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In vitro levels of cytokines in response to purified protein derivative (PPD) antigen in a population with high prevalence of pulmonary tuberculosis

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The control of mycobacterial infection by the host depends on cell-mediated immunity (CMI), involving activated macrophages, T cells, and type 1 cytokines (Th1). Here we evaluated the capacity of antigen-induced proliferation by peripheral blood mononuclear cells (PBMCs) and the production of proinflammatory tumor necrosis factor-alpha (TNF-α) and interleukin 12 (IL-12p40). The Th1 cytokine interferon-gamma (IFN–γ) and Th2 cytokines interleukin 4 (IL-4) and interleukin 5 (IL-5) in 62 pulmonary tuberculosis (TB) patients (40 Warao indigenous patients [WP] and 22 Creole nonindigenous patients [CP]) and 24 healthy controls (12 Warao indigenous controls [WC] and 12 Creole nonindigenous controls [CC]) at 24 and 48 hours in response to purified protein derivative (PPD) from *Mycobacterium tuberculosis*. The overall results revealed that testing of CP and CC' PBMCs for TNF-α, IFN-γ, and IL-12p40 production was higher compared with WP and WC' PBMCs after stimulating for 24 and 48 hours ($p < 0.0001$), within the WP group, the lower productions of IL-12p40 and IFN-γ significantly correlated ($r^2 = 0.91, p < 0.01$). Although in general there was interindividual variability in the observed responses of Th2 cytokines, especially with IL-4, there was a trend to produce higher PPD-induced IL-5 by WP' PBMCs compared with WC' PBMCs and CP' PBMCs at 24 and 48 hours, respectively. High IL-5 production correlated inversely with low IFN-γ production ($r^2 = -0.97, p < 0.002$). In conclusion, our results suggest that PPD-induced responses observed in patients from both populations can be divided into two groups: one group that activates Creole' PBMCs to preferentially secrete TNF-α, IL-12p40 and IFN-γ and another group that activates preferential secretion of IL-5 in Warao' PBMCs.
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1. Introduction

Approximately 2 billion persons—one third of the world's population—are infected with *Mycobacterium tuberculosis*. However, although a high number of individuals are infected with *M. tuberculosis*, only a minority of them will develop active tuberculosis [1,2]. In resistant individuals, control of the infection mainly requires development of a Th1 cell immunity response. This type of response involves participation of alveolar macrophages and T CD4$^+$, CD8$^+$, and T $\gamma\delta$ lymphocytes, and the production of interleukin 2 (IL–2), interferon-gamma (IFN–γ), IL-12, IL-18, and tumor necrosis factor-alpha (TNF–α), as well as some Chemokines CCL5 (such as RANTES), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1 alpha (MIP-1α), and IL-8. All of these play an important role in the chemotaxis of different cell subpopulations to the infection site for the formation of granulomes [3]. Studies of cytokine production in response to *M. tuberculosis* antigens in human beings have focused on the evaluation of T cell clones, peripheral blood mononuclear cells (PBMCs), and other cells present at the site of infection. Several reports have shown that pleural fluid lymphocytes stimulated with *M. tuberculosis* produced higher IFN-γ and IL-2 than peripheral blood lymphocytes [4,5]. These results provide strong evidence for selective concentration of cells such as Th1 at the site of infection in persons who develop a protective immune response, and support the concept that Th1 cells play an important role in human antimycobacterial defenses [4,5].

By contrast, extensive immunologic studies have been carried out to demonstrate the nonprotective role of the type Th2 immune response when high levels of IL-4 and IL-5 are produced in response to *M. tuberculosis* infection. It has been reported that an increase in IL-4 and IL-13 expression, together with IL-4delta2, the IL-4 splice variant occurs during the immune response to *M. tuberculosis*, and this correlates with lung damage. Moreover, data from flow cytometry and *in situ* hybridization indicate that a subversive T helper-2 (Th2) component in the response to *M. tuberculosis* may under-

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mine the efficacy of immunity and contribute to immunopathologic conditions [6].

The role of genetic and nongenetic factors in influencing the incidence of pulmonary tuberculosis in a population has been accepted [7]. In this context, it has been reported that the annual death rate from tuberculosis (TB) reached 10% when the disease first became prevalent in the Qu′appelle Valley Indian reservation in Canada, eliminating half of the Indian families in the first three generations; yet, 40 years later, the annual death rate had dwindled to 0.2%, suggesting selection for host resistance [7]. Different genetic backgrounds between the Venezuelan Warao and Creole populations exist. Both the Warao and Creole peoples have been tested for the human leukocyte antigen (HLA)–A, -B, -Cw, -DR and -DR/DW 1–8 antigens. For the Warao, the findings confirmed the existence of the DR/DW dissociation previously observed in North American Indian, Japanese, and Caucasian populations, and showed inheritance and segregation of DW specificities (DW 8.3, DW 16, DW 22) defined only with homozygous typing cells of Warao origin. These data illustrated HLA haplotypes, linkage-disequilibrium, and DR/DQ associations not seen previously in other human populations [8]. As regards the HLA class I and class II allele and haplotype distributions Venezuelan Creoles showed that genes of Mongoloid, Negroid, and Caucasoid origin have created a distinctive HLA genetic profile in this hybrid Creole population. The predominant HLA-B DQA1 DQB1 DRB1 haplotype is HLA-B44 DQA1*0201 DQB1*0201 DRB1*0701 (5.3%). It is noteworthy that the HLA-A3 B7 DR2 and the HLA-A1 B8 DR3 linkage groups are part of conserved or ancestral haplotypes [9].

Epidemiologic data reported by the Venezuelan Health Ministry on TB rates during the period 1997–2001 showed that these rates have been higher in indigenous populations (between 129.4 and 155.6 per 100,000 inhabitants) than nonindigenous populations (between 24.8 and 26.1 per 100,000 inhabitants) [10,11]. We therefore decided to study possible defects in the production of the type 1 and 2 cytokines in TB in a comprehensive fashion within an indigenous population in which TB is highly endemic compared with a nonindigenous population.

2. Subjects and methods

2.1. Study subjects

Total populations of 86 adults of both genders, 15–60 years of age, were enrolled in this study, including 52 Warao individuals (26 female and 26 male) living in 15 communities from Antonio Díaz, Pedernales, and Tucupita municipalities of the Delta Amacuro State, and 34 Creole or local individuals (23 female and 11 male). The total population was classified into four groups: group 1, Warao indigenous patients (WP, $n = 40$), group 2, Creole nonindigenous patients (CP, $n = 22$), both groups with active pulmonary TB; group 3, Warao controls (WC, $n = 12$), and group 4 Creole controls (CC, $n = 12$).

2.2. Inclusion criteria

Inclusion criteria for individuals with progressive TB (active infection) before treatment were as follows: volunteer patients with evidence of clinical and respiratory symptoms suggesting pulmonary TB and confirmed with Ziehl–Neelsen-stained smears, microbiological culture, and thorax X-ray studies according to standard technique in postanterior projection, and positive or negative for tuberculin skin test (TST). Induration of ≥10 mm was used as the criterion for infection with *M tuberculosis*. The control group included both TST+ and TST− subjects. Both groups had no evidence of clinical or respiratory symptoms suggesting pulmonary TB, and were negative for smears stained with Ziehl–Neelsen and/or microbiological culture. Individuals with the presence or absence of BCG scars were included.

2.3. Exclusion criteria

Exclusion criteria for either patients with active infection and controls without or with latent infection were as follows: patients and controls less than 15 years of age, with or without respiratory symptoms suggesting pulmonary TB; patients and controls who were human immunodeficiency virus (HIV) positive; patients taking immunosuppressive drugs (*e.g.*, corticosteroids, azathioprine, or cyclophosphamide); and individuals who did not sign an informed consent agreement.

2.4. Ethical approval

This study was approved by the Ethical Committee of the Biomedicine Institute (protocol number 09-00-6645-2007/23/05/07), and written informed consent was voluntarily signed by all patients and controls individuals.

2.5. The tuberculin skin test

The TST was performed on all the individuals of this study using 2 tuberculin units of purified protein derivative (PPD) of *M. tuberculosis* (strain RT-23, from the Statens Serum Institute in Copenhagen, Denmark) as published previously [12]. The diameters of indurations were measured 72 hour after inoculation.

2.6. Confirmatory microbiological studies, treatment and serologic marker

Smears from sputum were stained by Ziehl–Neelsen direct method. For each sputum specimen, two tubes of modified Ogawa egg medium and Lowënstein-Jensen were inoculated using the swab method of Kudoh and Kudoh [13]. Anti-TB treatment was initiated in all patients when radiologic evidence suggestive of tuberculosis was found or bacteriologic confirmation by smear or culture was obtained.

HIV testing was done with the Passive Particle Agglutination Test for the detection of antibodies to HIV-1 and/or HIV-2 of FUJIREBIO Diagnostics (Abbott Laboratorie-Dainabot, Tokyo, Japan).

2.7. Ex vivo stimulation of isolated cells

Blood samples were obtained from TB patients prior to treatment. PBMCs were prepared from whole heparinized blood by sedimentation over at Ficoll-Paque continuous density gradient (Pharmacia, Uppsala, Sweden.). Isolated PBMCs were resuspended at a final concentration of 2×10^6 cells/ml in complete medium (RPMI-1640, 10 mmol/l HEPES buffer, 200 mmol L-glutamine, and 5 U/ml of streptomycin–penicillin, all from Gibco-BRL, Rockville, MD) supplemented with 5% fetal calf serum, (Sigma, St. Louis, MO), cultured (2×10^5 per well) in microtiter plates (Immunolon, Birmingham, UK) and incubated with complete medium or with specific antigen (unstimulated and stimulated cultures, respectively) at 37°C in humidified atmosphere containing 5% CO_2 for 24 and 48 hours. Cell stimulations were performed with 20 μg/well of PPD (State Serum Institute, Copenhagen, Denmark). After this step of incubation, the cells and supernatants were harvested by centrifugation at 5000 rpm for 30 minutes and kept frozen at −70°C for subsequent cytokine determination.

2.8. Immunoassay for cytokines

Enzyme-linked immunosorbent assay (ELISA) was performed according to the manufacturer's protocol. For the IL-12p40 test, we used anti–human IL-12p40 (Dy1240, capture antibody) and IL-12p40 detection antibody (biotinylated-conjugated monoclonal antibody anti–human IL-12p40) (R&D Systems, Minneapolis, MN). Supernatants were always tested in duplicate (a quality control of duplicate variation was performed for standard deviations for each of the different points of the standard curve); this assay was sensitive to 7.8 pg of IL-12p40 activity per milliliter. For the IFN-γ test,

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we used mouse anti–human IFN-γ (Dy285, capture antibody) and biotinylated-conjugated monoclonal anti–human IFN-γ (R&D Systems, Minneapolis, MN) as a secondary antibody; this assay was sensitive to 15.6 pg of IFN-γ activity per milliliter. For IL-4, the coating antibody anti–human IL-4 and the detection antibody (biotinylated-conjugated monoclonal anti–human IL-4) were from Biosource (CytoSets, CHC0044); this assay was sensitive to 3.6 pg of IL-4 activity per milliliter. For IL-5 test, the reagents anti-human IL-5 (Dy205, capture antibody) and a biotinylated-conjugated rat anti-human IL-5 (R&D System, Minneapolis, MN, USA) were used; this assay was sensitive to 3.6 pg of IL-5 activity per milliter. For TNF-α test, anti–human TNF-alpha (MAB610, capture antibody) and biotinylated-conjugated goat anti-human TNF-α as detection antibody were acquired from R&D Systems (Minneapolis, MN); this assay was sensitive to 15.6 pg of TNF-α activity per milliliter. Standard curves were generated by using rIL-12, rIFN-γ, rIL-4, rIL-5, and rTNF-α over a concentration range from 3.6 to 1,000 pg/ml.

Sera were isolated from venous blood obtained from controls and TB patients before treatment. Serum cytokine concentrations were determined by a similar ELISA to that described for supernatant cytokines according to the instructions of the manufacturer.

2.9. Lymphoproliferative response

Similar to the procedure described for *ex vivo* stimulation of isolated cells, PBMCs were cultured for 3 or 5 days with 10 and 1 μg/well of phytohemaglutinin A (PHA; Sigma, San Diego, CA) and 20 and 2 μg/well of PPD antigen (optimal and suboptimal concentrations, respectively), for measurement of proliferation, cells were pulsed with 1 μCi/well [^{3}H] thymidine (Amersham, Aylesbury, UK; specific activity, 5 Ci/mmol) on days 3 and 5. Incorporation of [^{3}H] thymidine was determined with a beta counter and expressed as counts per minute (cpm).

2.10. Statistical analysis

A nonparametric test such as the Mann–Whitney test was used for comparing two unpaired groups. EPINFO 3.3.2 was used for correlations data analysis. The proliferation results are shown as stimulation index (SI). The latter was calculated as a ratio of counts per minute of stimulated PBMCs/counts per minute of unstimulated PBMCs from the same individual. SI ≥ 3 was used as the criterion for positive response to PHA or PPD. To establish the upper limits of normality for the SI, the cell response of a selected group of individuals from a nonendemic zone (none were known TB contacts, and all were TST negative) was plotted cumulatively, and the 90th percentile was determined. The Student *t* test was used to compare differences in cytokine concentrations between groups. A scatter gram was plotted using GraphPad Prism software version 5.02 (trial version, GraphPad Sofware, San Diego, CA). Statistically significant differences were determined using a *p* value of ≤0.01 when the Bonferroni correction was used.

3. Results

3.1. TST response in M tuberculosis–exposed versus nonexposed groups

Table 1 lists means ± SDs of different markers from patient and control groups and microbiological tests. Of the 52 Warao individuals studied, 26 were female (WP = 23 and WC = 3) and 26 male (WP = 17 and WC = 9), whereas of the 34 Creole individuals, 23 were female (CP = 13 and CC = 10) and 11 male (CP = 9 and CC = 2). There were no statistically significant differences among the percentage of female and male individuals in the patient groups (female: WP 23/40 and CP 13/22, male: WP 17/40 and CP 9/22). However, there was statistically significant difference between control groups (female: WC 3/12 and CC 10/12, $p < 0.01$; male: WC 9/12 and CC 2/12, $p < 0.01$); Table 1. From a total of 86 sputum samples analyzed by Ziehl–Neelsen direct method, in 20/40 and 17/22 of WP and CP patients, respectively, mycobacteria were identified. All sputum samples from both groups of patients were positive by culture. Mycobacteria were not detected in any of the Warao or Creole individuals of the control groups.

Table 1
Age and gender characteristics and immunologic and bacteriologic markers between Warao and Creole groups

Marker	Patients		Controls	
	Warao	Creole	Warao	Creole
Age	38.0 ± 13.7	32.2 ± 11.9	32.2 ± 11.9	35.0 ± 0.2
Female (%)	57.5	59.0	25.0[a]	83.3
Male (%)	42.5	40.9	75.5[b]	16.6
Tuberculin skin test + (%)	87.5	81.8	91.6	83.3
Smear$^+$ (%)	50.0	80.9	0	0
Smear$^+$ and culture (%)	100.0	100.0	0	0

WP ($n = 40$), WC ($n = 12$), CP ($n = 22$), and CC ($n = 12$). The TB cases were confirmed through Ziehl–Neelsen-stained smears or microbiological cultures. There was a significant difference among control groups: [a]female WC vs CC ($p < 0.01$) and [b]male WC vs CC ($p < 0.01$).

When skin test reactivity was carried out to study the delayed-type hypersensitivity, reactions of ≥10 mm were found in 35/40 (87.5%) and 18/22 (81.8%) of the untreated WP and CP patients, respectively and in 11/12 (91.6%) and 10/12 (83.3%) of the controls (WC and CC, respectively). There was no statistically significant difference between the TST groups (Table 1). Means of indurations among the groups were WP (TST+) = 16.7; WP (TST-) = 2.2; WC (TST+) = 14.1; WC (TST−) = 0; CP (TST+) = 13.0; CP(TST−) = 3.3; CC (TST+) = 16.3; CC (TST−) = 1.3 (data not shown). Patients and controls less than 20 years of age were highly likely to have received one BCG vaccination as neonates and/or during childhood as part of the increasingly effective Venezuelan national BCG vaccination program. There was no difference in PPD-induced cytokine production in patients or controls with or without BCG scars (data not shown).

3.2. Cytokine production

Measurement of IL-12p40 and IFN-γ productions after stimulation for 24 and 48 hours with PPD are shown in Fig. 1. The results are shown as absolute value mean ± SD of the cytokine concentration (pg/ml) in each group. PPD—stimulated PBMCs from WP produced significantly lower IL-12p40 production (108.8 ± 112.2) than in both Creole groups, CP (321.9 ± 246.6), $p<0.0001$, and CC (243.7±170.7), $p<0.002$, after stimulating for 24 hours, (Fig. 1A). This significantly lower production in WP (127.3 ± 153.6) than in both Creole groups CP (301.7 ± 243.4) and CC (295.6 ± 165.4), $p< 0.001$, persisted after stimulating for 48 hours (Fig. 1A). WC 'PBMC also presented low IL-12p40 production after stimulating for 24 and 48 hours (130.3 ± 153.5 and 131.6 ± 167.2), respectively; however according to the Bonferroni correction, this production did not show statistically significant differences between groups.

As in the case of IL-12p40 production, lower IFN-γ production was observed in WP (12.6 ± 25.4) than in CP (80.6 ± 163.0), $p<0.01$, especially in cells stimulated for 24 hour, (Fig. 1B). The lower IL-12p40 production correlated with lower IFN-γ production ($r2 = 0.91$, $p< 0.01$). Table 2 lists the levels of serum IL-12p40 and IFN-γ. Measurement of serum IL-12p40 showed increased levels of this cytokine in the Creole groups compared with Warao groups; whereas similar serum IFN-γ levels were found in both patient groups, WP and CP (Table 2).

The IL-4 contents in PPD—stimulated PBMCs from WP and CP were similar (4.4 ± 4.6 and 4.5 ± 6.0, respectively) after stimulation for 24 hours. Although there was interindividual variability in the obtained responses in the WP group after stimulating for 48 hours, the overall findings showed a trend to produce high IL-4 in WP

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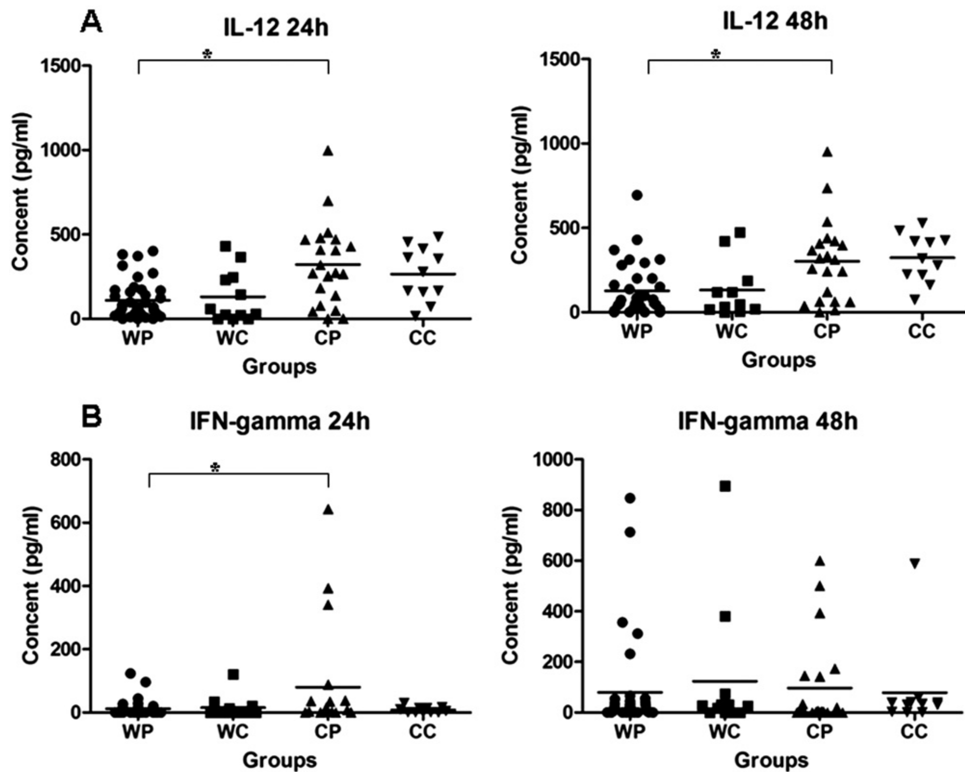

Fig. 1. Interleukin-12p40 (IL–12p40) and IFN-γ levels in peripheral blood mononuclear cells (PBMCs) exposed to purified protein derivative (PPD) at different times. (A) Data representing the concentration means ± SD of IL-12p40 after stimulating for 24 and 48 hours in the following groups: Warao patients, (WP, $n = 40$) and Creole patients (CP, $n = 22$). Healthy groups: Warao controls (WC, $n = 12$) and Creole controls (CC, $n = 12$). (B) Data representing the concentration means ± SD of IFN-γ after stimulating for 24 and 48 hours (*) indicates statistically significance differences among compared groups. Value of $p < 0.01$ was considered statistically significant.

compared with CP (5.8 ± 8.6 vs 4.1 ± 6.7); however, there was no statistically significant difference between groups (data not shown).

Measurement of IL-5 production was higher after stimulating for 24 hours in WP (195.5 ± 82.3) than in WC (100.7 ± 110.9), $p < 0.002$, and also CP (140.7 ± 73.1) and CC (124.9 ± 119.1). Concerning the IL-5 production after stimulation for 48 hours, even though there was a trend in WP to produce more IL-5 (212.3 ± 34.5) than in WC (155.2 ± 102.3), CP (167.2 ± 96.8), and CC (165.2 ± 108.6), there were no significant differences neither between Warao groups or between Creole groups (data not shown). As shown in Table 2, serum IL-5 levels did not show statistically significant differences among groups. Within WP, it was found that higher IL-5 production and lower IFN-γ production correlated inversely ($r^2 = -0.97$, $p<0.002$).

TNF-α production of stimulated PBMCs is reported in Fig. 2. Significantly lower TNF-α production was observed in WC (293.6 ± 274.1) than in CP (844.2 ± 424.4), $p < 0.003$, after stimulation for 24 hours. There was no statistically significant difference between Creole groups, CP (844.2 ± 424.4) and CC (676.7 ± 722.5) (Fig. 2). Significantly lower TNF-α production persisted in WC (246.5 ± 305.6) than in CP (883.8 ± 609.4), $p < 0.001$, after stimulation for 48 hours. Moreover, there was significant difference between WP (493.4 ± 464.9) and CP (883.8 ± 609.4), $p < 0.006$ (Fig. 2). As shown in Table 2, serum TNF-α showed statistically significantly decreased levels of TNF-α in WP compared with CP ($p<0.01$).

Table 2
Levels of serum IL-12p40, IFN-γ, IL-5, and TNF-α

Serum cytokine level	Warao patients	Warao controls	Creole patients	Creole controls
Serum IL-12	530.1 ± 399.2	532.0 ± 246.1	602.2 ± 233.7	910.6 ± 1088.0
Serum IFN-γ	417.5 ± 308.0	231.3 ± 155.9	417.0 ± 300.5	667.7 ± 1024
Serum IL-5	146.7 ± 62.6	148.4 ± 51.5	113.3 ± 50.0	126.8 ± 22.4
Serum TNF-α	119.2 ± 46.6[a]	189.2 ± 116.5	160.8 ± 47.9[b]	122.2 ± 66.0

Data representing the concentration means ± SD of serum cytokines. Statistically significant differences, WP vs CP: [a] vs [b] $p < 0.01$.

3.3. *Lymphocyte proliferation assay*

Table 3 lists means ± SDs of SI according the proliferation responses of PBMCs to PHA mitogen and PPD antigen. With regard to PHA concentrations, there was a trend toward decreased stimulation of PBMCs with suboptimal concentration of the T-cell mitogen PHA in Warao groups compared with Creole groups (Table 3). When the proliferation response to PPD antigen within the different groups was analyzed, it was found that the response of stimulated PBMCs to optimal PPD concentration was enhanced in WP compared with CP (Table 3). However, according to the Bonferroni correction, which was used for multiple comparisons, no statistically significant differences were observed among groups (Table 3).

4. Discussion

The present work describes studies in two ethnic groups with very different prevalence of TB; the indigenous Warao people have much higher TB rates than corresponding the Creole people living in the same geographic country [10,11]. Taking into account these findings, we decided to perform the experiments to analyze the productions of Th1 and Th2 cytokines in Warao indigenous patients with active TB, as it has been reported that immunity to mycobacteria is critically dependent on type 1 immunity involving IL-12, IFN-γ, and TNF-α cytokines [3,6,14–16].

Interestingly, we found that WP' PBMCs stimulated with PPD for 24 and 48 hours had a significant decrease in IL-12p40 and IFN-γ production compared with CP. In addition, these decreased produc-

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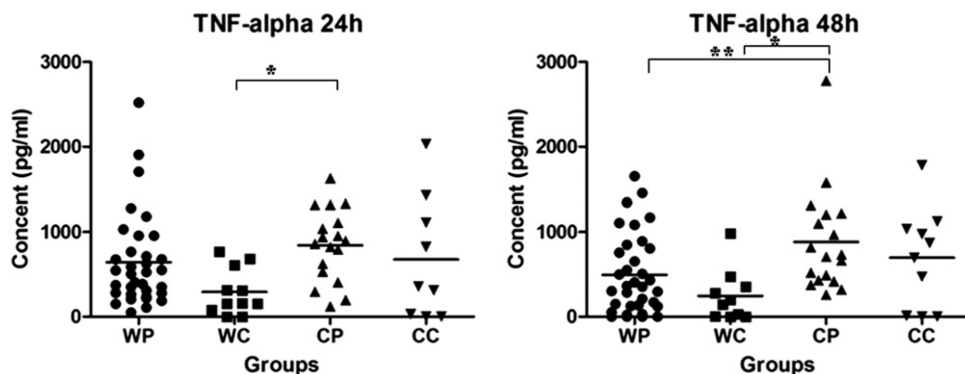

Fig. 2. Effect of PPD on TNF-α production by cultured PBMCs. Results represent the TNF-α mean values after stimulating for 24 and 48 hours in patient groups (WP and CP) and control healthy groups (WC and CC). For 24 hours (*) represents the significance differences among compared groups (WC vs CP) and for 48 hours (* and **) represent the significance differences among compared groups (WC vs CP and WP vs CP, respectively). Value of $p < 0.01$ was considered statistically significant.

tions significantly correlated. Several studies have reported that *M. tuberculosis* is associated with activation of cytokine circuits both at sites of active tuberculosis *in vivo* and in cultures of PBMCs stimulated by *M. tuberculosis* antigens *in vitro* [17–19]; these findings have been investigated through *ex vivo* studies with specific or crude mycobacterial antigens [20]. It has been accepted that both IL-12 and IFN-γ play a very important role in the control of TB. With regard to the role of IL-12 production, a previous study showed that when BALB/c mice, a strain highly susceptible to virulent *M. tuberculosis* infection, were given IL-12 at the initiation of infection with *M. tuberculosis*, their average survival time doubled from 58 to 112 days [21].

With respect to skin test reactivity, even though there was no statistically significant difference between the TST groups, a major percentage of Warao individuals demonstrated a latent infection, especially among WC. It is generally thought that most persons infected with *M. tuberculosis* have a clinically latent infection; that is, they are infected and are PPD positive by skin test but do not present with clinical symptoms and are not contagious to others [22]. Understanding latent and reactivation TB at the level of both the host and the bacillus is crucial to worldwide control of this disease. Taking these data together, and as it has been accepted that IL-12 is critical for the induction of IFN-dependent host control of *M. tuberculosis*, the diminished capacity of PBMCs from WC to synthesize these cytokines could disturb the mechanisms that prevent reactivation of latent *M. tuberculosis* infection in asymptomatic Warao individuals. In addition, it has been suggested that different genetic factors influence the predisposition of TB, such as racial or ethnic differences and the immune responses/suppression genes [7]. In this context, our findings in relation to the low IFN-γ production correlate with a recent study, which found that the PBMCs from Indonesian individuals with pulmonary TB stimulated with specific antigen produced lower IFN-γ, which was strongly depressed during active TB and inversely correlated with TB disease severity [23].

With regard to the activation of *M. tuberculosis*-induced cytokine production by PBMCs from individuals with or without reaction to tuberculin, there is a fair amount of evidence on mycobacteria-specific CD4 T cells isolated from TST + or active TB patients indicating that these cells produce IFN-γ [7,20,23]. In the present study Warao groups showed less proinflammatory cytokines (principally IL-12p40) compared with Creole groups, so the decrease in IFN-γ production might be related to lack of IL-12p40 production; thus these differences seem to relate more to the general responsiveness of their response to disease in this population. We observed within the Warao and Creole groups a significantly high proportion of individuals positive for the TST; however, the scarce number of individuals negative for the TST made the comparison among these groups difficult.

When we investigated the ability of PBMCs from different groups to secrete IL-4, we found that IL-4 data were not meaningful because all measurements fell very close to the lowest level of detection of the IL-4 assay; even though WP showed a trend toward producing more IL-4 compared with CP. Our results correlate with observations previously reported, which showed that IL-4 production measured in culture supernatants stimulated with PPD was low in pulmonary TB cases and did not differ among patients and controls individuals [24]. As regards IL-5 production, it was higher in WP compared with WC and CP. Furthermore, within WP, it was found that high IL-5 production correlated with low IFN-γ production. Further studies of cytokine productions measured in culture supernatants must be accompanied by intracellular cytokine staining and flow cytometry to confirm involvement of the IL-5 as responsible for the reduction of PPD-induced Th1 cytokine productions by PBMCs from WP. In addition, further insight into cytokine productions, such as IL-10 and transforming growth factor-beta (TGF-β), will be required, as these cytokines might act to downmodulate pulmonary immunity to *M. tuberculosis*, allowing *M. tuberculosis* to evade type 1 immunity [25].

By contrast, cell-mediated immunity, leading to *M tuberculosis*–constraining granuloma formation, is the major component of host defense against TB and is regulated by the balance of cytokines secreted mostly by mononuclear phagocytes and lymphocytes. Concerning this point, it is accepted that without TNF-α cytokine, effective granuloma formation is diminished [26]. Evidence on this point was reported previously; when investigators found that patients with recurrent pulmonary TB showed a significant depression of TNF-α production by PBMCs after *in vitro* stimulation with the 30-kDa antigen [27]. These findings correlated with the present results, in which a significant decrease in TNF-α production was

Table 3
Proliferative responses of PBMCs to PHA mitogen and PPD antigen

Group	PHA		PPD	
	Suboptimal	Optimal	Suboptimal	Optimal
Warao patients	23.0 ± 23.5	30.0 ± 23.7	19.0 ± 20.3	15.0 ± 20.4
Warao controls	23.3 ± 26.4	25.9 ± 26.6	9.4 ± 6.4	13.9 ± 32.9
Creole patients	44.6 ± 33.0	40.9 ± 34.3	6.3 ± 13.7	5.6 ± 9.8
Creole controls	60.2 ± 50.9	50.9 ± 68.5	8.5 ± 5.8	7.5 ± 6.6

Data represent SI mean ± SD of proliferative responses to suboptimal and optimal stimulus (10 and 1 μg/well of PHA and 20 and 2 μg/well of PPD antigen for optimal and suboptimal concentrations, respectively). SI ≥ 3 was used as the criterion for positive response to PHA or PPD. Even though that measurement of the proliferative response to suboptimal PHA (1 μg/well) and optimal PPD (20 μg/well) showed decreased and increased proliferative response, respectively, between WP and CP, even between WC and CC, no statistically significant differences were found after used the Bonferroni correction.

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found in Warao' PBMCs stimulated with PPD for 24 and 48 hours compared with Creole' PBMC groups, the latter correlating with decreased levels of serum TNF-α in WP compared with CP, even though it has been reported that serum concentrations of cytokines do not parallel PPD-induced cytokine levels [28].

From *in vitro* assays, the study of delayed-type hypersensitivity reaction using PHA and PPD shows that the nonspecific proliferate response was significantly decreased in WP compared with CP, whereas the PPD proliferate response was enhanced compared with both Creole groups. The specific proliferate response was not correlated with lower IFN-γ and IL-12p40 productions in the Warao groups. These findings are in concordance with results from other groups, which reported that PBMCs proliferation with mycobacterial antigens was not correlated with IFN-γ production [26,27,29]. Even though several studies suggest that different genetic factors, such as racial differences and the immune responses/suppression genes, influence the predisposition of tuberculosis [7,11,23], it has been accepted that different risk factors, such as deficient health systems, BCG vaccination status, malnutrition, overwhelming parasite infections, and severity of family TB exposure, cannot be discarded as contributing to TB within a population [1,7,11]. As regards these points, it has been reported that the different nutritional profile in a population results from the specific patterns of social, cultural and economic conditions of each population [29]. In Venezuela there are 28 different ethnic groups, of which Warao is one (no persons of mixed race or indigenous) whose socioeconomic status is low. In addition they do not have access to health care compared with the Creole people from the urban areas. Warao individuals also have recurrent or overwhelming parasite infections [11,30]. A significant association between intestinal helminthic infections and mycobacterial diseases, such as pulmonary TB and multibacillary leprosy, has been demonstrated by several authors [31,32]. A study showed that *A. lumbricoides* infections in endemic regions are associated with a highly polarized type 2 cytokine response [31]. Recently, it has been reported that concomitant intestinal helminthic infection in patients with diagnosed TB skews their cytokine profile toward a Th2 response [32]. Further studies are needed to understand the association between intestinal helminthic infections and a predominant cytokine profile in Warao with active TB, which could favor persistent *M. tuberculosis* infection in this population. Finally, overall the study reports an important correlation that is likely to be related to the extent of TB disease in these two groups of patients: one group that activates Creole' PBMCs to preferentially secrete TNF-α, IL-12p40 and IFN-γ, and another group that activates preferential secretion of IL-5 in Warao' PBMCs. Taken together, these findings comprise another important piece of evidence supporting the importance of the Th1 immune response for outcome in TB.

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