Interleukin 17 Production among Patients with American Cutaneous Leishmaniasis

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Interleukin 17 (IL-17) plays a critical role in inflammation and autoimmunity. Very little is known about IL-17 in protozoan infection. Here, we show that lymphocytes obtained from patients with mucosal leishmaniasis and cutaneous leishmaniasis produce higher levels of IL-17 than do lymphocytes obtained from uninfected control subjects (P < .01). There was a tendency for tissue obtained from patients with mucosal leishmaniasis to contain a higher number of cells expressing IL-17, compared with tissue obtained from patients with cutaneous leishmaniasis, and there was a direct correlation between the number of cells expressing IL-17 and the presence of cellular inflammation at the lesion site (r² = 0.86; P < .001). These data support the role of IL-17 in the pathogenesis of the inflammatory reaction in leishmaniasis.

Interleukin 17 (IL-17) is a member of a newly identified cytokine family that is produced predominantly by Th17 cells [1]. IL-17 enhances T cell priming and stimulates fibroblasts, endothelial cells, neutrophils, macrophages, and epithelial cells to produce multiple pro-inflammatory mediators, including IL-1, IL-6, tumor necrosis factor α (TNF-α), nitric oxide synthase 2, metalloproteinases, and chemokines [2]. IL-17 may protect against bacterial, fungal, and protozoal infection [3], but it has been linked predominantly to the pathogenesis of chronic inflammatory and autoimmune diseases. In humans, expression of IL-17 has been detected in serum samples and target tissue samples obtained from patients with systemic rheumatic diseases, multiple sclerosis, inflammatory bowel disease, and asthma [4, 5]. In arthritis, IL-17 is directly involved in the destruction of cartilage and bone [5].

Cutaneous leishmaniasis (CL) is a disease caused by protozoa of the genus Leishmania, characterized by the development of ulcerated skin lesions. In areas of Leishmania braziliensis transmission, a small percentage of patients develop mucosal leishmaniasis (ML), concomitantly or after CL. ML affects predominantly the nose, leading to tissue damage and, occasionally, to disfiguring facial lesions. Although diffuse cutaneous leishmaniasis is associated with impaired T cell responses against parasite antigen, patients with CL and ML have a strong type 1 immune response to Leishmania species, with high production of interferon γ (IFN-γ) and TNF-α [6]. However, the infection is only partially controlled, and the exaggerated T cell response participates in tissue damage observed in both CL and ML. High concentrations of TNF-α, IL-6, IFN-γ, and nitric oxide are documented in peripheral blood and tissue specimens and are characterized by an intense inflammatory infiltrate with few or no detectable parasites [7].

Only a few studies have assessed the role of IL-17 in human infectious diseases, and it is not known whether this cytokine participates as a defense mechanism or in...
the pathogenesis of these diseases. In this study, we showed that IL-17 is produced during _L. braziliensis_ infection. The direct correlation between TNF-α and IL-17 and the correlation between the number of cells expressing IL-17 and the intensity of the inflammatory infiltration indicate that IL-17 may be involved in the pathogenesis of leishmaniasis.

**Materials and methods.** This study included 15 patients with ML and 30 patients with CL; the 30 patients with CL were matched by age (± 5 years) and sex with the 15 patients with ML. All patients were seen at the health post of Corte de Pedra, Bahia, Brazil, which is a well-known area of _L. braziliensis_ transmission. The criteria for diagnosis were a clinical picture characteristic of CL and ML in conjunction with parasite isolation or a positive delayed-type hypersensitivity response to _Leishmania_ antigen, plus histological features of CL and ML. In all cases, the immunological analysis was performed before therapy. Ten uninfected control subjects were enrolled in the study as a control group; these individuals were healthy and lived in a location with no known history of _L. braziliensis_ transmission or infection. This research was conducted with the approval of the Ethical Committee of the Maternidade Clímério de Oliveira (Salvador, Bahia, Brazil), and informed consent was obtained from each participant.

For cell culture and IL-17 measurement, peripheral blood mononuclear cells (PBMCs) were obtained from heparinized venous blood layered over a Ficoll-Hypaque gradient (GE Healthcare), then washed and resuspended in Roswell Park Memorial Institute 1640 complete medium with 10% heat-inactivated human AB serum (Sigma) at a concentration of 3 × 10^6 cells/mL. These cells were added to 24-well plates and were kept unstimulated or were stimulated with soluble _Leishmania_ antigen (5 μg/mL) for 96 h at 37°C in 5% CO₂. After this time, the supernatants were collected and stored frozen until analyzed for cytokines. IL-17, IFN-γ, and TNF-α were measured by enzyme-linked immunoabsorbent assay (R&D Systems).

A total of 16 samples from patients with tegumentary leishmaniasis were evaluated by histological and immunofluorescence staining and confocal analysis. Ten patients presented with CL, and 6 presented with ML. Tissue biopsy samples were incubated for 30 min in 30% sucrose at 4°C and then transferred into TissueTek media and kept at −70°C until processing. Individual 4–5-μm cryosections were placed in saline-precoated slides, fixed for 10 min with acetone, washed with phosphate buffered saline for 15 min, and submitted to either hematoxylin–eosin staining or to immunofluorescence staining using specific monoclonal antibodies. Sections were incubated with an antibody mixture (anti-CD4 fluorescein isothiocyanate and anti–IL-17–phycoerythrin) and acquired in a laser-scanning confocal microscope (Zeiss), as described elsewhere [8]. Isotype controls were added to the immunofluorescence reactions to confirm the lack of nonspecific staining. Monoclonal antibodies were purchased from e-Biosciences.

Analyses were performed by counting the total number of cells in the fields and calculating the average of cells per section for each patient. The counts were performed blindly, and the results were expressed as mean values (± standard deviations). The results are representative of 2 experiments per patient.

Statistical analysis was performed using the Kruskal-Wallis test with post-hoc testing to compare IL-17 data between control subjects, patients with CL, and patients with ML (InStat). Statistical analysis of the confocal data was performed using the JMP statistical software from SAS. The comparisons of mean values between different treatments for a given parameter were performed with use of the nonparametric (1-tailed, considering unequal variance of groups) Student’s t test. Results were considered to be statistically significant at _P_ < .05.

**Results.** IL-17 is produced by cells from patients with CL or ML. The ability of PBMCs from 30 patients with CL and 15 patients with ML to produce IL-17 is shown in figure 1. IL-17 was detected in 70% of patients with CL and in all of the patients with ML. In cultures that did not receive stimulus with soluble _Leishmania_ antigen, the median IL-17 level was 0 pg/mL (range, 0–102 pg/mL) for patients with ML and 0 pg/mL (range, 0–59 pg/mL) for patients with CL, respectively. There was a tendency for patients with short duration of illness to have lower levels of IL-17, but there was no correlation between the level of IL-17 and the severity of the disease, evaluated

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**Figure 1.** Interleukin 17 (IL-17) production in patients with cutaneous leishmaniasis (CL), patients with mucosal leishmaniasis (ML), and uninfected control (UC) subjects. IL-17 production in peripheral blood mononuclear cell supernatants stimulated with soluble _Leishmania_ antigen was determined as described in Materials and Methods. IL-17 levels were measured by enzyme-linked immunosorbent assay. Data are represented as median values and interquartile range (whiskers) for 30 patients with CL, 15 patients with ML, and 10 UC subjects. *P_ < .001, comparing the ML group with the UC group, by the Kruskal-Wallis test. **P_ < .05, comparing the CL group with the UC group, by the Kruskal-Wallis test.
Figure 2. Interleukin 17 (IL-17) expression in cutaneous leishmaniasis (CL) and mucosal leishmaniasis (ML) lesions. Representative picture of the confocal microscopy analyses for determination of the number of CD4+IL-17+ cells. Tissue sections were stained with anti-CD4–fluorescein isothiocyanate (FITC) and anti–IL-17–phycoerythrin (PE) monoclonal antibodies and were counterstained with 4'-6-diamidino-2-phenylindole (DAPI), as described in Materials and Methods. The 3 optical sections for each patient were obtained simultaneously with 488 and 568 line of the argon/krypton laser and the proper set of filters to capture FITC, PE, and DAPI staining. Overlay images for CD4 (green) and IL-17 (red) in biopsy samples obtained from a representative patient with CL (A, left panel) and patient with ML (B, left panel) are shown. The cells that are double-positive for CD4 and IL-17 appear in yellow. Values represent the mean value ± standard deviation of CD4+IL17+ cells for each group. The bar indicates 10 mm. Correlative analysis between the total number of cells within the inflammatory infiltrate and the number of IL-17+ cells in a CL lesion (A, right panel) and an ML lesion (B, right panel), as indicated. (r², by nonparametric Student’s t test).2

Discussion. T helper cells that produce IL-17 have been shown to play a pivotal role in autoimmunity and chronic inflammatory diseases [4, 5]. Although IL-17 participates in defense mechanisms against certain pathogens, very little is known about IL-17 production in parasitic diseases in humans. Here, we show that IL-17 production by PBMCs is enhanced during the course of CL and ML infection and that IL-17 is present within the lesions caused by L. braziliensis.

IL-17 was secreted in supernatants of PBMC cultures after stimulation with Leishmania antigen in the majority of
patients with CL and in all patients with ML. The concentrations of IL-17 in patients with CL or ML were much lower than the concentrations of IFN-γ and TNF-α. As an inverse correlation between IFN-γ and IL-17 was documented, it is possible that IL-17 production could be more elevated for these patients. In tissue samples from CL and ML, the majority of IL-17 came from T helper cells that produce IL-17, but up to 30% of the cells that secreted IL-17 did not secrete CD4, indicating that other cell types are secreting IL-17 in these patients. Other investigators have shown that CD8⁺ T cells, γδ T cells, natural killer T cells, and monocytes can also secrete IL-17 [9], and in patients with CL or ML, a high percentage of cells that secrete IFN-γ are non-CD4⁺, non-CD8⁺ T cells [10]. Additional analysis should be performed to determine what other cells are secreting IL-17 in situ.

Several cytokines—including TNF-α, IFN-γ, and IL-10—play a role in the pathogenesis of CL and ML [6]. We have previously shown an association between TNF-α and IFN-γ production and lesion size in CL [11]. Alternatively, the severity of leishmaniasis may be determined by response to antimony therapy. Both variables are dependent on the duration of the illness. In the present study, patients with CL were evaluated 30–90 days after disease presentation, when a classical ulcerated lesion was already established, and a small number of patients with ML were observed. Therefore, we believe that the study did not have sufficient power to evaluate whether there was a correlation between IL-17 and the severity of the disease in this study; however, an ongoing study is evaluating the association of IL-17 and these variables.

In intracellular infections, IL-17 may participate in host defense mechanisms, as well as in tissue damage. In mice infected with Toxoplasma gondii, IL-17 produced by the innate immune response protects the animal, but it was also shown to induce tissue damage [12], and IL-17–deficient mice have impaired granuloma formation and impaired IFN-γ production upon Mycobacterium bovis bacillus Calmette-Guérin infection [3]. Moreover, a correlation between the self-healing of lesions and the frequency of CD4⁺IL-17⁺ cells was observed among mice infected with L. braziliensis [13]. CL and ML are associated with an exaggerated type 1 immune response [6, 14], parasites are few in the lesions, and drugs that inhibit TNF-α associated with antimony treatment induce ulcer healing in patients with CL or ML that is refractory to antimony therapy alone [15]. Because TNF-α has been considered to be an important molecule associated with the pathogenesis of CL and ML, the direct correlation between IL-17 and TNF-α gives support to the role of IL-17 in tissue damage associated with leishmaniasis.

This study shows that IL-17 is expressed in the peripheral blood and tissue of patients with leishmaniasis, and there was an association between the number of cells expressing IL-17 and the intensity of the inflammatory infiltration. These data, together with the observation of a correlation between IL-17 and TNF-α, point to the participation of IL-17 in the pathogenesis of leishmaniasis.

Acknowledgments
We thank Dr. Luiz Henrique Guimaraes, Dr. Paulo Machado, and Dr. Albert Schriefer for participation in clinical assistance of the patients.

References