Available online at www.sciencedirect.com
ScienceDirect
Veterinary Immunology and Immunopathology 115 (2007) 135–145
Veterinary immunology and immunopathology
www.elsevier.com/locate/vetimm

Analysis of the cytokine profile in spleen cells from dogs naturally infected by *Leishmania chagasi*

R.S. Lage [a], G.C. Oliveira [b,d,e], S.U. Busek [a,d], L.L. Guerra [a], R.C. Giunchetti [a,b], R. Corrêa-Oliveira [a], A.B. Reis [a,b,c,*]

[a] *Laboratório de Imunologia Celular e Molecular, Centro de Pesquisas René Rachou, Fundação Oswaldo Cruz, Belo Horizonte, Minas Gerais, Brazil*
[b] *Laboratório de Imunopatologia, Núcleo de Pesquisas em Ciências Biológicas/NUPEB, Instituto de Ciências Exatas e Biológicas, Universidade Federal de Ouro Preto, Ouro Preto, Minas Gerais, Brazil*
[c] *Departamento de Análises Clínicas, Escola de Farmácia, Universidade Federal de Ouro Preto, Ouro Preto, Minas Gerais, Brazil*
[d] *Laboratório de Parasitologia Celular e Molecular, Centro de Pesquisas René Rachou, Fundação Oswaldo Cruz, Belo Horizonte, Minas Gerais, Brazil*
[e] *Programa de Pós-Graduação e Pesquisa, Santa Casa de Belo Horizonte, Minas Gerais, Brazil*

Received 12 July 2006; received in revised form 22 August 2006; accepted 4 October 2006

Abstract

Recent studies suggest that asymptomatic dogs infected with canine visceral leishmaniasis (CVL) develop a Th1 immunological profile whilst oligosymptomatic and symptomatic CVL-infected animals present a Th2 profile. In the present study, an RT-PCR method has been standardised and employed to evaluate the frequency and the semi-quantitative level of expression of the cytokines IL-4, IL-10, IL-12, INF-γ and TNF-α in splenocytes of 30 dogs naturally infected with *Leishmania chagasi* and of 7 non-infected dogs (NID). An increase in the level of expression of IL-12 ($p = 0.059$) was detected in all CVL-infected dogs compared with NID. In dogs exhibiting high parasitism, the frequency of expression of IL-10 was higher ($p = 0.011$) than in animals presenting low parasitism or medium parasitism (MP) and in NID animals, whilst the level of expression of IL-10 was higher ($p = 0.0094$) than in animals exhibiting MP and in the NID group. Positive correlations between the levels of expression of IL-10 with respect to the progression of the disease (IL-10: $r = 0.3510$; $p = 0.0337$) and the levels of expression of IL-10 and INF-γ increase in parasitism (IL-10: $r = 0.3428$; $p = 0.0438$ and INF-γ: $r = 0.4690$; $p = 0.0045$) were observed. Such data suggest that CVL is marked by a balanced production of Th1 and Th2 cytokines, with a predominant accumulation of IL-10 as a consequence of an increase in parasitic load and progression of the disease, and INF-γ was related with the increase in parasitic load.

Keywords: Cytokines; RT-PCR; Canine diseases; Visceral leishmaniasis; *Leishmania chagasi*

1. Introduction

Leishmaniasis, which comprises a complex of diseases exhibiting significant clinical and epidemiological diversity, continues to represent a severe threat to public health. Whilst the prevalence of leishmaniasis is increasing significantly, the development of appropriate measures for its control is far from complete and

* Corresponding author at: Laboratório de Imunopatologia, Núcleo de Pesquisa em Ciências Biológicas, ICEB II, Morro do Cruzeiro, Universidade Federal de Ouro Preto, Ouro Preto, MG, CEP 35400-000, Brazil. Tel.: +55 21 31 3559 1694; fax: +55 21 31 3559 1680.
E-mail address: alexreis@nupeb.ufop.br (A.B. Reis).

0165-2427/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.vetimm.2006.10.001

remains dependent on further research in order to obtain better tools and a more cost-effective strategy for case management and vector control.

Visceral leishmaniasis (VL), which is caused by *Leishmania* (*Leishmania*) *infantum* [syn. *Leishmania* (*Leishmania*) *chagasi*], is endemic in over 88 countries within Europe and Latin America, and is transmitted by the bite of the female sand fly (phlebotomine) (Desjeux, 2001, 2004). Canine visceral leishmaniasis (CVL) is one of the most important emerging diseases with a high prevalence in Latin American countries. The main prophylactic procedures recommended by the World Health Organisation for the control of CVL include systematic treatment of human cases, vector control through the use of insecticide, and elimination of the domestic reservoir (Tesh, 1995).

Seropositive infected dogs are the zoonotic reservoir for parasite transmission in CVL. Following transmission, the parasites initially multiply in macrophages in the skin at the site of infection. From such a localised cutaneous infection, the parasite can be disseminated via lymphatic or blood vessels, infecting macrophages of the bone marrow, lymph node, liver and spleen, as well as the kidneys and gastrointestinal tract of the dog (Reis et al., 2006a).

The major signs of CVL include hepatosplenomegaly, lymphadenopathy, cutaneous lesions, keratoconjunctivitis, opaque bristles, alopecia, apathy, onychogryphosis, anorexia and severe weight loss (Reis et al., 2006a). According to Mancianti et al. (1988), CVL can be categorised into three distinct clinical forms on the basis of the major features observed in infected dogs. Asymptomatic (AD) animals show no indicative signs of the disease, those presenting a maximum of three clinical indications, including opaque bristles and/or localised alopecia and/or moderate weight loss, are classified as oligosymptomatic (OD), whilst animals exhibiting the most severe clinical signs of CVL are considered symptomatic (SD). Several epidemiological studies have indicated that about 50% of seropositive dogs are asymptomatic (Acedo-Sanchez et al., 1998; Fisa et al., 1999), although these animals are potentially infectious to sand flies (Molina et al., 1994; Giunchetti et al., 2006).

Dogs infected with the CVL parasite present high titres of anti-*Leishmania* antibodies, with a predominant IgG1 response (El Amin et al., 1986) and a depression of the T cell-mediated response (Pinelli et al., 1994; Martinez-Moreno et al., 1995; De Luna et al., 1999; Campino and Abranches, 2002). However, the nature of the cellular immune response in dogs is not fully understood. Studies using peripheral blood mononuclear cells (PBMCs) derived from experimentally infected dogs suggest an association between Th1 immune response and resistance to CVL (Pinelli et al., 1994; Santos-Gomes et al., 2002). On the other hand, a clear association between Th1 immune response, and humoral and cellular responses during the progression of the illness could not be demonstrated in a similar study using bone marrow aspirate from dogs infected with *L. chagasi* (Quinnell et al., 2001). Such studies, however, reveal only a partial description of the cell-mediated immunity and do not necessarily reflect that observed in the affected organs.

Canine experimental models are often used in order to investigate the immune mechanisms involved in diseases such as leishmaniasis and Chagas. Moreover, by virtue of the similarities between CVL and the human disease, the animal model has been of great value in the clinical testing of new drugs and vaccines (Cobbold and Metcalfe, 1994). However, the availability of tools and reagents for use in the study of the immunopathological mechanisms in this experimental model is still somewhat limited.

In the present paper, we describe the application of a cytokine-specific RT-PCR method in the determination of the cytokine profile in parasite target organ, such as the spleen, of dogs infected with *L. chagasi*. A balanced production of cytokine of Th1 and Th2 was revealed in splenocytes of infected dogs, with a predominant accumulation of mRNA for IL-10 and INF-γ that was related to the parasitic load and to clinical progression. The results obtained will permit a better understanding of the immune response in organs affected by CVL infection.

2. Materials and methods

Details of the proposed study were presented to and approved by the Ethical Committee for the Use of Experimental Animals (CETEA) of the Universidade Federal de Minas Gerais (UFMG, Belo Horizonte, Minas Gerais, Brazil).

2.1. Animals

The 37 dogs included in the study were selected from a population of stray or domiciled mongrel animals that had been captured by the Zoonosis Control Center in Belo Horizonte (Minas Gerais, Brazil) and confined under quarantine at the kennels of the Institute of Biological Sciences, UFMG. Prior to the commencement of the study, selected animals were treated for intestinal helminthic infections (Endal Plus®, Schering-Plough Coopers, São Paulo, SP, Brazil) and immunised

against parvovirosis, leptospirosis, distemper, parainfluenza and hepatitis (Vanguard® HTLP 5CV-L vaccine, Pfizer, New York, NY, USA). During confinement, all animals received drinking water and a balanced feed *ad lib.* After the quarantine period, blood samples were collected using 10 mL disposable sterile syringes, and 5 mL samples were transferred, in the absence of anticoagulant, to specimen tubes. Serum samples were stored in appropriate aliquots at −20° C until required for use in the serological tests.

2.2. Serological diagnosis of CVL

Diagnosis of CVL was carried out by assay for specific anti-*Leishmania* IgG reactivity using the indirect immunofluorescence antibody test (IFAT), which is considered to be the ''gold-standard'' diagnostic tool. Parasites, *L. amazonensis* (MHOM/BR/1960/BH6), were maintained in logarithmic growth in liver infusion tryptose (LIT) medium (Mancianti et al., 1988). Serum samples were added to slides coated with fixed promastigote forms of the parasite, and fluorescent-conjugated anti-dog IgG antibodies (Biomanguinhos-Fiocruz, Rio de Janeiro, Brazil) were used to reveal IgG reactivity. Observations were made using a microscope employing blue and ultraviolet incident light. Samples presenting fluorescence at dilutions ≥1:40 were considered positive. Positive and negative sera were assayed concomitantly for control purposes.

2.3. Determination of parasite load index

Seropositive dogs were submitted to parasitological examination for *Leishmania* and subsequently classified according to parasitic load. Euthanasia was performed by intravenous overdose of barbiturate (Thiopental®): following necropsy, fragments of spleen were collected and imprints prepared on two microscope slides. Slides were air-dried, fixed in methanol, stained with Giemsa, and examined under an optical microscopy in order to detect the amastigote forms of *Leishmania*. Parasite densities were determined according to Stauber (1955), and expressed as Leishman Donovan Units (LDU index), which correspond to the number of *Leishmania* amastigotes per 1000 nucleated cells. Parasitic loads were classified as low (LP; low parasitism) when LDU values were between 0 and 10, medium (MP) for LDU values 11–250, and high for LDU values ≥251. For control purposes, seven dogs who were shown to be non-infected with CVL according to the IFAT assay were also submitted to parasitological examination.

2.4. RT-PCR analyses

Total RNA was extracted from splenocytes obtained from the study dogs using Tri reagent (Sigma), and subsequently treated with deoxyribonuclease I (Invitrogen Brasil) and reverse-transcribed using the SuperScript™ II pre-amplification system with oligo(dT)$_{12–18}$ as primer (Invitrogen Brasil), all according to the manufacturer's instructions. Diluted (10×) canine cDNA (2 μL), obtained from 2.0 μg of total mRNA, was amplified by PCR in a reaction mixture containing 2 μL of 10× Taq buffer (Invitrogen Brasil, São Paulo, Brazil), 2.5 mM $MgCl_2$, 0.5 mM dNTP (Invitrogen Brasil), 0.5 pmol of specific primers, 0.16 U/μL of Taq DNA polymerase (Invitrogen Brasil), and DEPC-treated water to a final volume of 20 μL. Primers were adapted from published sources (Gröne et al., 1998) using canine sequences from GenBank with the accession numbers: IL-4, AF239917; IL-10, U33843; IL-12, U49100; TNF-α, S74068; INF-γ, AF126247; and β-actin, Z70044. The forward and reverse primers, respectively, were as follows: IL-4, CACTCACCAGCACCTTTGTCCACGG and TTGCCATGCTGCTGAGGTTCCTGTA; IL-10, CCCCGGGCTGAGAAACCACGAC and TGCGCTCTTCACCTGCTCCACCG; IL-12, CTCAGCAGTTGGTCATCTCC and CACTGCCTTCCTGACACTCC; TNF-α, CCAAGTGACAAGCCAGTAGC and TCTTGATGGCAGAGAGTAGG; INF-γ, CCAGATGTATCGGACGGTGG and TTATCGCCTTGCGCTGGACC; and β-actin, GACCCTGAAGTACCCCATTGAG and TTGTAGAAGGTGTGGTGCCAGAT. PCR amplification was performed in an MJ Research model PTC-100 thermocycler over 40 cycles (45 cycles for IL-4) each consisting of 1 min at 94 °C, 2 min at 56 °C for IL-4, INF-γ and TNF-α (59.5 °C for β-actin, IL-10 and IL-12), and 1 min at 72 °C, followed by a final extension at 72 °C for 5 min. Amplified fragments were analysed by electrophoresis on 6% polyacrylamide gel containing 0.5 μg/mL ethidium bromide (Sigma) (Fig. 1B). A set of primers corresponding to constitutive canine β-actin was used as positive controls and to adjust the efficiency of the cDNA synthesis.

In order to semi-quantify the intensity of the ethidium bromide signals, the gel images were captured using an Eagle Eye II (Stratagene, Cedar Creek, TX, USA) gel imaging system and analysed using a Stratagene image analysis software package with integrated density programme (Zero-Dscan version 1.3). The results obtained were expressed as [cytokine area (pixels)/β-actin area (pixels)] × 1000, and designated semi-quantitative levels of expression of cytokine.

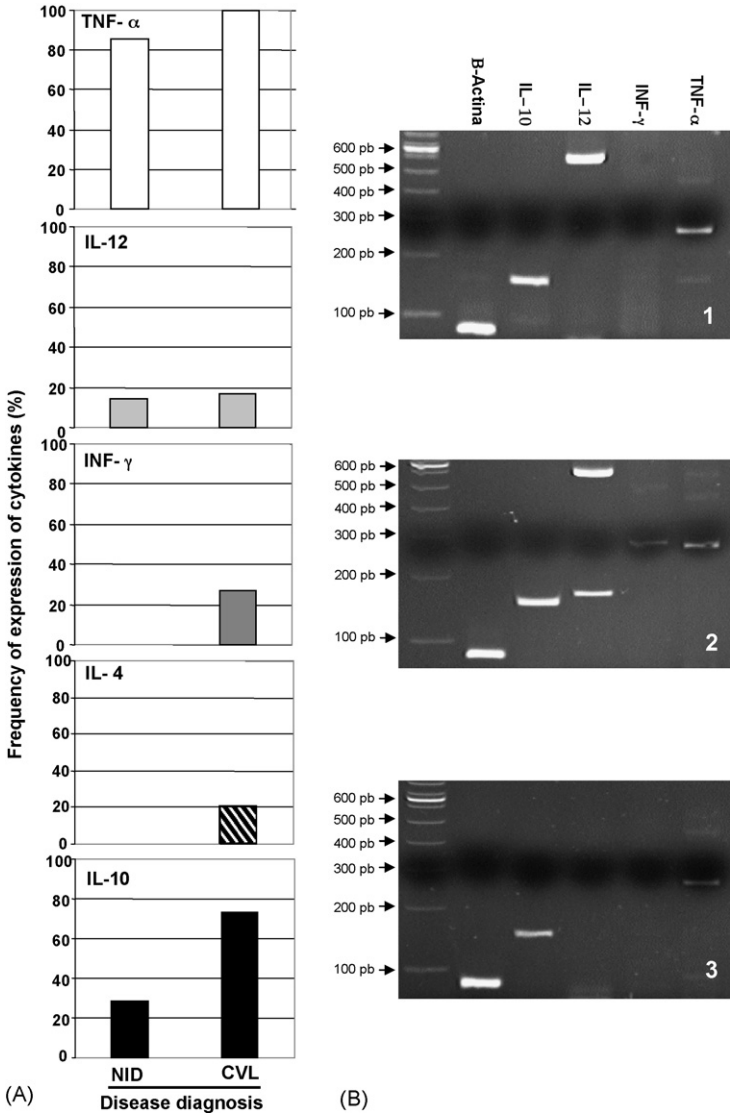

Fig. 1. (A) Frequency of expression (% of animals testing positive in each category) of canine cytokines in splenocytes from 30 dogs naturally infected by *Leishmania chagasi* (CVL-infected animals were IFAT+, ELISA+ and ELISA rK39+) and from 7 non-infected dogs (NID animals were IFAT, ELISA and with negative parasitological examination). Key to cytokines: TNF-α (□); IL-12 (▒); IFN-γ (▓); IL-4 (▨); IL-10 (■). (B) Electropherograms of RT-PCR products from total mRNA obtained from splenocytes of dogs naturally infected with *Leishmania chagasi* showing the presence of mRNA for IL-10, IL-12, IFN-γ and TNF-α, with β-actin as positive control.

2.5. Statistical analysis

Statistical analyses were performed using Minitab 9.2 (Minitab Inc., State College, PA, USA) and Prisma 3.0 software packages (Prism software, Irvine, CA, USA). The χ^2 test was used to determine the frequency of cytokine expressing cells. The Wilcoxon Signed Rank and the Mann–Whitney tests were used, respectively, to analyse cytokines IL-12 and IFN-γ, and IL-10 and TGF-β with respect to disease diagnosis, whilst the Kruskal–Wallis test was used to analyse cytokines in relation to parasite load and clinical status. Correlations between clinical status, parasite load and levels of IL-10 and IFN-γ were evaluated using the Spearman rank correlation test (r coefficient). In all cases, differences were considered significant for p-values ≤ 0.05.

3. Results

Thirty of the study dogs exhibited anti-*Leishmania* IgG antibody titres higher than 1:40 and were considered

Table 1
Semi-quantitative determination by RT-PCR of the levels of expression of canine cytokines in splenocytes of non-infected dogs (NID) and animals naturally infected by *Leishmania chagasi* (CVL-infected dogs tested positive in IFAT, ELISA and ELISA rK39 assays)

Groups	Level of expression of cytokines[a]			
	IL-10	IL-12	INF-γ	TNF-α
NID	0[a] (0–21[b])	0 (0–0)	0 (0–0)	42 (26–69)
CVL	27 (0–455)	0 (0–959)*	0 (0–40)*	44 (12–66)

[a] Median ratio of the expression levels of mRNA as determined from [cytokine area (pixels)/β-actin area (pixels)] × 1000: values labelled * are significantly different at $p \leq 0.05$.
[b] 25th–75th percentiles.

seropositive for CVL. Infection with *L. chagasi* was confirmed in all IFAT-positive dogs by at least one additional serological test, including ELISA-extract and ELISA rK39 assays previously described (Reis et al., 2006a), and/or by parasitological examination. CVL-infected dogs were clinically classified, according to the presence/absence of clinical signs (Mancianti et al., 1988), into groups AD ($n = 8$), OD ($n = 10$), and SD ($n = 12$). Seven non-infected dogs (NID), each of whom exhibited negative IFAT results at 1:40 dilution and presented negative results in the parasitological tests for *Leishmania*, were included as a control group.

RT-PCR analysis of splenocytes of the study animals was employed in order to determine the frequency and the level of expression of the cytokines IL-4, IL-10, IL-12, TNF-α and IFN-γ, as well as β-actin, in non-infected dogs and those naturally infected with *L. chagasi*. The frequency of expression of a cytokine was determined as the percentage of dogs in each group, classified according to the disease diagnosis, clinical status and parasitic load, expressing the cytokine. With respect to the NID group, the splenocytes showed expression of IL-12, IL-10, and TNF-α (Fig. 1A). None of the animals of this group exhibited expression of IL-4

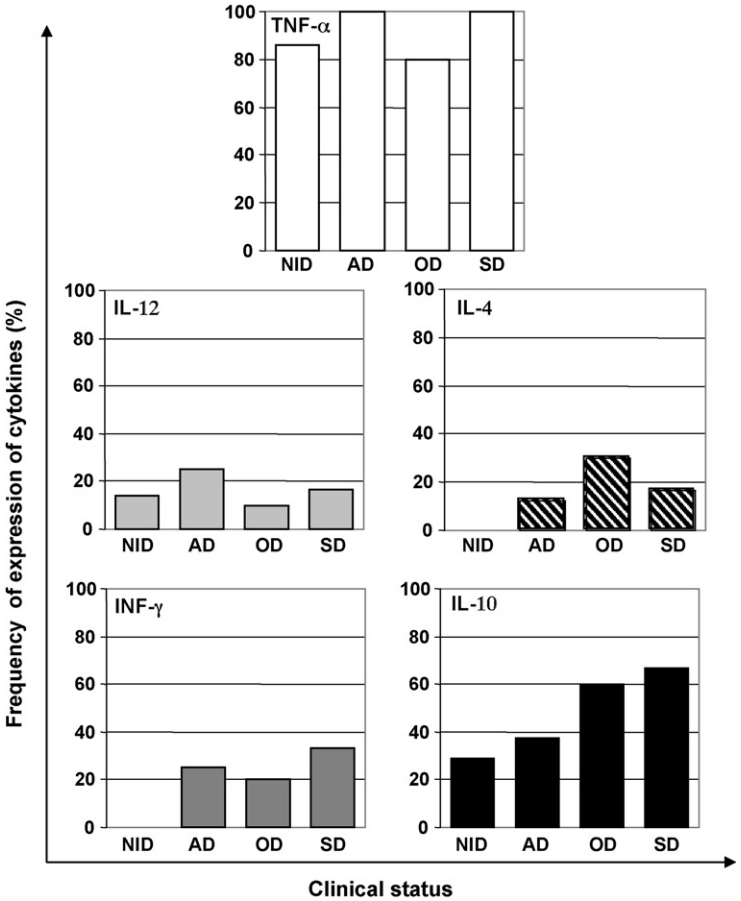

Fig. 2. Frequency of expression (% of animals testing positive in each category) of canine cytokines in splenocytes from 7 non-infected dogs (NID) and 30 dogs naturally infected by *Leishmania chagasi* and classified according to their clinical status as asymptomatic (AD, $n = 8$), oligosymptomatic (OD, $n = 10$) and symptomatic (SD, $n = 12$). Key to cytokines: TNF-α (□); IL-12 (▒); IFN-γ (▓); IL-4 (▨); IL-10 (■).

or IFN-γ. On the other hand, in CVL-infected dogs ($n = 30$), 6 (20.0%) animals showed expression of IL-4, and 8 (26.6%) of IFN-γ, whilst 5 animals (16.6%) exhibited expression of IL-12, 22 (73.3%) of IL-10, and 30 (100%) of TNF-α. However, statistical analysis did not reveal any significant differences ($p \leq 0.05$) in the frequencies of cytokine expression between the infected and the non-infected groups (Fig. 1A). The levels of cytokine expression, as determined semi-quantitatively by RT-PCR, in splenocytes of infected dogs were higher for IFN-γ ($p = 0.014$) and IL-12 ($p = 0.059$) than in splenocytes of the NID group. The corresponding differences between the two groups with respect to the expression of IL-10 ($p = 0.117$) and TNF-α ($p > 0.05$) were not, however, statistically significant (Table 1).

Fig. 2 shows the frequency of cytokine expression in splenocytes of CVL-infected dogs distributed according to the clinical status of the animals. In the AD group, expression of IL-4, IL-10, IL-12, IFN-γ and TNF-α was observed, respectively, in one (12.5%), three (37.5%), two (25.0%), two (25.0%) and eight (100%) of the animals. Within the OD group, positive expression of IL-4, IL-12 and IFN-γ occurred in less than 30% of the animals, whilst more than 59% of the dogs showed expression of IL-10 and TNF-α. In the SD group, expression of IL-4, IL-10, IL-12, IFN-γ and TNF-α was observed, respectively, in 2 (16.7%), 8 (66.7%), 2 (16.7%), 4 (33.3%) and 12 (100%) of the animals.

The levels of expression of selected cytokines, as determined semi-quantitatively by RT-PCR analysis of splenocytes of infected dogs, are presented in Table 2 according to the clinical status and parasitic load of the study animals. No statistically significant differences were observed between clinical groups with respect to IL-10 ($p = 0.321$), IL-12 ($p = 0.428$), IFN-γ ($p = 0.547$) and TNF-α ($p > 0.05$). However, there was a statistically significant increase in the accumulation of mRNA for IL-10 in dogs showing high LDU values (HP group) compared with the NID and MP groups ($p = 0.0094$). Moreover, when the frequency of expression of the cytokines was distributed according to parasitic load (Fig. 3), it was observed that expression of IL-10 mRNA occurred in 100% of animals in the HP group, a value that was significantly different from those determined in the NID, LP and MP groups ($p = 0.011$).

The results of an analysis on the correlations between semi-quantitative levels of expression of cytokines in splenocytes from CVL-infected dogs, clinical status and parasitic load are shown in Table 3. A positive correlation between the clinical status and levels of expression of IL-10 ($r = 0.3501$; $p = 0.0337$) was revealed. Furthermore, there was a positive correlation between the levels of expression of IL-10 ($r = 0.3428$; $p = 0.0438$) and IFN-γ ($r = 0.4690$; $p = 0.0045$) and parasitic load.

Table 2
Semi-quantitative determination by RT-PCR of the levels of expression of canine cytokines in splenocytes of non-infected dogs (NID) and of CVL-infected animals classified according to clinical status as asymptomatic (AD), oligosymptomatic (OD) and symptomatic (SD), and according to parasitic load as presenting low parasitism (LP; LDU = 1–10), medium parasitism (MP; LDU = 11–250) and high parasitism (HP; LDU ≥ 251)

Groups	Level of expression of cytokines[a]			
	IL-10	IL-12	INF-γ	TNF-α
NID	0[a] (0–21[b])[c]	0 (0–0)	0 (0–0)	42 (26–69)
Clinical status				
AD	0 (0–370)	0 (0–28)	0 (0–1)	48 (26–70)
OD	14 (0–332)	0 (0–0)	0 (0–0)	24 (6–47)
SD	116 (0–934)	0 (0–0)	0 (0–15)	48 (0–66)
Parasitic load				
LP	9[a] (0–221[b])	0 (0–0)	0 (0–0)	41 (32–59)
MP	0 (0–37)[c]	0 (0–0)	0 (0–0)	33 (7–61)
HP	391 (251–934)	0 (0–37)	0 (0–24)	48 (29–79)

[a] Median ratio of the expression levels of mRNA as determined from [cytokine area (pixels)/β-actin area (pixels)] × 1000.
[b] 25th–75th percentiles.
[c] Values statistically different ($p \leq 0.05$) from that of the HP group.

4. Discussion

The clinical manifestations that develop following infection with *Leishmania* are a consequence of complex interactions between the parasite and the immune response of the host. In this context, CVL presents a spectrum of clinical forms that have been shown to be correlated with immune mechanisms dependent on T cell and macrophage activities, and also on cytokine balance (Santos-Gomes et al., 2002). Whilst the expression of cytokines has been evaluated in humans, hamsters and several strains of mice (Awasthi et al., 2004), the cytokine response in CVL has received very little attention. Apart from one investigation that involved bone marrow (Quinnell et al., 2001), previous studies have been concerned primarily with cytokine expression in PBMCs (Pinelli et al., 1994, 1999; Chamizo et al., 2001, 2005; Santos-Gomes et al., 2002). Thus, whilst the spleen is responsible for the major immune response in leishmaniasis, and is also an important infection target in VL, our present knowledge of the cellular response in this immune compartment is very limited.

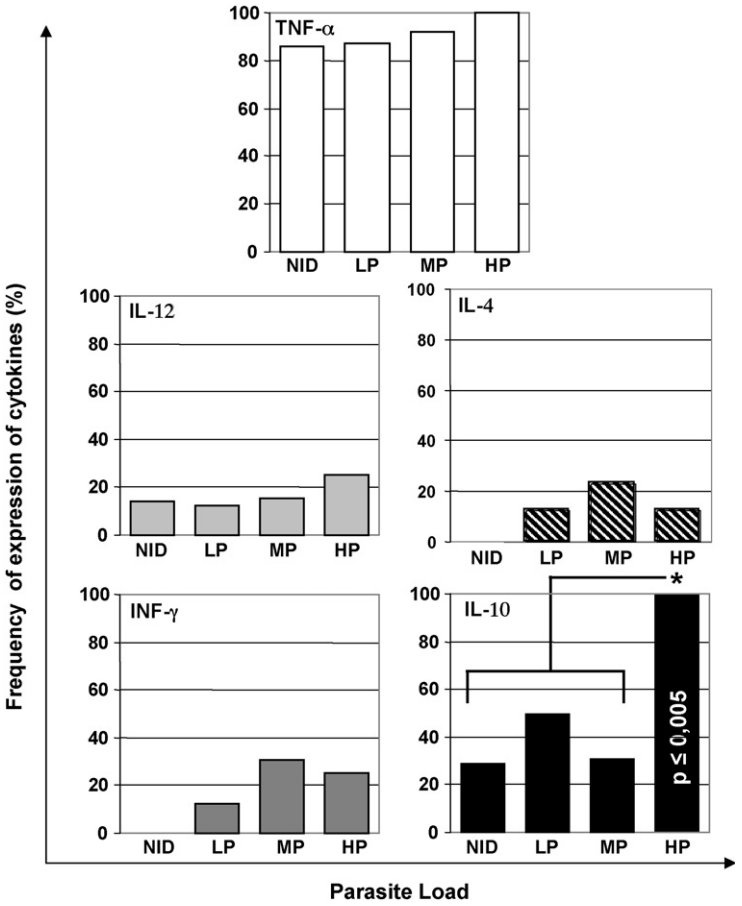

Fig. 3. Frequency of expression (% of animals testing positive in each category) of canine cytokines in splenocytes from 7 non-infected dogs (NID) and 30 dogs naturally infected by *Leishmania chagasi* and classified according to parasitic load as presenting low parasitism (LP; LDU = 1–10; $n = 8$), medium parasitism (MP; LDU = 11–250; $n = 13$) and high parasitism (HP; LDU ≥ 251; $n = 8$). Significant differences ($p < 0.05$) are identified by the symbol.

Initial reports concerning the profile of cytokine expression in CVL induced by *L. infantum* and/or *L. chagasi* have suggested a balance between Th1 and Th2 cytokines, with only a slight tendency towards one or the other (Pinelli et al., 1994, 1999; Chamizo et al., 2001, 2005; Santos-Gomes et al., 2002). Santos-Gomes et al. (2002), for example, investigated the expression of cytokines in active CVL and observed low expression during the asymptomatic phase, with the occurrence of a Th1 response, and low expression of both Th1 and Th2 during the symptomatic phase. The observed balance between cytokines reflects the complexity of the human and canine infection compared with systems studied in murines.

A specific cellular immunity that is protective against *L. infantum*/*L. chagasi* has been observed in dogs by several researchers (Cabral et al., 1992; Pinelli et al., 1994; Moreno et al., 1999; Reis et al., 2006c). It has been suggested that such resistance could be associated with low titres of specific anti-*Leishmania* IgG antibodies and with the production of cytokines IL-2 and TNF-α (Pinelli et al., 1994; Reis et al., 2006b), which appear to be related to the Th1 response.

Table 3
Correlation analysis between the semi-quantitative levels of expression of cytokines and clinical status and parasitic load in splenocytes of dogs naturally infected by *Leishmania chagasi*

Parameters	Level of expression of cytokines[a]			
	IL-10	IL-12	INF-γ	TNF-α
Clinical status	$r = 0.3501$ $p = 0.0337$	$r = 0.0872$ $p = 0.6079$	$r = 0.2816$ $p = 0.0913$	$r = 0.0508$ $p = 0.7653$
Parasitic load	$r = 0.3428$ $p = 0.0438$	$r = 0.2849$ $p = 0.1010$	$r = 0.4690$ $p = 0.0045$	$r = 0.0921$ $p = 0.5985$

[a] Statistically significant differences in semi-quantitative levels of expression of cytokines among clinical status and parasitic load are shown as $p \leq 0.05$.

However, the strong immunosuppression induced by the parasite in active CVL does not permit the determination of specific cytokine profiles in cells stimulated by soluble antigens of *Leishmania* sp. (Santos-Gomes et al., 2002). The reduced or absent proliferation response observed *in vitro* may be related either to the inhibition of IL-2 or to the expression of IL-2 and TNF-α receptors that are extremely important for the parasite-specific cytotoxic activity of T cells.

Recently, Chamizo et al. (2005) reported that the PBMCs of asymptomatic CVL-infected dogs, with or without *in vitro* stimulation, presented detectable levels of TNF-α, IL-2, INF-γ, IL-18, IL-4, IL-6 and IL-10 mRNAs, but with a significant reduction in the frequency of detection of mRNA for IL-4. PBMCs of healthy dogs exhibited detectable levels of all cytokines mRNAs except for IL-6.

In the present work, mRNAs for all of the cytokines studied, namely, IL-4, IL-10, IL-12, IFN-γ and TNF-α, were detected in at least one CVL-infected dog (i.e. IFAT + and ELISA+ or ELISA rK39+) in each of the three clinical groups, although expression of IFN-γ and IL-4 was not observed in non-infected dogs. These findings are similar to those reported by other researchers (Quinnell et al., 2001; Santos-Gomes et al., 2002) in which, for example, mRNA for IFN-γ and IL-12 could not be detected in healthy dogs, whilst animals naturally infected with *L. infantum* exhibited detectable levels of expression of IL-18, IL-10, IFN-γ and IL-4. In the present study the frequency of expression of IL-10 in dogs presenting HP was significantly different from that of NID, and LP and MP animals. An increase in IL-10 mRNA was also detected in human tissue (Ghalib et al., 1993; Karp et al., 1993; Kenney et al., 1998), hamsters (Melby et al., 1998) and murines (Engwerda et al., 1996) that had been infected by *L. infantum*. The predominant accumulation of IL-10 may play a role in the modulation of the Th1 response, inhibiting the microbiocidal activity of infected macrophages. The consequential splenomegaly could result from immunosuppression in the lymphoid compartments. It is important to note that IFN-γ may also be expressed, although in low quantities, by parasitised dogs, but this finding still needs confirmation.

The results of the serological analysis of CVL-infected dogs indicate an equilibrium between Th1 and Th2 responses since detectable levels of expression of IFN-γ, TNF-α and IL-12 together with IL-4 and IL-10 were found. However, when clinical indications are considered alongside the biochemical data, the Th1 response appears to be predominant since the expression of IL-4 decreased within the SD group, whilst the expression of IL-12 increased within the AD group. Hence the possible role of IL-4 in the clinical evaluation of CVL, and of IL-12 in the maintenance of the asymptomatic form of the disease, cannot be ruled out.

The frequency of expression of IL-12 and IFN-γ within the CVL groups was significantly different from that of the NID group ($p < 0.05$ and $p = 0.014$, respectively), although there were no significant differences between the CVL groups with respect to the expression of these cytokines. These results agree with those reported by Quinnell et al. (2001), who suggested that IFN-γ expression was not an appropriate indicator of resistance since asymptomatic and polysymptomatic dogs accumulated similar levels of this cytokine in tissues. Thus, as is the case in humans, mice and hamsters, the expression of IFN-γ may not be a suitable primary indicator of the development or cure of the disease in dogs. On the other hand, the inhibition of macrophage activation by IL-10 may have an important role in monitoring the progression of CVL.

A diminution in the expression of IFN-γ in Con-A stimulated PBMCs was observed by Santos-Gomes et al. (2002) immediately after infection of the dog, indicating that during this phase the parasite interferes with the ability of lymphocytes to express this cytokine. During the periods of incubation and emergence of infection, the capability of the non-stimulated cells of the animal to express IFN-γ is restored. The difficulty in detecting the expression of cytokines *in vitro* may be related to the capacity of the antigen to inhibit the proliferation of PBMCs in dogs undergoing active CVL, thus leading to a reduction in the expression of IFN-γ. The present study demonstrated that the significant increase in mRNA for IL-10 ($r = 0.3501$; $p = 0.0337$) was correlated with the progression of the disease. However, this effect is not related to IFN-γ deficiency, but is possibly due to the inhibition of the action of this cytokine on the activation of the macrophages, which is mediated by IL-10. Similar results were obtained by Richard et al. (1998).

In leishmaniasis, IL-10 has been associated with the suppression of Th1 cytokines, leading to the development of a Th2 immune response (Mosmann and Moore, 1991) and, consequently, to a reduction in macrophage activation (Bogdan et al., 1991). The demonstration that IL-10 inhibits the production of IFN-γ from IL-12 in PCMCs, stimulated with soluble antigen from *L. chagasi*, derived from patients suffering from VL suggests that this is the main cytokine involved in the development of the disease (Bacellar et al., 1996).

In the present study, the role of IL-10 in *Leishmania* infection was confirmed through the demonstration that

the frequency of expression of IL-10 increased significantly ($r = 0.4430$; $p = 0.0068$), along with that for IFN-γ ($r = 0.3499$; $p = 0.0365$), with the intensity of parasitism. Moreover, a significant increase in the level of IL-10 expression was found in HP dogs compared with those in the NID and MP groups. In contrast to the findings of Quinnell et al. (2001) and Santos-Gomes et al. (2002), our results present evidence for the first time that the increase in mRNA for IL-10 is directly proportional to the increase in clinical symptoms. According to Reis et al. (2006b) a positive correlation was observed between the spleen parasitic load and evolution of the clinical status in CVL ($r = 0.5048$; $p = 0.0010$). These results reinforce our data as we observed a positive correlation between IL-10, clinical status severity and parasite load. The co-existence of Th1 cytokines and IL-10 has been reported in cases of VL (Kenney et al., 1998), during the period that precedes the emergence of CVL (Santos-Gomes et al., 2002), and in non-stimulated PBMCs from asymptomatic CVL-infected dogs (Chamizo et al., 2005). In all of these cases, IL-10 may act as a regulator of potential damage occasioned by the increased expression of TNF-α, which occurs as a consequence of a predominant Th1 response (Titus et al., 1989). Furthermore, IL-10 may act as a regulatory factor responsible for maintaining the balance between Th1 and Th2 responses (Kemp et al., 1999). Such a regulatory function has been considered in some detail in a recent review by Awasthi et al. (2004). Initially IL-10 was characterised as a cytokine related to the Th2 response, but recent studies concerning the suppression of T cell populations suggest that the situation is more complex. Thus, IL-10 is not only produced by Th2 T $CD4^+$ cells, but also by macrophages, B cells and mastocytes, and hence the cytokine cannot be considered to be Th2 exclusively (Barral-Neto et al., 1998). Moreover, it is possible that IL-10, rather than IFN-γ, may be the most important macrophage regulator. An inhibitory activity over macrophages may also be attributed to TGF-β, since this cytokine is associated with an increase in IL-10 and with the inhibition of macrophages in murine leishmaniasis, characterising what is known as a Th2 response (Barral et al., 1993; Wilson et al., 1998). Recently, various other roles have been demonstrated for IL-10, such as suppression and regulation of the immune system in autoimmune diseases (Goudy et al., 2003), reaction of a host to organ transplant (Boehler, 2002), and susceptibility to parasitic infections (Kane and Mosser, 2001; Reed et al., 1994). The presence of IL-10 may also be important in explaining the high persistence of parasites in both C57BL/6 resistant mice and BALB/c susceptible mice (Belkaid et al., 2001; Viana da Costa et al., 2002).

As previously stated, CVL is characterised by the progressive deterioration of body functions, which culminates with the death of the animal. The drugs typically employed in the treatment of leishmaniasis including human VL, i.e. pentavalent antimony and amphotericin B, are not 100% efficient, and return of the disease is common (Baneth and Shaw, 2002; Moreno and Alvar, 2002). The results produced in the present study provide a valuable contribution to the understanding of CVL and to the immune response against the disease. The most significant aspects of the work are the standardisation of a RT-PCR technique for the determination of cytokine expression in the spleen compartment, and the clarification of the influence of parasitism on the immune response of dogs naturally infected with *L. chagasi*.

Acknowledgments

The authors wish to express their appreciation of the hard work carried out by the staff of the Fundação Nacional da Saúde during the execution of this project. The authors thank the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq; grant number 521124/98-0) and PAPESIIIb/FIOCRUZ/RJ for financial support. Two of us (GO and RCO) are particularly grateful to CNPq for fellowships.

References

Acedo-Sanchez, C., Morillas-Marquez, F., Sanchiz-Marin, M.C., Martin-Sanchez, J., 1998. Changes in antibody titres against *Leishmania infantum* in naturally infected dogs in southern Spain. Vet. Parasitol. 75, 1–8.

Awasthi, A., Mathur, R.K., Saha, B., 2004. Immune response to *Leishmania* infection. Indian J. Med. Res. 119, 238–258.

Bacellar, O., Brodskyn, C., Guerreiro, J., Barral-Neto, M., Costa, C.H., Coffman, R.L., Jonson, W.D., Carvalho, E.M., 1996. Interleukin-12 restores interferon-g production and cytotoxic responses in visceral leishmaniasis. J. Infect. Dis. 173, 1515–1518.

Baneth, G., Shaw, S.E., 2002. Chemotherapy of canine leishmanioses. Vet. Parasitol. 106, 315–324.

Barral, A., Barral-Neto, M., Yong, E.C., Brownell, C.E., Twardzik, D.R., Reed, S.G., 1993. Transforming growth factor-β as a virulencemechanism for *Leishmania braziliensis*. Proc. Natl. Acad. Sci. U.S.A. 90, 3442–3446.

Barral-Neto, M., Brodskyn, C., Carvalho, E.M., Barral, A., 1998. Braz. J. Med. Biol. Res. 31, 149–155., Human_Leishmaniasis@cytokines.bahia.br.

Belkaid, Y., Hoffmann, K.F., Mendez, S., Kamhawi, S., Udey, M.C., Wynn, T.A., Sacks, D.L., 2001. The role of interleukin (IL)-10 in the persistence of *Leishmania major* in the skin after healing and the therapeutic potential of anit-IL-10 receptor antibody for sterile cure. J. Exp. Med. 194, 1497–1506.

Boehler, A., 2002. The role of interleukin-10 in lung transplantation. Transpl. Immunol. 9, 121–124.

Bogdan, C., Vodovotz, Y., Nathan, C., 1991. Macrophage deactivation by interleukin 10. J. Exp. Med. 174, 1549–1555.

Cabral, M., O'Grady, J., Alexander, J., 1992. Demonstration of *Leishmania* specific cell mediated and humoral immunity in asymptomatic dogs. Parasite Immunol. 14, 531–539.

Campino, L., Abranches, P., 2002. Cutaneous leishmaniasis. Unusual disease in Portugal? Acta Med. Port. 15, 387–390.

Chamizo, C., Rubio, J.M., Moreno, J., Alvar, J., 2001. Semi-quantitative analysis of multiple cytokines in canine peripheral blood mononuclear cells by a single tube RT-PCR. Vet. Immunol. Immunopathol. 83, 191–220.

Chamizo, C., Moreno, J., Alvar, J., 2005. Semi-quantitative analysis of cytokine expression in asymptomatic canine leishmaniasis. Vet. Immunol. Immunopathol. 103, 67–75.

Cobbold, S., Metcalfe, S., 1994. Monoclonal antibodies that define canine homologues of human CD antigens: summary of the 1st International Canine Leukocyte Antigen Workshop (CLAW). Tissue Antigens 43, 137–154.

De Luna, R., Vuotto, M.L., Ielpo, M.T., Ambrosio, R., Piantedosi, D., Moscatiello, V., Ciaramella, P., Scalone, A., Gradoni, L., Mancino, D., 1999. Early suppression of lymphoproliferative response in dogs with natural infection by *Leishmania infantum*. Vet. Immunol. Immunopathol. 70, 95–103.

Desjeux, P., 2001. Worldwide increasing risk factors for leishmaniasis. Med. Microbiol. Immunol. (Berl) 190, 77–79.

Desjeux, P., 2004. Leishmaniasis: current situation and new perspectives. Comp. Immunol. Microbiol. Infect. Dis. 27, 305–318.

El Amin, E.M., Wright, E.P., Vlug, A., 1986. Characterization of the humoral immune response in Sudanese leishmaniasis: specific antibody detected by class- and subclass-specific reagents. Clin. Exp. Immunol. 64, 14–19.

Engwerda, C.R., Smelt, S.C., Kaye, P.M., 1996. An *in vivo* analysis of cytokine production during *Leishmania donovani* infection in scid mice. Exp. Parasitol. 84, 195–202.

Fisa, R., Gallego, M., Castillejo, S., Aisa, M.J., Serra, T., Riera, C., Carrio, J., Gallego, J., Portus, M., 1999. Epidemiology of canine leishmaniosis in Catalonia (Spain) the example of the Priorat focus. Vet. Parasitol. 83, 87–97.

Giunchetti, R.C., Mayrink, W., Genaro, O., Carneiro, C.M., Corrêa-Oliveira, R., Martins-Filho, O.A., Marques, M.J., Tafuri, W.L., Reis, A.B., 2006. Relationship between canine visceral leishmaniosis and the *Leishmania* (*Leishmania*) *chagasi* burden in dermal inflammatory foci. J. Comp. Path. 135, 100–107.

Ghalib, H.W., Piuvezam, M.R., Sheiky, Y., Siddig, M., Hashim, F.A., El Hassam, A.M., Russo, D.M., Reed, S.G., 1993. Interleukin-10 production correlates with pathology in human *Leishmania donovani* infections. J. Clin. Invest. 92, 324–329.

Goudy, K.S., Burkhardt, B.R., Wasserfall, C., Song, S., Campbell-Thompson, M.L., Brusko, T., Powers, M.A., Clare-Salzler, M.J., Sobel, E.S., Ellis, T.M., Flotte, T.R., Atkinson, M.A., 2003. Systemic overexpression of IL-10 induces $CD4^{+}CD25^{+}$ cell populations *in vivo* and ameliorates Th1 diabetes in non-obese diabetic mice in a dose-dependent fashion. J. Immunol. 171, 2270–2278.

Gröne, A., Frisk, A.L., Baumgartner, W., 1998. Cytokine mRNA expression in whole blood samples from dogs with natural canine distemper virus infection. Vet. Immunol. Immunopathol. 65, 11–27.

Kane, M.M., Mosser, D.M., 2001. The role of interleukin-10 in promoting disease progression in leishmaniasis. J. Immunol. 166, 1141–1147.

Karp, C.L., El Safi, S.H., Wynn, T., Satti, M.M., Kordafoni, A.M., Hashim, F.A., Hag-Ali, M., Neva, F.A., Nutman, T.B., Sacks, D.L., 1993. *In vivo* cytokine profiles in patients with Kala-azar. J. Clin. Invest. 91, 1644–1648.

Kemp, K., Kemp, M., Kharazmi, A., Ismail, A., Kurtzhals, J.A., Hviid, L., Theander, T.G., 1999. *Leishmania*-specific T cells expressing interferon-gamma (IFN-gamma) and IL-10 upon activation are expanded in individuals cured of visceral leishmaniasis. Clin. Exp. Immunol. 116, 500–504.

Kenney, R.T., Sacks, D.L., Gam, A.A., Murray, H.W., Sundar, S., 1998. Splenic cytokine responses in Indian Kala-azar before and after treatment. J. Infect. Dis. 177, 815–818.

Mancianti, F., Gramiccia, M., Gradoni, L., Pieri, S., 1988. Studies on canine leishmaniais control. I. Evolution of infection of different clinical forms of canine leishmaniasis following antimonsyl treatment. Roy. Soc. Trop. Med. Hyg. 82, 566–567.

Martinez-Moreno, A., Moreno, T., Martinez-Moreno, F.J., Acosta, I., Hernandez, S., 1995. Humoral and cell-mediated immunity in natural and experimental canine leishmaniasis. Vet. Immunol. Immunopathol. 48, 209–220.

Melby, P.C., Tryon, V.V., Chandrasekar, B., Freeman, G.L., 1998. Cloning of Syrian hamster (*Mesocricetus auratus*) cytokine cDNAs and analysis of cytokine mRNA expression in experimental visceral leishmaniasis. Infect. Immun. 66, 2135–2142.

Molina, R., Amela, C., Nieto, J., San-Andres, M., Gonzalez, F., Castillo, J.A., Lucientes, J., Alvar, J., 1994. Infectivity of dogs naturally infected with *Leishmania infantum* to colonized *Phlebotomus perniciosus*. Trans. Roy. Soc. Trop. Med. Hyg. 88, 491–493.

Moreno, J., Alvar, J., 2002. Canine leishmaniasis: epidemiological risk and the experimental model. Trends Parasitol. 18, 399–405.

Moreno, J., Nieto, J., Chamizo, C., Gonzalez, F., Blanco, F., Barrer, D.C., Alvar, J., 1999. The immune response and PBMC subsets in canine visceral leishmaniasis before, and after, chemotherapy. Vet. Immunol. Immnopathol. 71, 181–195.

Mosmann, T.R., Moore, K.W., 1991. The role of IL-10 in cross regulation of TH1 and TH2 responses. Immunol. Today 12, A49–A53.

Pinelli, E., Killick-Kendrick, R., Wagenaar, J., Bernadina, W., Del Real, G., Ruitenberg, J., 1994. Cellular and humoral immune responses in dogs experimentally and naturally infected with *Leishmania infantum*. Infect. Immun. 62, 229–235.

Pinelli, E., Van Der Kaaij, S.Y., Slappendel, R., Fragio, C., Ruitenberg, E.J., Bernadina, W., Rutten, V.P., 1999. Detection of canine cytokine gene expression by reverse transcription-polymerase chain reaction. Vet. Immunol. Immunopathol. 69, 121–126.

Quinnell, R.J., Courtenay, O., Shaw, M.A., Day, M.J., Garcez, L.M., Dye, C., Kaye, P.M., 2001. Tissue cytokine responses in canine visceral leishmaniasis. J. Infect. Dis. 183, 1421–1424.

Reed, S.G., Brownell, C.E., Russo, D.M., Silva, J.S., Grabstein, K.H., Morrissey, P.J., 1994. IL-10 mediates susceptibility to *Trypanosoma cruzi* infection. J. Immunol. 1535, 3135–3140.

Reis, A.B., Martins-Filho, O.A., Teixeira-Carvalho, A., Carvalho, M.G., Mayrink, W., Franca-Silva, J.C., Giunchetti, R.C., Genaro, O., Correa-Oliveira, R., 2006a. Parasite density and impaired biochemical/hematological status are associated with severe clinical aspects of canine visceral leishmaniasis. Res. Vet. Sci. 81, 68–75.

Reis, A.B., Teixeira-Carvalho, A., Vale, A.M., Marques, M.J., Giunchetti, R.C., Mayrink, W., Guerra, L.L., Andrade, R.A., Correa-Oliveira, R., Martins-Filho, O.A., 2006b. Isotype patterns of immunoglobulins: hallmarks for clinical status and tissue parasite

density in Brazilian dogs naturally infected by *Leishmania* (*Leishmania*) *chagasi*. Vet. Immunol. Immunopathol. 112, 102–116.

Reis, A.B., Teixeira-Carvalho, A., Giunchetti, R.C., Guerra, L.L., Carvalho, M.G., Mayrink, W., Genaro, O., Corrêa-Oliveira, R., Martins-Filho, O.A., 2006c. Phenotypic features of circulating leucocytes as immunological markers for clinical status and bone marrow parasite density in dogs naturally infected by *Leishmania chagasi*. Clin. Exp. Immunol. 146, 303–311.

Richard, T.K., David, L.S., Albert, A.G., Henry, W.M., Shyam, S., 1998. Splenic cytokine responses in Indian Kala-azar before and after treatment. J. Infect. Dis. 177, 815–819.

Santos-Gomes, G.M., Rosa, R., Leandro, C., Cortes, S., Romao, P., Silveira, H., 2002. Cytokine expression during the outcome of canine experimental infection by *Leishmania infantum*. Vet. Immunol. Immunopathol. 88, 21–30.

Stauber, L.A., 1955. Leishmaniasis in the hamster. In: Cole, W.H. (Ed.), Some Physiological Aspects and Consequence of Parasitism. Rugers University Press, New Brunswick, pp. 77–90.

Tesh, R.B., 1995. Control of zoonotic visceral leishmaniasis: is it time to change strategies? Am. J. Trop. Med. Hyg. 52, 287–292.

Titus, R.G., Sherry, B., Cerami, A., 1989. Tumor necrosis factor plays a protective role in experimental murine cutaneous leishmaniasis. J. Exp. Med. 170, 2097–2104.

Viana da Costa, A., Huerre, M., Delacre, M., Auriault, C., Correia Costa, J.M., Verwaerde, C., 2002. IL-10 leads to a higher parasite persistence in a resistant mouse model of *Leishmania major* infection. Parasitol. Int. 51, 367–379.

Wilson, M.E., Young, M.B., Davidson, B.L., Mente, K.A., Mcgowan, S.E., 1998. The importance of TGF-β in murine visceral leishmaniasis. J. Immunol. 161, 6148–6155.