



Immunological biomarkers of subclinical infection in household contacts of leprosy patients

Edson A. Queiroz^a, Nayara I. Medeiros^{b,c}, Rafael T. Mattos^c, Ana Paula M. Carvalho^a, Marina L. Rodrigues-Alves^c, Walderez O. Dutra^c, Francisco C. Félix-Lana^a, Juliana A.S. Gomes^{c,*}, Rodrigo Correa-Oliveira^{a,b}

^a Escola de Enfermagem, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil

^b Imunologia Celular e Molecular, Instituto René Rachou, Fundação Oswaldo Cruz - FIOCRUZ, Belo Horizonte, MG, Brazil

^c Laboratório de Biologia das Interações Celulares, Departamento de Morfologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil

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ABSTRACT

Hansen's disease (or leprosy) still persists as a serious public health issue. Its diagnosis is based primarily on the detection of clinical signs that are characteristic of the disease. Studies have pointed to the selection of a set of serological and cellular biomarkers of subclinical infection that result in an efficient diagnosis. The aim of this study was compare index cases and their household contacts to identify differentially expressed biomarkers of immune response in leprosy that could provide reliable evidence of subclinical infection in household contacts. The study population consisted of index cases with multibacillary form (IC, n = 13) and their household contacts (HC, n = 14). Serum cytokines and chemokines were quantified using the cytometric beads array (CBA) system. The humoral response was assessed by ELISA test. Flow cytometry was used to characterize the cellular immune response. Monocyte and CD4 + T lymphocytes frequency was significantly higher in IC. Both CD4+ and CD8 + T lymphocytes had a reduced CD25 expression in HC. The immunoglobulin (Ig)M profile anti- NDO-HSA, LID-1, and NDOLID antigens was significantly higher in IC. This study points to the monocyte and CD4 + lymphocyte frequency, as well as specific IgM profile, as predictors of subclinical infection in the household contacts.

1. Introduction

Leprosy (or Hansen's disease) still persists as a serious public health issue with an incidence rate of 210,671 reported cases in 2017 (WHO, 2018). It is a chronic, infectious disease caused by *Mycobacterium leprae*, which affects the skin and peripheral nerves, presenting varying degrees of clinical manifestation. Leprosy can be classified as paucibacillary (PB) or multibacillary (MB). Patients with up to five skin lesions are defined as PB, and those with more than five skin lesions are defined as MB.

Its diagnosis is based, primarily, on the detection of clinical signs that are characteristic of the disease (Scollard et al., 2006). A number of strategies have been used to eliminate it, including periodic clinical

examinations of household contacts aimed at establishing the earliest possible diagnosis (World Health Organization Regional Office for South-East Asia New, 2006). However, this measure alone has not been shown to be efficient in eliminating the disease, since its incidence and the number of new cases with Grade 2 disability continue to be significantly high (WHO, 2017).

The humoral response in Hansen's disease has been evaluated through a variety of serological tests designed to detect immunoglobulin (Ig) M against phenol-I glycolipid. These tests have been useful in identifying patients with the multibacillary form, as well as being potential tools in household contact follow-up (Bobosha et al., 2014; Carvalho et al., 2017; Cardona-Castro et al., 2005). An analysis of the cytokine and chemokine profile has also been an alternative

Abbreviations: IC, index cases of leprosy; HC, household contacts of leprosy patients; PB, paucibacillary; MB, multibacillary

* Corresponding author at: Departamento de Morfologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Avenida Presidente Antônio Carlos, 6627. Pampulha, Belo Horizonte, MG 31270-010, Brazil.

E-mail addresses: edalexqueiroz@yahoo.com.br (E.A. Queiroz), nayamedeiros@yahoo.com (N.I. Medeiros), rafaelteixeiramattos@hotmail.com (R.T. Mattos), anapaulamcarvalho@yahoo.com.br (A.P.M. Carvalho), marinarod.bio@gmail.com (M.L. Rodrigues-Alves), waldutra@gmail.com (W.O. Dutra), xicolana@gmail.com (F.C. Félix-Lana), juliana@icb.ufmg.br (J.A.S. Gomes), rodrigo.correa@fiocruz.br (R. Correa-Oliveira).

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strategy (Geluk et al., 2012; Hasan et al., 2004; Mendonca et al., 2008; Cassirer-Costa et al., 2017). However, the results have not yet shown standardized responses that allows for its regular use in monitoring. Moreover, the cell surface molecule profile involved in the disease's immune response shows signs of impairment in the cell-mediated immune response in MB patients (Sridevi et al., 2004; Chaves et al., 2018). Nonetheless, immunophenotypic characteristics still require an improved profile that considers the infected patients' household contacts.

Previous studies have pointed to the selection of a set of subclinical infection biomarkers, both serological and cellular, that may result in efficient diagnosis (Geluk et al., 2012; Nath et al., 2015) and thus apply the appropriate chemoprophylaxis needed to interrupt the infection transmission chain and reduce the number of cases. Subclinical infection is present when *M. leprae* is replicating and invading the host without causing clinical symptoms (yet), thus biomarkers for early infection would be detected (Geluk, 2013). Yet, there are no known biomarkers with reliable sensitivity and specificity, nor with the quantitative or qualitative immunological parameters, that can detect subclinical infection and predict the disease's evolution (Geluk, 2013).

The strategies employed by this study have the objective to compare the response index cases and their household contacts so as to identify immune response biomarkers in leprosy disease that could provide reliable evidence of subclinical infection in the household contacts.

2. Patients, materials, and methods

2.1. Ethical approval

This study was in accordance with the Helsinki Declaration and was approved and revised by the Research Ethics Committee of UFMG, protocol number 13639.

2.2. Study population

A cross-sectional study was carried out between 2014 and 2015 in the city of Almenara, located in northeastern region of Minas Gerais, Brazil. The study population consisted of Index Cases with multi-bacillary form (IC, $n = 13$) and their household contacts (HC, $n = 14$). The sample size was chosen for convenience.

Data collection was carried out through home visits, the use of a structured questionnaire, and collection of 20 mL blood. All individuals or your guardian (a parent or guardian of any child participant), provided their consent to participate, signed Informed Consent Form (ICF). The volunteers were recruited through the National System of Grievances and Notifications (SINAN) database from 2010 to 2015, along with secondary data related to the index cases.

The dermatoneurological clinical examination was carried out on the household contacts to exclude clinical signs of the disease at the time of the home visit. No household contact showed signs during the study.

Inclusion criteria for the index case group were: have a confirmed household contact, did not use corticosteroids, did not present any infectious diseases for at least two years, did not have any type of reaction event, and, lastly, did not have inflammatory diseases.

Inclusion criteria for the household contacts group were: to be a household contact in the index case group, to have no history of Hansen's disease, does not use corticosteroids, did not have an infectious disease for at least two years, and did not have any inflammatory diseases. Women who were pregnant or suspected of being pregnant were also excluded from the study.

2.3. Cytometric bead array (CBA)

Serum levels of the cytokines TNF, IL-6, IFN- γ , IL-2, IL-17A, IL-4 and IL-10 and the chemokines CXCL8 (IL-8), CCL2 (MCP-1 - monocyte chemoattractant protein-1), CXCL9 (MIG - monokine induced by

interferon- γ) and CXCL10 (IP-10 - interferon- γ -induced protein-10) were measured in all patient samples. The serum was centrifuged at 400 g for 40 min at 18 °C, aliquoted and then stored at -80 °C until the day of the experiment. Serum cytokines and chemokines were quantified using the CBA system (Becton Dickinson, BD-USA) in accordance with Peruhype-Magalhães et al. (Peruhype-Magalhaes et al., 2006). Data acquisition was performed by FACScanto flow cytometer (BD-USA) and analyzed in the BD FCAP Array software, where values were expressed in pg/mL for both analytes.

2.4. Enzyme-linked immunosorbent assay (ELISA)

Antigens were provided by the Infectious Disease Research Institute (IDRI). The reactivity of antibodies against the antigens Natural disaccharide linked to human serum albumin *via* octyl (NDOHSA), Leprosy IDRI diagnostic 1 (LID-1) and the Natural disaccharide octyl, Leprosy IDRI Diagnostic 1 (NDOLID) was performed by the ELISA assay as described by Lobato et al. (2006). Antigen reconstitution and dilution were performed as indicated by the IDRI. The samples and controls were tested in duplicate and the antibody titer was expressed as ELISA Index (EI), the cutoff was determined when EI equals the optical density of the sample (OD)/OD. The cutoff value was calculated as the mean OD of the three negative controls plus three times the standard deviation of the value. EI values greater than 1.1 were considered positive (Lobato et al., 2006). The ELISA test was repeated for samples that exhibited positive EI values in a single well of a duplicate and for samples that the EI values showed a 25% or greater variation between duplicates.

2.5. Flow cytometry

Ten (10) mL of lysis solution (50.000USP/L of heparin, 2.85 g/L of sodium citrate, 30 mL/L of diethyleneglycol, and 54 mL/L of formaldehyde) were added to the peripheral blood, incubated at room temperature for 15 min, centrifuged at 400 g for 7 min, poured into 10 mL of phosphate buffer (pH 7.4, containing 0.5% BSA and 0.1% sodium azide), and vortex-homogenized for one minute. It was centrifuged again at 400 g for 7 min, after which 5 mL of a freezing solution containing 2% formaldehyde solution was added. The aliquots were stored at -80 °C. The samples were thawed on the day of the experiment. One hundred μ L of blood were resuspended and placed in tubes with 2 μ L of undiluted monoclonal antibodies: anti-CD14 (clone M5E2) - conjugated with PerCP (Chlorophyll Peridinin Protein); anti-CD80 (L307.4) - conjugated with PE (phycoerythrin); anti-CD86 (clone 2331 (FUN-1) - conjugated with APC (Allophycyanin); anti-HLA-DR (G46-6) -conjugated with FITC (fluorescein isothiocyanate), anti-CD3 (17a2), aAnti-CD4 (RPA-T4) - conjugated with BVCC-21 (Shiny Violet), anti-CD8 (C8/144B) - conjugated to PerCP, anti-CD152 (BNI3), anti-CD25 (M-A251) - conjugated withAPCCy7 (Allophthoraine Cyanine 7), and anti-CD28 (MABF408) - conjugated with PECy7 for labeling surface molecules. The samples were incubated for 30 min at room temperature and in the absence of light. The erythrocytes were then lysed by incubation with 2 mL of a commercial lysis solution (FACS Lysing Solution-BD, USA) for 10 min at room temperature. The samples were washed with phosphate buffered saline (PBS) with 1% bovine serum albumin (BSA). Cells were acquired on the FACScantoII™ flow cytometer (BD Biosciences, Breda, The Netherlands). The antibodies were supplied by BD Pharmingen™ and BD Bioscience™ combined in a multicolor panel. Compensating controls were used for each fluorochrome to establish the compensation parameters. Analyses were performed using FlowJo 10.2 software (Tree Star, Inc., Ashland, OR, USA).

2.6. Data analysis

Statistical analyses were carried out using the GraphPad Prism 6.0 software package (San Diego, CA, USA). The Shapiro-Wilk test was used

Table 1
Clinical, epidemiological, and demographic characteristics of the index cases and their household contacts.

Variable	Index Case (IC)	Household Contacts (HC)
Sex		
Male	9 (69%)	6 (43%)
Female	4 (31%)	8 (57%)
Age^a		
Mean (± SD)	51 (± 18)	35.93 (± 36)
Median	54	30.5
Minimum	16	16
Maximum	76	65
Presence of BCG Scar		
0	7 (55%)	0 (0%)
1	4 (31%)	8 (57%)
2	1 (7%)	6 (43%)
Information not collected	1 (7%)	0 (0%)
Operational Classification		
Multibacilar	13 (100%)	NA
Madrid Classification		
Dimorph	10 (77%)	NA
Virchoviana	2 (15%)	NA
Information not collected	1 (8%)	NA
Treatment time^b		
In treatment	10 (77%)	NA
Treatment completed	3 (23%)	NA
Length of time of contact with the case (in years)		
Mean (± SD)	NA	18.85
Median	NA	16
Minimum	NA	4
Maximum	NA	36
Relationship to contact with the index case		
No kinship or relationship	NA	6 (43%)
Kinship or relationship	NA	8 (57%)

Note: ^aAge at the time of the interview. ^bTreatment time at the time of the interview/blood sample collection.

NA – Does not apply; SD – Standard deviation.

to test data normality. Statistical comparisons were performed using the non-parametric two-tailed Mann-Whitney test for each variable. To show the overall immunological profile between the index cases and household contacts, we used the multidimensional scaling approach (MDS) in unpaired samples to verify the similarity/dissimilarity between the two groups. The Euclidean distances and their correlation coefficients were calculated from correlation matrices as pre-processing for the MDS using the cmdscale in the R Software database, version 3.4.2. Significance was considered when $p < 0.05$ in all analyses.

3. Results

3.1. Clinical and demographic description of the population

The clinical and demographic description of study population was shown in Table 1. The IC group was composed of 13 patients (69% male) with a mean age of 51 years (range 16–76). These patients present the multibacillary form undergoing treatment ($n = 10$, 77%), or after treatment ($n = 3$, 23%). The HC group was composed of 14 patients (57% female) with a mean age of 35 years (range 16–65). In IC group 55% did not present BCG scar, whereas all patients from HC had one (57%) or two scars (43%).

The clinical and epidemiological characteristics was shown in Table 2.3. Our results showed that IC patients presented different treatment stages range 1–30 months from the start of treatment until the home visit. All patients received treatment for 12 months. The number of lesions ranged 2–20 and only one patient did not present lesion (Table 2). Most of household contacts (57%) show some relationship with IC. The average period of time of contact between HC

and IC was 16 years (range 4–36). We observed that 42% of HC shared the same room with IC patients. We also observed that 85% of HC lived in the same house of IC patients on the home visit date and diagnosis date (Table 3).

3.2. Leukocytes from household contacts present altered activation profile

To evaluate the influence of household contacts in the activation and co-stimulation profile we investigated the expression of CD80, CD86 and HLA-DR in neutrophils and monocytes (Fig. 1). Our results did not show significant differences in neutrophil frequency of IC and HC groups. The expression of CD80, CD86, and HLA-DR also has not changed (Fig. 1A). However, we observed a decrease of monocyte frequency in the HC compared to IC. Monocytes from HC showed lower expression of CD86 than IC (Fig. 1B).

The analysis of activation molecules in the CD4⁺ and CD8⁺ lymphocytes was presented in Fig. 2. We observed an increase of T CD4⁺ lymphocytes in the HC compared IC (Fig. 2B). Lymphocytes TCD4⁺ and T CD8⁺ from HC showed lower CD25 expression compared IC (Fig. 2B-C).

3.3. IgM profile distinguishes leprosy patients from their household contacts

To evaluate if household contacts alter cytokine and chemokine profile, we investigated the serum levels of TNF, IL-6, IFN- γ , IL-2, IL-17, IL-4, IL-10 (Fig. 3A), CXCL8, CCL2, CXCL9, CXCL10 (Fig. 3B). Our results showed that there are no significant differences between plasmatic levels of cytokine and chemokine from HC and IC groups.

We also evaluate the reactivity of IC and HC against the antigens Natural disaccharide linked to human serum albumin via octyl (NDOHSA), Leprosy IDRI diagnostic 1 (LID-1) and the Natural disaccharide octyl (NDOLID) by anti-IgM serum levels (Fig. 4). We observed that HC showed a decrease in the reactivity of anti-IgM serum levels against NDOHSA, LID-1 and NDOLID compared IC (Fig. 4A). The immunological distance was significantly different between the groups (Fig. 4B).

4. Discussion

Despite significant effort to reduce the prevalence rates and detect new cases, Hansen's disease still persists as a relevant public health issue in various countries in Asia, Africa, and Latin America (WHO, 2017). The main difficulties to an early diagnosis are the long incubation period and the manifestation of the disease's clinical signs, not to mention the absence of good subclinical infection markers (Nath et al., 2015). We looked for immunological biomarkers that could identify subclinical infection for early diagnostic purposes as a way to overcome this difficulty.

The main findings in this study, which best characterized the difference between the index cases and their household contacts, were elevated levels of IgM and the highest degree of activation of T CD4⁺ and T CD8⁺ lymphocytes in the index cases. Immunoglobulin levels of anti-IgM NDO-HSA, LID-1, and NDOLID antigens were significantly higher in the index cases than in their household contacts, corroborating findings in several other studies (Carvalho et al., 2017; Duthie et al., 2008; da Conceicao Oliveira Coelho Fabri et al., 2015; Lobato et al., 2011). The MDS approach was applied to the same reactivity parameters to the various antigens, which showed a clear and significant immunological distance between the groups. Thus, this immunological parameter can be considered as promising subclinical infection biomarker and should be further evaluated in larger studies as well as in other populations. The household contacts in this study were, basically, negative on these tests, which may be due to either, insufficient contact with the patient or an important and effective immune response to the infection. Although in this study we did not follow up the individuals that participated in it, the tendency of specific

Table 2
Details of the clinical and epidemiological characteristics of each index case.

Index Case (IC)	Age (years)	Sex	Madrid Class.	Treatment time (Months) ^a	No. of BCG Scars	No. of Lesions	No. of Nerves	Bacilloscopy
IC1	58	M	V	10	0	20	4	Positive
IC2	53	F	D	5	NI	10	NI	Positive
IC3	46	M	D	10	0	10	3	Positive
IC4	47	M	D	3	1	7	3	Positive
IC5	54	F	D	12	1	5	2	Positive
IC6	16	M	V	4	1	0	4	Positive
IC7	16	M	D	7	2	10	NI	Positive
IC8	75	F	D	30	0	12	2	Positive
IC9	62	M	D	1	0	NI	NI	Positive
IC10	58	M	D	1	0	15	2	Positive
IC11	76	M	D	11	0	2	1	Positive
IC12	48	F	D	8	0	8	6	Positive
IC13	54	M	NI	30	1	NI	NI	Positive

F: Female; M: Male; Madrid Class.: Madrid Classification; Borderline (B); Lepromatous (L); CGB: Bacille Calmette-Guérin; NI: Not Informed.

^a Time from the start of treatment until the home visit. All patients received MDT for 12 months.

Table 3
Details of the clinical and epidemiological characteristics of each household contact.

Household Contact (HC)	Age (years)	Sex	No. of BCG Scars	Relationship	Time of Coabitation ^a (years)	Type of Residence	Situation of contact ^b
HC1	65	F	1	Spouse	4	Room	Resides
HC2	16	F	2	Granddaughter	16	Domicile	At the diag.
HC3	36	F	1	Spouse	20	Room	Resides
HC4	23	F	2	Daughter	23	Domicile	In diag.
HC5	25	M	1	Son	NI	Domicile	Resides
HC6	18	M	1	Brother	16	Domicile	Resides
HC7	18	M	2	Brother	16	Room	Resides
HC8	36	M	1	Son	36	Domicile	Resides
HC9	27	F	2	Not Rel.	10	Domicile	Resides
HC10	62	M	1	Brother	13	Domicile	Resides
HC11	59	F	2	Spouse	32	Room	Resides
HC12	57	M	1	Spouse	32	Room	Resides
HC13	32	F	2	Sister	13	Domicile	Resides
HC14	29	F	1	Spouse	14	Room	Resides

F: Female; M: Male; BCG: Bacille Calmette-Guérin; Not Rel.: Not related; At the Diag: In diagnosis; NI: Not informed.

^a Time of Coabitation: total cohabitation time.

^b Contact situation at the time of the home visit.

IgM levels is toward a gradual decline after starting treatment in multibacillary cases and negativity in paucibacillary cases (Lobato et al., 2011).

Another differential finding was the higher degree of activation of T lymphocytes in the index cases. The phenotypic profile is more activated in CD4 + and CD8 + T lymphocytes in the index cases than in the household contacts, as evidenced by a higher MFI in CD4 + CD25 + and CD8 + CD25 + T lymphocytes. This increased activation is probably due to a response to antigenic stimulation by the bacillus, with consequent increased CD86 expression by the monocytes that activate these lymphocytes, generating effector and/or memory T cells. Thus, high levels of activated lymphocytes associated with positive levels of IgM may be biomarkers of subclinical infection in household contacts.

Blood monocytes migrate to the tissue and become macrophages, playing a key role in eliminating the bacillus through phagocytosis and participating in both innate and adaptative immune responses (Ohkawa, 1985). The phenotypic monocyte profile presented a lower frequency in the household contacts than in the index cases. The higher monocyte frequency in the index cases may be due to the disease's residual systemic consequences that may still be active, since 77% of the index cases had not yet finished treatment and in which bacterial or host factors may be responsible for the increase. Although we did not evaluate monocyte subpopulations, the frequency of non-classical and intermediate monocytes might be higher in the case group than in the contacts, as found by Castano et al. (2011) in studies with tuberculous patients, explaining, at least in part, the higher monocyte count. Another aspect that could explain the increased number of monocytes in

the index cases is the high number of tissue macrophages in Hansen's disease lesions in both clinical forms, resulting from the formation of granulomas (Van Voorhis et al., 1982; Kaplan et al., 1983). This could reflect an increase in monocytes circulating in the peripheral blood, given the need for macrophagic interaction with the bacillus at the tissue level. Therefore, an increase in monocytes in the household contacts' peripheral blood could be an expression of bacillus combat and, consequently, subclinical infection.

We also evaluated the secondary signs of immune response, important for T-cell survival and expansion, and, consequently, the cell-mediated immune response against the bacillus. We found similar levels in the expression of CTLA-4, CD80, CD28, and HLA-DR molecules in monocytes, neutrophils, and lymphocytes, in both the index cases and household contacts, which may indicate a similar level of intensity of secondary sign immune response, at least at the time of analysis. In untreated multibacillary index cases, some studies have shown a reduction in the CD80 and CD86 co-stimulatory molecules as compared to the paucibacillary cases (Schlienger et al., 1998; Agrewala et al., 1998; Palermo Mde et al., 2012) and CD28 in healthy subjects (Fafutis-Morris et al., 1999), indicating cell anergy, with resulting impairment in antigen presentation. The similarity between the index cases and household contacts may be due to the effects of treatment. Surprisingly, the levels of another co-stimulatory molecule, CD86 in monocytes, are higher in the index cases than in the household contacts, contrary to the above-cited studies. However, the CD80 molecule appears to be more important in maintaining a stronger immune response relative to the bacillus (Schlienger et al., 1998). Due to the paucity of studies involving

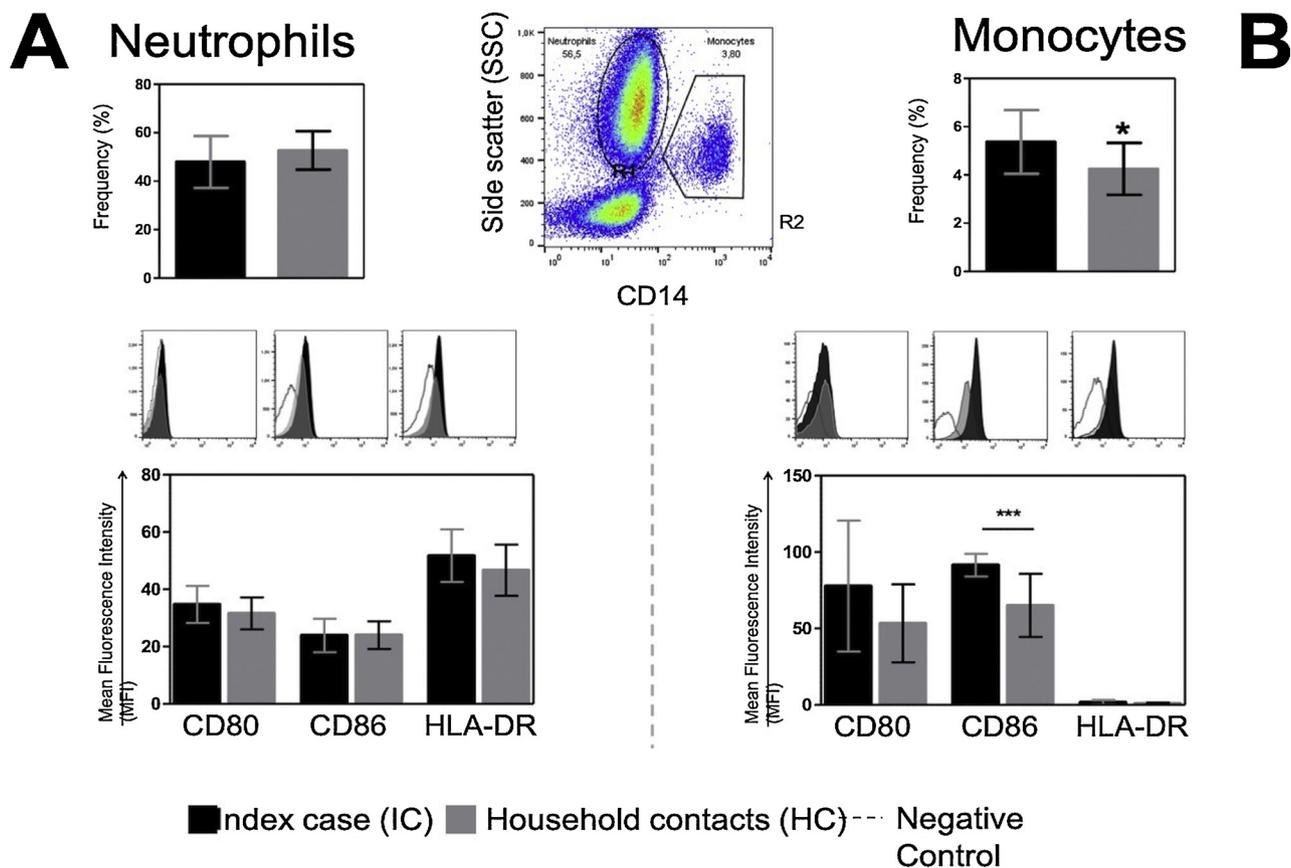


Fig. 1. Expression of activation and co-stimulation molecules in neutrophils and monocytes from leprosy patients. Activation and co-stimulation profile of neutrophils (A) and monocytes (B) was evaluated by *ex vivo* assay in leprosy patients with multibacillary form named Index Cases (IC, n = 13, black bars) and subclinical infection in household contacts (HC, n = 14, gray bars). Mean fluorescence intensity of CD80, CD86 and HLA-DR was determined by flow cytometry. Data were shown in bar charts highlight median values and interquartile range. The Mann-Whitney test was used for comparative analyses between two groups and asterisk and connector lines highlight significant differences ($p < 0.05$).

surface molecules, along with the different methods used in this one concerning the index cases under treatment and their household contacts, it is hard to establish a clear explanation for the high CD86 expression in monocytes. We have hypothesized that the destruction of bacilli by MDT (multidrug therapy) could result in a greater antigen presentation from exposure to new antigenic molecules, with different epitopes, which could lead to a late stimulation wave of antigen-presenting cells, among them monocytes, thus activating an effector T or memory cells, which could reflect an increase in the frequency of CD4+CD25+ and CD8+CD25+ cells observed in the index cases in this study.

Another differential parameter was the lower frequency of peripheral blood CD4 + T lymphocytes in the index cases. This reduction may be due to the migration of these cells to the tissue infection site (Tsao et al., 2002) with resulting participation in the granuloma formation (Gonzalez-Juarrero et al., 2001; Modlin et al., 1988).

Neutrophil recruitment is crucial for an initially successful defense against microbial infection by the host. The frequency profile and expression molecules were the same in both groups, for both co-stimulatory molecules and antigen presentation molecules. This may be due to the time elapsed after disease onset, in which the acquired immunity starts to combat the bacillus, and in the case of household contacts, the innate immune cells, such as neutrophils, may be able to eliminate the infection agent such as *M. leprae*. Another aspect that may indicate the absence of active infection, or at least the absence of Hansen's disease Type 2 reactions (ENL – *Erythema Nodosum Leprosum*) (Lee et al., 2010), is the similarity of the level of the neutrophil recruiting chemokines, CXCL8, between the index cases and household contacts, given that this

chemokine is at levels similar to those of the contacts, which did not present any clinical signs suggestive of the disease.

Cytokines and chemokine levels have been candidates for subclinical infection biomarkers (Geluk et al., 2012; Martins et al., 2012), in addition to serving as a parameter for classifying and monitoring treatment effectiveness and indicative of Hansen's disease reactions (Cassirer-Costa et al., 2017; Belgaumkar et al., 2007). However, cytokine and chemokine levels in this study did not present a differential expression, which limits the search for plasma biomarkers. This may be due to the fact that the index cases were still under treatment, thus reducing cytokine and chemokine serum levels, as shown in other studies (Cassirer-Costa et al., 2017; Moubasher et al., 1998), in both multibacillary and paucibacillary patients. It is difficult to state that the cytokine and chemokine levels were low or normal, only that they had a similar inflammatory and pro-inflammatory profile. The absence of *in vitro* stimulation of the cells with *M. leprae* antigens may be another factor that influenced the similarity in the cytokine and chemokine profile. Studies using *in vitro* stimulation indicate the potentiality of some chemokines and cytokines, such as CCL2 (MCP-1) and IFN- γ , with regard to evaluating subclinical infection (Geluk et al., 2012). However, a study by Saini et al., which assessed some cytokines at baseline (non-stimulated) and with specific stimulation, found very similar results between these two methods, whereas baseline assays had lower cytokine levels as compared to specific stimulation with bacillus fractions (Saini et al., 2014).

When we evaluate the various immunological parameters, we observed that most of them are similar in the groups analyzed, reflecting a lack of immunological distance between the index cases and household

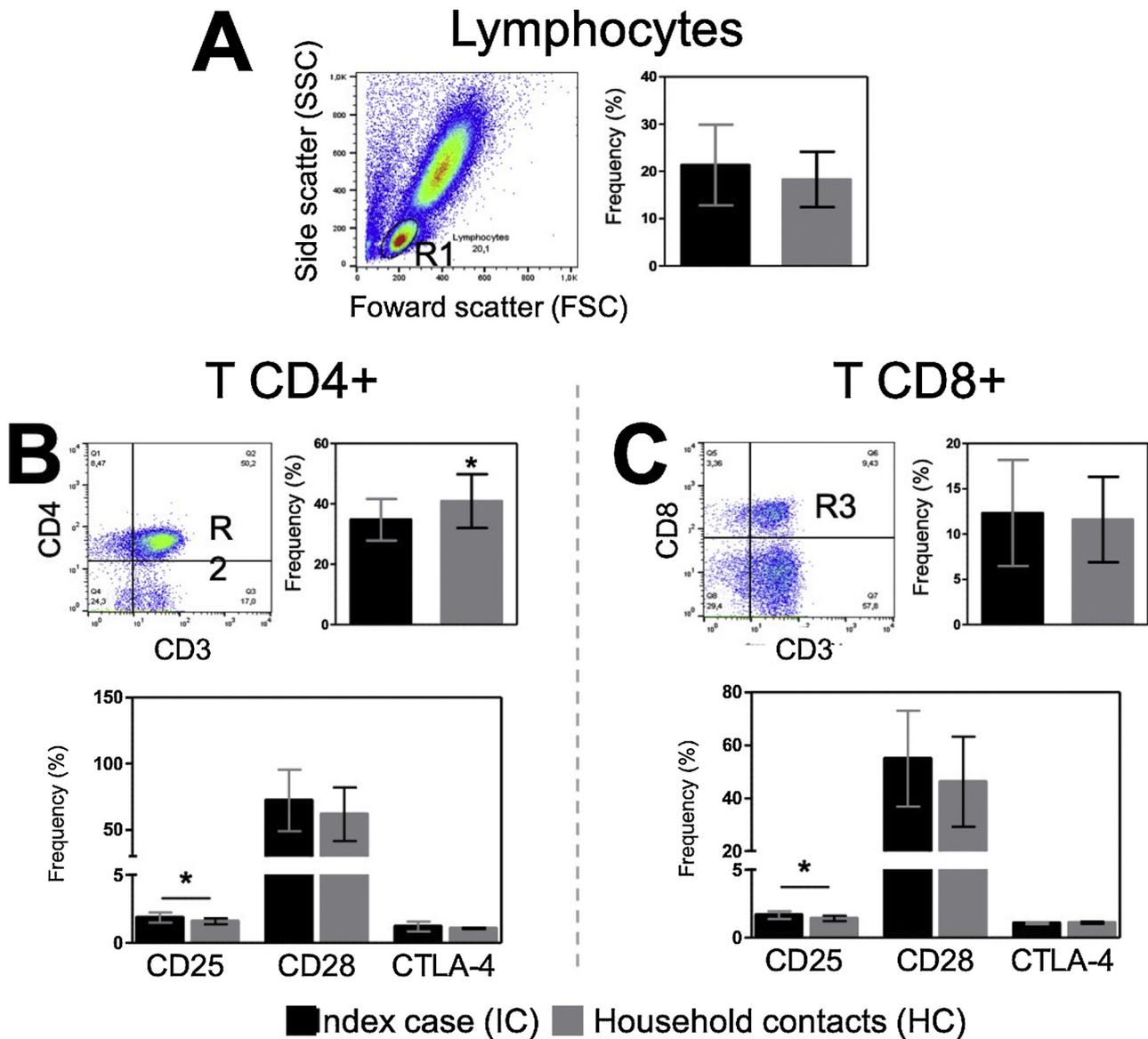


Fig. 2. Expression of activation and co-stimulation molecules in lymphocytes from leprosy patients. Activation and co-stimulation profile of lymphocytes (A) and their subpopulations CD4⁺ (B) and CD8⁺ (C) was evaluated by *ex vivo* assay in leprosy patients with multibacillary form named Index Cases (IC, n = 13, black bars) and subclinical infection in household contacts (HC, n = 14, gray bars). The frequency of CD25, CD28 and CTLA-4 was determined by flow cytometry. Data were shown in bar charts highlight median values and interquartile range. The Mann-Whitney test was used for comparative analyses between two groups and asterisk and connector lines highlight significant differences (p < 0.05).

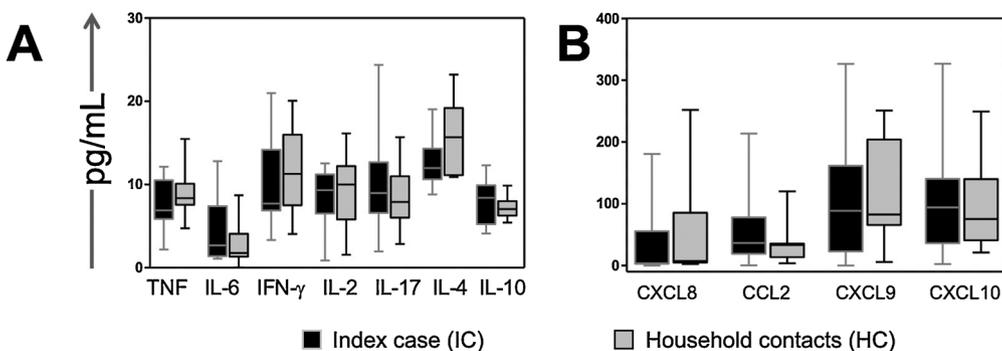


Fig. 3. Evaluation of cytokines and chemokines plasma levels from leprosy patients. Cytokines (A) and chemokines (B) was evaluated by plasma levels in leprosy patients with multibacillary form named Index Cases (IC, n = 13, black bars) and subclinical infection in household contacts (HC, n = 14, gray bars). The concentration of TNF, IL-6, IFN-γ, IL-2, IL-17, IL-4, IL-10, CXCL8, CCL2, CXCL9, CXCL10 was determined by CBA. Data were shown in box plot charts highlight median values and interquartile range. The Mann-Whitney test was used for comparative analyses between two groups and asterisk and connector lines highlight significant differences (p < 0.05).

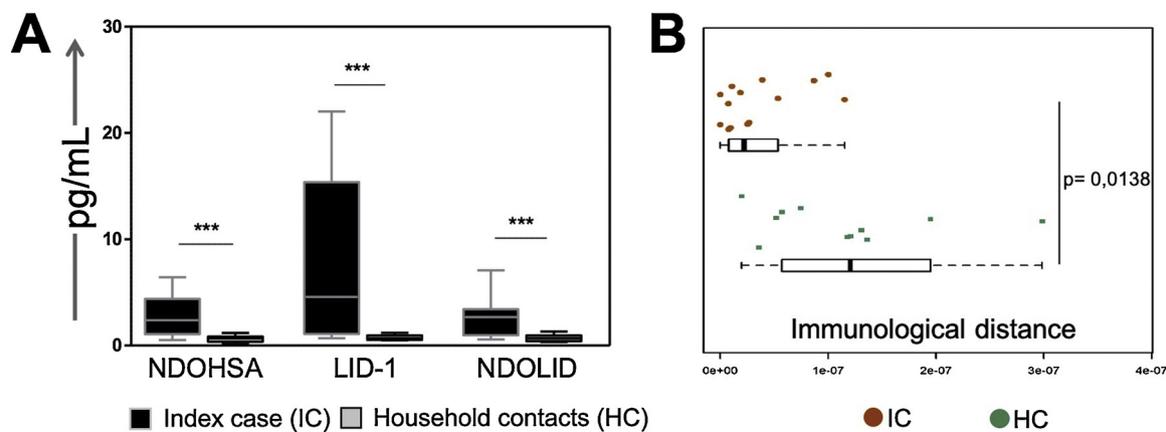


Fig. 4. Evaluation of IgM reactivity from leprosy patients against the antigens NDOHSA, LID-1 and NDOLID. IgM reactivity against the antigens Natural disaccharide linked to human serum albumin *via* octyl (NDOHSA), Leprosy IDRI diagnostic 1 (LID-1) and the Natural disaccharide octyl (NDOLID) in leprosy patients with multibacillary form named Index Cases (IC, $n = 13$, black bars/ orange dots) and subclinical infection in household contacts (HC, $n = 14$, gray bars/ green dots) was evaluated by ELISA. Data were shown in box plot charts highlight median values and interquartile range. The Mann-Whitney test was used for comparative analyses between two groups and asterisk and connector lines highlight significant differences ($p < 0.05$).

contacts. However, some of them stand out and could be used as subclinical infection predictors in contacts that could be in the course of disease evolution and had not yet presented clinical signs of the disease. The immunological distance between both groups is evident in terms of the humoral response, considering this immunological parameter clearly and specifically differentiates the index cases from their household contacts.

Our data suggest that the level of contact between the household contacts and their index cases was not very intense or the contacts developed an immune response that was able to fight the infection. However, at the time of the evaluation, there were no sufficiently perceptible immunological response that could provide evidence of previous subclinical infection nor they had clinical signs suggestive of the disease at the time of screening. Our study presents internal validity, since the immune response depends on several factors, among them genetic variations presented by populations of different geographical areas of the world (Geluk et al., 2012), which probably hinders the identical replication of results. A limiting factor of the study was the verification of possible adjacent infections only from the verbal information of the volunteer of the study according to the questionnaire.

We conclude that, from the differentially expressed parameters in the index cases, such as increased humoral response, a higher level of lymphocyte activation (with probable proliferation and differentiation in effector and memory cells), and a greater frequency of monocytes circulating in the peripheral blood, the cellular and serological biomarker options used as subclinical infection predictors in the household contacts could be expanded.

Conflict interest

None.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.imbio.2019.05.002>.

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