

Analysis of Cytokine Production in Household Contacts of Leprosy

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ABSTRACT

Analyze cytokine production in household contacts of leprosy, paucibacillary (CPB) and multibacillary (CMB) after one year of follow-up. Eighty-three individuals attended the second evaluation. Peripheral blood mononuclear cells (PBMC) of the subjects were stimulated with *Mycobacteriu leprae*, PHA and cytokines were assessed by ELISA and Cytometry. The results showed significant correlation between TNF- α and age of the CMB in unstimulated PBMC culture. Grouping these individuals is ≤ 20 and ≥ 21 years it was observed that ≥ 21 years had higher levels of TNF- α . It was found higher levels of IL-10 in female CPB ≤ 20 years in cultures stimulated with *M. leprae*. CMB showed a higher frequency of TCD8+IL-10+ cells stimulated in culture with *M. leprae* when compared with CPB. It was found that CMB whose index case had a treatment time ≤ 2 years, showed a higher frequency of TCD8+IL-10+ cells stimulated in culture with *M. leprae* when compared with CPB. It was found that female's ≥ 21 years of CMB showed a higher frequency of TCD4+IL-4+ cells in culture without stimulation than female ≤ 20 years. In conclusion, CMB displays a modulatory response with increased IL-10; in contrast, CPB shows a pro-inflammatory response with high levels of IFN- γ and TNF- α .

Key words: Leprosy, Household contacts and Cytokines.

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INTRODUCTION

Leprosy caused by *Mycobacterium leprae* has several clinical forms that are correlated with distinct immunologic patterns, ranging from a vigorous immune response of the Th1 type in the tuberculoid pole, contrasting with a heightened Th2-type response in lepromatous pole (Sieling and Modlin, 1994; Esquenazi et al., 2015; Sampaio et al., 2011; Geluk et al., 2011). Whereas the transmission occurs especially among household contacts, it is important to monitor actions that can minimize or control the risks of transmission, studying the

cellular immune response to *M. leprae* antigens and BCG in leprosy patients and household contacts, classified these individuals into high and low responders to *M. leprae* based on IFN- γ production by mononuclear cells from peripheral blood (Sarno et al., 1988). These authors found that household contacts high responder constituted 54.8% of the analyzed group, three times higher percentage when compared with the percentage in the group of healthy subjects (control). These authors suggested the possible presence of active infection

among contacts. Later, Sundar et al. (1989) showed that household contacts had relative risk of developing the disease 3 to 6 times higher for contacts of multibacillary patients (CMB) and 2 to 4 times higher for contacts of paucibacillary patients (CPB) relative to population. Interestingly, Godal and Negassi (1973) had reported that occupational or household contacts had immunologic evidence of exposure to the bacteria without yet showing clinical signs of the disease. Thus, these authors suggested that detection of subclinical infection becomes extremely important in those individuals at high risk of developing the disease. Sampaio et al. (1991) showed that five household contacts of MB patients who developed the disease after two years of monitoring showed reduced levels of IFN- γ in response to *M. leprae* antigen.

These data reinforce the importance of following household contacts not only by clinical examination as well by evaluating immunological markers that can show the presence of subclinical infection. According to Moet et al. (2004) the age of household contact, the classification of the clinical form of index case, the physical distance and the genetic factors are associated with the contact's risk of developing the disease. In 2006, Moet et al. (2006) found that individuals between 5 and 15 years old and over 30 years of age, had a higher risk of developing the disease. Sales et al. (2011) showed that the main risk factors were associated with the clinical form of the index case (paucibacillary or multibacillary), the age of contacts, physical proximity of housing, and the degree of kinship (consanguinity) with the patient. Martins et al. (2012) suggested that low level of IFN- γ in CMB compared to CPB is responsible for the development of a latent infection of the disease in its active form. The disease was therefore associated with progressive reduction in specific IFN- γ production to the pathogen, probably due to the higher degree of exposure to *M. leprae* bacilli. In light of the studies carried out in Governador Valadares, a hyperendemic county in eastern of Minas Gerais State in Brazil, we decided to identify and monitor the household contacts of patients evaluated in the Center of Reference-CREDEN-PES of the Public Health Department, in Governador Valadares. The data obtained by Marçal (2012), showed differences in the cytokine production in response to the stimulus of *M. leprae* in household contacts. The CPB group responded with higher frequency of IFN- γ high-producing cells, on the other hand, the CMB group responded with higher frequency of IL-4 high-producing cells. The study of Marçal (2012) was extended by Gama (2012) who detected *M. leprae* DNA in dermal shaved the earlobe of the same household contact group. It was found that 23.89% of the contacts presented bacterial DNA by qPCR, indicating strong presence of sub-clinical infection. These data reinforced the importance of monitoring contacts, which although they were clinically healthy, had

a high risk of developing the disease over time. Accordingly, the purpose of this study was to follow these household contacts for one-year period and to evaluate the immune response in order to identify any immunological marker associated with susceptibility to develop leprosy in the future. We believe that the monitoring of household contacts exposed to increased risk of infection, will contribute to the early detection of new cases of leprosy, enabling a more effective control of the disease. This study is interesting and valuable to understand the development of an immune response of people who are exposed to leprosy cases.

MATERIALS AND METHODS

Study Group

The study was developed in the city of Governador Valadares, eastern Minas Gerais, Brazil. Leprosy patients are treated at the Department of Public Health of Gov. Valadares - CREDEN-PES. Household contacts were individuals who lived with leprosy patients in the last five years and did not show any symptoms of the disease. All contacts were subjected to careful clinical evaluation before being considered asymptomatic, by physicians of CREDEN-PES. Household contacts were selected who had participated in an earlier study conducted in 2011/2012 by Marçal (2012) and Gama (2012) when evaluated immunological and molecular aspects of household contacts of paucibacillary (CPB) and multibacillary (CMB) patients. In this study, three criteria were adopted for inclusion of participants: (i) have participated in the first clinical and laboratory evaluations occurred at baseline in 2011 (ii) have not been treated for leprosy in a time prior to first assessment and (iii) have not been diagnosed for leprosy between the first and the second evaluation. Given these criteria, 83 household contacts were selected who agreed to participate in this follow-up study. Eight individuals who reported no contact with patients nor history of leprosy in the family were included in the group taken as negative control.

Ethical Approval

We hereby declare that this study was approved by the Ethics Committee of Univale, filed under N^o. PQ 022 / 09-009. All participants signed a free and informed consent (IC) at the first evaluation.

Separation of Mononuclear Cells from Peripheral Blood

Peripheral blood mononuclear cells (PBMC) were

obtained from the blood of individuals selected for the study. Briefly, whole blood was diluted 1: 2 in phosphate buffered saline (PBS) for separating the cells by density gradient on Ficoll Hypaque (GE Healthcare, Sweden). After centrifugation the cells were collected, washed and suspended in RPMI (Gibco Invitrogen Corporation 1640, USA) supplemented with 10% fetal bovine serum (FBS, Gibco Invitrogen Corporation, USA), L-glutamine (Gibco Invitrogen Corporation, USA) at 2 mM, penicillin 100 U / mL and streptomycin (Gibco Invitrogen Corp., USA) at 100 µg / ml (complete medium). A small aliquot of this cell suspension was diluted with 0.4% Trypan Blue (1: 2) (Bio Whittaker USA) and counted in Neubauer chamber for estimating the concentration of viable cells.

PBMC Culture Supernatant

PBMC were cultured in duplicate at a concentration of 2×10^5 cells/well in 96-well plates (Falcon, BD) for 24 h to produce (IL-4, IL-10 and TNF- α) and 05 days (IFN- γ), incubated at 37°C in an oven containing 5% CO₂ (End Electron), in a humid atmosphere in the presence and absence of antigenic stimulation, using a suspension of *M. leprae* in a proportion of 10 bacilli for each cell, and phytohemagglutinin mitogen (PHA). Supernatants from each duplicate were recovered and kept at -20°C until use (ANTAS et al., 2004). *M. leprae*, killed by irradiation (3.48×10^8 bacilli/ml), was kindly provided by leprosy laboratory of the FIOCRUZ/ RJ and Instituto Lauro de Souza Lima - ILSL/SP.

ELISA for Detection of Cytokines in the Culture Supernatant

The levels of cytokines IL-4, IL-10, TNF- α and IFN- γ in the supernatant of PBMC cultures were determined by ELISA test, in accordance with the protocol established by the manufacturer Kits – Pharmingen. At the end of the protocol, the reaction was quenched with 2N solution of sulfuric acid and the optical density measured at 490 nm in an automatic ELISA reader. Cytokine levels were determined from the standard curve data.

Flow Cytometry for Detection of Intracellular Cytokines

PBMC were incubated in RPMI supplemented in the absence or presence of *M. leprae* antigen for 20 h, according to Antas et al. (2004). Five hours before the end of the incubation period, the anti-CD28 (3 µg/ml) was added to the culture and one hour after, it was added brefeldin A (10 ng/ml). After this incubation, cells were washed with PBS, incubated with 1.0 ml of Fc Blocker (3% normal goat serum, 2% fetal bovine serum in PBS-FACS) for 30 min, protected from light, at a temperature of 4°C. Subsequently, these cells were centrifuged and

suspended in 100 µl of FACS-PBS. They were then incubated with 1µl of monoclonal antibodies CD4 PerCP, CD8 PE DY590 and CD69 FITC (Becton Dickson) for 30 min, protected from light, at room temperature. The cells were washed with PBS- FACS, centrifuged and then washed again with PBS-FACS. After fixation, cells were permeabilized with 500 µl saponin solution (0.3%) for 10 min at room temperature and then washed with 500 µl saponin solution. The cells were suspended in 100 µl saponin solution and incubated with 5 µl of anti-cytokine monoclonal antibodies (IL-4, IL-10, TNF- α and IFN- γ) labeled with PE (Becton Dickson) for 30 min protected from light, at room temperature). After incubation, the cells were washed with PBS and then were added 200 µl of 1% paraformol aldehyde. The data acquisitions were made in the flow cytometer EPICS XL Beckman Coulter-MCL and the results were analyzed using Expo-32 software. It was collected 50,000 events for analysis. The identification of cell populations of interest, as well as determining the percentage of cytokine producing cell populations was performed by a computer system coupled to the flow cytometer.

Analysis of Cytokine Production in the Supernatant

Analysis of cytokine production (IL-4, IL-10, TNF- α and IFN- γ) in PBMC culture supernatant was performed considering the following variables: the group CPB and CMB, antigenic stimuli (*M. leprae* and PHA), age, gender, and the treatment time of index case at the second assessment (2012). The results shown refer to data that showed significant difference $p < 0.05$.

Statistical Analysis

Analysis of cytokine production by ELISA and flow cytometry was performed using a quantitative method applying non-parametric tests, since the data did not meet a normal (Gaussian) distribution. The Mann-Whitney test was used to compare the medians between the groups. For the analysis we considered outliers all values above the mean ± 2 standard deviation. All outliers values for cytokine analysis were removed. It was considered statistically significant p values < 0.05 and the analysis were performed with the GraphPad Prism v.5.0 software.

RESULTS

Characterization of Household Contacts

Eighty-three household contacts of leprosy were reassessed and underwent clinical examinations after one year of follow-up. Of these contacts, 47% (n=39) were contacts of paucibacillary (CPB) and 53% (n=44) of

Table 1. Characterization of household contacts.

Variable	CPB N (%)	CMB N (%)	p value
Gender			
Male	12 (30.77)	22 (50.00)	
Female	27 (69.23)	22 (50.00)	0.07 ^a
Age			
Mean \pm SD	32.46 \pm 18.59	31.47 \pm 19.96	0.82 ^b
Min – Max	6 – 65	5 – 92	
Time of treatment of the index cases in 2012			
\leq 2 years	25 (64.1)	32.0 (70.45)	
>2 years	14 (35.9)	12.0 (29.55)	0.39 ^a
Degree of kinship of contacts in relation to the index cases			
Parents	14 (35.9)	17 (38.64)	
Offspring	06 (15.38)	02 (4.65)	
Brothers	02 (5.13)	05 (11.36)	0.44 ^a
Spouse	06 (15.38)	10 (22.73)	
Number of cases in family			
1	23 (58.97)	29 (67.44)	
>1	16 (41.03)	14 (32.56)	0.42 ^a

^a Test Q-square. ^b Test *Mann-Whitney*. CPB total n= 39; CMB total n=44. Excluded individuals without information.

multibacillary (CMB) patients. For the CPB group, 30.77% (n = 12) were male and 69.23% (n=27) female. The average age of these individuals was 32.46 \pm 18.59 years. Regarding the CMB group, 50% (n= 22) were males and 50% (n=22) female. The average age of these individuals was 31.47 \pm 19.96 years. Taking the index cases treatment time relative to the time of the second evaluation of household contacts which occurred in 2012, it was found that 64.1% (n = 25) of the CPB group and 70.45% (n = 32) of CMB group were evaluated over time less than two years of treatment of the respective index cases. Furthermore, 35.9% (n = 14) of the CPB group and 29.55% (n = 12) CMB group were evaluated when the respective index cases had over 2 years of treatment. The degree of kinship of contacts in relation to their index cases was investigated. It was observed that 35.9% (n=14) of the CPB group and 38.64% (n=17) of the CMB group declared that the father or mother had the disease. While 15.38% (n=6) of the CPB group and 4.65% (n=2) of the CMB group indicated at least one of his sons as the index case. A total of 5.13% (n = 2) of the CPB group and 11.36% (n=5) of the CMB group were brothers of cases indices. Among the spouses it was found that 15.38% (n=6) of the CPB group and 22.73% (n=10) of the CMB group were married with sick individuals. It was observed that 58.97% (n=23) of the CPB group and 67.44% (n=29) of the CMB group have or have had at least one case of leprosy in the family, while 41.03% (n=16) of the CPB and 32.56% (n=14) of the CMB group had more than one case of leprosy in the family, as shown in Table 1. By comparing the number of

BCG scars on CPB and CMB in both evaluations (2011 and 2012), it was observed that there was a significant reduction in the frequency of the CMB group having only one BCG scar in the second assessment (p = 0.0038) . Of those 17 contacts showed a BCG scar in the first evaluation, only four individuals remained with one scar. The other 13 subjects received the second dose of BCG and therefore migrated to the group with two BCG scars. With respect to the CPB group was not observed a significant difference between the frequencies of individuals with one or two BCG scars. Thus, we find that for the CMB group there was a change in the vaccination plan following the diagnosis of their respective index case

Cytokine Production in the Supernatant of PMBC Culture

TNF-A Production by CPB and CMB

TNF- α levels between CPB and CMB groups after PHA stimulation were different (p=0.0067) as shown in Figure 1.

Correlation between TNF-A Level and Age

Considering that the age factor is important in the epidemiology of leprosy, we set out to evaluate the levels of cytokines IL-4, IL-10, TNF- α and IFN- γ among household contacts spread over age group. Using Spearman correlation analysis, a positive correlation was found (r = 0.47 / p = 0.008) between TNF- α levels and age of the CMB group in unstimulated culture (Figure 2).

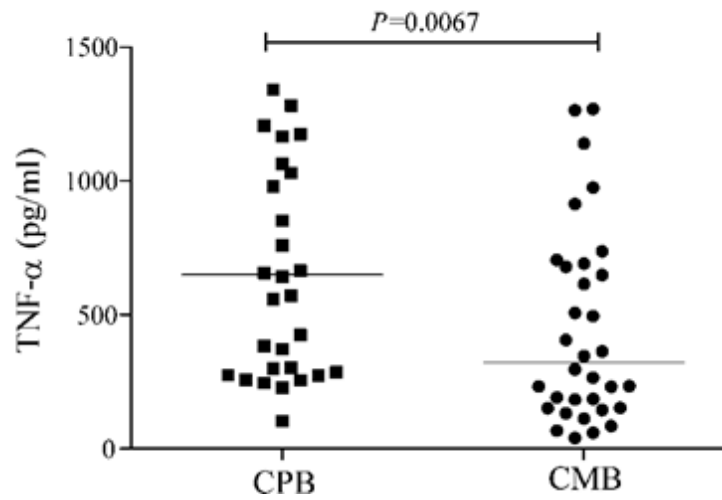


Figure 1. TNF- α level in PHA stimulated culture of PBMC of household contacts. CPB: total n=39, CMB: total n=44. Negative values and outliers were removed from the analysis. The bars represent the median. CPB: 650.06 CI: 303.57 – 1064.23; CMB: 322.00 CI: 186.91 – 649.47. Mann-Whitney test $p=0.0067$. The median of the controls was 708.87 pg/ml.

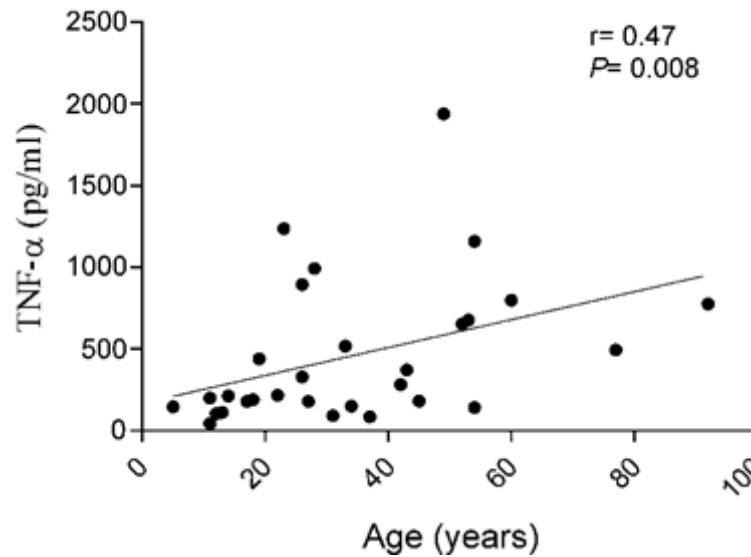


Figure 2. Correlation between the level of TNF- α in unstimulated culture of PBMC of CMB and age. Each point represents the sample of an individual culture supernatant. Spearman correlation ($p=0.008$). Negative values and outliers were removed from the analysis.

No significant correlation was found among the other groups

High Levels of TNF- α in CMB More Than Twenty-One Years Old

Considering that age positively correlated with TNF- α

levels in the CMB group, it occurred to us to evaluate the cytokine profile in terms of age, distributing the participants in two groups ≤ 20 and ≥ 21 years old. In this case it was observed that individuals ≥ 21 years had an increased production of TNF- α in relation to those ≤ 20 years of age ($p = 0.0071$) only in the CMB group, as shown in Figure 3. No significant differences were

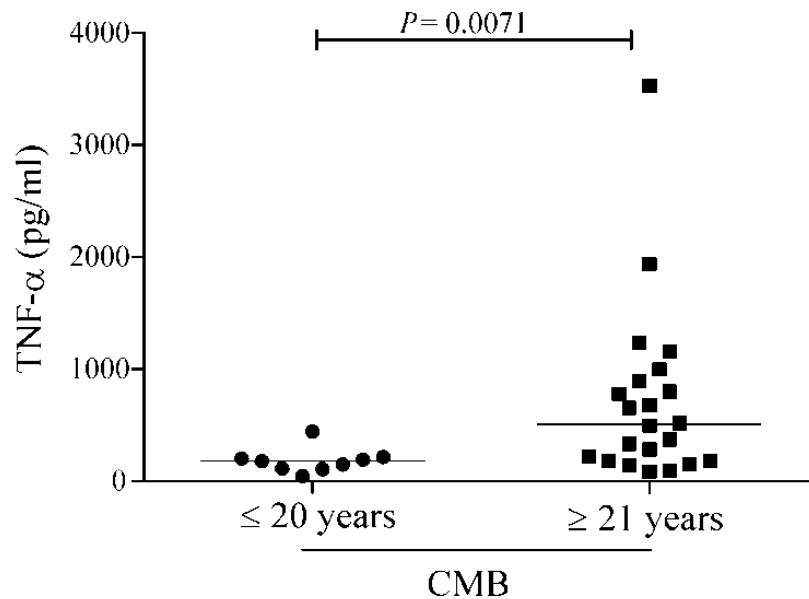


Figure 3. TNF- α level in unstimulated culture of PBMC of CMB by age. CMB: total n=44. Negative values and outliers were removed from the analysis. The bars represent the median. ≤ 20 years: 179.42 CI: 106.11 to 213.79; ≥ 21 years: 506.76 CI: 182.19 to 894.64. Mann-Whitney test $p=0.0071$. The median of the controls was 764.15 pg/ml.

observed among the other groups.

High Levels of IFN- γ and TNF- α in CPB Less Than Twenty Years Old

The association analysis between age and cytokine production in both groups of CPB and CMB showed a significant difference in IFN- γ production in culture without stimulation ($p = 0.0039$) and TNF- α in culture stimulated with PHA ($p = 0.0009$) only for those individuals ≤ 20 years old, as shown in Figures 4a and b. No significant differences were observed among the other groups.

High Levels of IL-10 among Women Less Than Twenty Years Old Of CPB Group

The analysis of cytokine production relating gender and age showed a significant difference ($p= 0.0352$) of IL-10 levels among women of CPB in the age ≤ 20 and ≥ 21 years, using supernatant from PBMC culture stimulated with *M. leprae* antigen (Figure 5). No significant differences were observed among the other groups.

High Levels of IL-10 in the CPB Group Whose Index Case Had More Than 2 Years of Treatment

Considering the time of treatment of index cases,

household contacts were stratified into (a) if index case had lower treatment time not exceeding two years and (b) if index case had treatment time more than two years. There was a significant difference in IL-10 production in PBMC cultures unstimulated ($p=0.0221$), stimulated with PHA ($p=0.0047$) and stimulated with *M. leprae* ($p=0.0184$) in accordance with treatment time of the index cases. IL-10 production was higher in CPB of index cases that had treatment time > 2 years (Figure 6a, b and c). No significant differences were observed among the other groups.

Intracytoplasmatic Cytokine Production by CD4 and CD8 T Cells

The analysis of the production of intracytoplasmatic cytokines IL-4, IL-10, TNF- α and IFN- γ was performed considering the following parameters: lymphocyte subpopulation of CD4+ and CD8+, age, gender and treatment time of index case at the second assessment (2012). The results presented refer to data that showed significant difference $p \leq 0.05$.

High Frequency of T CD8 + IL-10 + Cells in CMB

The frequency of T CD8+IL-10+ cells was higher ($p=0.0202$) in culture stimulated with *M. leprae* antigens, in CMB as shown in Figure 7.

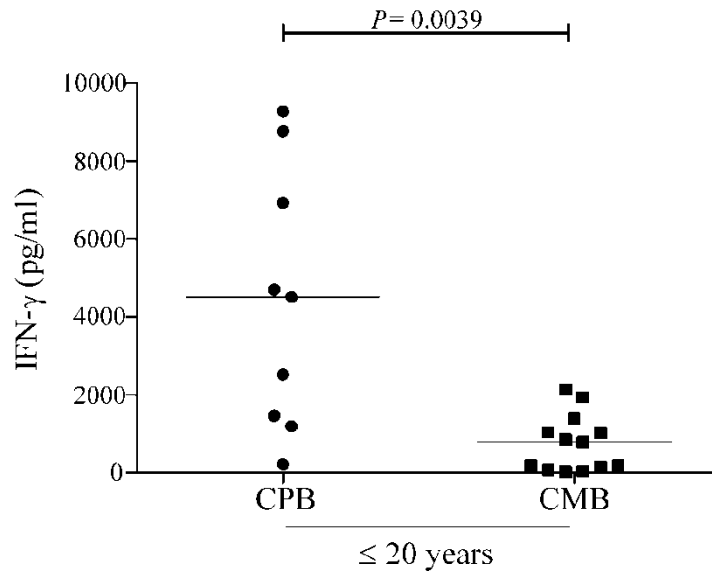


Figure 4a. IFN- γ level in unstimulated culture of PBMC of CPB and CMB ≤ 20 years. CPB: total n= 13, CMB: total n=17. Negative values and outliers were removed from the analysis. The bars represent the median. CPB: 4.512,00 CI:1.198,25 – 8.771,56; CMB: 788.65 CI: 68.48 – 1.030,32. Mann-Whitney test $p=0.0039$. The median of the controls was 1.495pg/ml.

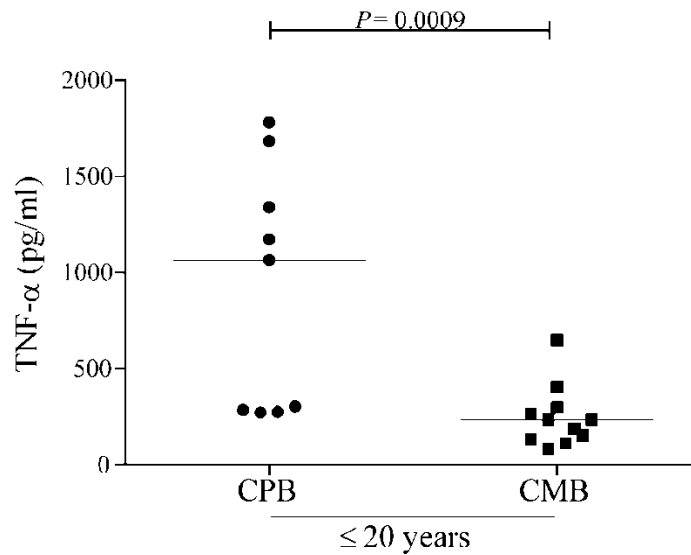


Figure 4b. TNF- α level in PHA stimulated culture of PBMC of CPB and CMB ≤ 20 years. CPB: total n= 13, CMB: total n=17. Negative values and outliers were removed from the analysis. The bars represent the median. CPB: 1.064,23 CI: 276.20 – 1.682, 64; CMB: 233.73 CI: 112.78 – 406.30. Mann-Whitney test $p=0, 0009$. The median of the controls was 708.87 pg/ml.

High Frequency of T CD4+ IL-4+ Cells Among Women More Than Twenty One Years Old in CMB Group

Considering the variables gender and age it was observed that women over the age of 21 years had a higher frequency ($p=0.0389$) of T CD4+IL-4+ cells in the

absence of antigenic stimulation in the CMB group (Figure 8).

High Frequency of TCD8+ IL-10+ Cells in the CMB Group Whose Index Case Had Less Than 2 Years of Treatment

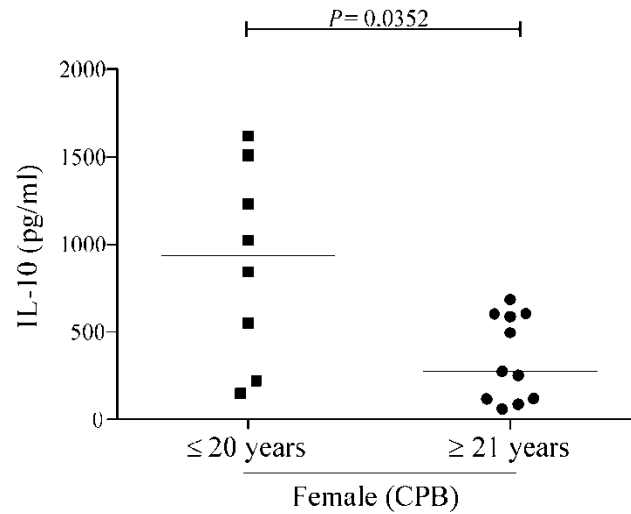


Figure 5. IL-10 level in *M. leprae* stimulated culture of PBMC of CPB, female, ≤ 20 years and ≥ 21 years. CPB: total n=27. Negative values and outliers were removed from the analysis. The bars represent the median. ≤ 20 years: 933.95 CI: 149.65 – 1.620,39; ≥ 21 years: 275.71 CI: 87.53 – 604.38. Mann-Whitney test $p=0.0352$. The median of the controls was 204.5pg/ml.

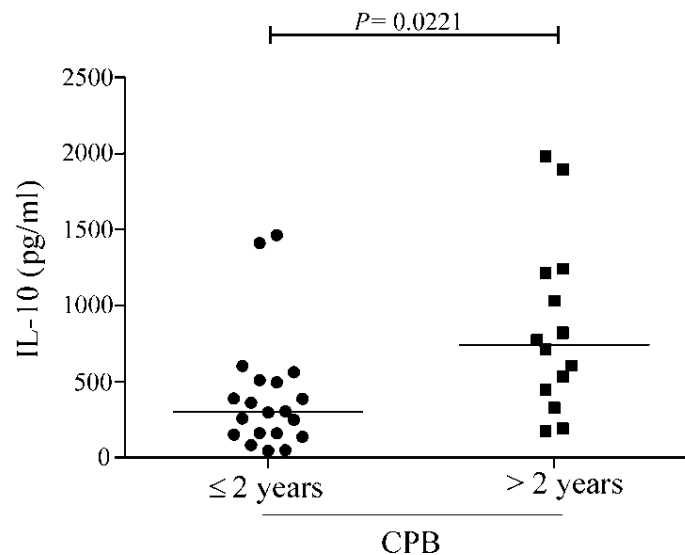


Figure 6a. IL-10 levels in unstimulated culture of PBMC of CPB according to the treatment time ≤ 2 years and > 2 years of their index cases. CPB: total n=39. Negative values and outliers were removed from the analysis. The bars represent the median. ≤ 2 years: 300.47 CI: 158.56 – 494.84; > 2 years: 743.49 CI: 327.71 – 1.242,85. Mann-Whitney test $p=0.00221$. The median of the controls was 355.58 pg/ml.

Considering the time of treatment of the index case it was observed a higher frequency ($p=0.0019$) of T CD8+IL-10+ cells stimulated in culture with *M. leprae* antigen in CMB group whose index case had less than 2 years of treatment (Figure 9).

DISCUSSION

Analysis of cytokine production (IL-4, IL-10, TNF- α and IFN- γ) in household contacts of leprosy, after one year of follow-up, contemplates the assumption that different

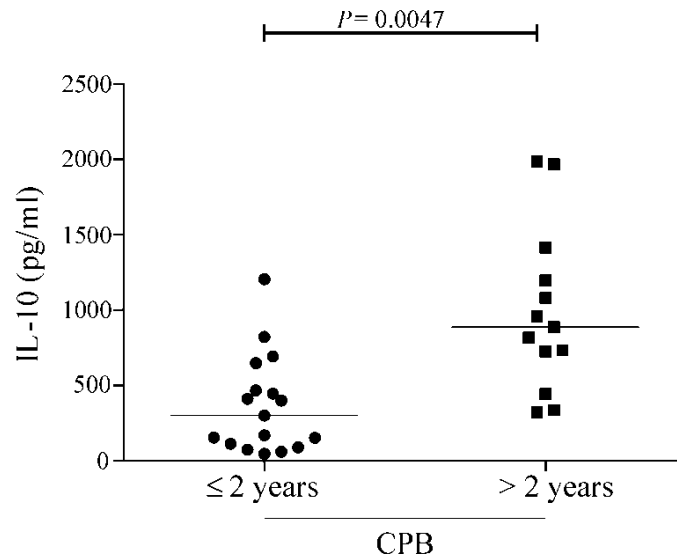


Figure 6b. IL-10 levels in PHA stimulated culture of PBMC of CPB according to the treatment time ≤ 2 years and > 2 years of their index cases. CPB: total n=39. Negative values and outliers were removed from the analysis. The bars represent the median. ≤ 2 years: 300.47 CI: 112.92 – 648.66; > 2 years: 888, 75 CI: 444.20 – 1.414, 69. Mann-Whitney test $p=0.0047$. The median of the controls was 493.46 pg/ml.

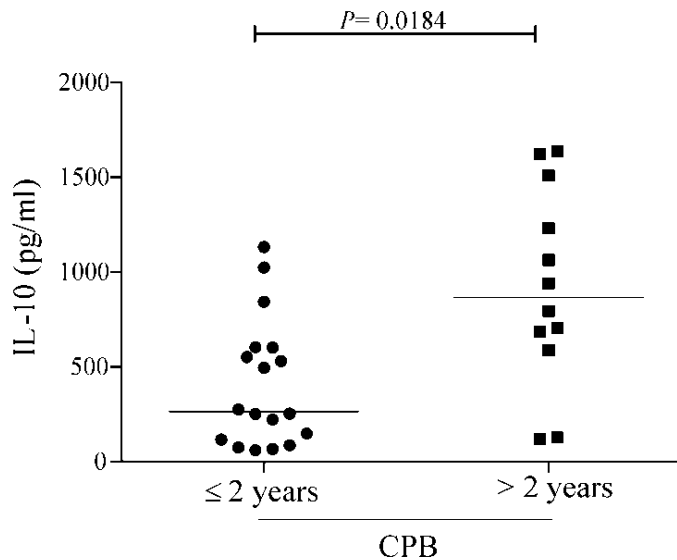


Figure 6c. IL-10 levels in *M. leprae* stimulated culture of PBMC of CPB according to the treatment time ≤ 2 years and > 2 years of their index cases. CPB: total n=39. Negative values and outliers were removed from the analysis. The bars represent the median. ≤ 2 years: 264.69 CI: 149.65 – 602.02; > 2 years: 867.24 CI: 586.72 – 1.508,57. Mann-Whitney test $p=0.0184$. The median of the controls was 204.5 pg/ml.

levels of these cytokines may be associated with the subclinical disease and accordingly function as infection susceptibility markers. Initially, it was found that CPB

group had higher levels of TNF- α in culture supernatants stimulated with PHA when compared with individuals of the CMB group (Figure 1). It is known that paucibacillary

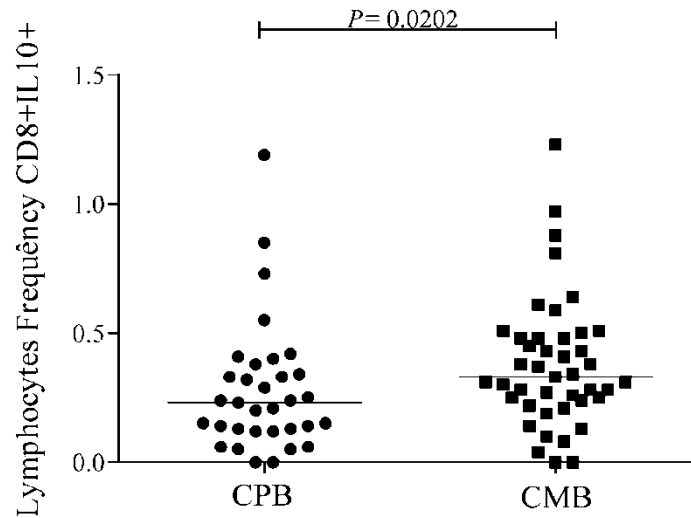


Figure 7. Frequency of lymphocytes T CD8+IL-10+ in *M. leprae* stimulated culture of PMBC of CPB and CMB. CPB: total n=39, CMB: total n=44. The bars represent the median. CPB: 0.23 CI: 0.14 – 0.33; CMB: 0.33 CI: 0.27 – 0.43. Mann-Whitney test $p=0.0202$. The median of the controls was 0.13 %.

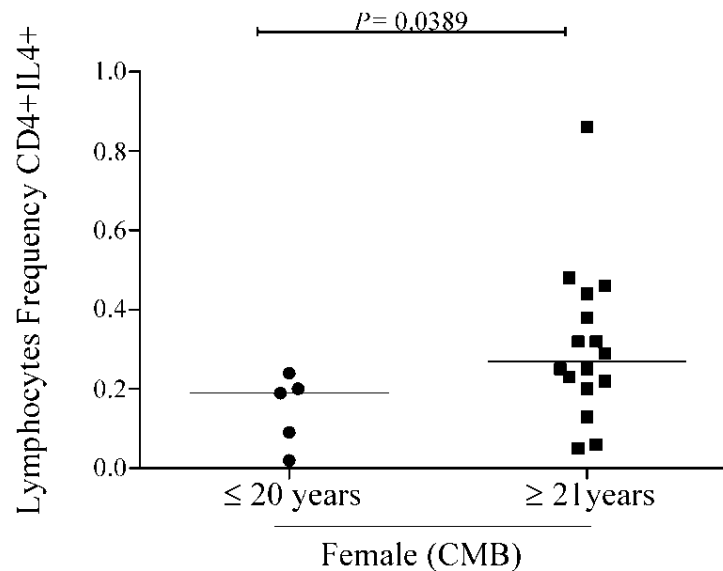


Figure 8. Frequency of lymphocytes T CD4+IL-4+ in unstimulated culture of PBMC of CMB, female, age ≤ 20 years and ≥ 21 years. CMB: total n=22. The bars represent the median. ≤ 20 years: 0.19 CI: 0.02– 0.24; ≥ 21 years: 0.27 CI: 0.20 – 0.44. Mann-Whitney test $p=0.0389$. The median of the controls was 0.205%.

subjects tend to develop a Th1 response with TNF- α and IFN- γ production, among other cytokines (Foss, 1997; Esquenazi et al., 2015). Yamamura et al. (1991) demonstrated an increased expression of genes to Th1 cytokin (TNF- α , IFN- γ and IL-2) in skin biopsies of patients with tuberculoid leprosy. Antans et al. (2004), showed in tuberculoid leprosy patients T-cells producing IFN- γ and T CD4 and T CD8 cells producing TNF- α when

stimulated by PPD. Interestingly, in our study, although we used mitogenic stimulation (PHA) in the culture of PBMC, we observed a different behavior for TNF- α production between the two groups (CPB and CMB). We also observed a higher frequency of CD8 + IL-10 + cells stimulated in culture with *M. leprae* in CMB group (Figure 7). It is known that multibacillary patients preferably have a profile of Th2 cytokines, IL-4 and IL-10 (Foss, 1997;

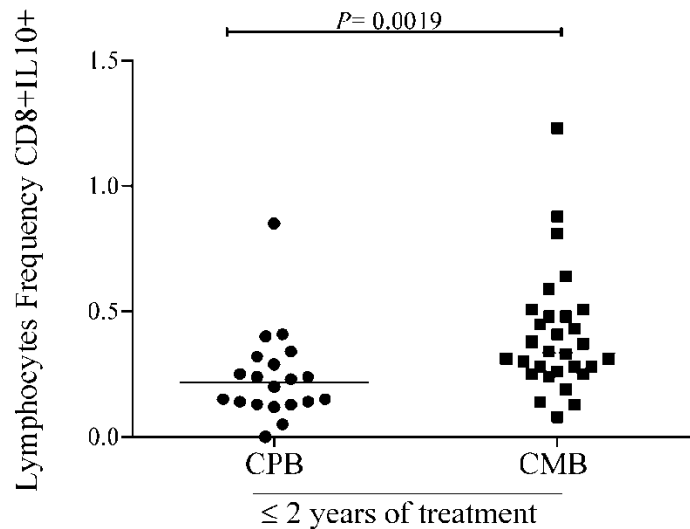


Figure 9. Frequency of lymphocytes T CD8+IL-10+ in *M. leprae* stimulated culture of PBMC of CPB and CMB according to the treatment time ≤ 2 years and > 2 years of their index cases. CPB: total n=27, CMB: total n=30. The bars represent the median. CPB: 0.22 CI: 0.14 – 0.29; CMB: 0.34 CI: 0.28 – 0.45. Mann-Whitney test $p=0.0019$. The median of the controls was 0.135%.

Walker and Lockwood, 2006; Sampaio et al., 2011). Yamamura et al. (1991) found that increased expression of genes for cytokines (IL-4, IL-5 and IL-10) in skin biopsies was strongly associated with the development of multibacillary form. Interestingly, our results indicate increased frequency of CD8+IL-10+ lymphocytes, suggesting that CMB which live with MB patients have a tendency to develop a type of modulatory immune response with a significant production of IL-10. Studying subpopulations of T CD4 and CD8 cells *in situ* in patients with leprosy, Modlin et al. (1986) Modlin and Rea (1988) Modlin et al. (1988) have shown that T CD4 cells predominate in skin lesions of tuberculoid patients, in contrast, T CD8 cells predominate in skin lesions of lepromatous patients. Moreover, these authors observed that T CD8 cells from lesions of lepromatous but not tuberculous lesions, can be activated specifically by *M. leprae* and suppress the proliferation of T CD4 cells *in vitro*. It is believed that this suppression is due to the presence of IL-10 produced by T CD8 cells. It is important to note that this result corroborate with our data, which demonstrated an increased frequency of T CD8+IL-10+ cells in peripheral blood of CMB (Figure 7). Considering that the age factor is important in the epidemiology of leprosy (Amador et al., 2001; Brasil, 2010; Lana et al., 2013), we evaluated cytokine levels among household contacts grouped by age (≤ 20 years and ≥ 21 years). In this sense, we identified a positive correlation ($r = 0.47/p=0.008$) between TNF- α levels and age group, only in the CMB group, and we detected that

CMB ≥ 21 years had a higher TNF- α level compared to ≤ 20 years of the same group (Figure 3). There was also a significant difference of IFN- γ production ($p=0.0039$) and TNF- α ($p=0.0009$) between groups (CPB and CMB) to only those individuals ≤ 20 years (Figures 4a and b), with higher levels of both cytokines in the CPB group ≤ 20 years. These data indicate a possible "match" between the profile of CPB group cytokine response and their cases indexes (Andrade et al., 1994; Duraes et al., 2010). In contrast, individuals ≥ 21 years of CMB group had a higher frequency of T CD4+IL-4+ cells (Figure 8), which leads us to suggest that just as with the MB patients, the disease manifests itself in the oldest age, and the Th2 cytokine profile is also expressed in both groups (Durães et al., 2010). Although the literature of leprosy does not present association of cytokine production with age, especially in relation to CPB and CMB groups, it is important to consider that in our study, some significant differences in cytokine production were only observed from the segregation of individuals by age. And, when we include gender in the analysis, we found higher levels of IL-10 ($p= 0.0352$) among women ≤ 20 years in the CPB group (Figure 5). Rather, women ≥ 21 years showed high frequency ($p = 0.0389$) of T CD4+IL-4+ cells in the CMB group (Figure 8). Lana et al. (2003) analyzed the distribution of leprosy according to gender in Governador Valadares and found a higher proportion of leprosy cases in females (55.3%) with a greater number of tuberculoid, borderline and indeterminate clinical forms among women, and a predominance of lepromatous form among

men. Assessing the degree of exposure of the contacts to infection based on the treatment time of their index cases, it was found that the CMB whose index cases possessed ≤ 2 years of treatment, showed increased frequency of T CD8+IL-10+ cells (Figure 9). Interestingly, Goodal and Negassi (1973) detected significant difference in the pattern of lymphocyte proliferation response of leprosy patients and their household contacts. These authors grouped contacts according to the treatment time of their index cases of < 6 months and > 6 months. They found a low frequency in the number of household contacts of patients with lepromatous form, that were responders to the antigens used in the assays, when the index case had a treatment time < 6 months. Thus, the authors suggested that these household contacts, non-responsive, would be super exposed to *M. leprae* and therefore with a suppressed immune response possibly due to intense exposure to the bacillus. They concluded that the increased risk of these individuals may be related to decreased resistance caused by over-exposure to *M. leprae*. Consistent with this hypothesis, recently, Martins et al. (2012), showed that the household contacts of PB patients were seropositive for PGL-I produced lower levels of IFN- γ compared to subjects negative for PGL-I. However, these authors observed that there was no influence of the levels of anti-PGL-I antibodies among contacts of multibacillary patients, and concluded that this fact could be due to low levels of IFN- γ caused by modulation of the immune system due to the high exposure to the bacillus. And suggest that low production of IFN- γ by the CMB group can contribute to the latent infection (subclinical) in these individuals developing into active disease.

Conclusion

In conclusion, our data regarding the highest frequency of T CD8+IL-10+ cells in the CMB group corroborate the data from Martins et al. (2012), in the sense that the cytokine IL-10 modulates IFN- γ levels. Therefore, it is consistent to suggest that there is a trend of CMB group to display a profile of modulatory response given by an increase of IL-10 and consequently a reduction of IFN- γ .

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