# Interleukin-10-dependent down-regulation of interferon-gamma response to *Leishmania* by *Mycobacterium* leprae antigens during the clinical course of a coinfection

R.B.G. Azeredo-Coutinho, D.C.S. Matos, J.A.C. Nery, C.M. Valete-Rosalino and
S.C.F. Mendonça

# Interleukin-10-dependent down-regulation of interferon-gamma response to *Leishmania* by *Mycobacterium leprae* antigens during the clinical course of a coinfection

R.B.G. Azeredo-Coutinho[1], D.C.S. Matos[2], J.A.C. Nery[3],
C.M. Valete-Rosalino[4] and S.C.F. Mendonça[1]

[1]Laboratório de Imunoparasitologia, Instituto Oswaldo Cruz,
Fundação Oswaldo Cruz, Rio de Janeiro, RJ, Brasil
[2]Laboratório de Tecnologia Imunológica, Instituto de Tecnologia em Imunobiológicos,
Fundação Oswaldo Cruz, Rio de Janeiro, RJ, Brasil
[3]Centro de Referência Nacional em Hanseníase, Instituto Oswaldo Cruz,
Fundação Oswaldo Cruz, Rio de Janeiro, RJ, Brasil
[4]Departamento de Doenças Infecciosas, Instituto de Pesquisa Clínica Evandro Chagas,
Fundação Oswaldo Cruz, Rio de Janeiro, RJ, Brasil

## Abstract

We have described a case of a patient with an intriguing association of mucocutaneous leishmaniasis with lepromatous leprosy, two opposite polar forms of these spectral diseases. In the present follow-up study, we investigated the effect of the addition of *Mycobacterium leprae* antigens on interferon-gamma (IFN-γ) production in *Leishmania* antigen-stimulated cultures of peripheral blood mononuclear cells (PBMC) from this patient. For this purpose, PBMC cultures were stimulated with crude *L. braziliensis* and/or *M. leprae* whole-cell antigen extracts or with concanavalin A. In some experiments, neutralizing anti-human interleukin (IL)-10 antibodies were added to the cultures. IFN-γ and IL-10 levels in culture supernatants were measured by ELISA. During active leprosy, *M. leprae* antigens induced 72.3% suppression of the IFN-γ response to *L. braziliensis* antigen, and this suppression was abolished by IL-10 neutralization. Interestingly, the suppressive effect of *M. leprae* antigen was lost after the cure of leprosy and the disappearance of this effect was accompanied by exacerbation of mucosal leishmaniasis. Considered together, these results provide evidence that the concomitant lepromatous leprosy induced an IL-10-mediated regulatory response that controlled the immunopathology of mucosal leishmaniasis, demonstrating that, in the context of this coinfection, the specific immune response to one pathogen can influence the immune response to the other pathogen and the clinical course of the disease caused by it. Our findings may contribute to a better understanding of the *Leishmania*/*M. leprae* coinfection and of the immunopathogenesis of mucosal leishmaniasis.

Key words: Interferon-gamma; Interleukin-10; Mucocutaneous leishmaniasis; Lepromatous leprosy; *Leishmania braziliensis*; *Mycobacterium leprae*

## Introduction

Leprosy and American tegumentary leishmaniasis are both caused by intracellular organisms. These diseases exhibit similar spectra of clinical forms, which are dependent on antigen-specific cell-mediated immunity. Diffuse cutaneous leishmaniasis and lepromatous leprosy are considered to be the anergic pole. These clinical forms have been associated with high expression of interleukin (IL)-10 and low expression of interferon (IFN)-γ (1,2). At the other end of the spectrum, mucosal leishmaniasis and tuberculoid leprosy are associated with a conspicuous cell-mediated immune response in which proinflammatory cytokines, such as IFN-γ, predominate (3,4). Although the immunopathogenesis of these diseases has been extensively investigated, there are very few immunological studies on leishmaniasis/lep-

rosy coinfection, especially in HIV-negative patients (5,6). We have previously described an unusual association of mucosal leishmaniasis and lepromatous leprosy, opposite clinical forms in the immunopathological spectra of these diseases, and have characterized the cytokine response to *Leishmania* and *Mycobacterium leprae* antigens during coinfection. Although the stimulation of peripheral blood mononuclear cells (PBMC) with *Leishmania* and *M. leprae* antigens induced comparable levels of tumor necrosis factor (TNF)-α, IL-5 and IL-10, the IFN-γ response to *Leishmania* antigens was extremely high while the response to *M. leprae* antigens was nearly absent (6). The contrasting responses to *Leishmania* and *M. leprae* antigens seen in this patient, leading to divergent polar forms of leishmaniasis and leprosy, prompted us to investigate a possible effect of the immune response to one infection on the course of the other. Thus, we evaluated the effect of the addition of *M. leprae* antigens on IFN-γ production in *L. braziliensis* antigen-stimulated PBMC cultures.

## Material and Methods

### Case presentation

A 46-year-old male patient presented with a clinically active mucosal leishmaniasis and lepromatous leprosy coinfection, the clinical picture of which has been previously described in detail (6). The diagnosis of polar lepromatous leprosy was based on positive results from smears of skin samples from the earlobes, elbows, and knees, with an average bacterial index of 4+ with Ziehl-Nielsen staining. Mucosal leishmaniasis was diagnosed by isolation of *Leishmania* from the nasal mucosal lesion. The isolated parasites were characterized as *Leishmania braziliensis* by isoenzyme analysis (7). The patient was treated for leishmaniasis with meglumine antimoniate for 30 days with a low-dose schedule of 5 mg/kg per day of pentavalent antimony and for leprosy with a multidrug therapy regimen (6) with 24 monthly supervised doses. Leprosy treatment was started 10 months before the beginning of leishmaniasis therapy and ended 13 months after its completion. Nine months after diagnosis, the leishmaniasis lesion was considered to be healed, leaving partial mutilation of the nose. Relapse was noticed 13 months after cure of the mucosal lesion, with erosion of the nose and large edema and infiltration of the nose and lips extending to the malar region. On that occasion, *Leishmania* parasites were again isolated from a biopsy specimen from the nasal lesion, but the patient had no clinical sign of leprosy and skin smears obtained bilaterally from earlobes, elbows, and knees showed a bacterial index of 0 (zero).

### Methods

Venous blood samples were obtained twice before and twice after the cure of leprosy. The patient was clinically cured of mucosal leishmaniasis when the experiments were performed before the cure of leprosy. PBMC were obtained, cultured and stimulated with crude *L. braziliensis* and/or *M. leprae* whole-cell antigen extracts (50 mg/mL) or with concanavalin A (ConA) (20 mg/mL). The antigen extracts were prepared as previously described (6). Briefly, *L. braziliensis* (WHO reference strain MHOM/BR/75/2903) promastigotes were separately cultured in Novy-McNeal-Nicolle medium (8), supplemented with RPMI 1640 medium (Sigma, USA) and 10% heat-inactivated fetal calf serum (Sigma). Stationary phase promastigotes were washed three times by centrifugation at 900 $g$ for 10 min at 4°C in PBS and were disrupted by 10 repeated cycles of freezing and thawing, followed by ultrasonication (Ultra-tip Labsonic System; Lab-Line, USA) at 40 watts for 15 min in an ice bath. An irradiated, armadillo-derived whole *M. leprae* antigen preparation ($2 \times 10^9$ bacteria/mg; kindly provided by Dr. M.C. Pessolani, Laboratório de Hanseníase, Oswaldo Cruz Foundation, Rio de Janeiro, Brazil) was sonicated according to the same protocol as used for the *Leishmania* antigen extracts. Previous publications have shown that the antigen extracts used in this study stimulate production of TNF-α and IL-10 in PBMC cultures from healthy subjects (9-11). The antigen concentration in each preparation was adjusted to 1 mg/mL protein nitrogen. All samples were kept at -20°C until use.

Neutralizing anti-human IL-10 antibodies (BD Pharmingen, USA) were added to some cultures at a final concentration of 50 μg/mL, concomitantly with the antigens. IFN-γ and IL-10 levels in PBMC culture supernatants were estimated by ELISA as previously described (6). The results are reported as the differences between the mean cytokine levels in stimulated and unstimulated (with medium alone) triplicate cultures. The detection limit of the assays was 8 pg/mL for both IFN-γ and IL-10. This research protocol was carried out in compliance with the Helsinki Declaration, and was approved by the Ethics Review Committee of Instituto de Pesquisa Clínica Evandro Chagas, Fundação Oswaldo Cruz, Brazilian Ministry of Health, under the reference No. CAAE 0016.0.009.000-02.

## Results

Figure 1 shows the results of representative experiments performed before (Figure 1A and C) and after (Figure 1B and D) the cure of leprosy. On both occasions, the patient's PBMC produced high levels of IFN-γ in response to the *L. braziliensis* antigen extract and no IFN-γ response when stimulated with *M. leprae* antigens (Figure 1A and B). The neutralization of IL-10 (Figure 1C and D) did not reverse the inability of the patient's cells to secrete IFN-γ in response to *M. leprae* (Figure 1A and B). The levels of IFN-γ and IL-10 in unstimulated cultures were 0 (Figure 1A), 39.3 pg/mL (Figure 1B), 130.9 pg/mL (Figure 1C), and 31.2 pg/mL (Figure 1D).

We investigated whether the *M. leprae* antigens could

inhibit the IFN-γ production stimulated by *L. braziliensis* antigens. This was indeed the case in the two experiments performed before the cure of leprosy, when cultures stimulated with both antigen preparations produced less than one third of the amount of IFN-γ measured in supernatants of cultures stimulated with *L. braziliensis* antigens alone. Figure 1A shows the results of one of these experiments. In an experiment performed only once, the *M. leprae* antigens were unable to inhibit the production of IFN-γ induced by ConA, and the addition of neutralizing anti-IL-10 antibodies had no effect on the IFN-γ levels in this particular experiment (data not shown).

The *M. leprae* antigen-induced suppression of the IFN-γ response to the *L. braziliensis* extract observed before the cure of leprosy was blocked by neutralizing anti-IL-10 antibodies (Figure 1A). Interestingly enough, the suppressive effect of *M. leprae* antigens in the *L. braziliensis* antigen-induced IFN-γ production could not be seen after the cure of leprosy, when the cultures stimulated with both antigen preparations produced as much IFN-γ as the cultures stimulated with *L. braziliensis* antigens alone. Figure 1B shows the results of one of two experiments with similar results performed after the cure of leprosy. The loss of the *M. leprae* antigen-induced suppression of the IFN-γ response to *L. braziliensis* antigens observed *in vitro* was clinically accompanied by the reactivation and exacerbation of mucosal leishmaniasis (Figure 2).

## Discussion

The high production of IFN-γ after stimulation with *L. braziliensis* antigens and the absence of this cytokine after stimulation with *M. leprae* antigens observed both before and after the cure of leprosy are in accordance with previous results obtained

with this patient (6). The absence of an IFN-γ response to the *M. leprae* antigen extract was not due to IL-10-mediated suppression since neutralization of IL-10 did not change the inability of the patient's cells to produce IFN-γ in response to *M. leprae* antigens, as previously reported (6). During active leprosy, *M. leprae* antigens were able to markedly inhibit the IFN-γ production in *Leishmania* antigen-stimulated PBMC cultures, which was restored by the addition of neutralizing anti-IL-10 antibodies, indicating that this cytokine plays a role in the observed immunosuppression. The down-regulatory effect of the *M. leprae* antigens on the IFN-γ response induced by *Leishmania* antigens was completely lost after the cure of leprosy, and the loss of this effect was accompanied
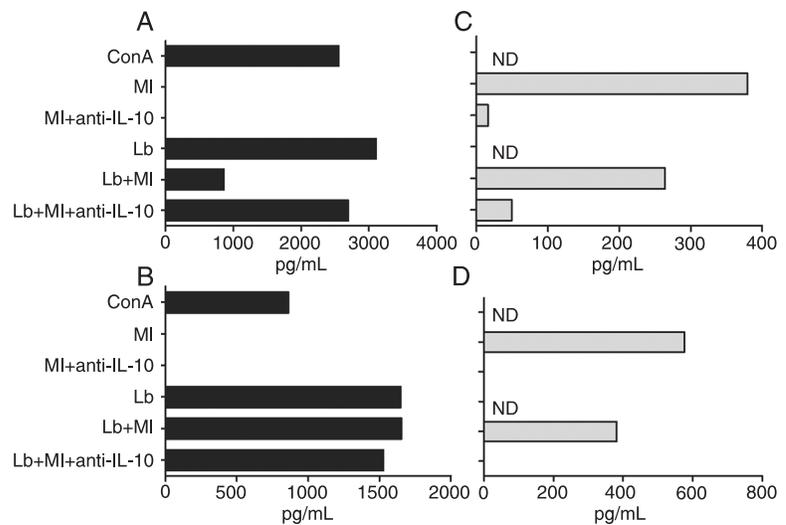
**Figure 1.** IFN-γ and IL-10 levels in PBMC culture supernatants estimated by ELISA. *A, B,* IFN-γ levels in PBMC cultures in the presence of *Mycobacterium leprae* and *Leishmania braziliensis* whole antigen extracts (Ml and Lb, respectively), concanavalin A (ConA), and neutralizing anti-IL-10 antibody (anti-IL-10), alone or in combination. *C, D,* IL-10 levels in cultures stimulated with Ml alone or with Lb, in the presence or absence of anti-IL-10. *A, C,* During active leprosy; *B, D,* after cure of leprosy. The results shown represent the differences between the levels obtained in stimulated and unstimulated cultures (with medium alone). IFN-γ = interferon-γ; IL-10 = interleukin-10; PBMC = peripheral blood mononuclear cells; ND = not done.
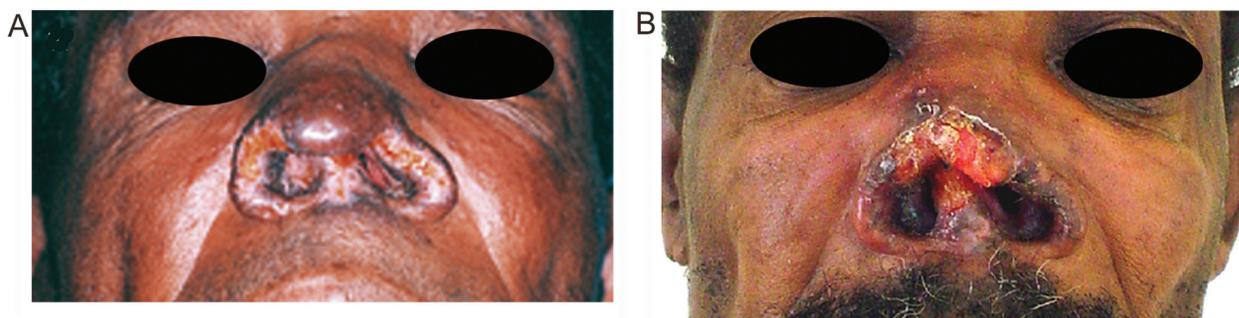
**Figure 2.** Clinical appearance of the leishmaniasis mucosal lesion. *A,* During active leprosy. *B,* Exacerbation after the cure of leprosy.

by worsening of mucosal leishmaniasis lesions. Besides IL-10, other factors and cells such as IL-4, indoleamine 2, 3-dioxygenase and CD8+T cells have also been suggested to participate in the specific T-cell anergy found in patients with lepromatous leprosy (2,12,13). Although we cannot rule out the participation of other suppressive mechanisms that could be absent after the healing of leprosy, it is possible that the exacerbation of mucosal leishmaniasis was responsible for the lack of responsiveness to the IL-10-induced suppression of IFN-γ production. It has been reported that PBMC from mucosal leishmaniasis patients are poorly responsive to IL-10-mediated suppression of IFN-γ production (14). It should be noted that, in contrast to the reactivation of the mucosal lesion seen when the experiments were performed after the cure of leprosy, the patient was clinically cured of mucosal leishmaniasis when the previous experiments were performed before the cure of leprosy.

Taken together, these observations indicate that coinfection with *M. leprae* interfered with the course of mucosal leishmaniasis. Lepromatous leprosy is characterized by the absence of specific cellular immunity to *M. leprae*, resulting in uncontrolled proliferation of bacilli (12). A number of factors have been implicated as causes of the specific T-cell anergy found in patients with lepromatous leprosy, including CD8+ and regulatory T cells, IL-4 and IL-10 (2,3,12,15). However, we have found that neutralization of IL-10 did not increase the production of IFN-γ in *M. leprae* antigen-stimulated cultures of PBMCs from this multibacillary patient, indicating that the lack of IFN-γ production by the patient's T cells was not due to IL-10-mediated suppression. Mucosal leishmaniasis patients have up-regulated IFN-γ and TNF-α secretion and decreased IL-10 secretion when compared to patients with uncomplicated cutaneous leishmaniasis (4). It has been shown that mucosal leishmaniasis lesions possess a higher number of IFN-γ-producing cells compared to cutaneous leishmaniasis lesions. Here and in a previously published article (6) we showed that the IFN-γ response of this patient to *Leishmania* antigens was remarkably high, as expected in mucosal leishmaniasis patients. It was even higher than the response obtained with polyclonal stimulation using ConA (Figure 1A and 1B). This result agrees with the data shown in our previous study with this patient, in which the levels of proinflammatory cytokines (IFN-γ and TNF-α) were higher with *Leishmania* antigen stimulation than with ConA stimulation (6).

Moreover, a decreased *in situ* expression of the IL-10 receptor was proposed as a possible mechanism for the exacerbated inflammatory and cytotoxic responses involved in tissue damage in the mucosal lesions caused by *L. braziliensis* infection (16). IL-10 is a potent inhibitor of the production of cytokines involved in the inflammatory response, such as TNF-α, IL-1, IFN-γ, and IL-12 (17). There is solid evidence that live *L. braziliensis* parasites persist after the clinical cure of American tegumentary leishmaniasis (18). Relapse of leishmaniasis mucosal lesions is frequent (19) and is a challenge for clinicians. Recently, local immunological factors associated with recurrence of mucosal leishmaniasis were described using immunohistochemical analysis of cell phenotypes and cytokines present in the inflammatory infiltrate of mucosal lesions. IFN-γ-producing cells were detected in active lesions and decreased after therapy (19). On the other hand, IL-10 expression was markedly increased in cured patients. These observations suggest that IL-10 plays a role in the control of pathology in mucosal leishmaniasis.

Most reports on immunomodulation during coinfection refer to associated HIV/AIDS infections. It has also been shown that helminth infections can induce immunomodulation and interfere with the course of several infectious diseases (20), including leprosy (21) and leishmaniasis (22). Interestingly, the influence of this association with helminthic infections may either be beneficial or deleterious to the clinical course of protozoan diseases such as malaria (20).

Studies on the association of leprosy and leishmaniasis are rare, and, to our knowledge, in this coinfection, this is the first report of the influence of one infection (with *M. leprae*) on the immune response to the other pathogen (*L. braziliensis*) and on the clinical course of the disease caused by it (mucosal leishmaniasis). Our results indicate that concomitant leprosy induced an IL-10-mediated regulatory response that might have controlled the immunopathology of mucosal leishmaniasis. It should be remembered, however, that this patient had an unusual association of two opposite polar forms of these spectral diseases: lepromatous leprosy (anergic, multibacillary) and mucosal leishmaniasis (hyperergic, pauciparasitic). The study of other cases of association of leprosy and tegumentary leishmaniasis may increase our knowledge about the interference that one infection can have on the immunopathogenesis and clinical course of the other.

## Acknowledgments

## References

1. Akuffo H, Maasho K, Blostedt M, Hojeberg B, Britton S, Bakhiet M. *Leishmania aethiopica* derived from diffuse leishmaniasis patients preferentially induce mRNA for inter-

leukin-10 while those from localized leishmaniasis patients induce interferon-gamma. *J Infect Dis* 1997; 175: 737-741.
2. Sieling PA, Abrams JS, Yamamura M, Salgame P, Bloom

BR, Rea TH, et al. Immunosuppressive roles for IL-10 and IL-4 in human infection. *In vitro* modulation of T cell responses in leprosy. *J Immunol* 1993; 150: 5501-5510.

3. Modlin RL. Th1-Th2 paradigm: insights from leprosy. *J Invest Dermatol* 1994; 102: 828-832.

4. Bacellar O, Lessa H, Schriefer A, Machado P, Ribeiro de JA, Dutra WO, et al. Up-regulation of Th1-type responses in mucosal leishmaniasis patients. *Infect Immun* 2002; 70: 6734-6740.

5. Delobel P, Launois P, Djossou F, Sainte-Marie D, Pradinaud R. American cutaneous leishmaniasis, lepromatous leprosy, and pulmonary tuberculosis coinfection with downregulation of the T-helper 1 cell response. *Clin Infect Dis* 2003; 37: 628-633.

6. Matos DS, Azeredo-Coutinho RB, Schubach A, Conceição-Silva F, Baptista C, Moreira JS, et al. Differential interferon-gamma production characterizes the cytokine responses to *Leishmania* and *Mycobacterium leprae* antigens in concomitant mucocutaneous leishmaniasis and lepromatous leprosy. *Clin Infect Dis* 2005; 40: e5-12.

7. Cupolillo E, Grimaldi G Jr, Momen H. A general classification of New World *Leishmania* using numerical zymotaxonomy. *Am J Trop Med Hyg* 1994; 50: 296-311.

8. Nicolle C. Culture des corps de Leishman isolés de la rate dans trois cas d'anémie splénique infantile. *Bull Soc Pathol Exot* 1908; 1: 121-126.

9. Telino E, De Luca PM, Matos DC, Azeredo-Coutinho RB, Meirelles MN, Conceição-Silva F, et al. *In vitro* responses of human peripheral blood mononuclear cells to whole-cell, particulate and soluble extracts of *Leishmania* promastigotes. *Clin Exp Immunol* 2006; 143: 338-344.

10. Matta NE, Nogueira RS, Franco AM, de Souza E Souza, Mattos MS, Oliveira-Neto MP, et al. *Leishmania* (*Viannia*) *guyanensis* induces low immunologic responsiveness in leishmaniasis patients from an endemic area of the Brazilian Amazon Highland. *Am J Trop Med Hyg* 2009; 80: 339-344.

11. Lima MC, Pereira GM, Rumjanek FD, Gomes HM, Duppre N, Sampaio EP, et al. Immunological cytokine correlates of protective immunity and pathogenesis in leprosy. *Scand J Immunol* 2000; 51: 419-428.

12. Salgame P, Modlin R, Bloom BR. On the mechanism of human T cell suppression. *Int Immunol* 1989; 1: 121-129.

13. de Souza Sales J, Lara FA, Amadeu TP, de Oliveira Fulco T, da Costa Nery JA, Sampaio EP, et al. The role of indoleamine 2,3-dioxygenase in lepromatous leprosy immunosuppression. *Clin Exp Immunol* 2011; 165: 251-263.

14. Carvalho LP, Passos S, Dutra WO, Soto M, Alonso C, Gollob KJ, et al. Effect of LACK and KMP11 on IFN-gamma production by peripheral blood mononuclear cells from cutaneous and mucosal leishmaniasis patients. *Scand J Immunol* 2005; 61: 337-342.

15. Britton WJ, Lockwood DN. Leprosy. *Lancet* 2004; 363: 1209-1219.

16. Faria DR, Gollob KJ, Barbosa J Jr, Schriefer A, Machado PR, Lessa H, et al. Decreased *in situ* expression of interleukin-10 receptor is correlated with the exacerbated inflammatory and cytotoxic responses observed in mucosal leishmaniasis. *Infect Immun* 2005; 73: 7853-7859.

17. D'Andrea A, Aste-Amezaga M, Valiante NM, Ma X, Kubin M, Trinchieri G. Interleukin 10 (IL-10) inhibits human lymphocyte interferon gamma-production by suppressing natural killer cell stimulatory factor/IL-12 synthesis in accessory cells. *J Exp Med* 1993; 178: 1041-1048.

18. Schubach A, Cuzzi-Maya T, Oliveira AV, Sartori A, de Oliveira-Neto MP, Mattos MS, et al. Leishmanial antigens in the diagnosis of active lesions and ancient scars of American tegumentary leishmaniasis patients. *Mem Inst Oswaldo Cruz* 2001; 96: 987-996.

19. Tuon FF, Gomes-Silva A, Da-Cruz AM, Duarte MI, Neto VA, Amato VS. Local immunological factors associated with recurrence of mucosal leishmaniasis. *Clin Immunol* 2008; 128: 442-446.

20. van Riet E., Hartgers FC, Yazdanbakhsh M. Chronic helminth infections induce immunomodulation: consequences and mechanisms. *Immunobiology* 2007; 212: 475-490.

21. Diniz LM, Zandonade E, Dietze R, Pereira FE, Ribeiro-Rodrigues R. Short report: do intestinal nematodes increase the risk for multibacillary leprosy? *Am J Trop Med Hyg* 2001; 65: 852-854.

22. O'Neal SE, Guimarães LH, Machado PR, Alcântara L, Morgan DJ, Passos S, et al. Influence of helminth infections on the clinical course of and immune response to *Leishmania braziliensis* cutaneous leishmaniasis. *J Infect Dis* 2007; 195: 142-148.