Published in final edited form as:
Parasite Immunol. 2011 February ; 33(2): 132–136. doi:10.1111/j.1365-3024.2010.01256.x.

IL-17 and Regulatory Cytokines (IL-10 and IL-27) in *L. braziliensis* Infection

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Abstract

Cutaneous leishmaniasis (CL) is characterized by high production of pro-inflammatory cytokines and development of pathology. Individuals with subclinical *L. braziliensis* infection (SC) have a positive skin test to leishmania but do not develop disease. We evaluated if the downregulation of inflammatory response in SC is mediated by IL-10 and IL-27 and if IL-17 is associated with control of infection. Participants include SC individuals, CL patients and healthy subjects. Cytokines protein and mRNA were detected by ELISA and real-time PCR. IFN-γ and TNF-α levels were higher in CL than in SC group. The IL-10 levels and mRNA for IL-10 were similar in both SC and CL. mRNA for IL-27 was increased in cells from SC after stimulation with *L. braziliensis* antigen. There was a tendency for increased levels of IL-17 in SC compared to CL. The weak type 1 immune response observed in SC *L. braziliensis* infection is not due to the regulatory effects of IL-10 and IL-27. The control of *Leishmania* infection may be mediated by innate immune response with participation of IL-17. The results from this pilot study warrant further larger studies to investigate the potential contributions of IL-17 and IL-27 to the control of *L. braziliensis* infection.

Keywords

Leishmania braziliensis; subclinical infection; cutaneous leishmaniasis; IL-17; regulatory cytokines

INTRODUCTION

Cutaneous leishmaniasis (CL) due to *Leishmania braziliensis* is characterized by well delimited ulcerated skin lesions with raised borders. About 3% of CL patients develop mucosal leishmaniasis (ML) (1). Patients with CL and ML have a strong type 1 immune response to *Leishmania* antigen, with high production of IFN-γ and TNF-α and decreased ability of IL-10 in downmodulating IFN-γ production (2,3). In endemic areas of *L. braziliensis* about 10% of the individuals have a positive delayed type hypersensitivity (DTH) skin test to leishmania antigen but do not have a previous history of CL or a typical scar of CL. These individuals are considered as having a subclinical (SC) *L. braziliensis* infection (2). Individuals with SC *L. braziliensis* infection produce significantly lower levels

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Disclosures: There is nothing to disclose

of IFN-γ and TNF-α than CL patients (3). Others have shown that levels of IL-10 are greater in SC *L. braziliensis* infected individuals when compared with those from CL patients (4). IL-27 is a cytokine that can initiate a Th1 response but can also regulate inflammatory response (5,6). Therefore we evaluated if the downregulation of TNF-α and IFN-γ production in individuals with SC *L. braziliensis* infection is mediated by IL-10 and IL-27.

The mechanisms by which individuals with subclinical *L. braziliensis* infection achieve control over the infection are as yet not understood. In a highly endemic area for *L. major*, DTH to *Leishmania* antigen was associated with protection to CL (7). Recently it was shown that IL-17 may protect against visceral leishmaniasis (VL) (8). In this report, we characterized the immune response in individuals with SC *L. braziliensis* infection, focusing on the role of IL-10 and IL-27 in the modulation of immune response and evaluating whether IL-17 production was associated with control of infection.

MATERIAL AND METHODS

Study subjects

This study was performed in the village of Corte de Pedra, an endemic area of *L. braziliensis* transmission that is located in the state of Bahia, Brazil. Patients with CL (n = 15) were limited to individuals with a single typical ulcerative skin lesion of 1–3 months duration, without evidence of mucosal involvement, and without a history of previous therapy. CL was diagnosed by detection of parasites from culture aspirate or histopathology or by the presence of a typical CL lesion plus a positive DTH reaction to *L. braziliensis* antigen (9) and histopathologic findings of CL. SC individuals (n = 15) were recruited among household contacts of CL patients. They did not have past or current history of leishmaniasis, but had a positive skin test to *Leishmania* antigen. Patients with CL and controls were paired by age (+ / − 5 years) and gender with individuals with SC infection. The groups had similar socioeconomic conditions. In the SC individuals, the time between the detection of a positive skin test and realization of *in vitro* studies ranged from 6 months to two years. Ten healthy controls, from non-endemic area of leishmaniasis, were also evaluated. All patients provided an informed consent and the study followed the guidelines of the Ethical Committee of the Federal University of Bahia.

Cytokines production

Peripheral blood mononuclear cells (PBMC) from SC, CL patients and controls were isolated by density gradient centrifugation with Ficcoll-Hypaque. The cell were cultured in RPMI 1640 (Life Technologies GibcoBRL, Grand Island, N.Y, USA), 10% human AB serum (Sigma, St. Louis, MO., USA), glutamine, HEPES and antibiotics. Briefly 3×10^6 cells/mL were plated in 24-well flat botton microtiter plates (Falcon, Becton Dickinson, Lincoln Park, N.J., USA) and kept only with media (unstimulated) or were stimulated with 5 μg/mL of soluble *Leishmania* antigen (SLA). To determine if neutralization of IL-10 enhanced IFN-γ production, monoclonal antibody anti-IL-10 (BD-Bioscience-Pharmigen, USA), at 100ng/mL was added to PBMC cultures from 6 individuals with SC *L. braziliensis* infection. To determine if IL-27 downmodulates IFN-γ production, recombinant human IL-27 (rIL-27) (R&D Systems, Minneapolis, MN) at 100ng/mL (10) was also added to PBMC cultures from 6 CL patients. Cell cultures were incubated at 37°C with 5% CO2 for 72 hours and 96 hours in the case of determination of IL-17 levels. IFN-γ, TNF-α, IL-10 and IL-17 levels were determined in supernatants using the ELISA sandwich technique (BD Bioscience Pharmingen, San Jose, Calif., USA). The results were expressed in pg/mL.

RNA isolation and cDNA synthesis

Total RNA from cell lysates was isolated using Tri Reagent Soln. (Ambion, Applied Biosystems, Foster City, CA, USA). The concentration and purity of RNA were measured using the Eppendorf BioPhotometer plus spectrophotometer. Absorbance ratio at 260/230nm above 1.8 indicated that RNA samples were devoid of impurities. RNA integrity was assessed by gel eletrophoresis. First strand cDNA was synthesized from 1μg total RNA using the M-MLV reverse transcriptase (Invitrogen) and oligo dTprimers. The reverse transcription step was performed at 37° C for 60 minutes and 95° C for 5 minutes.

Relative quantitative Real-Time PCR

cDNA specific primers for IL-17, IL-10, IL-12 and IL-27 and reference gene HPRT were purchased from RealTimePrimers.com (Real Time primers, LLC, 7304 Mountain Ave, Elkins Park, PA 19027, USA). Relative quantitative PCR reactions were performed on a Real-Time PCR system Step One Plus AB Applied Biosystems in a 96-well microtiter plate and a final volume of 10μL using 0,5μL of cDNA, 2,5μL of 2X SYBR Green Master mix (AB Applied Biosystems) and 30 pM specific primer mix. The cycling conditions were as follows: 10 minutes polymerase activating at 95°C followed by 50 cycles of 15 seconds at 95°C and 1 minute at 60°C. All samples were amplified in duplicate and two non template negative controls per primer pair were included in each run. Melting curve analysis was performed immediately after amplification. HPRT mRNA expression was used for normalization. Relative expression levels were obtained as mean ΔCT for each gene using the software StepOnePlus Software v2.0.

Statistical analysis

Statistical analysis was performed with Mann-Whitney test, using the GraphPad Prism 3 (GraphPad Prism 3 Software, San Diego, CA, USA). The results were expressed as median (interquartile range). Statistical significance was assigned to $p<0.05$.

RESULTS

The cytokines production in SC *L. braziliensis*-infected individuals and in patients with CL is shown in Figure 1. Both TNF-α and IFN-γ levels were higher ($p<0.01$) in CL in comparison with SC *L. braziliensis* infection. The median (interquartile range) of IL-17 in SC *L. braziliensis* infection was 26pg/mL (0 -278pg/mL) and in CL patients was 0 (0–144pg/mL) p=0.06. IL-10 production was similar in SC *L. braziliensis* infection (median 9 pg/mL, 0 - 461pg/mL) and in patients with CL (median 0 pg/mL, 0 - 75), p= 0.6. Cytokines were either undetectable or detected at low levels in supernatants of culture stimulated with media and in culture from non infected individuals (controls) stimulated with *L. braziliensis* antigen (data not shown). The mRNA for cytokines in cells *ex vivo* and after stimulation with SLA is shown in Figure 2. In panel 2A we observed that expression of IL-10 *ex vivo* or in cultures of SC *L. braziliensis*-infected individuals was similar to that observed in CL patients (p= 0.2). The expression of mRNA for IL-27 *ex vivo* or in cultures with medium, and after stimulation with SLA was higher in CL patients compared with SC group (Figure 2B). No difference was observed in the expression of IL-12 (Figure 2C) and in the expression of IL-17 (Figure 2D). In 6 individuals with SC *L. braziliensis* infection, we evaluated if neutralization of IL-10 would enhance IFN-γ production. The median of IFN-γ production was 46pg/mL (24–2560pg/mL). The addition of neutralizing monoclonal antibody anti-IL-10 did not increase the production of this cytokine (median 61pg/mL, 22 - 1920pg/mL, $p>0.05$). We also evaluated the ability of IL-27 in modulating IFN-γ production. The addition of rIL-27 to cells from patients with CL stimulated with SLA decreased IFN-γ production, although there was no significant difference in cultures with or without IL-27. The median of IFN-γ production after stimulation with SLA was 10040pg/

mL (249 - 20580pg/mL) and after addition of SLA plus rIL-27 was 3500pg/mL (65 - 20360pg/mL).

DISCUSSION

The understanding of the role of the host immune response in the control of *Leishmania* and in the pathology associated to *L. braziliensis* infection is highly relevant to the pathogenesis of CL and ML and has implications on vaccine development and control of leishmaniasis. It is known that inflammatory response in CL and ML is associated with an exaggerated production of TNF-α and IFN-γ (11), but little is known about the immune response in individuals exposed to these parasites but control *L. braziliensis* infection. A few concerns should be pointed out in the immunological evaluation of SC *L. braziliensis*-infected individuals. First, it is not known when they were exposed to *L. braziliensis* infection. Second, the time between the positivity of the skin test and the determination of the immunological response may interfere in the results obtained. In fact, the lowest values of TNF-α, IFN-γ and IL-17 occurred in individuals, in whom immunological studies were performed after one year from detection of a positive skin test.

As patients with CL and ML have an impairment in downmodulating IFN-γ production (11), we evaluated if the decreasing in the production of pro-inflammatory cytokines in SC *L. braziliensis*-infected individuals was related to the ability of regulatory cytokines IL-10 and IL-27 in controlling the immune response, and consequently prevent development of pathology. IL-10 levels were low in the supernatants of SC group and were similar to that found in the CL group. Moreover, neutralization of IL-10 did not enhance IFN-γ production in SC individuals. There is no previous data regarding IL-27 in human leishmaniasis. IL-27-receptor deficient mice infected with *Mycobacterium tuberculosis* or *Toxoplasma gondii* develop a lethal inflammatory disease, despite controlling the microbial multiplication (12–14). In human cells stimulated with anti-CD3, IL-27 enhances IFN-γ and IL-10 production and decrease IL-17 (10). In IL-27R-defficient mice infected with *L. major*, a decrease in IL-10$^+$ and IFN-γ$^+$CD4$^+$ T cells and an increase in IL-17$^+$ CD4$^+$ T cells is observed (15). Here the expression of IL-27 *ex vivo* and in cells cultured with media or stimulated with SLA was higher in patients with CL than in SC *L. braziliensis*-infected individuals. Moreover, exogenous addition of IL-27 in cells from CL patients tends to decrease IFN-γ production. It is possible that IL-27 may contribute to a type 1 immune response in patients with CL.

The mechanisms by which SC *L. braziliensis*-infected individuals control parasite growth are unknown. In CL, both CD4$^+$ and CD8$^+$ T cells activation and production of cytokines have been associated with pathology (11,16,17). As the adaptative immune response in SC *L. braziliensis*-infected individuals is weak, it is possible that in such case, control of *Leishmania* may be dependent of the innate immune response. Both neutrophils/macrophages interaction and NK cells may participate in the control of *Leishmania* infection (18,19), and ongoing studies are evaluating the role of neutrophils and NK cells in SC *L. braziliensis* infection.

We recently detected IL-17 production in PBMC and in tissue from CL patients (20). While in BALB/C mice infected with *L. major* IL-17 promotes progression of CL (21), we have not observed a pathological role for IL-17 in CL and ML (20). In fact, IL-17 has been associated with protection against human VL (8). As IL-17 tends to be higher in SC *L. braziliensis* infections than in CL, further studies are needed to better evaluate the role of this cytokine in the control of *L. braziliensis* infection. This study did not show that decreased production of inflammatory cytokines in SC *L. braziliensis* infection is due to a modulator effect of IL-10 and IL-27. In such case, it is likely that the weak adaptative

immune response in these individuals is due to their ability to control quickly parasite infection by the innate immune response.

Acknowledgments

This study was supported by NIH grant AI-30639 and Brazilian Council for Scientific and Technological Development (CNPq) 302729/2007-0.

We thank Renato Santos Lima for helping in the determination of the RT-PCR.

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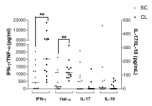

Figure 1. Protein levels of IFN-γ, TNF-α, IL-10 and IL-17 in the supernatants from peripheral blood mononuclear cells (PBMC) from SC individuals and patients with CL

PBMC were stimulated with Soluble *Leishmania* Antigen (SLA) (5μg/mL) for 72 hours. The levels of cytokines were determined by ELISA. Each point represents a different patient, and each bar represents the median. For statistical analysis, Mann-Whitney test was used. ** $p < 0.01$ compared results of SC versus CL.

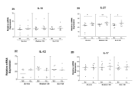

Figure 2. Cytokines mRNA levels from peripheral blood mononuclear cells (PBMC) of SC individuals and patients with CL

Total cytokines mRNA *ex vivo* and in response to Soluble *Leishmania* Antigen (SLA), after 12 hours of culture. Each point represents a different patient, and each bar represents the median. **(A)** Total IL-10 mRNA. (**B)** Total IL-27 mRNA, (**C)** Total IL-12 mRNA. **(D)** Total IL-17 mRNA. For statistical analysis, Mann-Whitney test was used. The one * indicate $p<0.05$ and two ** indicate $p=0.01$.