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Cytokines and NO in American tegumentary leishmaniasis patients: Profiles in active disease, after therapy and in self-healed individuals

Marina de Assis Souza [a,*], Maria C.A. Brelaz de Castro [a], Andresa Pereira de Oliveira [a], Amanda Ferreira de Almeida [a], Thays Miranda de Almeida [a], Luiza C. Reis [b], Ângela Cristina Rapela Medeiros [c], Maria Edileuza Felinto de Brito [a], Valéria Rêgo Alves Pereira [a]

[a] Departamento de Imunologia, Centro de Pesquisas Aggeu Magalhães (CPqAM/FIOCRUZ), Recife, PE, Brazil
[b] Instituto de Medicina Tropical de São Paulo, Universidade de São Paulo (IMT/USP), São Paulo, SP, Brazil
[c] Hospital Universitário Oswaldo Cruz, Universidade de Pernambuco (HUOC/UPE), Brazil

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ABSTRACT

Studies suggest the influence of immune response on the successful treatment of American tegumentary leishmaniasis (ATL), and indicate the existence of protective immunity in self-healed patients. Thus, the aim of this work was to quantify interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α), interleukin (IL-) 10, IL-17, IL-22 and nitric oxide (NO) in culture supernatants of PBMC from patients with active disease (AD), after treatment (AT), and from self-healed (SH) and healthy subjects (CT), in response to *Leishmania (Viannia) braziliensis* insoluble antigen (AgIns). All groups of patients produced IFN-γ, indicating a predominant proinflammatory profile. AD and AT patients presented TNF-α levels, with a slight increase after therapy, whereas it was weakly quantified in SH. Interestingly, NO secretion was significant in these individuals, whereas IL-17 appeared in low levels and seems to be regulated by NO. Although IL-22 was detected in AD, its role is still questionable. The presence of IL-10 in all groups of patients suggests that the cytokine plays distinct roles in the disease. These results indicate that specific cellular immunity takes part against *Leishmania*, but with some similarities between the different clinical states herein described; these mediators seem to be necessary for the cure to occur.

1. Introduction

American tegumentary leishmaniasis (ATL) is an anthropozoonosis caused by several species of *Leishmania*. It is considered an endemic disease in Brazil, where the main causative agent is *Leishmania (Viannia) braziliensis*. The emergence of the diverse clinical forms of ATL depends on characteristics of the parasite and the vector, in addition to the immune status and genetic constitution of the vertebrate host [1–3]. In humans, the infection can be subclinical or it can present manifestations ranging from localized, disseminated or diffuse skin lesions to aggressive and mutilating mucocutaneous lesions [1]. The treatment is executed through chemotherapy for all clinical forms, and the first line drug used is meglumine antimoniate (Glucantime®).

The diversity of clinical manifestations in human ATL is strongly influenced by the host immune response [4]. In all clinical forms, there is an expansion characterized by T $CD4^+$ cells, presenting Th1 or Th2 cytokines profiles [5]. Interferon-gamma (IFN-γ) and tumor necrosis factor (TNF) -α and -β, from the Th1 profile, are known to be involved in the resistance and elimination of the parasites, while Th2 cytokines such as IL-4 and IL-10 are linked to susceptibility to infections by *Leishmania* [6,7].

In addition to these cytokines, recent studies suggest that IL-17 is involved in processes that lead to chronicity of the disease [8,9]. Moreover, IL-22 is known to be involved in immunity at the epithelium and mucosal surfaces [10]. Both cytokines were produced in human Kala-Azar caused by *Leishmania donovani*, and were also associated to the resistance to infection [11]. Furthermore, IL-10 may develop a wider role in leishmaniasis, once it is related not only to the improved survival of the parasite in the host, leading to macrophage deactivation, but also to counterbalance mechanisms necessary to the resolution of the disease [12].

* Corresponding author. Departamento de Imunologia, Centro de Pesquisas Aggeu Magalhães/FIOCRUZ, Av. Moraes Rego, s/n, Cidade Universitária, 50670-420 Recife, PE, Brazil. Tel.: +55 81 2101 2631; fax: +55 81 2101 2640.
E-mail addresses: marinaasouza@gmail.com, marinaasouza@cpqam.fiocruz.br (M. de Assis Souza).

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As previously demonstrated by some authors, cellular immune response is involved with the healing process after the treatment with antimonials. In attempt to detect possible parameters in the immune response associated with healing after treatment, the cellular immunity of patients with ATL was previously evaluated using total antigen of *L. (V.) braziliensis*, before and after the chemotherapy [13,14].

Once the search for immunogenic fractions of *Leishmania* is necessary for vaccine synthesis and development of prognostic tests, our group previously assessed the cellular immune response of patients with active disease and after clinical cure, with or without chemotherapy (self-healed individuals), in response to the soluble antigen of *L. (V.) braziliensis* [15]. The results demonstrated a specific immune response developed by the patients, with some similarities in cytokine production among the different groups. Considering that this response may be diverse in the presence of different fractions, this work evaluated IFN-γ, TNF-α, IL-10, IL-17, IL-22 and nitric oxide (NO) production of patients with active ATL and post-therapy or spontaneous clinical cure against the insoluble (particulate) antigen of *L. (V.) braziliensis*.

2. Materials and methods

2.1. Study population

Individuals of both gender and older than 15 years old were selected from the municipalities of Moreno, Araçoiaba, Amaraji, Vicência and Chã de Alegria, endemic areas for ATL. Fourteen patients with active disease (AD) were chosen based on criteria such as: presence of cutaneous lesions, confirmed diagnosis by the Reference Service in leishmaniasis of CPqAM and no previous chemotherapy treatment. The history of previous ATL, presence of characteristic scars, positive Montenegro skin test (MST) and absence of chemotherapy were the considered criteria to select eleven self-healed patients (SH). The ones with active disease were submitted to blood collection prior to chemotherapy treatment with Glucantime® and then 12 months after the end of treatment. Therapeutic scheme was composed by doses of 20 mg/kg/day by subcutaneous injections during 20 and 30 days (1 cycle). After the end of treatment, patients (AT) were followed up for a period of 12 months to confirm clinical cure and to avoid the appearance of new lesions and relapse. At the end of this period, all treated individuals were submitted to a new blood collection. In the self-healing group the blood was collected in only one moment. Nine healthy individuals represented the control group (CT) from non-endemic areas and without previous ATL infection were selected. All individuals signed the "Term of Free and Informed Consent" and CPqAM/Fiocruz Research Ethics Committee (Protocol No. 123/08) approved the experimental protocols.

2.2. Insoluble antigen of L. (V.) braziliensis

As described by Brito et al. [16], promastigote forms of *L. (V.) braziliensis* (MHOM/BR/75/M2903), cultured in vitro, were expanded in Schneider's medium (Sigma) supplemented with 10% of fetal calf serum (Cultilab) and 1% of antibiotics (100 IU/ml penicillin and 100 mg/ml streptomycin; Sigma) until they reached the exponential phase. Afterwards, they were sedimented by centrifugation at 800× *g* for 15 min at 4 °C and three times washed with phosphate-buffered saline (PBS; pH 7.2). Proteases inhibitors such as 0.1 mM methyl-phenyl-fluoride and 2 mM ethylenediaminotetraacetic acid (Sigma), pepstatine A 0.001 M (Sigma) were added and, right after, the parasites were ultrasonicated. The parasitic suspension was centrifuged at 10,000× *g* for 10 min at 4 °C. The resultant supernatant was removed and submitted to a new centrifugation at 100,000× *g* for 1 h at the same temperature. Protein concentration was determined [17] from the pellet, the insoluble antigen (AgIns), which was stored at −20 °C for further use.

2.3. Cell culture

PBMC obtained from venous blood (10^6 cells/ml) were cultured (37 °C/5% CO_2) in 24-well plates (TPP) with RPMI medium (Sigma) in the presence of AgIns (2.5 μg/ml) of *L. (V.) braziliensis*. Wells containing phytohemagglutinin mitogen (PHA; 5.0 μg/ml) were the positive control of the assay, and cells only in the presence of the culture medium were used as the negative control. After incubation during 48 h and 6 days, the plates were centrifuged (1800× *g* for 10 min, at RT) and the culture supernatants were collected and stored at −70 °C.

2.4. Cytokine determination in culture supernatants

Cytokines in the supernatants of cultures were assayed with capture ELISA kits according to the manufacturer's instructions. IFN-γ (BD Biosciences), IL-17 (R&D Systems), IL-10 (BD Biosciences) and IL-22 (R&D Systems) levels were measured at 6 days. TNF-α (BD Biosciences) was quantified at 48 h. The lower limits of detection for the ELISA analyzes were as follows: 1.95 pg/ml for IFN-γ, TNF-α, IL-17, IL-10 and 3.9 pg/ml for IL-22. The final concentrations were expressed in pg/ml using the Microplate Manager Version 4.0 software (Bio-Rad Laboratories).

2.5. Nitrite detection by Griess method

ELISA plates (96-well-Costar half-area plate) were filled with 25 μl of culture supernatants (two replicates), followed by the Griess reagent in the same volume. A standard curve was made using sodium nitrite at 200 μM, and submitted to serial dilution (factor 2) in RPMI medium (Sigma) supplemented with 2% of fetal calf serum (Cultilab). After incubation for 10 min in the dark, the reading in the spectrophotometer was carried out at 450 nm. The absorbances were compared to the standard curve (threshold set in 0.19 μM), and the results were expressed as the replicate means ± standard error, using the Microplate Manager Version 4.0 software (Bio-Rad Laboratories).

2.6. Statistical analysis

The data were analyzed using nonparametric tests. For intra-group comparative analysis (AD × AT), the Wilcoxon test was used and to detect differences between groups the Mann–Whitney *U*-test. The results were considered significant when $P < 0.05$.

3. Results

Before treatment, the AD patients presented ulcerated skin lesions with raised borders and granulomatous bottom, distributed mostly by uncovered areas of the body. The disease evolution time, calculated from the lesion appearance until the patient visits the health surveillance service in the municipal districts, varied from eight days to three months. After treatment with Glucantime® and subsequent monitoring of the patients for until a year, all patients showed complete healing of the lesions. All the SH patients presented typical scars indicating previous disease at the time of clinical evaluation. The period between the emergence of the lesion and the scar formation varied from fifteen days to nine months. MST result above 5 mm was observed in all patients. As shown in

Table 1
Demographic, clinical and therapeutic data of the patients with ATL.

Patient	Age	Gender	Location	Clinical form	No lesions	Evolution	Local lesions	Lesion size (mm^2)	Circles
01	53	F	Amaraji	LCL	01	03 months	R leg	12.25	02
02	47	M	Amaraji	LCL	01	03 months	L leg	21	02
03	21	F	Moreno	LCL	01	02 months	Body	32	02
04[a]	25	M	Moreno	LCL	08	01 month	R and L legs/L hand	2/24.5	04
05	41	F	Moreno	LCL	02	08 days	R leg	0.25/5	02
06	39	M	Moreno	LCL	01	01 month	L leg	36	02
07	33	F	Moreno	LCL	01	01 months	R leg	24/27.5	02
08	52	M	Moreno	LCL	02	02 months	Body/Shoulder	24.75/15	01
09	58	F	Moreno	LCL	01	01 month	R leg	18	01
10	20	M	Chã de Alegria	LCL	02	02 months	R leg	8/7.5	02
11	56	M	Araçoiaba	LCL	01	03 months	R leg	39	03
12	28	F	Moreno	LCL	01	15 days	R foot	NI	02
13	26	F	Moreno	LCL	01	02 months	R leg	36	02
14	34	M	Moreno	LCL	01	03 months	L leg	6	02

NI = not informed; F = female; M = male; CL = localized cutaneous leishmaniasis; L = left; R = right.
[a] Patient presented 01 lesion measuring 24.5 mm^2, and seven others measuring 2 mm^2.

Tables 1 and 2, in both groups gender proportion was statistically similar ($P > 0.05$).

PBMC from all groups were efficiently induced to secrete cytokines and NO in response to PHA, and the non-stimulated cultures secreted minimal or any levels of all mediators (data not shown). In relation to IFN-γ, PBMC of AD (7742 ± 7884, $P = 0.003$), AT (8129 ± 7934, $P = 0.01$) and SH (9317 ± 10.290, $P = 0.001$) presented significant levels of the cytokine in comparison to CT (167.3 ± 196.2). On the other hand, it was not observed statistic difference when comparing patients prior, post-treatment and self-healed (Fig. 1).

TNF-α production in active disease (310 ± 362.1; $P = 0.007$) and after therapy (358.4 ± 406.1; $P = 0.003$) was significant in comparison to the healthy donors (48.67 ± 77.18). The AT patients ($P = 0.035$) exhibited significant levels of this cytokine when compared to the self-healed group (124.6 ± 197.3).

All groups of patients produced IL-10 in response to AgIns, and significant levels of the cytokine were observed in AD (261.9 ± 220) when comparing with AT (139.1 ± 98.11; $P = 0.03$). In contrast, significant levels of IL-17 were observed after therapy (139.9 ± 54.36) in comparison to the control group (1.77 ± 0.89; $P = 0.04$), as well as in AD patients (37 ± 13.78) in comparison to SH group (5.5 ± 5.5; $P = 0.04$). Concerning IL-22, significant concentrations of the cytokine were observed in AD group (571.8 ± 219.7) in comparison to the healthy subjects (71.4 ± 34.1; $P = 0.02$).

AD patients exhibited higher and significant levels of NO (10.29 ± 15.2, $P = 0.048$) in comparison to AT (1.429 ± 3.48). Both SH (16.36 ± 21.85, $P = 0.04$) and AD groups (10.29 ± 15.72, $P = 0.012$) produced NO in greater levels in relation to CT (1.4 ± 4.3), with statistical difference between groups. SH produced NO with statistical significance ($P = 0.008$) when comparing with AT (Fig. 1).

Table 2
Demographic and clinical data of the self-healed (SH) patients.

Patient	Age	Gender	Location	No scars	Local of scars	Healing time	MST (mm)
01	77	M	Moreno	01	L leg	3 months	12
02	71	M	Moreno	01	L forearm	NI	10
03	81	M	Moreno	01	L leg	15 days	10
04	28	M	Moreno	01	L thigh	3 months	12
05	NI	F	Amaraji	01	L foot	1 month	05
06	47	F	Vicência	04	Neck, L shoulder, R thigh, L leg	9 months	12
07	17	F	Vicência	01	L leg	NI	08
08	30	M	Vicência	01	R foot	4 months	10
09	39	F	Vicência	01	R leg	8 months	07
10	60	F	Moreno	01	L ankle	3 months	10
11	57	F	Moreno	01	L leg	2 months	15

NI = not informed; F = female; M = male; L = left; R = right; MST = Montenegro skin test.

4. Discussion

American tegumentary leishmaniasis presents a spectrum of manifestations that ranges from localized skin lesions to the impairment of mucous regions [18,19]. In addition, the occurrence of self-healing in patients with cutaneous leishmaniasis has been documented in endemic areas of *L. (V.) braziliensis* infection [16,20,21]. Considering the importance of the cellular immune response in ATL, and also that few studies on self-healed individuals were developed, this work evaluated cytokine and nitric oxide production in patients with active and post-healed American tegumentary leishmaniasis, with or without chemotherapy.

The immune response in cutaneous leishmaniasis has been assessed by many authors using different antigens [6,8,12–14,40]. In an attempt to evaluate a more specific immune response, we have previously used the soluble antigen of *L. (V.) braziliensis* for PBMC stimulation [15]. We demonstrated that in active disease and after clinical cure, with or without chemotherapy, specific cellular immunity takes part against *Leishmania*, but with some similarities between the clinical states. Sequentially, in this work, we proceeded to evaluate the cellular immunity of these patients after exposure to the insoluble antigen of *L. (V.) braziliensis*, once particulate fractions are known to be constituted by membrane fractions of the parasite [22].

From the increased IFN-γ production observed herein, we can suggest that the patients have established an immune response in order to eliminate the parasite. The levels of the cytokine found after treatment and in self-healed patients tended to be higher than in the active disease, in contrast with the results previously presented by our group [15]. There are evidences that the decreasing in parasite load after the use of antimonials may contribute to the development of a Th1 response, once it is no longer suppressed by the presence of *Leishmania* parasites [23]. Thus, IFN-γ production is maintained post-chemotherapy [6,14,24]. In addition, although in SH patients the chemotherapy has not been administered, we can suggest that their immune response underwent a modulation, reaching clinical cure [21].

The same increasing IFN-γ levels after clinical cure were previously observed, although the total antigen of *L. (V.) braziliensis* was used for PBMC stimulation [13,14]. Once this antigen is not

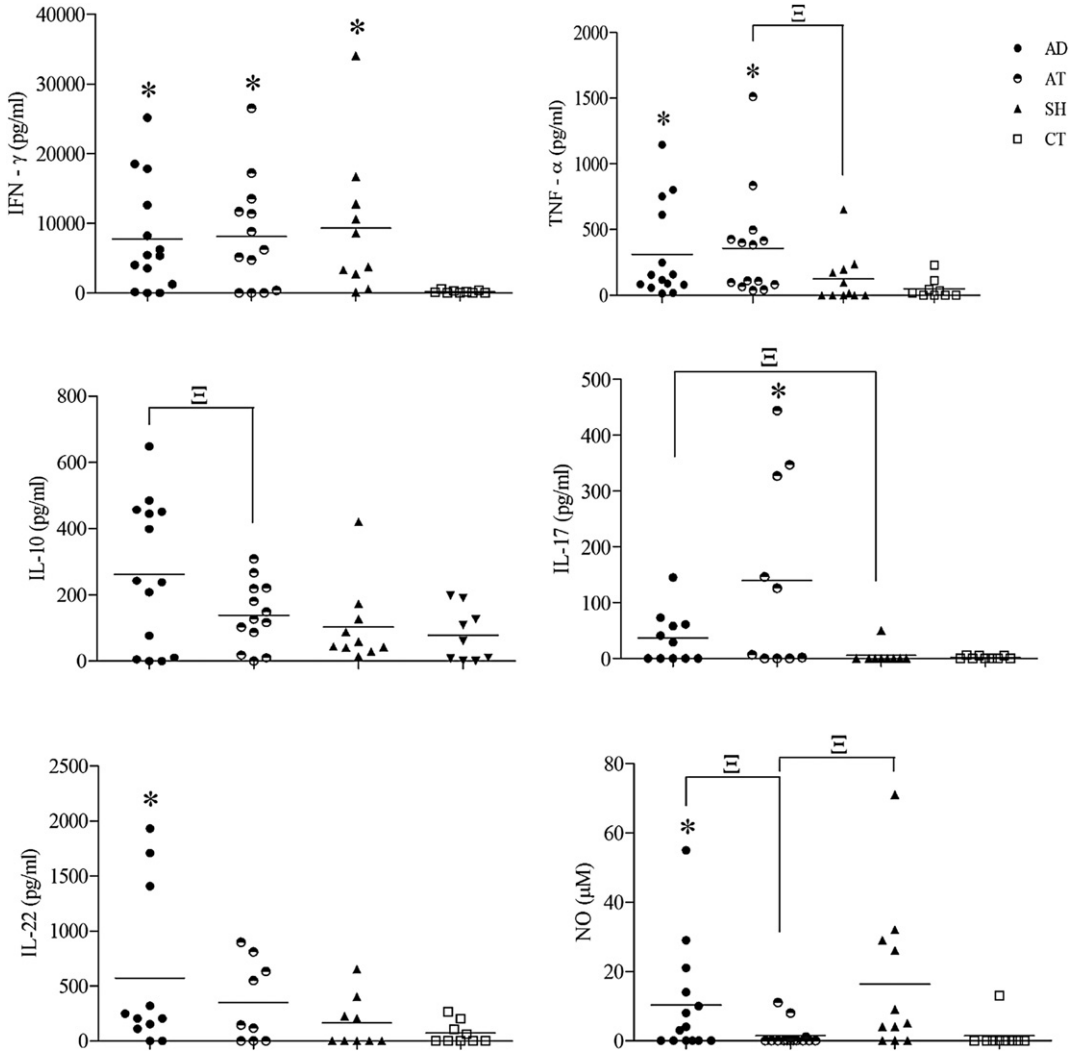

Fig. 1. Cytokine and nitric oxide production among patients with active disease (AD), after treatment (AT), self-healed patients (SH) and control group, in response to insoluble antigen. The asterisk represents the significant differences between patients and control group, and the Greek letter Ξ (ksi) shows the significances between the groups of patients ($P < 0.05$). The horizontal bars represent the mean of the groups.

submitted to differentiated centrifugation process to separate the membrane components from the cytoplasmic ones, we can suggest that the immunogenicity of the total fraction arises from the existence of portions of membrane of the parasite antigenic preparation [22].

In addition to IFN-γ, TNF-α levels were also measured in this study. Under antigenic stimulation, the treated patients produced significant levels of TNF-α in relation to the self-healed ones. These results could be explained by the possible influence of antimonial treatment on the secretion of proinflammatory cytokines [25,26]. Furthermore, the different levels of TNF-α observed in these groups may exist due to the genetic constitution of the host. It is known that the presence of polymorphisms in tumor necrosis factor genes contributes to functional differences in the cytokine levels, as it was shown by Cabrera et al. [27]. According to the authors, susceptibility to the mucocutaneous form of disease may be directly associated with regulatory polymorphisms affecting TNF-α production.

In relation to NO, some stable, measurable products come from its decomposition process, such as nitrite (NO_2^-). In response to AgIns, the self-healed individuals exhibited significant nitrite levels in relation to the treated patients, as well as a considerable production of proinflammatory cytokines. Thus, it is reasonable to suggest that IFN-γ and TNF-α may be necessary to NO production [28–30]. Nevertheless, nitrite levels exhibited after therapy were rare, despite the great concentration of IFN-γ and TNF-α secreted by these patients. Once the nitric oxide production tends to be elevated by influence of antimonials [31], we believe that the reduction observed after treatment occurs by influence of the decreased parasite load, as well as due to the period of re-evaluation of the patients, which occurred a year after the end of the treatment.

Besides the benefits of Th1 response, Gollob et al. [32] suggest that IL-10 production may be critical for the disease control. Thus, it is postulated that IL-10 levels presented by the patients in this study regulate the production of proinflammatory cytokines, consequently leading to the clinical resolution of the disease. However, studies point to the reminiscence of parasites after the wound healing [33], and IL-10 seems to play a role in this event [34]. Thus, it is constantly discussed whether a patient who has achieved clinical cure remains healthy due to a memory immune response or by the existence of constant stimulation of the immune system by *Leishmania* antigens present in the individual (concomitant immunity). Amato et al. [35], who observed the persistence of

T lymphocytes in lesions of healed patients, suggest that this event may work as a defense mechanism against reinfection in regions endemic for leishmaniasis.

Concerning IL-17, this cytokine has been increasingly studied in protozoal, fungal and bacterial infections in mice [36], but the role of this cytokine in human infections, especially in leishmaniasis, is still undefined. Herein, the significant levels of IL-17 observed in AD patients in comparison to SH could be explained by the proinflammatory role of this cytokine. In a previous study, Bacellar et al. [8] verified that in lesions of cutaneous and mucosal leishmaniasis patients, the intensity of the inflammatory infiltration is directly correlated to the number of cells expressing this cytokine. In addition, a decreased quantity of IL-17 exhibited by SH patients under antigenic stimulus may be, at least in part, due to a suppressive effect exerted by NO levels, which impair the polarization and the stabilization of IL-17-producing cells [37].

The inverse situation seems to occur with the treated patients, which exhibited a considerable quantity of IL-17 and low levels of NO. Similar behavior was observed in the production of TNF-α in these groups of patients, although it was not possible to evaluate the existence of a direct correlation between the secretion of IL-17 and TNF-α. In contrast, Bacellar et al. [8] observed that the production of these two cytokines was directly proportional in response to *Leishmania* infection. Due to the different counterbalance among TNF-α, IL-17 and NO observed after therapy and in self-healed patients, both resulting in clinical cure, we can suggest that the chemotherapy process can exert some influence in the immune response.

Another cytokine evaluated in this study was IL-22, which belongs to the IL-10 family [38]. IL-22 levels were observed in all groups of patients, being significant in the AD group in comparison to the healthy donors. As previously seen [15], the cytokine was observed in SH patients, but not IL-17. As it happens to IL-17, little is known about the role of IL-22 in *Leishmania* infections, although protective and harmful roles are attributed for this cytokine. These mechanisms may occur under influence of IL-17, which absence or presence seems to govern the proinflammatory versus tissue-protective properties of IL-22 [39]. In contrast, Pitta et al. [11] verified an increasing in IL-22 and also IL-17 production in patients who presented resistance to kala-azar. According to the authors, IL-22 and IL-17, along with Th1 cytokines, are supposed to play complementary roles in human protection against the disease. Moreover, the production of these cytokines may point the presence of Th17 profile in the defense against the parasite. However, more investigation about the cytokines and their producing cells from this profile is needed to better understand its role in the pathogenesis of leishmaniasis.

From the data herein demonstrated, we can postulate that the patients with active disease and after clinical cure, with or without chemotherapy, exhibit specific cellular immune response profiles, but with some similarities between them, as it previously occurred using other antigen [15]. IFN-γ production by the three groups of patients suggests an influent inflammatory response against the parasite, as well as in relation to TNF-α. The secretion of TNF-α showed a tendency to be higher after treatment, may be by influence of pentavalent antimonials. Moreover, IL-17 and IL-22 may also contribute to mount an effective response against the parasite. The former seems to be regulated by NO levels, which is known to be regulated by cytokines, and this mechanism may be important to enable the self-healing process. Nonetheless, the presence of IL-10 suggests the induction of immunomodulatory mechanisms necessary for the establishment of an effective immune response against *Leishmania*.

Thus, we can suggest that specific cellular immunity takes part against *Leishmania*, but with some similarities between the different clinical states herein described. Thus, the results suggest that the mediators assessed are necessary for the cure to occur.

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