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Evaluation of the influence of skin color on the immunological response to tuberculosis

Nayanne Gama Teixeira Dantas[1], Tânia Mara Pinto Guimarães Dabés[2], Vicente de Paulo Coelho Peixoto de Toledo[2], Maria Luiza Silva[3], Ricardo Toshio Fujiwara[4] and Silvana Spindola-de-Miranda[1,*]

[1]Departamento de Clínica Médica, Programa de pós-graduação em Ciências de Saúde do Adulto, Faculdade de Medicina da Universidade Federal de Minas Gerais (UFMG), [2]Departamento de Análises Clínicas e Toxicológicas, Faculdade de Farmácia da UFMG, [3]Setor de Citometria de Fluxo do Laboratório Central do Hospital das Clínicas da UFMG, [4]Laboratório de Imunologia e Genômica de Parasito, Instituto de Ciências Biológicas da UFMG, Belo Horizonte, Brazil

ABSTRACT

Tuberculosis is a serious infectious disease with extensive importance for public health. Currently about 2 billion people are infected with the etiological agent of tuberculosis, *Mycobacterium tuberculosis*. Skin color has been described as a risk factor in susceptibility for development of this disease. The objective of this study was to evaluate the cellular and humoral immunological profile of self-referred white individuals bearing tuberculosis, in comparison with self-referred black individuals bearing the disease. Statistically significant differences in cellular aspects such as cytokines and humoral profiles were not found between the two groups. The obtained results do not demonstrate association between skin color and immunological responses inducing susceptibility to tuberculosis.

KEYWORDS: tuberculosis, immunology, skin color, immunophenotyping, cytokines

*Corresponding author: Dr. Silvana Spíndola de Miranda, Departamento de Clínica Médica, Faculdade de Medicina da Universidade Federal de Minas Gerais, Av. Professor Alfredo Balena, 190, Santa Efigênia, Belo Horizonte, Brazil.
silvanaspindola@gmail.com

INTRODUCTION

Tuberculosis (TB) is a chronic infectious disease responsible for approximately one million and a half yearly obits; it has an incidence of nine million cases per year all over the world and its occurrence is more frequent in underdeveloped and developing countries [1]. The prevalence of TB could be influenced by environmental, socioeconomic, and individual factors. Among individual factors, gender, age, skin color, and influence of comorbidities have been described, besides the influence of the organism's immunocompetence. With respect to skin color, it is estimated that TB infection presents a higher prevalence in non-white subgroups as compared to white subgroups, within the different studied populations. All over the world, TB infection is at least twice more frequent in non-white subgroups [1, 2, 3].

Cells mainly mediate immunity in TB. Cells, when activated, produce cytokines that regulate the immune response to *Mycobacterium tuberculosis (M. tuberculosis)*. $CD4^+$ T lymphocytes (TL) are the main effective cells involved in protection against *M. tuberculosis.* They are part of the adaptive immune response and may contribute for the amplification and regulation of immunity through the production of several cytokines. The $CD8^+$ TL are cytotoxic cells acting directly on

infected target cells and participating in the memory immune response to *M. tuberculosis* [4]. Therefore, the $CD4^+$ TL with higher CD25 marker levels ($CD3^+CD4^+CD25^{HIGH}$) can act like regulatory cells reducing the production of certain cytokines and the proliferation of $CD4^+$ and $CD8^+$ TL [5, 6, 7].

Proinflammatory monocytes are the main source of tumor necrosis factor alpha (TNF-α) in TB, which together with other immunoregulatory cytokines, are essential for the maintenance and limitation of bacillus dissemination. The NK cells constitute a distinct subpopulation of lymphoid cells that are useful for the destruction of target cells without the necessity of previous sensitization or restriction of the Major Histocompatibility Complex (MHC). Some studies suggest the participation of NKT cells in TB, by way of involvement in the protection and production of interferon-gamma (IFN-γ) and interleukin (IL) 4 [8, 9]. These cells participate in innate immune response. The Th1 profile cytokines (IFN-γ and IL-2) increase the microbicide activity of macrophages and the cellular response. TNF-α is important in the granuloma formation, contributing to the organism's protective response against *M. tuberculosis* infection. On the contrary, Th2 profile cytokines (IL-4, IL-5, IL-6, IL-10 and TGF-β) inhibit the production of Th1 profile cytokines and thus contribute to the development of TB [10].

A better understanding of the influence of the immunological system in the interactions between *M. tuberculosis* and its host could help in TB diagnosis, treatment, and cure evaluation. The purpose of this study is to evaluate in the context of TB susceptibility, the immunological profile of TB infected non-white individuals compared to that of infected white individuals, by means of immunophenotyping of peripheral blood mononuclear cells (PBMC) and quantification of cytokines and immunoglobulins (Ig).

MATERIALS AND METHODS

Studied population

The studied population consisted of 87 patients diagnosed with tuberculosis by clinical and epidemiological history and confirmed through bacteriological (bacterioscopy of clinical samples and/or cultures in Lowenstein-Jensen medium positives for *M. tuberculosis*) or histopathological examinations. All of these individuals underwent a chest X-ray (XR). The tuberculin skin test (TST) was utilized as a diagnostic ancillary, when necessary.

The studied population was recruited from the TB Reference Clinic of the Hospital of Clinics (*Hospital das Clínicas* – HC) of the Federal University of Minas Gerais (*Universidade Federal de Minas Gerais* – UFMG) in the period between 2009 May and 2011 May, with non-probabilistic sampling. The study individuals were divided into 2 groups, based on self-referred race/skin color: white TB patients (WP) and non-white TB patients (NWP).

The patients were classified with respect to the severity of pulmonary TB as described by AL-Moamary and co-workers, for which two doctors and a radiologist to evaluate the thorax radiogram (RX) were involved [11]. The severity of the disease was classified based on the extent to which the lungs were affected. Each lung was classified into three zones: upper, middle and lower. Considering both the lungs, the involvement of one or two zones was classified as localized disease, three or four zones as moderate disease, and five or six zones, as extensive disease. Based on this, patients displaying localized or moderate disease were classified as having non-severe tuberculosis and those displaying extensive disease and/or cavitations were classified as having severe tuberculosis [12].

The inclusion criteria were adult patients with pulmonary TB (positive baciloscopy and/or positive culture), of age between 18 to 60 years, both sex, non-immunocompromised, those agreed to participate in the study, answered the socioeconomic questionnaire, and signed an informed written consent. The project was approved by the UFMG Institutional Review Board/Independent Ethics Committee, under the registration number ETIC 491/06. The exclusion criteria were patients with extrapulmonary TB, those with nontuberculosis mycobacterium infections, immunosuppressive diseases/conditions, usage of immunosuppressives, and those who already had established usage of TB drugs.

Peripheral blood (PB) samples were collected, using ethylenediamine tetraacetic acid (EDTA) and heparin as anticoagulants. The samples were collected from patients immediately before the commencement of antituberculous treatment.

Immunophenotyping by flow cytometry

An aliquot of 50 µL of PB with EDTA was added to polystyrene tubes containing predetermined volumes of monoclonal antibodies, allocated to each tube to evaluate the following populations of PBMC: CD3-FITC/CD4-PE/CD8-TC (subpopulations $CD4^+$ TL and $CD8^+$ TL); CD3-FITC/CD25-PE/CD4-TC (regulatory T cells); CD4-FITC/HLA-DR-PE (activated $CD4^+$ TL); CD8-FITC/HLA-DR-PE (activated $CD8^+$ TL); CD3-FITC/CD19-PE (B lymphocytes - BL); CD16-FITC/CD56-PE/CD3-APC (NK, NKT, pre-NK and mature NK cells); and CD16-FITC/HLA-DR-PE/CD14-TC (macrophages). The monoclonal antibodies used in this study were purchased from different companies. CD4-FITC and CD4-PE (clone RPA-T4), CD8-FITC (clone G42-8), CD25-PE (clone M-A251), CD3-FITC (clone HIT3a), CD3-APC (clone UCH-T1) were purchased from Diatec (Oslo, Norway); CD56-PE (clone MEM-188) was purchased from Dako (CA, USA); and CD16-FITC (clone 3G8), CD14-TC (clone M5E2), CD19-PE (clone SJ25-C1), HLA-DR-PE (clone G46-6), CD4-TC (clone RPA-T4) and CD8-TC (clone HIT8a) were from Becton, Dickinson and Company (NJ, USA). After homogenization and incubation for 30 minutes at room temperature, protected from light, the hemolysis was performed with 2.0 mL of lysis solution (Becton, Dickinson and Company – BD Biosciences, San Jose, USA). The preparations were homogenized and incubated for 10 minutes at room temperature, protected from light, and then they were centrifuged (400 g, 10 minutes, at 18 °C). The supernatant was discarded and the samples were washed with 2 mL of saline solution buffered with phosphate (0.015 M, pH 7.4). Then, the samples were fixed with 300 µL of fixer solution (10 g/L of paraformaldehyde, 1% sodium cacodylate, 6.67 g/L of sodium chloride, pH 7.2). The phenotypical expression of leukocytes was determined with the help of a flow cytometer (FACSCalibur – BD Biosciences) and analyzed using the CellQuest™ software. The conventional analysis was performed through cellular population selection based on morphometric aspects, by punctual distribution sized graphics (Forward Scatter – FSC) *versus* granulosity (Side Scatter – SSC). The selection of the region of interest was made for obtaining the percentage of fluorescent cellular subpopulations within the selected population. Thus, the size punctual distribution of fluorescence in bidimensional graphics was analised two by two as follows, FL1 versus FL2, FL2 versus FL3, or FL1 versus FL3 (Figure 1).

Preparation of *M. tuberculosis* antigen

For obtaining *M. tuberculosis* antigen (H37Rv), the colonies were inactivated at 80 °C for 1 hour

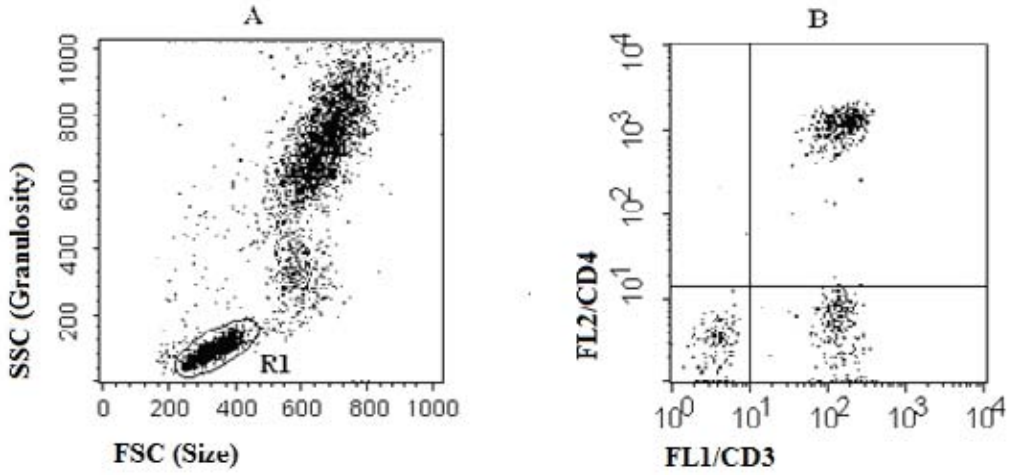

Figure 1. Sequence of procedures used for the percent analyzes of cellular populations by flow cytometry: (A) Identification of population of interest – lymphocytes – in region R1 through punctual distribution size graphic *versus* granulosity. (B) To quantify the percentage of specific cellular population in R1, FL1/CD3FITC punctual distribution size graphic *versus* FL2/CD4PE was performed.

and sonicated in 2 cycles of 20 seconds, at 40 Hertz. Then, the suspension was sterilized with gamma radiation (dose of 5000 Gray for 2 hours and 15 minutes).

Quantification of cytokines by Cytometric Bead Array

For obtaining PBMC, 16 mL of heparinized PB was carefully added to 8 mL of Histopaque® (density of 1.077 g/mL) (SIGMA Chemical Co.; St. Louis, MO), and then centrifuged at 550 g for 40 minutes, at 18 °C. The ring of mononuclear cells formed in the Histopaque®-plasma interface was removed and washed three times in RPMI-1640 medium, and then they were centrifuged at 370 g for 7 minutes at 4 °C. The cells were resuspended in 1.0 mL of RPMI-1640 medium and counted in a Neubauer's hemocytometric chamber, adjusting the concentration to 1.0×10^7 cells/mL.

The PBMC were incubated in 24-well culture plates (Corning Costar Corporation, Cambridge, MA) containing 800 µL of RPMI-1640 medium supplemented with 5% human AB Rh-positive serum, 200 IU/mL of penicillin and 1 mM L-glutamine (SIGMA Chemical Co.), for 48 hours, at 37 °C, with 5% of CO_2 in the absence of H37Rv antigen (control culture) and in presence of H37Rv antigen (20 µL/mL) (stimulated culture). The supernatant was then harvested and stored at -70 °C until cytokine quantification was performed through an assay with fluorescent microspheres using flow cytometry, Cytometric Bead Array (CBA) - Kit Th_1/Th_2 (IL-2, IL-4, IL-6, IL-10, TNF-α and IFN-γ), according to manufacturer's manual (BD Biosciences).

Quantification of immunoglobulins by immunoenzymatic assay

The polystyrene plates (Falcon™, BD Biosciences) were sensitized with 2 µg/100 µl/H37Rv antigen per well overnight, at 4 °C. After blockage with 150 µL of PBS-casein and washing with distilled water, 100 µl of plasma (diluted 1/10) from evaluated individuals were added and the tubes were incubated for 60 minutes at 37 °C. After another washing with distilled water, 100 µl of 1/300 diluted human-peroxidase anti-IgG conjugate (SIGMA Chemical Co.) was added and then ortho-phenylenediamine (OPD) and H_2O_2 were added. The absorbance values were determined with the help of an automatic reader for microwaves, equipped with a filter of 492 nm (BIORAD – 550 model).

Statistical analysis

The statistical analyses were carried out using the Graphpad Prism 5.0 and SPSS 12.0 software programs. The first step to analyze the data was to identify the possible occurrence of normal distribution between the variables. The data presenting normal distribution were analyzed with Student's *t*-test. Whereas, the data not presenting normal distribution were analyzed using the Mann-Whitney non-parametric test. For all the tests, a significance level of 95% was used.

RESULTS

Studied population

Among the 87 patients only 15 satisfied the criteria of inclusion (8 white and 7 non-white patients) (Table 1). The patients excluded (72) were: Those

Table 1. Clinical variables of TB patients.

	WP (n = 8)	NWP (n = 7)
Age (Average)	36.1	51.1
Gender (%)		
Male	85	75
Female	15	25
Classification pulmonary involvement (%)		
Non severe tuberculosis	37.5	57
Severe tuberculosis	62.5	43

TB = tuberculosis; WP = white patients group; NWP = non-white patients group; n = sampling.

with Extrapulmonary TB; those with non-mycobacteria tuberculosis; those using immuno-suppressed drugs and those with autoimmune diseases.

Immunophenotyping by flow cytometry

Immunophenotypic evaluation of PBMC in TB patients did not show any significant difference in TL between WP and NWP groups, and also, there was no significant difference in the $CD4^+$ and $CD8^+$ TL subpopulations (p-value > 0.05). The immunophenotypic results are presented in Table 2.

No significant difference was found in HLA-DR expression in TL subpopulations as well as in regulatory T cells $CD3^+CD4^+CD25^{HIGH}$ and $CD3^+CD4^+CD25^{LOW}$ subpopulations (p-value > 0.05). In addition, significant difference (p-value > 0.05) was not observed in the frequency analysis of monocytes ($CD14^+CD16^+$) and their subpopulations.

In the evaluation of NK cells, the frequency of pre-NK, mature NK, NKT, $CD3^-CD56^{DIM}CD16^-$, $CD3^-CD56^{DIM}CD16^+$, $CD3^-CD56^{BRIGHT}CD16^-$ and $CD3^-CD56^{BRIGHT}CD16^+$ cell subpopulations observed in the results did not demonstrate significant differences (p-value > 0.05) between the studied groups. Also, no difference was found between the two groups in BL evaluation (p-value > 0.05).

Quantification of cytokines by Cytometric Bead Array

There were no statistically significant differences in the values of IFN-γ, TNF-α, IL-2, IL-4 IL-6 and IL-10 among the groups evaluation. The results of quantification of cytokines are shown in Table 3.

Table 2. Immunophenotyping evaluation of peripheral blood mononuclear cells of TB patients.

		TB Patients		
		WP (n = 8)	**NWP (n = 7)**	
Marker	Identified cell	Average ± SE	Average ± SE	ρ-value
$CD19^+$	B Lymphocyte	8.98 ± 0.85	10.65 ± 2.24	0.4795
$CD3^+$	TL	73.13 ± 3.18	65.57 ± 3.96	0.1529
$CD4^+$	$CD4^+$ TL	44.74 ± 4.01	39.25 ± 1.49	0.2474
$CD8^+$	$CD8^+$ TL	26.06 ± 2.45	24.26 ± 4.72	0.7310
CD4-HLA-DR	Activated $CD4^+$ TL	5.82 ± 0.83	7.59 ± 1.10	0.1893
CD8-HLA-DR	Activated $CD8^+$ TL	9.86 ± 2.35	15.21 ± 1.94	0.1836
$CD14^+CD16^+HLA^-DR^+$	"Macrophage like" Monocyte	58.18 ± 10.01	70.39 ± 7.84	0.3645
$CD14^+CD16^+$	Proinflammatory Monocyte	12.83 ± 2.94	12.94 ± 1.86	0.9762
$CD3^+CD4^+CD25^{HIGH}$	Regulatory $CD25^{HIGH}$ TL	0.63 ± 0.07	0.72 ± 0.15	0.9551
$CD3^+CD4^+CD25^{LOW}$	Regulatory $CD25^{LOW}$ TL	2.47 ± 0.30	2.42 ± 0.31	0.9062
$CD3^+CD16^+CD56^-$	Pre-NK cells	3.39 ± 0.61	2.71 ± 0.84	0.3357
$CD3^+CD16^+CD56^+$	Mature NK cells	0.65 ± 0.13	1.35 ± 0.52	0.6943
$CD3^+CD56^+$	Total NK cells	5.20 ± 1.14	10.48 ± 4.7	0.4634
$CD3^-CD56^{DIM}CD16^-$	DIM $CD16^-$ NK cells	4.93 ± 2.46	4.47 ± 2.57	0.6126
$CD3^-CD56^{DIM}CD16^+$	DIM $CD16^+$ NK cells	77.31 ± 4.66	86.61 ± 4.31	0.1520
$CD3^-CD56^{BRIGHT}CD16^-$	BRIGHT $CD16^-$ NK cells	9.88 ± 2.80	4.44 ± 2.12	0.1893
$CD3^-CD56^{BRIGHT}CD16^+$	BRIGHT $CD16^+$ NK cells	7.87 ± 1.28	4.47 ± 1.46	0.1179

TB = tuberculosis; WP = white patients group; NWP = non-white patients group; TL = T Lymphocytes; NK = Natural Killer cells; CD = Cluster of Differentiation; SE = Standard Error; n = sampling.

Table 3. Concentration of cytokines (pg/mL) represented by the difference between the concentration of stimulated versus non-stimulated PBMC supernatant from TB patients, in presence of 20 µg/mL of *Mycobacterium tuberculosis* (H37Rv) antigen, after incubation for 48 hours at 37 °C, 5% CO_2.

	Patients TB		
Cytokine	**WP (n = 8)**	**NWP (n = 7)**	
	Average ± SE	Average ± SE	ρ value
IFN-γ	37.14 ± 20.22	196.5 ± 127.9	0.1749
TNF-α	1200 ± 636.3	1982 ± 799.40	0.5350
IL-6	2640 ± 350.4	3944 ± 3342	0.7104
IL-4	1086 ± 1086	1.41 ± 1.70	0.3566
IL-2	10.17 ± 5.4	4.29 ± 11.24	0.6416
IL-10	245.4 ± 124.1	40.10 ± 10.80	0.1254

PBMC = peripheral blood mononuclear cells; TB = tuberculosis; WP = white patients group; NWP = non-white patients group; IFN-γ = Interferon gamma; TNF-α = Tumor Necrosis Factor alpha; IL = Interleukin; SE = Standard Error; n = sampling.

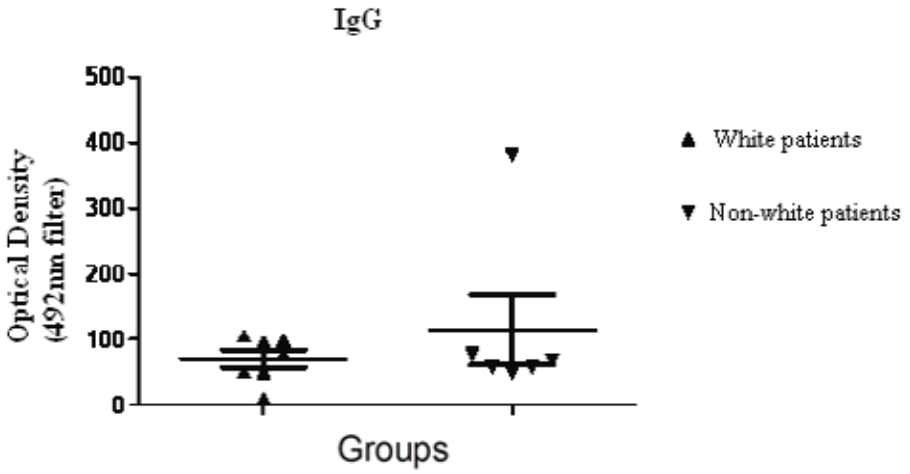

Figure 2. Evaluation of serum concentration of immunoglobulin G (IgG) specific for *M. tuberculosis* antigens, by ELISA method using plasma obtained from white patients (n = 8) and non-white patients (n = 7).

Quantification of immunoglobulins by immunoenzymatic assay

The results found in the ELISA reaction for immunoglobulins did not demonstrate significant differences between the groups (*p*-value > 0.8975) (Figure 2).

DISCUSSION

Epidemiological data observed in several populations around the world demonstrate a higher prevalence of TB in non-white individuals compared to white individuals [13]; for example in the United States of America, TB incidence is approximately 8 times higher in non-white individuals than in white individuals [14]. Several factors could be contributing for this ethnical disparity, which include differences in the exposure to tuberculosis and socioeconomic status [15], besides the normal immunological differences [16]. In our results, the socio-epidemiological profile analysis of the participants has demonstrated that the majority of TB patients consisted of adult males in both racial groups, as described by other authors [17]. In most parts of the

world, the majority of individuals diagnosed for TB are males, and they die more in numbers due to this disease compared to females [18]. With respect to age range, TB is a disease affecting the adult population in their economically productive phase, and our results reflect the situation in Brazil and all over the world, Brazil being an endemic region for this disease [19].

In terms of cellular immune response to TB, there were no significant differences among the evaluated groups in the results of total TL, $CD4^+$ TL and $CD8^+$ TL, as well as in NK cells and their subpopulations, and Regulatory T cells. These results are in accordance with a comparative study between Afro-Americans and Caucasians, which did not demonstrate a significant difference in CD3 TL levels [20]. Also in a study with NK cells and their subpopulations no differences were observed comparing individuals of different ethnic groups (Han Chinese, Caucasians and black individuals) [21].

This study has not showing any difference in the amount of monocytes ($CD14^+CD16^+$) and their subpopulations, in both groups of TB patients. An *in vitro* study [22] suggests that macrophages from black people are more permissive to multiplication of Koch bacilli, than those from white people, and that the incubation of such macrophages in autologous serum causes increase of such permissibility. Differences between $CD4^+$ TL and $CD8^+$ TL cells were not observed in this study in comparison between both groups of TB patients. Other search suggests that the transitory activation state of $CD8^+$ TL during active infectious diseases, like tuberculosis, possibly reflects cytotoxic activity against intracellular pathogens [23]. Studies in animal models have shown that $CD8^+$ TL deficiency can result in susceptibility to tuberculosis, although they suggest that it is important to do more studies to better understand this situation of human hosts against the pathogen [24]. According to the results of immunofenotyping, no difference in the concentration of any evaluated cytokine, such as IL-2, IL-4, IL-6, IL-10, TNF-α and IFN-γ, was found. Studies investigating differences in the level of serum cytokines between black and white populations have shown the genetic predisposition of black subjects for polymorphism in several genes, such as IL-6, IL-10, TNF-α and IFN-γ [25, 26, 27].

Significant differences were not observed in humoral response, neither in frequency of BL, nor in the levels of immunoglobulins. Studies showed an increase of immunoglobulin G in patients with tuberculosis [28, 29], although the function of immunoglobulins in providing protective immunity against tuberculosis is still under investigation, and other studies have reported that humoral response does not play an important role in immunological response to TB [8]. Immunological studies involving different ethnical/skin color groups, comparing humoral immune response to TB could not be found in the literature. Immunologic evaluation of patients from similar socio-economic groups did not reveal significant differences in the profiles of cellular and humoral responses.

CONCLUSION

The results of this study do not show any evidence of difference in the susceptibility to tuberculosis, between TB patients of different skin colors, white and non-white. There are few studies in the literature similar to that evaluated in this article, carrying out an integrated comparison of immunological response through immunophenotyping, and a cytokines and humoral response determination among groups of individuals.

The main limitation of our study is the small number of sample that precluded the use of paired statistical tests. Hence, further studies with larger sample numbers are necessary such as in investigations on immunological profile and evaluation of others factors, like serum of vitamin D and receptor polymorphisms analysis, to better understand the complex mechanism of interaction between the host and the *M. tuberculosis*. This could help in further comprehension of the mechanisms involving environmental and individual factors, which can lead to the susceptibility towards or protection against TB with better clarity.

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CONFLICT OF INTEREST STATEMENT

There are no specific conflicts of interests involved in this study to disclosure.

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