Cytokines and visceral leishmaniasis: a comparison of plasma cytokine profiles between the clinical forms of visceral leishmaniasis

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It is not well established whether cytokine production differs in response to different clinical forms of visceral leishmaniasis (VL). In this work, we performed a cross-sectional study to investigate the plasma levels of cytokines [interferon (IFN)-γ, tumour necrosis factor (TNF)-α, interleukin (IL)-2, IL-4, IL-10 and IL-12] involved in the pathogenesis of VL in 80 subjects from VL endemic areas, including subjects with active VL, subjects with asymptomatic infection, subjects with cured VL and uninfected controls. The patients were recruited by sampling from a referral hospital and by random selection from a population-based cohort study. The results showed significant differences in the plasma concentration of all cytokines between the groups ($p < 0.05$). Patients with the active disease had higher plasma levels of IL-10, IL-4, INF-γ and TNF-α relative to the other groups and they produced more IL-12 than asymptomatic and cured subjects. Only the IL-2 concentration was higher in the asymptomatic and cured subjects relative to the patients with active disease ($p < 0.05$). Our results suggest that these cytokines can be used as markers in epidemiological studies conducted in endemic areas to distinguish between different clinical forms of VL. However, their usefulness should be confirmed in investigations conducted in other endemic areas.

Key words: cytokines - visceral leishmaniasis - clinical forms

Visceral leishmaniasis (VL), or kala-azar, is a serious public health problem with worldwide distribution and is among the seven priority endemic diseases in the world. In Latin America, this disease has been described in at least 12 countries. Approximately 90% of the cases occur in Brazil and the disease is endemic in the state of Maranhão (MA) (Michalick & Genaro 2005, Maia-Elkhoury et al. 2008, MS/SVS 2009, WHO 2009).

Infection with *Leishmania chagasi*, the etiological agent of VL, is characterised by a broad spectrum of clinical manifestations that range from asymptomatic infection to discrete (oligosymptomatic), moderate and severe manifestations, which if not treated can result in death. These clinical forms reflect the balance between the multiplication of the parasite in mononuclear phagocytes, the host immune response and the degenerative conditions resulting from the infection (Medeiros et al. 1998, Peruhype-Magalhães et al. 2005, MS/SVS 2009). The factors that determine the development of VL have not been completely identified, but the specific cellular immune response to *Leishmania* seems to play a key role in the control of infection (Holaday 2000, Bacelar & Carvalho 2005, Michalick & Genaro 2005). In experimental mouse models using *Leishmania major*, protection against the disease is attributed to a T helper (Th)1 immune response that is characterised by the production of interferon (IFN)-γ and interleukin (IL)-12, whereas susceptibility to the disease is attributed to a Th2 type response with the production of IL-4 and IL-10 (Park et al. 2000, Belkaid et al. 2002, Stetson et al. 2002, Goto & Prianti 2009). However, this dichotomy is not evident in mice infected with *Leishmania donovani* (Melby et al. 2001, Peruhype-Magalhães et al. 2005). In humans, VL is considered to be the prototype of immune dysfunction, compromising both cellular and humoral immunity. The immunological mechanism involved in the infection and the relationship between the immune system and the clinical forms of the disease have been studied by evaluating cellular profiles and cytokine production in bone marrow, spleen and lymph node aspirates, as well as in peripheral blood mononuclear cells and in plasma samples from patients with different clinical forms of VL. Analysis of plasma samples is a suitable alternative to the other methods because it is simpler and less invasive (Hailu et al. 2004, Peruhype-Magalhães et al. 2005, Nylen & Sacks 2007, Khoshdel et al. 2009).

The plasma concentrations of both IFN-γ and IL-10 are higher in patients with the active disease relative to asymptomatic individuals and are also higher in asymptomatic individuals compared to uninfected individuals (Ansari et al. 2006, Khoshdel et al. 2009). However, the basal levels of these cytokines have been detected in asymptomatic cases from Brazil, a finding that requires

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further investigation (Peruhype-Magalhães et al. 2006). A significant reduction in these cytokines, particularly a decline in IFN-γ, which has been indicated to be a reliable marker of the cured disease, has been observed in patients after treatment of VL (Caldas et al. 2005, Ansari et al. 2006).

Knowledge of the mechanisms involved in the regulation of cytokine production during the various clinical forms of VL infection and after treatment may contribute to a better understanding of the immunological phenomena that occur in the disease. Therefore, the objective of the present study was to compare the plasma cytokine profiles [INF-γ, tumour necrosis factor (TNF)-α, IL-2, IL-4, IL-10 and IL-12] between patients with active VL, asymptomatic individuals, individuals with a history of the disease and uninfected (healthy) controls from endemic areas in the state of MA.

PATIENTS, MATERIALS AND METHODS

A cross-sectional study of subjects from VL endemic areas in MA was conducted between June 2008-2009. The sample consisted of 80 subjects divided into four groups (n = 20): group 1, subjects with active VL (patients), group 2, subjects with asymptomatic infection, group 3, subjects with a history of VL (cured), and group 4, subjects not infected with *L. chagasi* (control).

Patients in group 1 were recruited from a referral hospital and the other three groups (asymptomatic infection, history of VL and control) were randomly selected from a cohort study conducted on the island of São Luís, MA, in 2008. That population-based study involved 365 subjects (relatives and neighbours of 50 cases).

The following clinical and laboratory criteria were used to define the four groups: (i) active VL, subjects presenting clinical and laboratory signs of the disease (MS/SVS 2006) and a positive bone marrow aspirate who were at the beginning of treatment, (ii) asymptomatic, subjects showing no clinical manifestations of the disease, but had a positive Montenegro skin test and anti-*Leishmania* antibodies [enzyme-linked immunosorbent assay (ELISA)], (iii) post-treatment of VL/cured, subjects with confirmation of the disease by a positive bone marrow aspirate (myelogram) who were considered to be clinically cured after treatment (12 months after the end of treatment), and (iv) uninfected (healthy) controls, subjects without clinical manifestations of the disease who had a negative Montenegro skin test and no anti-*Leishmania* antibodies (ELISA). Cytokines were measured by a sandwich ELISA using commercial kits according to the manufacturer's instructions (EBioscience). Absorbance was read at 450 nm using a spectrophotometer and the results are expressed in pg/mL based on a standard curve. The sensitivity of the test was 4 pg/mL for IFN-γ, TNF-α, IL-2 and IL-12 and 2 pg/mL for IL-4 and IL-10.

The statistical analysis was performed using GraphPad Prism software, version 5.0 (GraphPad Software, Inc, 2007). Because the cytokine levels did not show a normal distribution, nonparametric tests were used. The Kruskal-Wallis test was first applied to compare three or more independent samples and the Dunn's post-test was used to compare the mean ranks. Differences were considered to be statistically significant when $p \leq 0.05$.

The study was approved by the Human Research Ethical Committee of the University Hospital, Federal University of Maranhão (protocol 33104-1295/2006).

RESULTS

The study population was mainly composed of female subjects (56.25%) older than 15 years (41.25%) from rural areas (72.5%) (Table).

A significant difference ($p < 0.001$) in cytokine production was observed between the four groups. A comparison of the mean ranks showed higher levels of IFN-γ, TNF-α, IL-4 and IL-10 ($p < 0.05$) in patients with the active disease relative to those in the other groups (Figure). However, the plasma IL-2 levels were higher in the asymptomatic and cured subjects than in the subjects with the active disease ($p < 0.05$). The plasma IL-12 levels were higher in the group with the active disease relative to the asymptomatic and cured subjects ($p < 0.05$), but no significant difference in IL-12 levels was observed when the active disease group was compared to the uninfected controls (Figure).

TABLE

Demographic characteristics of uninfected subjects and subjects with active visceral leishmaniasis (patients), asymptomatic infection and a history of the disease (cured), São Luís, state of Maranhão, 2011

Variable	Uninfected n (%)	Patients n (%)	Asymptomatic n (%)	Cured n (%)	Total n (%)
Gender					
Male	5 (25)	14 (70)	9 (45)	7 (35)	35 (43.7)
Female	15 (75)	6 (30)	11 (55)	13 (65)	45 (56.3)
Age (years)					
≤ 5	6 (30)	6 (30)	2 (10)	18 (90)	32 (32)
5-10	3 (15)	1 (5)	5 (25)	0 (0)	9 (11.3)
10-15	3 (15)	1 (5)	1 (5)	1 (5)	6 (7.5)
≥ 15	8 (40)	12 (60)	12 (60)	1 (5)	33 (41.2)

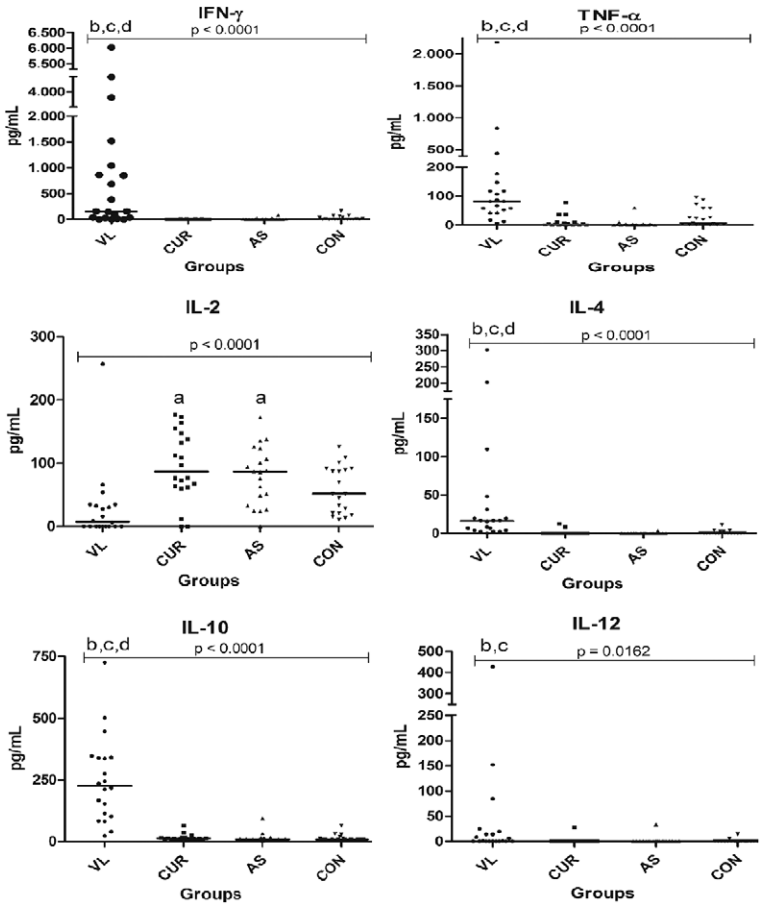
DISCUSSION

Although the majority of the study population were females, most of the subjects in the active VL group were males. Previous studies have indicated that hormonal changes can influence susceptibility to VL, as the disease has the highest prevalence in adolescents after puberty and adult men (Jerônimo et al. 2004). It has been experimentally shown that testosterone increases replication of the parasite (Liu et al. 2006).

In our study, the majority of the subjects were older than 15 years. The second largest demographic population consisted of subjects who were younger than five years old; this young demographic was highly represented in the group with a history of the disease. In contrast to the present findings, national statistical data reveal a predominance of VL in children up to 10 years old (58%).

The plasma concentrations of most of the analysed cytokines (IFN-γ, TNF-α, IL-4 and IL-10) were higher in the subjects with active VL relative to the other groups, demonstrating an exacerbation of the immune response in these patients; this elevation in cytokine levels is in agreement with results from other studies (Hailu et al. 2004, Peruhype-Magalhães et al. 2005, Kurkjian et al. 2006, Khoshdel et al. 2009). Here, a mixed cytokine profile (Th1 and Th2) was detected in the patients with the active disease. This mixed profile has also been reported by other investigators (Goto & Lindoso 2004, Peruhype-Magalhães et al. 2005, Goto & Prianti 2009). The increase in inflammatory cytokines could be associated with the physiopathological alterations seen during the active disease, whereas the production of IL-10 has been suggested to be important for the survival and persistence of the parasite inside macrophages (Peruhype-Magalhães et al. 2006, Nylen & Sacks 2007).

Additionally, higher concentrations of IFN-γ were detected in the patients with the active disease, which is in agreement with results from other studies (Hailu et al. 2004, Caldas et al. 2005, Ansari et al. 2006). IFN-γ is likely produced in lymphoid organs where *Leishmania* proliferates (Goto & Prianti 2009). However, few studies support this hypothesis because it is difficult to obtain tissue samples from patients with the active disease. Several investigators, however, have detected the expression of IFN-γ mRNA in aspirates of bone marrow (Karp et al. 1993), lymph nodes (Ghalib et al. 1993) and spleen (Nylen



Plasma levels of interferon (IFN)-γ, tumour necrosis factor (TNF)-α and interleukin (IL)2, , IL-4, IL-10 and IL-12 in subjects with active visceral leishmaniasis (VL), cured subjects (CUR), asymptomatic subjects (AS) and uninfected controls (CON). Results are expressed as pg/mL. The horizontal line indicates the median. p value was obtained by the Kruskal-Wallis test. The letters (a, b, c, d) indicate significant differences ($p < 0.05$) for VL, CUR, AS and CON, respectively, obtained by Dunn's post-test.

et al. 2007). The simultaneous detection of IFN-γ and IL-10 in the group with active disease indicates a lack of counter-regulation by these cytokines and that IL-10 is the main cytokine involved in the establishment and progression of infection (Caldas et al. 2005, Peruhype-Magalhães et al. 2005, Ansari et al. 2006). In addition, IL-10 protects against the side effects of an exaggerated inflammatory response, playing an important role in the regulation of the inflammatory response (Trinchieri 2007).

The concentration of IL-4, a cytokine that generally plays an immunosuppressive role (Miralles et al. 1994), was higher in patients with the active disease. However, its role in the induction of Th2 responses is has not been resolved. In vitro, when IL-4 is added after cell stimulation, the production of IFN-γ is reduced (Biedermann et al. 2001), whereas the addition of IL-4 to cells before stimulation increases IFN-γ production (Alexander et al. 2000, Biedermann et al. 2001, Peruhype-Magalhães et al. 2005). A synergism between IL-4 and IL-10 has been reported in the literature. High concentrations of these cytokines have been shown to promote the deactivation of macrophage leishmanicidal activity and, consequently, favour the multiplication of the parasite and the development of the disease and are accompanied by the expression of Th2 (Peruhype-Magalhães et al. 2005). In the present study, high levels of IL-4 and IL-10 were observed in patients with the active disease.

Another cytokine found at high concentrations in patients with active VL was TNF-α, which was detected in 100% of these subjects. This cytokine plays an important role in the progression of infectious diseases because it induces the excessive production of nitric oxide. In addition, TNF-α is responsible for the symptoms of the disease, such as fever, anorexia, weight loss, increased energy expenditure and cutaneous and mucosal pallor, and mediates the polyclonal activation of B cells (Engwerda et al. 2004). In contrast, Ansari et al. (2006) found minimal levels of this cytokine during the active disease in Indian patients with VL. In experimental models using *L. donovani* (Tumang et al. 1994), TNF-α is generally increased during the active disease. In addition, this cytokine has been reported to be a reliable marker of VL that has been cured (Barral-Netto et al. 1991), which explains the detection of basal levels of TNF-α in the cured group.

Here, the concentration of IL-12 was higher in patients with the active disease relative to asymptomatic and cured subjects, but not when compared to healthy controls. IL-12, which is produced by cells of the mononuclear phagocyte system, is important for the induction of IFN-γ production because it interacts with natural killer cells and T cells. IL-12 also plays a key role in the initiation and maintenance of Th1 responses and the subsequent production of IFN-γ (Ghalib et al. 1995). However, studies using the sera of patients with VL from Iran (Khoshdel et al. 2009), southeast Ethiopia (Hailu et al. 2005) and Brazil (Caldas et al. 2005) have shown significantly higher levels of IL-12 in patients with the active disease relative to those in asymptomatic and healthy subjects.

In the present study, lower IL-2 levels were observed in patients with active VL relative to asymptomatic and cured subjects. IL-12, together with IFN-γ, plays an important role in the immune response and contributes to parasite death. Its absence may indicate a defect in the immune response (Murray et al. 1993, Bacellar & Carvalho 2005). Basal levels of IL-2 were detected in patients with active VL from India (Ansari et al. 2006) and Brazil (Peruhype-Magalhães et al. 2005), suggesting the absence of effective lymphocyte activation during the active disease.

A comparison of cured and asymptomatic subjects showed no significant difference in the serum levels of the cytokines assayed, which is in agreement with studies conducted in Iran (Khoshdel et al. 2009) and Brazil (Peruhype-Magalhães et al. 2005). These findings indicate that, after the infection is cured, the immune response is effective in controlling the disease, followed by the establishment of protective mechanisms similar to those that occur in asymptomatic individuals (Peruhype-Magalhães et al. 2005).

In conclusion, the production of Th1 and Th2 cytokines is increased in patients with VL during the active disease. Cured subjects have a cytokine profile similar to that seen in subjects with asymptomatic infection, including basal levels of IFN-γ. These findings show that cytokine levels should be evaluated in combination with the clinical and epidemiological characteristics of VL. Taken together, our results suggest that these cytokines can be used as markers in epidemiological studies conducted in endemic areas to distinguish between the different clinical forms of VL. However, their usefulness should be confirmed in investigations conducted in other endemic areas.

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REFERENCES

Alexander J, Carter KC, Al-Fasi N, Satoskar A, Brombacher F 2000. Endogenous IL-4 is necessary for effective drug therapy against visceral leishmaniasis. *Eur J Immunol 30*: 293-243.

Ansari NA, Saluja S, Salotra P 2006. Elevated levels of interferon-gamma, interleukin-10 and interleukin-6 during active disease in Indian kala-azar. *Clin Immunol 119*: 339-345.

Bacellar O, Carvalho E 2005. Imunopatogênese da leishmaniose visceral. *Gaz Med Bahia 75*: 24-34.

Barral-Netto M, Badaró R, Barral A, Almeida RP, Santos SB, Badaró F, Pedral-Sampaio D, Carvalho EM, Falcoff E, Falcoff R 1991. Tumor necrosis factor/cachectin in human visceral leishmaniasis. *J Infect Dis 163*: 853-857.

Belkaid Y, Von Stebut E, Mendez S, Lira R, Caler E, Bertholet S, Udey MC, Sacks D 2002. $CD8^+$ T cells are required for primary immunity in C57BL/c mice following low-dose, intradermal challenge with *Leishmania major*. *J Immunol 168*: 3992-4000.

Biedermann T, Zimmermann S, Himmelrich H, Gumy A, Egeter O, Sakrauski AK, Seegmüller I, Voigt H, Launois P, Levine AD, Wagner H, Heeg K, Louis JA, Röcken M 2001. IL-4 instructs Th1 responses and resistance to *Leishmania major* in susceptible BALB/c mice. *Nat Immunol 2*: 1054-1060.

Caldas A, Favali C, Aquino D, Vinhas V, Van Weyenbergh J, Brodskyn C, Costa J, Barral-Netto M, Barral A 2005. Balance of IL-10 and interferon-gamma plasma levels in human visceral leishmaniasis: implications in the pathogenesis. *BMC Infect Dis 5*: 113.

Engwerda CR, Ato M, Stager S, Alexander CE, Stanley AC, Kaye PM 2004. Distinct roles for lymphotoxin-alpha and tumor necrosis factor in the control of *Leishmania donovani* infection. *Am J Pathol 165*: 2123-2133.

Ghalib HW, Piuvezam MR, Skeiky YA, Siddig M, Hashim FA, El-Hassan AM, Russo DM, Reed SG 1993. Interleukin-10 production correlates with pathology in human *Leishmania donovani* infections. *J Clin Invest 92*: 324-329.

Ghalib HW, Whittle JA, Kubi M, Hashim FA, El-Hassan AM, Grabstein KH, Trinchieri G, Reed SG 1995. IL-12 enhances Th1-type responses in human *Leishmania donovani* infections. *J Immunol 154*: 4623-4629.

Goto H, Lindoso JA 2004. Immunity and immunesuppression in experimental visceral leishmaniasis. *Braz J Med Biol Res 37*: 615-623.

Goto H, Prianti MG 2009. Immunoactivation and immunopathogeny during active visceral leishmaniasis. *Rev Inst Med Trop Sao Paulo 51*: 241-246.

Hailu A, Van Baarle D, Knol GJ, Berhe N, Miedem F, Kager PA 2005. T cell subset and cytokine profiles in human visceral leishmaniasis during active and asymptomatic or sub-clinical infection with *Leishmania donovani*. *Clin Immunol 117*: 182-191.

Hailu A, Van Der Poll T, Berhe N, Kager PA 2004. Elevated plasma levels of interferon (IFN)-gamma, IFN-gamma inducing cytokines and IFN-gamma inducible CXC chemokines in visceral leishmaniasis. *Am J Trop Med Hyg 71*: 561-567.

Holaday BJ 2000. Role of $CD8^+$ cells in endogenous interleukin-10 secretion associated with visceral leishmaniasis. *Mem Inst Oswaldo Cruz 95*: 217-220.

Jerônimo SM, Duggal P, Braz RF, Cheng C, Monteiro GR, Nascimento ET, Martins DR, Karplus TM, Ximenes MF, Oliveira CC, Pinheiro VG, Pereira W, Peralta JM, Sousa J, Medeiros IM, Pearsoni RD, Burns TL, Pugh EW, Wilson ME 2004. An emerging peri-urban pattern of infection with *Leishmania chagasi*, the protozoan causing visceral leishmaniasis in Northeast Brazil. *Scand J Infect Dis 36*: 443-449.

Karp CL, El-Safi SH, Wynn TA, Satti MM, Kordofani AM, Hashim FA, Hag-Ali M, Neva FA, Nutman TB, Sacks DL 1993. *In vivo* cytokine profiles in patients with kala-azar. Marked elevation of both interleukin-10 and interferon-gamma. *J Clin Invest 91*: 1644-1648.

Khoshdel A, Alborzi A, Rosouli M, Taheri E, Kiany S, Javadian MH 2009. Increased levels of IL-10, IL-12 and IFN-γ in patients with visceral leishmaniasis. *BJID 13*: 44-46.

Kurkjian KM, Mahmutovic AJ, Kellar KL, Haque R, Bern C, Secor WE 2006. Multiplex analysis of circulating cytokines in the sera of patients with different clinical forms of visceral leishmaniasis. *Cytometry A 69*: 353-358.

Liu L, Wang L, Zhao Y, Wang Y, Wang Z, Qiao Z 2006. Testosterone attenuates p38 MAPK pathway during *Leishmania donovani* infection of macrophages. *Parasitol Res 99*: 18-193.

Maia-Elkhoury ANS, Alves WA, Sousa-Gomes ML, Sena JM, Luna EA 2008. Visceral leishmaniasis in Brazil: trends and challenges. *Cad Saude Publica 24*: 2941-2947.

Medeiros IM, Castelo A, Salomão R 1998. Presence of circulating levels of interferon-γ, interleukin-10 and tumor necrosis factor-α in patients with visceral leishmaniasis. *Rev Inst Med Trop Sao Paulo 40*: 31-34.

Melby PC, Chandrasekar B, Zhao W, Coe JE 2001. The hamster as a model of human visceral leishmaniasis: progressive disease and impaired generation of nitric oxide in the face of a prominent Th1-like cytokine response. *J Immunol 166*: 1912-1920.

Michalick MSM, Genaro O 2005. Leishmaniose visceral americana. In D Neves, AL Melo, PM Linardi, RWA Vitor, *Parasitologia humana*, 11th ed., Atheneu, São Paulo, p. 56-72.

Miralles GD, Stoeckle MY, McDermott DF, Finkelman FD, Murray HW 1994. Th1 and Th2 cell-associated cytokines in experimental visceral leishmaniasis. *Infect Immun 62*: 1058-1063.

MS/SVS - Ministério da Saúde/Secretaria de Vigilância em Saúde 2006. Manual de vigilância e controle da leishmaniose visceral. Available from: portal.saude.gov.br/portal/arquivos/pdf/manual_leish_visceral2006.pdf.

MS/SVS - Ministério da Saúde/Secretaria de Vigilância em Saúde 2009. Guia de vigilância em saúde (leishmaniose visceral). [cited 2009 Nov 11]. Available from: portal.saude.gov.br/portal/arquivos/pdf/gve_7ed_web_atual_lv.pdf.

Murray HW, Miralles GD, Stoeckle MY, McDermott DF 1993. Role and effect of IL-2 in experimental visceral leishmaniasis. *J Immunol 151*: 929-938.

Nylen S, Maurya R, Eidsmo L, Manandhar KD, Sundar S, Sacks D 2007. Splenic accumulation of IL-10 mRNA in T cells distinct from $CD4^+CD25^+$ (Foxp3) regulatory T cells in human visceral leishmaniasis. *J Exp Med 204*: 805-817.

Nylen S, Sacks D 2007. Interleukin-10 and the pathogenesis of human visceral leishmaniasis. *Trends Immunol 28*: 378-384.

Park AY, Hondowicz BD, Scott P 2000. IL-4 is required to maintain a Th1 response during *Leishmania major* infection. *J Immunol 165*: 896-902.

Peruhype-Magalhães V, Martins-Filho OA, Prataz A, Silva LA, Rabello A, Teixeira-Carvalho A, Figueiredo RM, Guimarães-Carvalho SF, Ferrari TCA, Correa-Oliveira R 2005. Immune response in human visceral leishmaniasis: analysis of the correlation between innate immunity cytokine profile and disease outcome. *Scand J Immunol 62*: 487-495.

Peruhype-Magalhães V, Martins-Filho OA, Prataz A, Silva LA, Rabello A, Teixeira-Carvalho A, Figueiredo RM, Guimarães-Carvalho SF, Ferrari TCA, Van Weyenbergh J, Correa-Oliveira R 2006. Mixed inflammatory/regulatory cytokine profile marked by simultaneous raise of interferon-gamma and interleukin-10 and low frequency of tumour necrosis factor-alpha(+) monocytes are hallmarks of active human visceral leishmaniasis due to *Leishmania chagasi* infection. *Clin Exp Immunol 146*: 124-132.

Stetson BD, Mohrs M, Mattlet-Designe V, Teyton L, Locksley RM 2002. Rapid expansion and IL-4 expression by *Leishmania*-specific naïve help T cells *in vivo*. *Immunity 17*: 191-200.

Trinchieri G 2007. Interleukin-10 production by effector T cells: Th1 cells show self control. *J Exp Med 204*: 239-243.

Tumang MCT, Keogh C, Moldawer LL 1994. Role and effect of TNF-α in experimental visceral leishmaniasis. *J Immunol 153*: 768-775.

WHO - World Health Organization 2009. *Leishmaniasis*: magnitude of the problem. [cited 2009 Dec 15]. Available from: who.int/leishmaniasis/burden/magnitude/burden_magnitude/en/index.html.