusual clinical manifestation [4]. Furthermore, it was observed the presence of specific TCD8+IDO+ cells that could be inhibiting the proliferation of other cells involved in the immune response to *M. tuberculosis*. Although we cannot assure the immune statuses of the patient, these cytokines/molecules are known by their ability to down modulate macrophage and T cell functions [4,21], suggesting that the activity of these cells might be modified in the CTB patient.

6. CONCLUSION

A case of CTB patient with a delayed diagnosis is presented here. Unusual clinical manifestation was the main reason for the late diagnosis. The assessment of the *M. tuberculosis* specific TCD8+ immune response revealed a regulatory/modulatory phenotype with a reduced IFN-γ response when compared to a healthy control.

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