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

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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].

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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