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Inflammatory cytokines in leprosy reactions and periodontal diseases

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ABSTRACT

The inflammatory cytokines involved in the immune response to chronic periodontal disease (CPD) in the context of leprosy reactions (LR) were analyzed in 57 new cases of multibacillary leprosy (MBL). They were stratified by the presence of CPD and LR. Messenger RNA (mRNA) expression of inflammatory mediators was determined by qRT-PCR using skin biopsy and by ELISA using serum samples, maintaining 5% of significance level in ANOVA and correlation analyses. Twenty-three (40.4%) patients presented the first LR, whereas 22 (45.0%) patients presented CPD. IL-4 and IL-6 serum levels were significantly lower in patients with CPD and LR than in patients without CPD but with LR; IFN-γ serum levels were higher in patients with CPD and LR than in patients with no CPD and no LR; IL-4 serum levels were negatively correlated with TNF-α gene expression, while IL-6 serum levels were positively correlated with IFN-γ gene expression, in the skin of subjects with CPD and LR. The presence of DPC in individuals with LR immunoregulated IL-6, IFN-γ, and IL-4 concentrations. The presence of DPC decreased serum levels of IL-6 and IL-4 in reactional individuals. CPD concomitant to LR resulted in increased IFN-γ serum levels.

KEYWORDS: Multibacillary leprosy. Reactions. Odontogenic infections. Cytokines. Chronic periodontal diseases.

INTRODUCTION

The leprosy reactions (LR) Type 1 (T1R), reverse reaction and type 2 (T2R) and erythema nodosum leprosum (ENH) are the most common clinical forms of LR observed in 20–50% of the cases of leprosy. They require immediate treatment as they cause disabilities and physical deformities[1].

There are evidences that oral coinfections are among the predisposing factors for LR[2]. However, there are gaps in the literature regarding which types of odontogenic infections could influence the occurrence of reactional episodes.

The chronic periodontal disease has been identified as a risk factor to cardiovascular disease, stroke and diabetes by increasing systemic inflammatory cytokines, among them IFN-γ, TNF-α, IL-6[3-6]. The periodontium, when affected, may persist as a reservoir of bacteria, bacterial products and inflammatory cytokines, favoring their interaction with structures or organs distant from the oral cavity. Such products and/or mediators may induce or perpetuate systemic effects[7,8].

Important pro-inflammatory and anti-inflammatory mediators, such as tumor necrosis factor (TNF)-α, interferon (IFN)-γ and interleukin (IL)-1β, IL-6, IL-4, and IL-10 are pathophysiologically interrelated not only in LR but also in chronic periodontal disease (CPD)[9].

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Appropriate innate and adaptive immune response interactions can influence the clinical outcome of leprosy, and recent studies suggest that higher serum IFN-γ concentrations are related to the development of leprosy reactions in individuals with the multibacillary form[10,11].

Considering the plausibility of the relationship between CPD and LR, this study aimed to analyze the relationship between inflammatory cytokines involved in the immune response to CPD and LR. In terms of practical implications, the relationship between CPD and LR could lead to prioritized dentistry assistance for leprosy patients, consequently reducing the risk of physical disabilities.

METHODS

Fifty-seven new cases of leprosy were selected by the non-probabilistic sampling of convenience type. All the selected subjects were multibacillary (MB) according to bacilloscopic examination and were diagnosed as new cases by specialized physicians in reference centers from three endemic municipalities from Mato Grosso State, Brazil, from August the 1st, 2012 to January the 31st, 2014 (18 months).

Group I constituted new cases experiencing the first onset of LR (n=23); Group II was composed of new leprosy cases without LR during their first month of leprosy treatment (n=34). They were classified as LR type 1 (T1R) or reverse reaction and type 2 (T2R) or erythema nodosum leprosum (ENH).

The following patients were excluded from the study: those who were being treated for LR, were LR recurrent, used other medication for the last 6 months besides the multidrug therapy (WHO/MDT), or who presented any comorbidity during medical evaluation.

Data related to dental and gingival-periodontal conditions were collected at the first dental appointment in a specialized dentistry center. CPD was defined in patients who presented at least one (1) site with clinical insertion loss equal to 3 mm and probing depth equal to 4 mm[12].

Blood, skin and gum tissues were collected. Blood samples collection and skin biopsy were performed on the day of leprosy diagnosis or the first LR.

Blood samples (10 mL) were obtained by venous puncture and collected in a vacuum tube containing a coagulation inhibitor for the serological analysis of inflammatory cytokines, such as TNF-α, IFN-γ, IL-1β, IL-6, IL-4, IL-10 and IL-12p70. After collection, the tubes were placed in an upright position at room temperature for clot retraction (approximately 60 min) and centrifuged (400 x g for 10 min). The obtained serum (2 mL) was stored in cryogenic tubes at -80 °C.

Qualified professionals performed punch skin biopsy of 5.0 mm diameter of tissue from the edge of the lesion. The gingival tissue biopsy was performed 7 and 14 d after blood collection and skin biopsy. The site for the gingival tissue collection represented the worst clinical condition after performing the periodontal clinical examination. The anatomical region adjacent to the dental element, i.e., the interproximal wedge, was used for removal of the gingival tissue. In case of patients who needed to undergo dental extraction or cystic removal, the biopsy sample was collected at the time of the surgical procedure.

RNA from the skin and gingival tissue samples was stored in a cryogenic tube and frozen at -80 °C prior to analysis of gene expression of inflammatory cytokines, TNF-α, IFN-γ, IL-1β, IL-6, IL-4, IL-10 and IL-12p70. Quantitative reverse transcription- polymerase chain reaction (qRT-PCR) was performed for mRNA analysis. Total RNA extraction was performed using TRIzol (Thermo Fisher Scientific, Carlsbad, CA, USA), according to the manufacturer's protocol. The yield and purity of extracted RNA were evaluated using the NanoDrop spectrophotometer (Thermo Fisher Scientific, Carlsbad, CA, USA). Samples with appropriate quantities of RNA, above 100 ng/μL, and purity between 1.7 and 1.9 were considered for further analysis. The cDNA synthesis was performed from 1 μg of mRNA with SuperScript III (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instruction. Gene expression was calculated by the relative standard curve method using the GAPDH gene expression as the endogenous control. Quantification of the target gene and the GAPDH gene expression was obtained from their Ct (threshold cycle) on the standard curve. Thus, the relative expression was calculated normalizing the target data by the GAPDH data in each sample.

The qPCR reactions were performed using TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's recommendations in a StepOne Plus real-time PCR equipment (Applied Biosystems, Foster City, CA, USA). Serum levels of inflammatory cytokines was measured by ELISA, using a Quantikine® ELISA Kit (R&D Systems, Minneapolis, MN 55413, USA) and a microplate reader (MultiSkan FC; ThermoScientific®) at 450 nm or 490 nm, according to the manufacturer's protocol, specific for each investigated cytokine.

The Spearman correlation coefficient was used for exploratory analysis. Data of the mediators resulting from blood samples and biopsies were evaluated regarding the distribution of normality by the Shapiro-Wilk test, normalized by a logarithmic function. The Student's *t*-test was performed to determine differences between mean values, and the analysis of variance (ANOVA)

was carried out in case of variables with more than two comparison groups using the Prism software version 5, at 5% significance level.

Ethical considerations

This research was approved by the Ethics Committee in Research of the Hospital Universitario Julio Müller of the Federal University Foundation of Mato Grosso, Protocol Nº 930/CEP-HUJU/2010. After explaining the purpose, procedure and importance of the study, written informed consent was obtained from all patients examined. For participants under the age of 18 years written informed consent was systematically obtained from the family heads or guardians. All study participants received instructions on oral hygiene, prevention of caries and periodontal disease. When necessary, they underwent dental and surgical treatment.

RESULTS

From the total of 57 multibacillary subjects studied, 23 (40.4%) new cases were evaluated to have their first LR, of which 5 (21.7%) presented T1R and 18 (78.3%) presented T2R; 34 (59,6%) were new cases without LR; 22 (45.0%) patients presented CPD; 34 were (59.6%) male patients; the average age of the study group was 40.0 years (male: SD = ±18.2; female: SD = ±20.63); 29 (50.9%) patients declared themselves as African descendants; 28 (49.1%) attended high school or had a college degree; 47 (82.5%) lived in urban areas; 41 (71.9%) reported a family income inferior or equal to 2 minimum wages (US$ 602). Considering the clinical characteristics, 25 (43.9%) were BT; 10 (17.5%) BB; 5 (8.8%) BV and 17 (29.8%) were virchowian[13]; 31 (53.6%) patients showed no neural impairment, 24 (46.2%) had grade 1 physical disability, and none of the patients had grade 2 physical disability.

Results of the relative mRNA expression analysis of IFN-γ, TNF-α, IL-10, IL-6 and IL-1β from the skin biopsies of MB patients revealed a higher than average expression of IL-6, IFN-γ and IL-1β; in gingival tissue biopsies of subjects with gingival-periodontal impairment, mRNA expression of IFN-γ and IL-1β reached higher than average values. In serum samples, the level of the mediator IL-6 was found to be the highest, followed by IL- 10 and TNF- α (Table 1).

Table 1 - Descriptive analysis of inflammatory mediators in the skin and gingiva of multibacillary leprosy cases according to variables related to gene expression and serum quantitation (pg/mL)

Inflammatory mediators	n	Minimum	Maximum	Mean	SD[c]
Gene expression of inflammatory mediators					
Skin biopsy					
IFN-γ	26	0.127	185.800[a]	16.560	42.320
TNF-α	17	0.384	33.751	3.816	7.938
IL-10	20	0.451	32.724	7.480	10.418
IL-6	20	0.053	1143.542	58.653	255.384
IL-1β	22	0.023	288.852	13.453	61.513
Gingival tissue biopsy					
IFN-γ	7	0.011	416.801	60.118	157.284
TNF-α	7	0.079	0.800	0.307	0.265
IL-10	8	0.067	2.763	1.155	1.076
IL-6	9	0.014	5.940	1.158	1.936
IL-1β	9	0.052	19.867	3.737	6.318
Inflammatory mediators in serum (pg/mL)					
IFN-γ	16	0.001	0.088	0.013	0.021
TNF-α	26	0.002	0.135	0.018	0.027
IL-10	34	0.001	0.105	0.020	0.026
IL-6	51	0.003	0.651[b]	0.078	0.120
IL-4	29	0.001	0.005	0.002	0.001
IL-12p70	33	0.001	0.014	0.003	0.003

[a,b]outliers (a = 10779.85; b = 3025.98); [c]SD= standard deviation

In skin biopsies, IL-4 and IL-12p70 were not detected. In case of patients with CPD and LR, there was a negative correlation between serum IL-4 and TNF-α expressed in the skin ($r = 0.83$; $p < 0.05$); a positive correlation between IL-4 and IFN-γ present in serum ($r = 0.89$; $p < 0.05$) and between serum IL-6 and IFN-γ expressed in the skin ($r = 0.79$; $p < 0.05$) (Table 2).

Higher concentrations of IL-6 were found in serum samples of patients with LR ($p = 0.036$). There was no significant difference in the expression of IFN-γ, TNF-α, IL-10, IL-6 and IL-1β in the skin and results found the gingiva biopsies of patients with and without LR, even though the gene expression in skin biopsies was observed among the cases with LR. In case of gingiva, only IL-1β gene expression was high (Table 3).

Patients with CPD and LR presented higher IL-1β gene expression in skin than those without CPD ($p = 0.007$) (Table 3). No statistically significant difference was observed for other mediators (Table 4).

In patients with CPDLR, serum levels of IL-4 were significantly lower than those in patients with no CPDLR. On the contrary, these cases showed higher levels of IL-4 than patients with no CPD and no LR (Figure 1A).

Levels of IL-6 in patients with CPDLR were significantly lower than those in patients with no CPDLR (Figure 1B). In contrast, the levels of IL-6 in patients with no CPDLR were higher than those in patients with no CPD and no LR (Figure 1B).

Serum levels of IFN-γ in patients with CPDLR were significantly higher than those in patients with no CPD and no LR. However, no significant difference in serum levels of TNF-α was found among the groups (Figure 2B).

DISCUSSION

Accumulating evidence suggests the involvement of similar inflammatory cytokines in the development of PD and of LR[9], and in this study, it was demonstrated that patients with CPD have a modified serum profile of IL-4, IL-6 and IFN-γ.

It is known that CD8+ cells, along with Th2 cells, produce predominantly IL-4. IL-4 has an antagonistic effect on IFN-γ and its main activity is to suppress the immune response mediated by cells owing to no optimization of TLR1 activation, as well as downregulation of the expression of these receptors on the surface of monocytes[14]. Contrary to the action mechanism of IL-4 on the cell activity, IL-6, IFN-γ and TNF-α promote the activation of the immune response mediated by Th1 cells[15].

In this study, IL-4 detected in serum samples showed a negative correlation with TNF-α gene expression in skin biopsies of patients with CPDLR. Additionally, low and significant levels of IL-4 were found in serum samples of patients with CPDLR as compared to those in patients without PD, and higher levels of IFN-γ were observed in serum samples of patients with CPDLR than in serum samples of patients with no CPD and no LR. These results suggest an immunoregulatory role of PD in these conditions and a possibility of Th1 cell-mediated activation of mediators in the skin, which would be consistent with

Table 2 - Spearman correlation coefficient between inflammatory mediators (IFN-γ, TNF-α, IL-10, IL-6, IL-4 and IL-12p70) in the skin and those in serum samples of multibacillary leprosy cases with chronic periodontal disease and leprosy reaction

	Skin biopsy					Serum					
	IFN-γ	TNF-α	IL-10	IL-6	IL-1β	IFN-γ	TNF-α	IL-10	IL-6	IL-4	IL-12p70
Skin											
IFN-γ	1										
TNF-α	0.60	1									
IL-10	0.68	0.66	1								
IL-6	0.60	0.50	0.30	1							
IL-1β	0.00	0.20	0.49		1						
Serum											
IFN-γ	0.10		0.00	-0.50	-0.10	1					
TNF-α	0.80	-0.50	0.40		0.20	0.56	1				
IL-10	-0.30		0.40		0.60	0.76	0.95	1			
IL-6	**0.79***	-0.20	0.36	0.40	0.04	0.61	0.50	-0.03	1		
IL-4	0.00	**-0.83***	-0.41	0.00	0.00	**0.89***	0.63	0.34	0.55	1	
IL-12p70	0.80	0.60	-0.40	0.50	0.20	0.40			0.30	0.45	1

*p-value ≤ 0.05

Table 3 - Descriptive analysis of inflammatory mediators in the skin, gingiva and serum samples in cases of multibacillary leprosy according to leprosy reactions (Yes/No)

Inflammatory mediators	Leprosy reactions	N	Mean	IC* (95%)		F**	p-value
				Inferior	Superior		
Skin							
IFN-γ	Yes	13	3.808	1.040	13.736	0.063	0.804
	No	13	2.981	0.554	15.959		
TNF-α	Yes	8	2.059	0.726	5.870	0.645	0.434
	No	9	1.305	0.571	3.004		
IL-10	Yes	10	3.390	1.336	8.499	0.031	0.861
	No	10	3.046	1.105	8.331		
IL-6	Yes	8	1.789	0.139	22.874	3.331	0.085
	No	12	0.290	0.122	0.691		
IL-1β	Yes	10	0.485	0.083	2.829	2.314	0.144
	No	12	0.142	0.083	0.307		
Gingival tissue							
IFN-γ	Yes	5	0.297	0.024	3.633	1.734	0.245
	No	2	8.577	0.000	2.3e+22		
TNF-α	Yes	6	0.199	0.085	0.468	1.209	0.322
	No	1	0.519	-	-		
IL-10	Yes	6	0.570	0.092	3.525	0.004	0.949
	No	2	0.622	0.002	228.149		
IL-6	Yes	7	0.467	0.071	3.096	1.251	0.300
	No	2	2.578	0.038	177.683		
IL-1β	Yes	7	1.379	0.239	7.925	0.016	0.904
	No	2	1.154	0.021	64.715		
Serum (pg/mL)							
IFN-γ	Yes	10	0.008	0.003	0.024	4.125	0.062
	No	6	0.002	0.001	0.004		
TNF-α	Yes	12	0.012	0.007	0.020	0.371	0.548
	No	14	0.009	0.005	0.018		
IL-10	Yes	15	0.010	0.005	0.022	0.006	0.939
	No	19	0.010	0.006	0.017		
IL-6	Yes	20	0.082	0.022	0.304	4.668	**0.036**
	No	31	0.023	0.014	0.038		
IL-4	Yes	12	0.002	0.001	0.003	1.547	0.224
	No	17	0.001	0.001	0.002		
IL-12p7	Yes	13	0.003	0.002	0.005	1.271	0.268
	No	20	0.002	0.002	0.003		

*IC (95%) (95% confidence interval); **F (F test)

fostering of LR and impairment of the patient's general condition[16].

Santos *et al*.[10] observed that reactional multibacillary patients presented highest concentrations of serum IFN-γ. Likewise, Motta *et al*.[17] observed that the odontogenic infection in subjects with leprosy could increase the pro-inflammatory response mediated by IFN-γ, while the contrasting effect would occur on the immunoregulatory activity of IL-4, triggering exacerbation of the inflammatory reaction.

Both, LR and PD, independently present a complex network of immune activity that culminates with the participation of various cell types and inflammatory cytokines, both in serum samples and tissues affected by the disease[9].

The coexistence of infections, such as leprosy and

Table 4 - Descriptive analysis of inflammatory mediators in the skin and gingiva of multibacillary leprosy cases with leprosy reaction in the presence of chronic periodontal disease (CPD: Yes/No)

Inflammatory mediators	CPD	n	Mean	IC (95%)		F**	p-value
				Inferior	Superior		
Skin							
IFN-γ	Yes	8	5.002	0.878	28.789	0.205	0.661
	No	3	2.484	0.001	3395.799		
TNF-α	Yes	6	2.270	0.497	10.381	0.240	0.645
	No	1	1.051	-	-		
IL-10	Yes	7	3.221	1.116	9.300	0.287	0.609
	No	2	5.870	2.2e-9	1.5e+10		
IL-6	Yes	5	0.664	0.053	8.331	3.305	0.129
	No	2	43.380	4.3e-17	4.4e+19		
IL-1β	Yes	7	0.232	0.120	0.445	13.895	**0.007**
	No	2	1.3e-13	1.4e-13	3.6e+15		
Gingival tissue							
TNF-α	Yes	4	0.139	0.063	0.307	3.799	0.123
	No	2	0.415	0.000	2275.602		
IL-10	Yes	4	0.407	0.015	10.805	0.407	0.558
	No	2	1.127	0.000	7942.632		
IL-6	Yes	5	0.343	0.018	6.488	0.361	0.574
	No	2	1.010	0.000	4865.866		
IL-1β	Yes	5	1.076	0.073	15.959	0.262	0.630
	No	2	2.563	0.000	2.2e+4		

*IC (95%) (95% confidence interval); **F (F test)

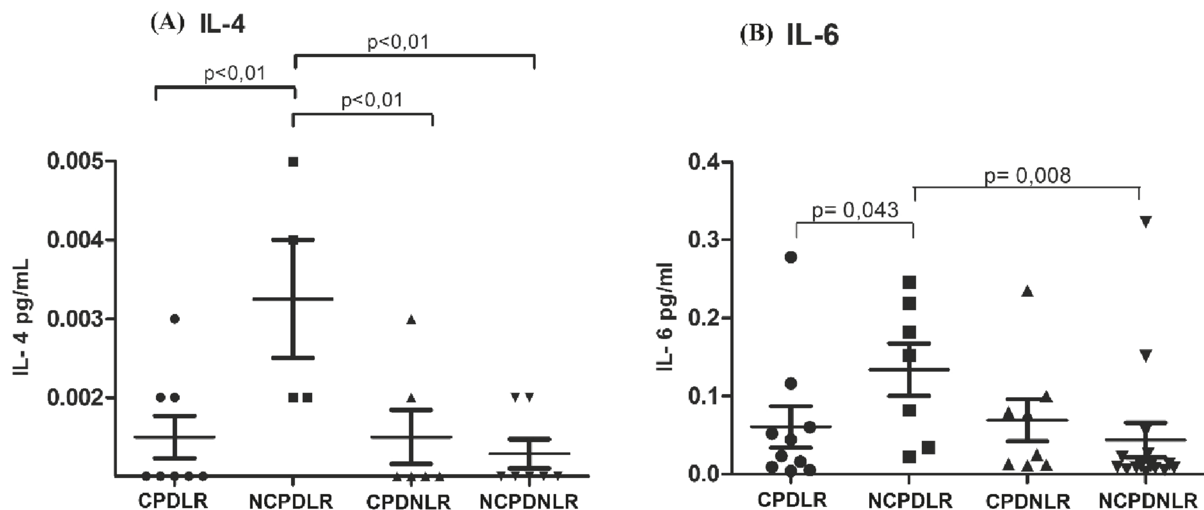

Figure 1 - Comparison between serum levels of (A) IL-4 and (B) IL-6 among patients with CPD and those with no CPD with LR and no LR. Mann-Whitney test; 5% significance level. CPDLR (chronic periodontal disease and leprosy reaction); NCPDLR (no chronic periodontal disease and leprosy reaction); CPDNLR (chronic periodontal disease and no leprosy reaction); NCDPNLR (no chronic periodontal disease and no leprosy reaction).

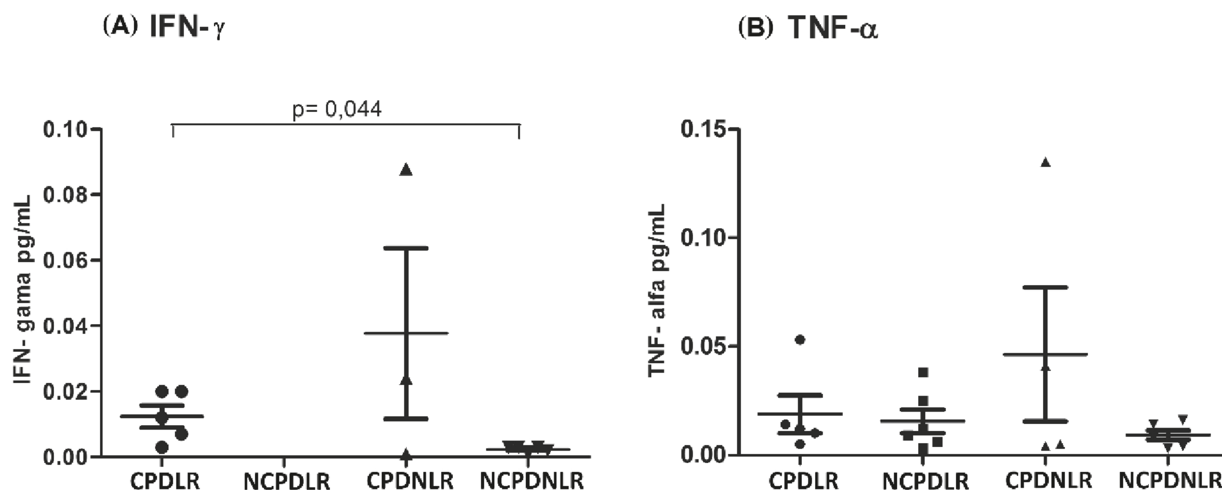

Figure 2 - Comparison between serum levels of (A) IFN-γ and (B) TNF-α among patients with CPD and no CPD and with LR and no LR. Mann-Whitney test; 5% significance level. CPDLR (chronic periodontal disease and leprosy reaction); NCPDLR (no chronic periodontal disease and leprosy reaction); CPDNLR (chronic periodontal disease and no leprosy reaction); NCDPNLR (no chronic periodontal disease and no leprosy reaction).

PD, promotes a change in the pattern of inflammatory response by either activating, inhibiting, or enhancing these processes[2,17,18]. The same systemic condition was observed in diabetes and PD[19].

The balance between Th1 and Th2 cells and a change in the mediator profile found in serum samples and of those expressed in skin, such as IFN-γ, TNF-α, IL-1β, IL-6 and IL-4, both during the occurrence of T1R and T2R, seems to be closely related to the clinical spectrum of the disease. In PD, although there is no consensus on the immunological standards involved in the pathophysiology, a combined function of Th1 and Th2 cells is possible[20].

The complex association between the inflammatory cytokines and the immune cells system during LR and CPD manifestation has prompted researchers to conduct more specific studies on the cell type and cytokine determinants of these processes[9], as well as on other cell types, such as keratinocytes and fibroblasts, as well as cytokine producers in response to bacterial stimulation or other cytokines[16,21].

Particularly in the case of leprosy, in patients with borderline clinical features, the participation of other cell subtypes, such as the subpopulations of Th0 cells that seem to produce both IFN-γ and IL-4 in occurrence of T1R has been described. However, Th1 polarization along with IFN-γ production could also occur in the face of T1R recurrence[22]. Other cell subtypes, such as the regulatory T cells (Tregs), Th3 cells with an immunosuppressive profile and Th17 cells with a pro-inflammatory profile, may participate in the determination of the cell profile and the involved mediators[23,24]. However, the understanding of the association between molecular and cellular paths and the pathogenesis of CPD and LR is still incomplete.

Considering the involvement of IL-6 in the development of LR, the results of this study are consistent with those in the literature, in which patients with LR showed higher levels of IL-6 in serum samples than the levels in patients with no LR. Recognized as a potential inflammatory marker of LR, IL-6 stands out due to its multifunctional profile[9,18,25].

In case of patients with CPD and reactional states, a positive correlation between IL-6 in the serum and IFN-γ gene expression in the skin was found. Therefore, an increased serum level of IL-6 may contribute to an increase in gene expression of IFN-γ in the skin, which could justify the intensification of Th1 type cell response with triggering or maintenance of the LR. However, in case of patients with CPD or reactional episodes, the serum level of IL-6 was lower than that in patients without CPD and reactional episodes, suggesting an IL-6 downregulation in the presence of PD. On the contrary, as described previously, other cell types, such as Th0 cells, Tregs, and keratinocytes, may influence the inflammatory mechanisms.

The influence of PD on the manifestation of LR was strengthened by the clinical improvement of reactional symptoms after subjects with odontogenic infections and LR received dental treatment[18]. Additionally, the probability of occurrence of LR may increase when the patient presents poor oral health[26].

It was not possible to verify significance values of gene expression of mediators in the gingival tissue related to the presence of CPD and LR. This fact may have been influenced by the difficulty in participants' allocation, since the patients had already begun treatment for LR when the

biopsy was performed, or even due to the sample size. Although this represents a study limitation, it should be emphasized that blood collection was performed on the same day of skin biopsy, before initiation of the reactional treatment, and in the initial weeks of leprosy treatment.

Considering the gingival tissue, IFN-γ and IL-1β showed higher mean values for gene expression, although not statistically significant, than that of other mediators studied. This is in agreement with the literature, since both IFN-γ and IL-1β are important mediators involved in bone resorption during the development of PD and the extent and severity of these diseases[9]. It is possible that treatment of reactional episodes or even polychemotherapy, favor the reduction of pathogenic microorganisms responsible for the development of CPD, and consequently, the expression of inflammatory cytokines in the gingiva. More specific studies are necessary to clarify this approach.

As an original study on CPD and LR, it could be stated that the results suggested an interaction between CPD and immunoregulatory mechanisms involved in the triggering, exacerbation and maintenance of LR. These interactions can promote the expression of TNF-α and IFN-γ in the skin, while downregulating IL-4 and IL-6 in the serum.

Considering the previous approaches, questions about the association between molecular and cellular paths and the pathogenesis of these diseases suggest the need of future investigations.

CONCLUSION

The presence of DPC in individuals with LR immunoregulated the concentrations of IL-6, IFN-γ and IL-4. The presence of DPC decreased serum levels of IL-6 and IL-4 in reactional individuals. CPD concomitant to LR resulted in increased IFN-γ serum levels.

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