

Interleukin-12 Restores Interferon- γ Production and Cytotoxic Responses in Visceral Leishmaniasis

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American visceral leishmaniasis (AVL) is associated with the absence of lymphocyte proliferative responses and interleukin (IL)-2 and interferon- γ (IFN- γ) production upon stimulation with *Leishmania* antigen. In contrast, cure of AVL is associated with restoration of these T cell functions. In the present study, the ability of IL-12, a cytokine that acts on NK and T cells to restore cellular immune responses in AVL, was evaluated. Participants of the study included 12 patients with AVL and 7 subjects cured of AVL. The [3 H]thymidine uptake and IFN- γ production in cultures of peripheral blood mononuclear cells (from AVL patients) stimulated with *Leishmania chagasi* antigen were 882 ± 1393 cpm and zero, respectively. Addition of IL-12 enhanced the proliferative response to 5097 ± 6429 cpm ($P < .001$) and IFN- γ production to 305 ± 325 pg/mL ($P < .01$). IL-12 also restored cytotoxic activity against the K562 cell line. These results indicate that IL-12 has an important role in the regulation of the cellular immune response in human leishmaniasis.

American visceral leishmaniasis (AVL) is a systemic protozoan infection caused by *Leishmania chagasi*, an intracellular parasite that infects and multiplies in macrophages. Monocyte and T cell defects have been associated with *Leishmania* infection. Macrophages infected with leishmania have decreased ability to express MHC class II molecules [1] and produce interleukin (IL)-12 [2]. In patients with AVL, IL-2 and interferon (IFN)- γ production are absent from supernatants of mononuclear cells stimulated with *Leishmania* antigen [3]. Furthermore, NK cell activity is deficient in AVL patients [4]. Since Th2 cell activation may be involved in progression of AVL [5] and IFN- γ production is associated with the ability to control *Leishmania* infection [6], the aim of the present study was to evaluate the ability of IL-12 to restore in vitro lymphoproliferative responses, IFN- γ production, and NK cell activity following stimulation with *Leishmania* antigen.

Material and Methods

Patients. Participants included 12 patients with AVL. In all cases, amastigote forms of leishmaniae were detected in Giemsa-

stained spleen or bone marrow aspirates. All immunologic studies were done prior to therapy. As a control, 7 subjects cured of AVL were studied.

Leishmania antigen. *L. chagasi* mammalian *Homo sapiens* (MHOM Ba 62) antigen was prepared by freeze-thaw lyses. After sonication, the lysate was centrifuged (12,000 g) for 20 min, the supernatant was collected, and protein content was determined by the Lowry method.

Lymphocyte culture, proliferation, and cytokine assays. Peripheral blood mononuclear cells (PBMC) were obtained from venous blood layered over a ficoll-hypaque gradient (lymphocyte separation medium; Organon Teknica, Durham, NC). For in vitro proliferation, 2×10^5 cells/well were cultured in RPMI 1640 (Life Technologies GIBCO BRL, Grand Island, NY) supplemented with penicillin, streptomycin, and 10% heat-inactivated normal AB human serum in 96-well flat-bottomed plates. Cells were stimulated with 5 μ g/mL *L. chagasi* antigen in the presence (10–500 U/mL) or absence of recombinant human IL-12. Recombinant human IL-10 (10 ng/mL; DNAX Research Institute) was used as stated. Anti-IL-10 (250 μ g/mL), monoclonal antibody (MAb), and rat IgG1 (GL-113) isotype control (DNAX) were also added to some cultures. After 5 days, cells were pulsed with 1 Ci of [3 H]thymidine for the final 6 h of culture. Data are given as mean \pm SE counts per minute (cpm) of triplicate cultures or as stimulation index, calculated by dividing the cpm of stimulated culture by the cpm of unstimulated culture.

For determination of cytokine production, PBMC (3×10^6 /mL) in complete RPMI were stimulated with *L. chagasi* antigen (10 μ g/mL) with (10–500 U/mL) or without IL-12. In some experiments, recombinant IL-10 (10 ng/mL) was also added to cultures. Supernatants were collected at 72 h, and IFN- γ levels were determined by ELISA using a sandwich technique. A standard curve was used to express the results in picograms per milliliter.

Evaluation of cytotoxic responses. Patient PBMC (3×10^6 cells/mL) stimulated with *Leishmania* antigen (10 μ g/mL) in the

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Informed consent was obtained from the patients or their parents, and human experimentation guidelines of the Hospital Universitário Professor Edgard Santos were followed in the conduct of the clinical research.

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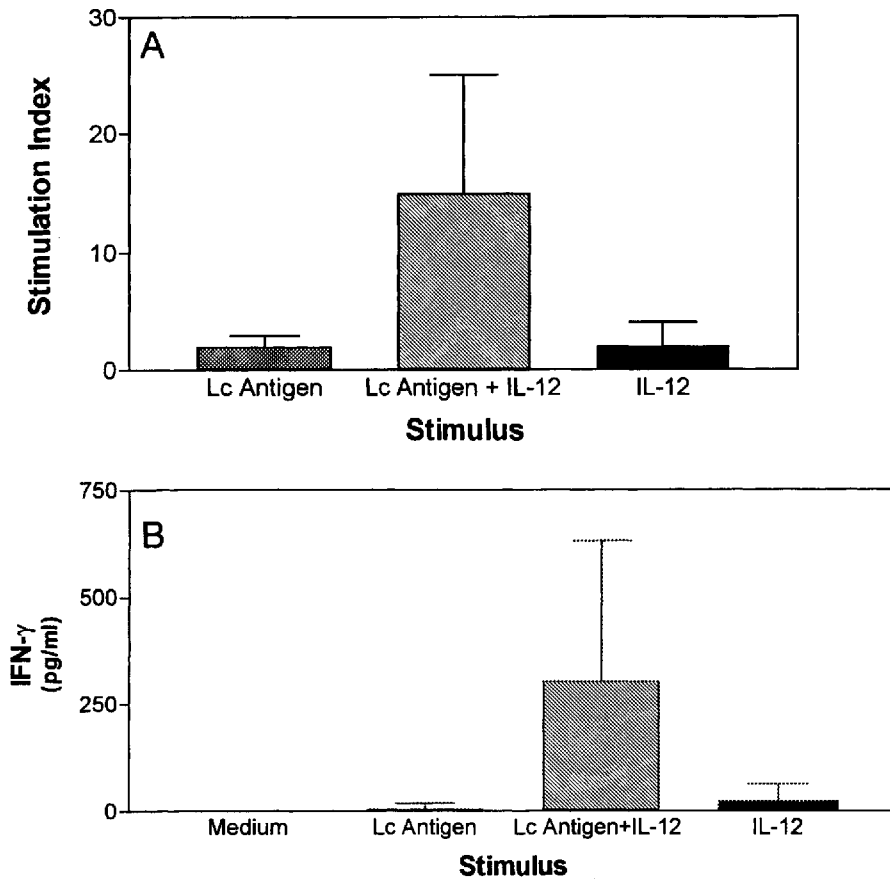


Figure 1. A, Restoration of lymphoproliferative response by IL-12 in patients with American visceral leishmaniasis (AVL). Data represent mean \pm SE stimulation index (cpm of cultures with antigen divided by cpm of control cultures) in 12 patients after stimulation with *Leishmania chagasi* (Lc) antigen (10 μ g/mL), *L. chagasi* plus IL-12 (10 U/mL), and IL-12 (10 U/mL). B, IL-12 increases IFN- γ production in cells from patients with AVL. Peripheral blood mononuclear cells (3×10^6 cells/mL) were cultured for 72 h in presence or absence of 10 μ g/mL *L. chagasi* antigen with or without 10 U/mL of IL-12. Data (mean \pm SE) are from 9 patients.

presence (500 U/mL) or absence of IL-12 for 5 days were used as effector cells. The K562 tumor cell line target cells were radiolabeled with 200 Ci/mL [51 Cr]sodium chromate for 30 min at 37°C. After being washed, cells were resuspended in RPMI 1640 with 10% fetal calf serum. Aliquots (2×10^3 cells/well) were incubated with different numbers of effector cells (effector-to-target [E:T] ratio, 3:1 to 100:1) in round-bottomed plates for 4 h at 37°C. Supernatants were harvested and radioactivity was counted using a gamma counter (model 5500; Beckmann, Fullerton, CA). The percent specific lysis was calculated using the following formula: $100 \times [(experimental\ release - spontaneous\ release)/(maximum\ release - spontaneous\ release)]$ for a mean of triplicate wells. Spontaneous release was obtained by target cells in the presence of medium alone; maximum release was obtained by the addition of 0.1 M HCL.

Results

*IL-12 restores lymphoproliferative response and IFN- γ production to *L. chagasi* antigen.* The proliferative responses and IFN- γ production of PBMC from patients with AVL in cultures stimulated with *L. chagasi* antigen in the presence or absence of recombinant IL-12 are shown in figure 1. The [3 H]thymidine uptake in cultures stimulated with *L. chagasi* antigen was 882 ± 1393 cpm compared with 5097 ± 6429 cpm ($P < .001$) in

cultures with antigen plus IL-12. The proliferative response in cultures containing *Leishmania* antigen and IL-12 was similar ($P > .05$) to that observed in subjects cured of AVL (8831 ± 7379 cpm). At the concentration used (10 U/mL), IL-12 did not induce lymphocyte proliferation in the absence of *L. chagasi* antigen. The cpm in cultures with medium alone was 403 ± 627 and with IL-12 alone was 549 ± 433 . In a dose-response curve (data not shown), the enhancement observed with IL-12 at concentrations of 100 and 500 U/mL was similar to that observed at 10 U/mL.

The mean IFN- γ level in lymphocyte cultures (of 9 patients) stimulated with *L. chagasi* antigen was zero. IL-12 increased IFN- γ levels to 305 ± 325 pg/mL ($P < .01$). IFN- γ levels in cultures with IL-12 alone were 24 ± 13 pg/mL. The amount of IFN- γ production in cultures containing leishmania antigen and IL-12 was similar ($P > .05$) to that produced by lymphocytes from subjects cured of AVL (498 ± 428 pg/mL).

The role of IL-10 in modulating the immune response mediated by IL-12. Anti-IL-10 MAb restores lymphocyte proliferation and IFN- γ production in response to leishmania antigen in patients with AVL [7]. When the enhanced lymphocyte proliferation and IFN- γ production mediated by anti-IL-10 MAb were compared with those observed with IL-12, IL-12 was shown to be more potent (at least twice as much as the

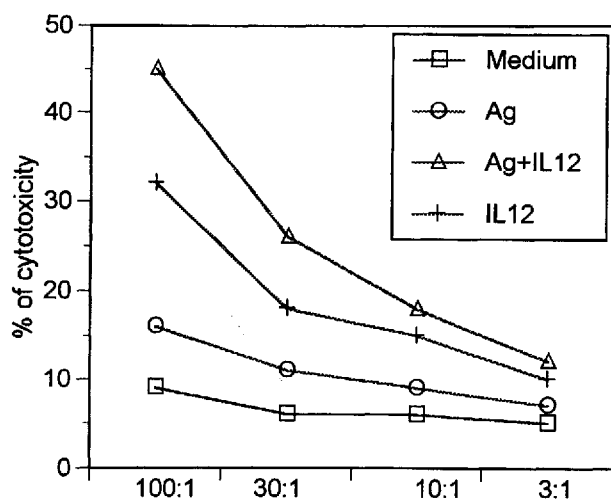


Figure 2. IL-12 induces cytotoxic activity in AVL patients. Peripheral blood mononuclear cells (3×10^6) previously stimulated with *L. chagasi* antigen (Ag, 10 $\mu\text{g}/\text{mL}$) in presence or absence of IL-12 (500 U/mL) for 5 days were incubated with K562 tumor target cells. NK cell cytotoxicity was assayed as described in Material and Methods. Results are expressed as % specific ^{51}Cr release at several effector-to-target ratios.

anti-IL-10) in restoring these T cell functions (data not shown). Additionally, anti-IL-10 MAb and IL-12 had a synergistic effect on lymphocyte proliferation and IFN- γ production (data not shown). Indeed, the addition of IL-10 suppressed the lymphoproliferative response and IFN- γ production induced by *L. chagasi* antigen plus IL-12. In 4 patients the mean \pm SE of the [^3H]thymidine uptake in cultures stimulated with *L. chagasi* antigen plus IL-12 was 6581 ± 8020 cpm. Addition of IL-10 (10 ng/mL) decreased the response to 682 ± 623 cpm.

IL-12 induces cytotoxic activity in AVL patients. The functional activity of NK cells is impaired in visceral leishmaniasis [4]. The effect of IL-12 on the cytotoxic activity in PBMC from 5 AVL patients is shown in figure 2. The results are expressed as percent lysis. The addition of 500 U/mL of IL-12 enhanced cytotoxic activity at four E:T ratios tested ($P < .05$).

Discussion

The major immunologic dysfunction in patients with visceral leishmaniasis is the inability of T cells to produce IL-2 and IFN- γ upon stimulation with *Leishmania* antigen and the inability of lymphocytes to activate macrophages to kill leishmaniae [3, 8]. These abnormalities allow parasites to multiply and contribute to the pathogenesis of the disease. In contrast, lymphocyte proliferation in response to leishmania antigen and IFN- γ production in the early stages of *L. chagasi* infection are associated with the host's ability to prevent the progression of the infection to disease [6]. In the present study, IL-12, a

cytokine that stimulates NK activity and differentiation of T cells toward Th1 cell development [9, 10], restored IFN- γ production and lymphocyte proliferation. These results confirm the observation by Ghalib et al. [2] in patients with visceral leishmaniasis from Sudan. In addition, IL-12 increased cellular cytotoxicity, a function that is also suppressed in visceral leishmaniasis [4].

There are indications in vitro of the activity of lytic cells against macrophages parasitized by leishmaniae [11]. Since the main cell responsible for the cytotoxicity against K562 cells is the NK cell and cytotoxic response in this study was enhanced even in cultures without *Leishmania* antigen, it is conceivable that the main effect of IL-12 was on NK cells. In fact, IL-12 is the most important NK cell stimulatory factor and induces accumulation of perforin mRNA in most NK cells [12]. In the case of restoration of proliferative response and IFN- γ production, we observed that both NK cells and T cells participated. First, IL-12 restored lymphocyte proliferation, a function related predominantly to T cells; second, proliferation and IFN- γ production were predominantly observed in cultures containing both IL-12 and leishmania antigens. It is likely that in this situation, IL-12 and IFN- γ improved antigen presentation and, consequently, T cell activation. Then IL-12 acting directly in activated T cells induced lymphocyte proliferation and cytokine production.

Marked expression of mRNA for IL-10 has been documented in patients with visceral leishmaniasis, and the in vitro production of IFN- γ and lymphocyte proliferation can be restored by anti-IL-10 MAb [7]. IL-10 down-regulates macrophage activity and inhibits IFN- γ production by human lymphocytes by suppression of IL-12 synthesis in accessory cells [13]. Here we show that IL-10 abrogates the enhancement of the lymphocyte proliferation and IFN- γ production mediated by IL-12. We have previously shown [7] that IL-10 suppressed IFN- γ production in subjects cured of visceral leishmaniasis, and others have shown that anti-IL-12 abrogates the IFN- γ production observed when anti-IL-10 is added to PBMC cultures stimulated with *L. chagasi* antigen [2]. These data indicate that IL-10 plays a major role in the suppression of T cell responses and NK cell activity in visceral leishmaniasis. This suppression can be caused by inhibition of IL-12 synthesis at the accessory cell level and by suppression of cytokine production at the T cell level.

IL-12 plays a crucial role in the resolution of cutaneous leishmaniasis in mice, initiating a Th1 cell response that is essential for inducing a protective host immune response; IL-12 also decreases the parasite burden in mice infected with *Leishmania donovani* [14]. Since control of intracellular infection is related to the ability of macrophages to produce IL-12, which then stimulates IFN- γ production, these cytokines are strong candidates as therapeutic agents in leishmaniasis. In fact, there is evidence in both cutaneous and visceral leishmaniasis that IFN- γ , in association with pentavalent antimony, induces healing of patients who were previously refractory to antimo-

nia therapy [15]. The evidence that IL-12 restores functions (cytotoxicity and IFN- γ production) that are essential for control of leishmanial multiplication provides a rational basis for the use of IL-12 as an immunotherapeutic adjuvant for the treatment of visceral leishmaniasis.

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References

1. Reiner NE, Ng W, Ma T, McMaster WR. Kinetics of interferon binding and induction of major histocompatibility complex class II mRNA in *Leishmania*-infected macrophages. *Proc Natl Acad Sci USA* **1988**;85:4330–4.
2. Ghalib HW, Whittle JA, Kubin M, et al. IL-12 enhances Th1-type responses in human *Leishmania donovani* infections. *J Immunol* **1995**;154:4623–9.
3. Carvalho EM, Badaró R, Reed SG, Jones TC, Johnson Jr WD. Absence of gamma interferon and interleukin-2 production during active visceral leishmaniasis. *J Clin Invest* **1985**;76:2066–9.
4. Harms G, Pedrosa C, Omena S, Feldmeier H, Zwingenberger K. Natural killer cell activity in visceral leishmaniasis. *Trans R Soc Trop Med Hyg* **1991**;85:54–5.
5. Ghalib HW, Piuvezam MR, Skeiky YA, et al. Interleukin-10 production correlates with pathology in human *Leishmania donovani* infections. *J Clin Invest* **1993**;92:324–9.
6. Carvalho EM, Barral A, Pedral-Sampaio D, et al. Immunologic markers of clinical evolution in children recently infected with *Leishmania donovani* chagasi. *J Infect Dis* **1992**;165:535–40.
7. Carvalho EM, Bacellar O, Brownell C, Régis T, Coffman RL, Reed SG. Restoration of IFN- γ production and lymphocyte proliferation in visceral leishmaniasis. *J Immunol* **1994**;152:5949–56.
8. Carvalho EM, Bacellar O, Reed S, Barral A, Rocha H. Visceral leishmaniasis: a disease associated with inability of lymphocytes to activate macrophages to kill leishmania. *Braz J Med Biol Res* **1988**;21:85–92.
9. Chehimi J, Starr SE, Frank I, et al. Natural killer (NK) cell stimulatory factor increases the cytotoxic activity of NK cells from both healthy donors and human immunodeficiency virus-infected patients. *J Exp Med* **1992**;175:789–96.
10. Manetti R, Parronchi P, Guidizi MG, et al. Natural killer cell stimulatory factor (interleukin-12 [IL-12]) induces T helper type 1 (Th1)-specific immune responses and inhibits the development of IL-4-producing Th cells. *J Exp Med* **1993**;177:1199–204.
11. Farrell JP, Muller I, Louis JA. A role of Lyt-2⁺ T cells in resistance to cutaneous leishmaniasis in immunized mice. *J Immunol* **1989**;142:2052–6.
12. Salcedo TW, Azzoni L, Wolf SF, Perussia B. Modulation of perforin and granzyme messenger RNA expression in human natural killer cells. *J Immunol* **1993**;151:2511–20.
13. D'Andrea A, Aste-Amezaga M, Valiante NM, Ma X, Kubin M, Trinchieri G. Interleukin-10 (IL-10) inhibits human lymphocyte interferon-production by suppressing natural killer cell stimulatory factor/IL-12 synthesis in accessory cells. *J Exp Med* **1993**;178:1041–8.
14. Murray HW, Harisprasad J. Interleukin-12 is effective treatment for an established systemic intracellular infection: experimental visceral leishmaniasis. *J Exp Med* **1995**;181:387–91.
15. Badaró R, Johnson, WD. The role of interferon- γ in the treatment of visceral and diffuse cutaneous leishmaniasis. *J Infect Dis* **1993**;167(suppl):S13–7.