



## Cytokine and transcription factor profiles in the skin of dogs naturally infected by *Leishmania (Leishmania) chagasi* presenting distinct cutaneous parasite density and clinical status

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### ARTICLE INFO

#### Article history:

Received 13 October 2009

Received in revised form 7 November 2010

Accepted 10 November 2010

#### Keywords:

Canine visceral leishmaniasis

Cytokines

Transcription factors

Real-time PCR

*Leishmania chagasi*

Parasitism

### ABSTRACT

The immune response in the skin of dogs infected with *Leishmania chagasi* and its association with distinct levels of tissue parasitism and clinical progression of canine visceral leishmaniasis (CVL) are poorly understood and limited studies are available. A detailed analysis of the profiles of cytokines (IFN- $\gamma$ , IL-4, IL-5, IL-10, IL-12, IL-13, TGF- $\beta$ 1 and TNF- $\alpha$ ) and transcription factors (T-bet, GATA-3 and FOXP3) in the skin of 35 naturally infected dogs was carried out using real-time PCR alongside determinations of skin parasite density and the clinical status of CVL. A mixed cytokine profile with high levels of expression of IFN- $\gamma$ , TNF- $\alpha$  and IL-13 was determined in asymptomatic dogs. Additionally, the levels of transcription factors GATA-3 and FOXP3 were correlated with the asymptomatic disease. A mixed cytokine profile was also observed during active CVL. Moreover, high levels of IL-10 and TGF- $\beta$ 1, concomitant with the low expression of IL-12, may represent a key condition that allows persistence of parasite replication in the skin. The results obtained indicate that in asymptomatic disease or lower levels of skin parasite density, a mixed inflammatory, regulatory immune response profile may be of major relevance for both the maintenance of the clinical status of the dogs as well as for parasite persistence and replication at low levels.

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### 1. Introduction

Visceral leishmaniasis (VL) caused by the protozoan *Leishmania (Leishmania) chagasi* [syn. *Leishmania (Leishmania) infantum*], is one of the most important of zoonotic diseases affecting dogs and humans in Europe and Latin America (Desjeux, 2004). Dogs are considered to be excel-

lent models for the study of human VL because the natural history of the canine disease is very similar to that observed in human (Moreno and Alvar, 2002). A number of reports are available concerning the parasite load found in different tissues and the immunopathological changes related to the progression of clinical forms of canine visceral leishmaniasis (CVL) (Chamizo et al., 2005; Reis et al., 2006a,b,c; Giunchetti et al., 2006; Lage et al., 2007; Giunchetti et al., 2008a,b; Alves et al., 2009; Carrillo and Moreno, 2009; Guerra et al., 2009; Manna et al., 2009; Reis et al., 2009).

It has been established that the skin is an important reservoir for parasites in asymptomatic and symptomatic *Leishmania*-infected dogs, and the high parasite loads found in this organ suggest that the skin may play an important role in the transmission and epidemiology of the disease (Abranches et al., 1991). Previous investigations have revealed that symptomatic CVL-infected dogs exhibit an intense diffuse dermal inflammatory infiltrate and high parasitic burden in comparison with their asymptomatic counterparts (Giunchetti et al., 2006). On this basis it was proposed that the immunopathological changes in the skin and the levels of cutaneous parasitism are directly related to the clinical severity of the disease.

Earlier evaluations of the immune response pattern in *Leishmania*-infected dogs have been based on the analysis of cytokines profiles in peripheral blood mononuclear cells (PBMCs), skin, lymph nodes, bone marrow and spleen. Thus, Pinelli et al. (1994) found higher levels of IL-2 and TNF- $\alpha$  in supernatants from *in vitro*-stimulated PBMCs derived from asymptomatic dogs, and proposed that these cytokines could be used as markers of disease progression. Furthermore, Chamizo et al. (2005) reported that PBMCs of asymptomatic CVL-infected dogs exhibited preferential expression of T<sub>H</sub>1 cytokines (Chamizo et al., 2005). Some authors have demonstrated the ability of IL-12 to augment the production of IFN- $\gamma$  by PBMCs derived from dogs with experimental or natural symptomatic CVL, and stressed the importance of these cytokines in the resolution of the disease (Dos-Santos et al., 2004; Strauss-Ayali et al., 2005). In a recent study, both type 1 and 2 immune responses were demonstrated to occur in the spleen during CVL (Strauss-Ayali et al., 2007), while Lage et al. (2007) suggested that CVL is marked by the balanced splenic production of type 1 and 2 cytokines with the predominant accumulation of IL-10 and IFN- $\gamma$  as a consequence of increased parasitic load and progression of the disease.

In the present study, the immunopathology of CVL has been further investigated by performing a detailed analysis of the expression of type 1 (IL-12, IFN- $\gamma$  and TNF- $\alpha$ ), type 2 (IL-4, IL-5 and IL-13) and immunoregulatory (IL-10 and TGF- $\beta$ 1) cytokines in the skin of dogs naturally infected by *Leishmania (L.) chagasi*. In addition, the levels of the transcription factors T-bet, GATA-3 and FOXP3 have been assessed during CVL. Attention was particularly focussed on the possible association between clinical status and skin parasite density, but the key objective of the study was to explore novel biomarkers, including the relationship between type 1 and 2 cytokine patterns and transcription factors that might influence susceptibility and resistance to infection.

## 2. Materials and methods

### 2.1. Study population and clinical evaluation

The investigation was approved by the Ethics Committee on Animal Experimentation (CETEA) of the Universidade Federal de Minas Gerais, Brazil. The study population comprised 51 adult dogs (aged between 2 and 6 years) of both genders that had been captured by the Center of Zoonosis Control in Belo Horizonte (Minas Gerais, Brazil), a region with a high prevalence of CVL and human VL. The animals were maintained under quarantine at the kennels of the Institute of Biological Sciences (Universidade Federal de Minas Gerais) and treated for intestinal helminthic infections (Endal Plus<sup>®</sup>; Schering-Plough Coopers, Brazil) and immunised against parvovirus, leptospirosis, distemper, parainfluenza and hepatitis (Vanguard<sup>®</sup> HTLP 5/CV-L vaccine; Pfizer, New York, NY, USA). Experimental animals were categorised on the basis of serological results from an indirect immunofluorescence assay test (IFAT), the “gold standard” immunological test in Brazil for the diagnosis of CVL. Sixteen dogs presenting negative IFAT assays with serum samples diluted 1:40, and negative parasitological examinations for *Leishmania* in tissue smears (bone marrow, ear skin, spleen, liver and popliteal lymph node), were considered to be non-infected and were employed as the control group (CD,  $n = 16$ ). Thirty-five animals with positive IFAT titres  $\geq 1:40$  were considered CVL-positive and were included in the groups of infected animals. *Leishmania*-infected dogs were sub-divided on the basis of the presence or absence of signs of infection according to Mancianti et al. (1988) as follows: absence of indicative signs of the disease – asymptomatic group (AD,  $n = 10$ ); presence of a maximum of three clinical signs of the disease including opaque bristles and/or localised alopecia and/or moderate loss of weight – oligosymptomatic group (OD,  $n = 10$ ); presence of characteristic clinical signs of the disease including cutaneous lesions, onychogryphosis, opaque bristles, severe loss of weight, apathy and keratoconjunctivitis – symptomatic group (SD,  $n = 15$ ).

### 2.2. Sample collection and assessment of skin parasite load

Animals were euthanised with sodium thiopental (Abbott Laboratories, Abbott Park, IL, USA; 30 mg/kg body weight) and samples of skin tissue were collected from the ears without lesions. One fragment of the skin was used for tissue imprints on microscopic slides. The samples were fixed in methanol, stained with Giemsa and examined under an optical microscope. *Leishmania* amastigote stages were counted and parasite densities were expressed as Leishman Donovan Units (LDU) as described by Stauber (1955) with some modifications. Parasite densities were categorised statistically into tertiles according to Reis et al. (2006a) as absent (LDU = 0; CD group,  $n = 16$ ), low (LDU = 1–9; LP group,  $n = 12$ ), medium (LDU = 10–130; MP group,  $n = 11$ ) and high (LDU = 131–7246; HP group,  $n = 12$ ).

**Table 1**

Sequences of primers used for quantification of mRNA expression by real-time PCR. F: forward primer, R: reverse primer. GeneBank accession number of the sequence used to design primers and their product length are shown as well as each PCR efficiency and  $R^2$ .

Gene	Primer sequence (5'–3')	Product length (bp)	Reaction efficiency (%)	$R^2$
GAPDH				
F	TTCCACGGCACAGTCAAG	115	99.1	0.996
R	ACTCAGCACCAGCATCAC			
IL-12p40				
F	CAGCAGAGAGGGTCAGAGTGG	109	96.5	0.989
R	ACGACCTCGATGGGTAGGC			
IFN- $\gamma$				
F	TCAACCCCTTCTCGCCACT	113	95.4	0.967
R	GCTGCCTACTTGGTCCCTGA			
TNF- $\alpha$				
F	CGTCCATTCTTGCCCAAAC	94	97.2	0.983
R	AGCCCTGAGCCCTTAATTC			
IL-4				
F	CACCTCCCAACTGATCCAA	123	96.9	0.991
R	CTCGCTGTGAGGATGTTCAA			
IL-5				
F	GCCTATGTTTCTGCCTTTGC	106	95.3	0.979
R	GGTTCCTATCGCCTATCA			
IL-13				
F	CCTCCTCAGAGCAAAGTG	148	96.7	0.973
R	CCCAGCACAAACAAGAC			
IL-10				
F	AGAACCACGACCCAGACATC	129	97.1	0.993
R	CCACCGCTTGCTCTTATTC			
TGF- $\beta$ 1				
F	AGGATCTGGGCTGGAAGTG	134	95.1	0.981
R	CGGGTTGTGCTGTTGTA			
T-bet				
F	GCTTCCAACACACACATC	80	96.0	0.977
R	TGAGTGATCTCCGCAITTC			
GATA3				
F	ATGACACGCTGGAGGACTTC	106	98.5	0.969
R	TGGCTGGAGTGGCTGAAA			
FOXP3				
F	AAACAGCACATTCCCAGAGTTC	102	95.1	0.981
R	AGGATGGCCCAGCGGATCAG			

### 2.3. Extraction of total RNA and synthesis of first strand cDNAs

The second fragment of ear skin was stored at  $-80^{\circ}\text{C}$  until required for RNA analysis. Total RNA was extracted by homogenising approximately 20 mg of skin tissue with 1 mL of TRIzol reagent (Invitrogen Brasil, São Paulo, SP, Brazil) in a rotor stator. The lysate was incubated at room temperature for 10 min, mixed with chloroform (200  $\mu\text{L}$ ) by tube inversion, and centrifuged at  $12,000 \times g$  for 10 min at  $4^{\circ}\text{C}$ . The aqueous phase was collected and RNA extraction continued using the SV Total RNA Isolation System (Promega, Madison, WI, USA) according to the recommendations of the manufacturer, which included a DNase treatment step. The efficiency of DNase treatment was evaluated by PCR amplification of the cDNA reaction mix without the addition of the ThermoScript enzyme. Finally, each q-PCR run was performed with 2 internal controls assessing both potential genomic DNA contaminations (no reverse transcriptase added) and purity of the reagents used (no cDNA added). Strand cDNAs were synthesised from 1.0  $\mu\text{g}$  of total RNA using the ThermoScript<sup>TM</sup> RT-PCR

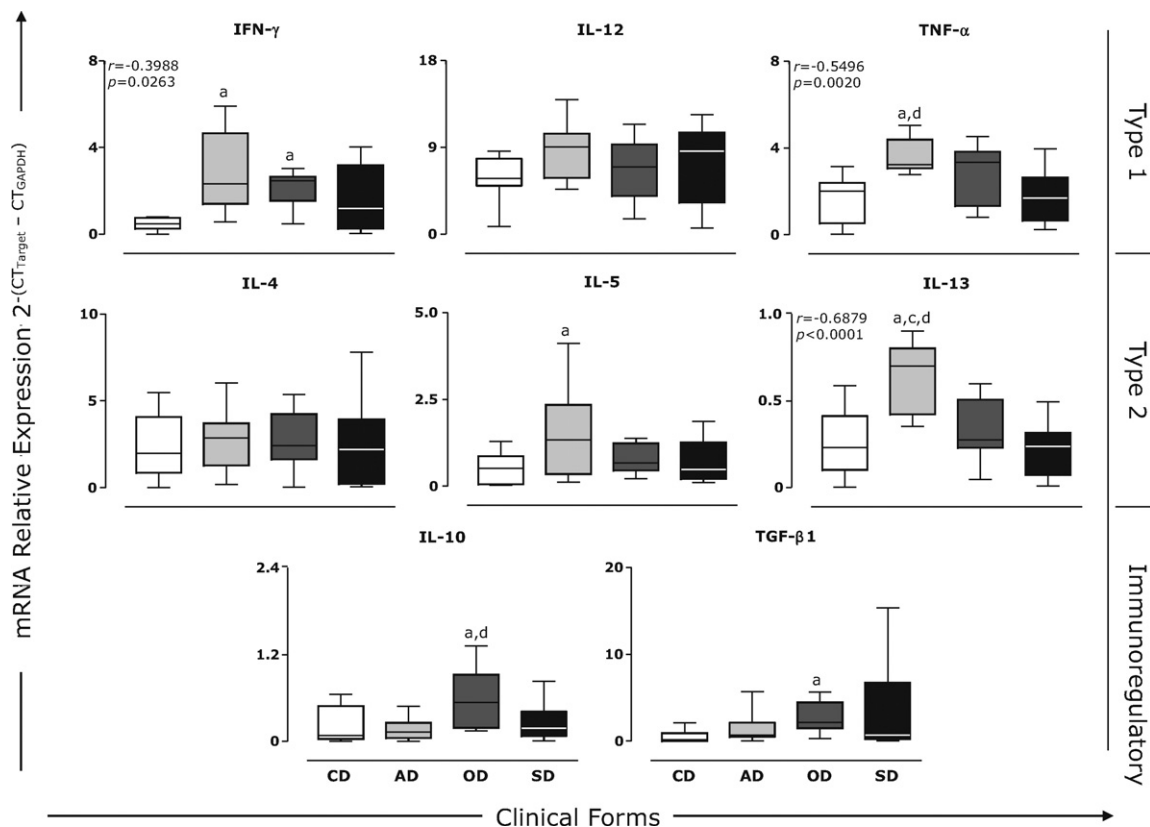
System (Invitrogen Brasil, São Paulo, SP, Brazil) with oligo-dT primers according to the manufacturer's instructions.

### 2.4. Design of primers for gene evaluation

Primers were designed with the aid of Gene Runner version 3.05 (copyright Hasting Software Inc. 2004) using specific canine sequences obtained from GenBank with accession numbers GAPDH (AB038240), IL-4 (AF239917), IL-5 (AF331919), IL-10 (U33843), IL-12p40 (U49100), IL-13 (AF244915), IFN- $\gamma$  (AF126247), TGF- $\beta$ 1 (L34956), TNF- $\alpha$  (DQ923808), FOXP3 (XM.548996), GATA-3 (XM.844060) and T-bet (XM.548164). The sequences of the primers employed are listed in Table 1. The primers were synthesised by Eurogentec (Southampton, U.K.) and reconstituted in nuclease free water.

### 2.5. Real-time PCR, cloning and sequencing of amplicons

PCR was performed on an ABI Prism 7000 DNA Sequence Detection System using SYBR<sup>®</sup> Green PCR Master Mix (PE Applied Biosystems, Foster City, CA, USA), 100 mM of each



**Fig. 1.** Relationship between clinical status and the expression of mRNAs for cytokines in the skin of dogs naturally infected with *Leishmania chagasi*. Animals were categorised as asymptomatic (AD), oligosymptomatic (OD) and symptomatic (SD) according to the clinical progression of CVL: the control group (CD) comprised uninfected animals. Box plots show the median value (horizontal line across the box), the interquartile ranges (horizontal ends of the box), and the highest and lowest values (lines extending from the box and terminating in horizontal lines). The log number of messenger RNA relative expression for IFN- $\gamma$ , IL-4, IL-5, IL-10, IL-12, IL-13, TGF- $\beta$  and TNF- $\alpha$  are shown. Significant differences ( $p < 0.05$ ) compared with CD, AD, OD and SD are indicated by the letters 'a', 'b', 'c' and 'd', respectively. Spearman's correlation indexes ( $r$  and  $p$ -values) are shown on the graphs where applicable. The data was also evaluated as mean fold-differences relative to the each messenger RNA expression of the cytokines in the clinical groups in comparison to the values of the control group. Statistically significant increase in the target transcript levels of AD to TNF- $\alpha$ , IL-13 and IL-10 as compared to SD ( $p = 0.0491$ ;  $p = 0.0225$  and  $p < 0.05$ , respectively) were observed. Moreover, there was an increase in the target transcript levels of OP to IL-10 as compared to SD ( $p < 0.05$ ).

primer and cDNA diluted at 1:5. The samples were incubated at 95 °C for 10 min and then submitted to 40 cycles of 95 °C for 15 s and 60 °C for 1 min, during which time fluorescence data were collected. The efficiency of each pair of primers was evaluated by serial dilution of cDNA according to the protocol developed by PE Applied Biosystems. In order to evaluate gene expression, three replicate analyses were performed and the amount of target RNA was normalised with respect to the control (housekeeping) gene GAPDH and expressed according to the  $2^{-\Delta Ct}$  method. PCR products were cloning with pGEM<sup>®</sup>-T Easy Vector (Promega) and sequenced to check specificity using an ABI 3100 Automated Sequencer (PE Applied Biosystems) and a Dye Terminator Kit.

## 2.6. Statistical analysis

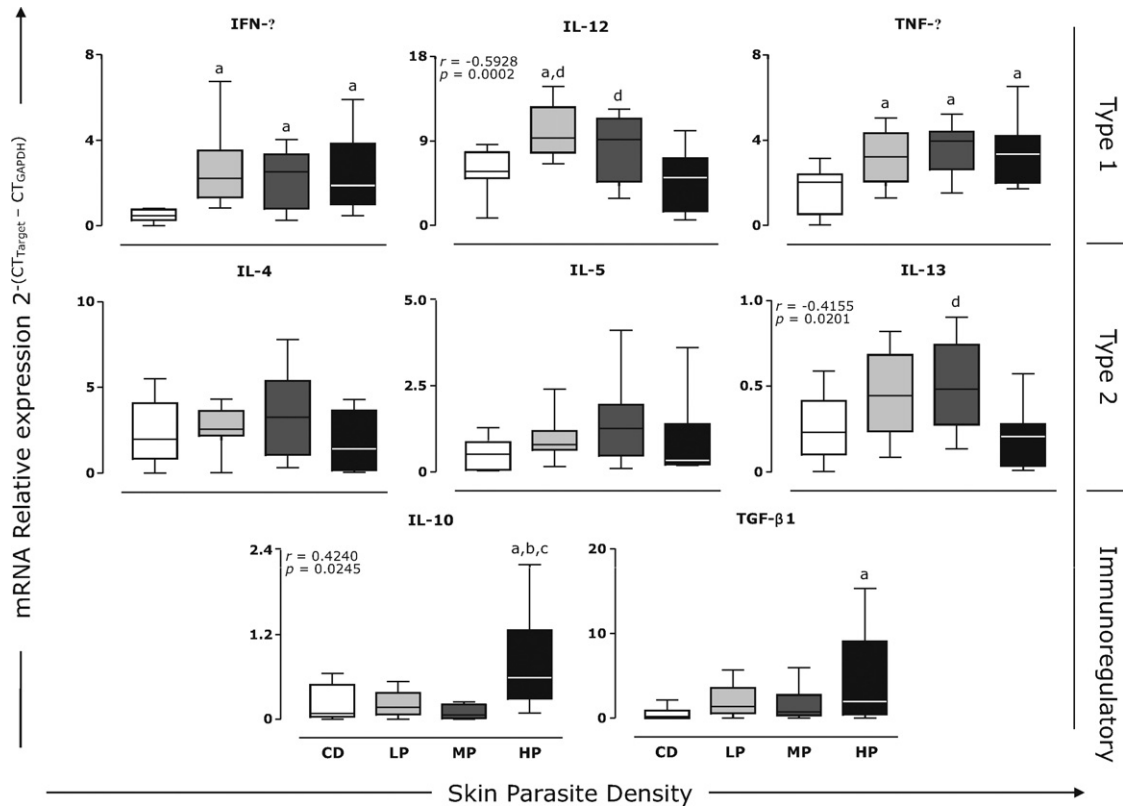
Statistical analyses were performed with the aid of GraphPad Prism software package version 5.0 (GraphPad Software, San Diego, CA, USA). Normality of the data was established using the Kolmogorov–Smirnov test. In the

parametric data, one-way analysis of variance was used for the comparative study between groups, followed by Tukey's test. In the nonparametric data, Kruskal–Wallis test was used for between group comparative study, followed by Dunns' test for multiple comparisons. Spearman's rank correlation was also computed in order to investigate relationships between the expression of cytokine and transcription factor mRNAs with clinical forms and skin parasite density. In all cases, differences were considered significant when the probabilities of equality,  $p$  values, were  $\leq 0.05$ .

## 3. Results

### 3.1. Asymptomatic dogs show high expression of IFN- $\gamma$ , TNF- $\alpha$ and IL-13 in the skin

The expression of cytokine genes was assessed in the skin of dogs naturally infected with *Leishmania chagasi* and exhibiting different clinical forms of the disease (Fig. 1). IFN- $\gamma$  showed higher expression in the AD



**Fig. 2.** Relationship between skin parasite density and the expression of mRNAs for cytokines in the skin of dogs naturally infected with *Leishmania chagasi*. Animals were categorised with low (LP), medium (MP) and high (HP) parasite densities according to Reis et al. (2006a); the control group (CD) comprised uninfected animals. Box plots show the median value (horizontal line across the box), the interquartile ranges (horizontal lines ends of the box), and the highest and lowest values (lines extending from the box and terminating in horizontal lines). The log number of messenger RNA relative expression for IFN- $\gamma$ , IL-4, IL-5, IL-10, IL-12, IL-13, TGF- $\beta$  and TNF- $\alpha$  are shown. Significant differences ( $p < 0.05$ ) compared with CD, LP, MP and HP are indicated by the letters 'a', 'b', 'c' and 'd', respectively. Spearman's correlation indexes ( $r$  and  $p$ -values) are shown on the graphs where applicable. The data was also evaluated as mean fold-differences relative to the each messenger RNA expression of the cytokines in the clinical groups in comparison to the values of the control group. Increase in the target transcript levels of LP and MP to IL-12,  $p = 0.0337$  and  $p = 0.0307$ , respectively as well as MP to IL-13,  $p = 0.0420$  as compared to HP were observed. Moreover, there was an increase in the target transcript levels of HP to IL-10 as compared to LP ( $p = 0.0311$ ) and MP (0.0070), respectively.

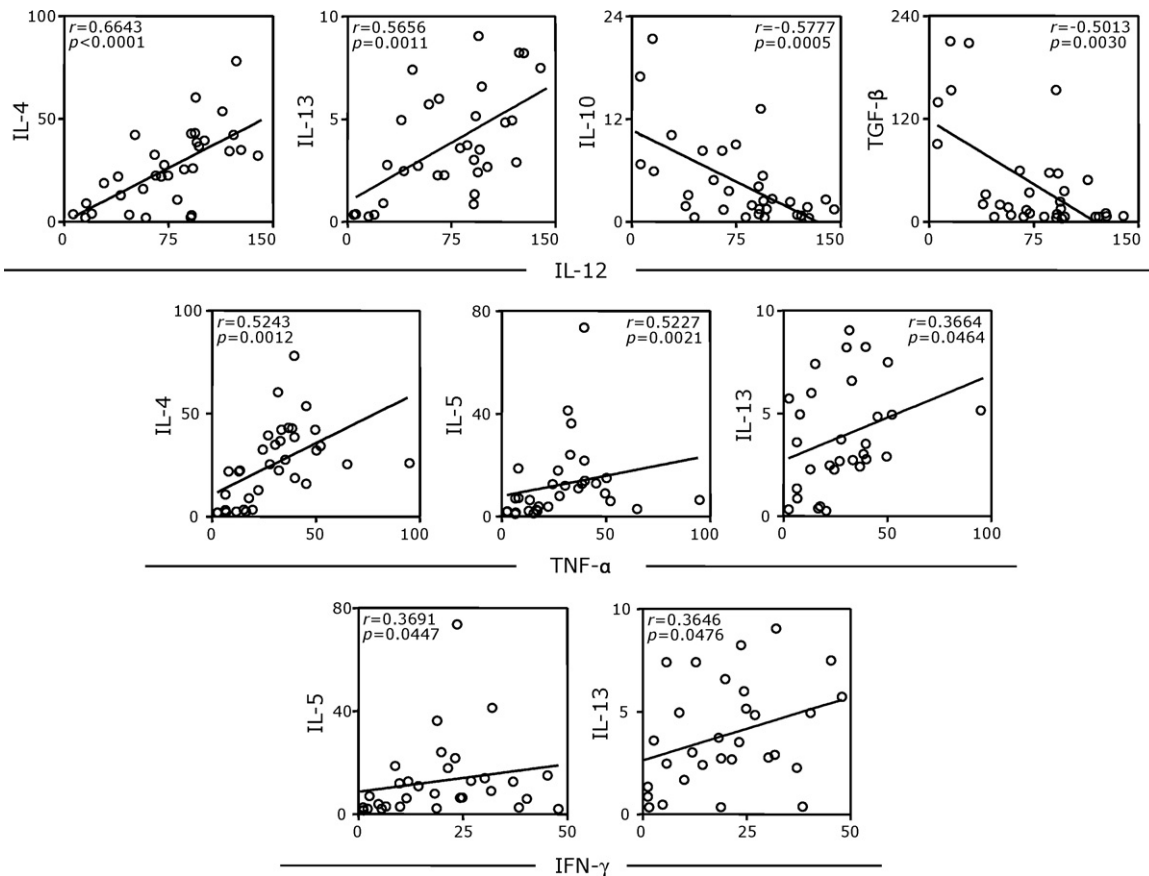
and OD groups when compared with the CD group ( $p < 0.05$ ). TNF- $\alpha$  was highly expressed in AD in relation to CD and SD ( $p < 0.05$ ). The data revealed that the impaired expression of IFN- $\gamma$  and TNF- $\alpha$  correlated ( $r = -0.3988/p = 0.0263$  and  $r = -0.5496/p = 0.0020$ , respectively) with the morbidity of the disease. Interestingly, asymptomatic animals presented increased levels of IL-13 in comparison with all other groups ( $p < 0.05$ ), and this was significantly negatively correlated with clinical progression ( $r = -0.6879/p < 0.0001$ ). Additionally, AD showed a significant increase in IL-5 expression in comparison with CD ( $p < 0.05$ ), while OD exhibited an enhanced expression ( $p < 0.05$ ) of IL-10 when compared with CD and AD. Analysis of TGF- $\beta$ 1 expression showed levels were significantly higher in OD than in CD ( $p < 0.05$ ).

The data was also evaluated as mean fold-differences relative to the each messenger RNA expression of the cytokines according to clinical groups in relation to the values of the control group. Similar findings were found in comparison to those evaluated during the analysis of the expression of cytokine genes with statistically significant increase in the target transcript levels of AD to TNF- $\alpha$ , IL-13 and IL-10 as compared to SD ( $p = 0.0491$ ;  $p = 0.0225$  and

$p < 0.05$ , respectively). Moreover, there was an increase in the target transcript levels of OP to IL-10 as compared to SD ( $p < 0.05$ ).

### 3.2. Enhanced IL-10 and TGF- $\beta$ 1 cytokines and a concomitant low expression of IL-12 may represent a key condition for parasite replication in the skin of infected dogs

In order to determine whether the cytokine profiles of dogs naturally infected with *L. chagasi* were associated with dermal parasite density, the expression of cytokine genes was assessed in experimental animals classified according to parasitism (Fig. 2). The data revealed a high expression of IL-10 in HP in relation to LP and MP groups ( $p < 0.05$ ), accompanied by a positive correlation ( $r = 0.4240/p = 0.0245$ ) with an increase in skin parasite density. Interestingly, TGF- $\beta$  expression was significantly higher ( $p < 0.05$ ) in HP compared with CD, although no correlation ( $r = 0.0979/p = 0.5937$ ) with increased parasite load was observed. In addition, a positive correlation ( $r = 0.4940/p = 0.0004$ ) was observed between the increases in IL-10 and TGF- $\beta$ 1 (data not shown).



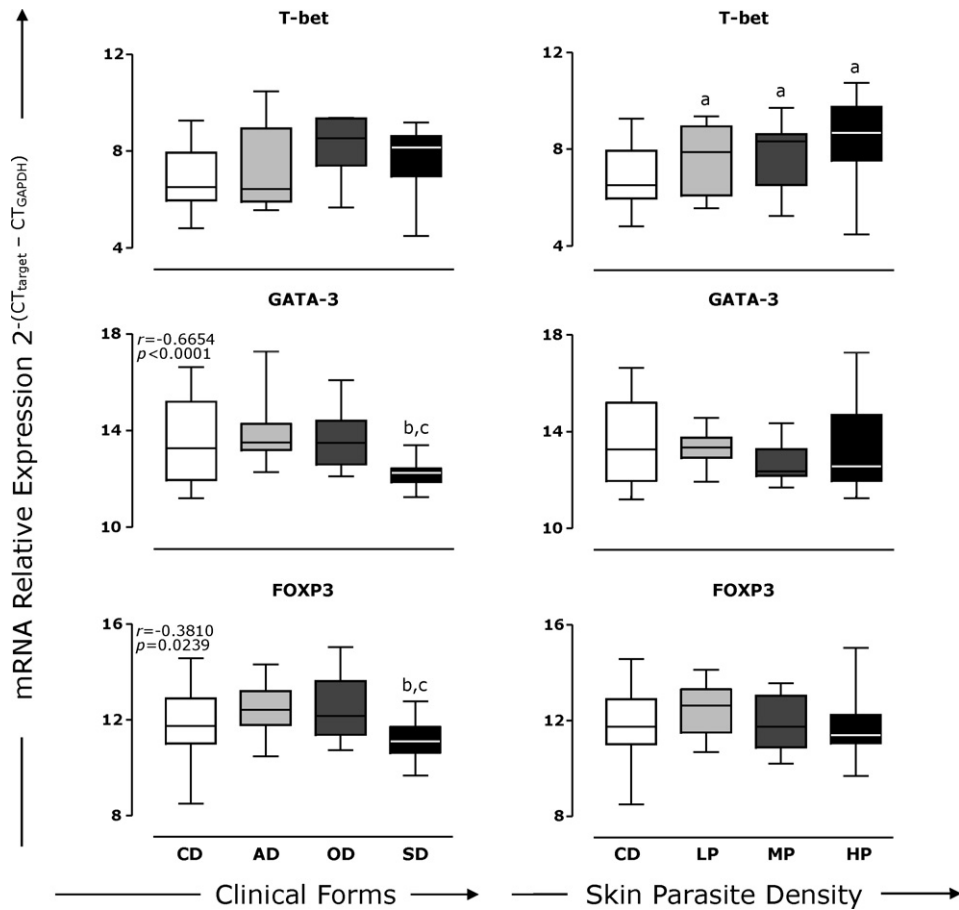
**Fig. 3.** Correlations between the expression of the mRNAs of mixed cytokines in the skin of dogs presenting CVL (Infected dogs,  $n=35$ ). The results are displayed as scatter diagrams of individual values. Spearman's correlation indexes ( $r$  and  $p$ -values) are shown on the graphs while connecting lines illustrate positive and negative correlation indexes.

Analysis of IL-12 expression indicated that a significant up-regulation of this cytokine occurred in the LP and MP groups in comparison with the HP group ( $p<0.05$ ). Moreover, there was a significant negative correlation ( $r=-0.5928/p=0.0002$ ) between the decrease in the relative expression of IL-12 and the increase in parasite load (Fig. 2), and a negative correlation between the levels of IL-12 and those of IL-10 or TGF- $\beta$  ( $r=-0.5777/p=0.0005$  and  $r=-0.5013/p=0.0030$ , respectively; Fig. 3). Consistent with these observations, a significant increase in the ratio of expression of IL-12 to IL-10 was observed in groups with a lower ( $p<0.05$ ) parasite burden (LP:  $69.95 \pm 85.06$ ; MP:  $90.80 \pm 97.24$ ; HP:  $16.13 \pm 31.06$ ). The relationship between inflammatory and regulatory responses was confirmed by the ratio of expression of IFN- $\gamma$ /IL-10, which was found to be significantly higher ( $p<0.05$ ) in LP and MP when compared with HP (LP:  $1845 \pm 6138$ ; MP:  $1780 \pm 4169$ ; HP:  $40.58 \pm 128.2$ ). The presence of the parasite was associated with an increase in the pro-inflammatory cytokines IFN- $\gamma$  and TNF- $\alpha$  ( $p<0.05$ ) in all infected groups when compared with the control group, although no correlation could be established between the expression of these cytokines and skin parasite density (Fig. 2).

The data was also evaluated as mean fold-differences relative to the each messenger RNA expression of the cytokines according to parasitism in relation to the values of the control group. Similar findings were found in comparison those evaluated during the analysis of the expression of cytokine genes with statistically significant increase in the target transcript levels of LP and MP to IL-12,  $p=0.0337$  and  $p=0.0307$ , respectively as well as MP to IL-13 as compared to HP,  $p=0.0420$ . Moreover, there was an increase in the target transcript levels of HP to IL-10 as compared to LP and MP ( $p=0.0311$  and  $0.0070$ ), respectively.

### 3.3. Mixed cytokine profile is a hallmark of active CVL following *L. chagasi* infection

A detailed analysis of the correlations between of type 1 and type 2 cytokines expressed in the skin of dogs naturally infected by *L. chagasi* are depicted in Fig. 3. Correlation analyses revealed that the type 1 cytokines IFN- $\gamma$ , IL-12 and TNF- $\alpha$  were positively correlated with IL-13 expression ( $r=0.3646/p=0.0476$ ;  $r=0.5656/p=0.0011$ ;  $r=0.3664/p=0.0464$ , respectively). Interestingly, concomitant expression of IFN- $\gamma$ , TNF- $\alpha$  and



**Fig. 4.** Analyses of the expression of mRNAs for transcription factors FOXP3, GATA-3 and T-bet in the skin of dogs naturally infected with *Leishmania chagasi*. In left plate, animals were categorised as asymptomatic (AD), oligosymptomatic (OD) and symptomatic (SD) according to the clinical progression of CVL. In right plate, animals were categorised with low (LP), medium (MP) and high (HP) parasite densities according to Reis et al. (2006a). In each case the control group (CD) comprised uninfected animals. Box plots show the median value (horizontal line across the box), the interquartile ranges (horizontal ends of the box), and the highest and lowest values (lines extending from the box and terminating in horizontal lines). The log number of messenger RNA relative expression for T-bet, GATA-3 and FOXP3 are shown. Significant differences ( $p < 0.05$ ) compared with CD, AD or LP, OD or MP and SD or HP are indicated by the letters 'a', 'b', 'c' and 'd', respectively. Spearman's correlation indexes ( $r$  and  $p$ -values) are shown on the graphs where applicable. The data was also evaluated as mean fold-differences relative to the each messenger RNA expression of the transcription factors in the clinical groups in comparison to the values of the control group. Statistically significant decrease in the target transcript levels of SD to GATA-3 and FOXP3 has been observed as compared to the transcript levels of the AD ( $p = 0.0188$  and  $p < 0.05$ ) or OD ( $p = 0.0296$  and  $p = 0.0256$ ), respectively.

IL-13 was observed in AD (Fig. 1). Simultaneous expression of IL-5 with IFN- $\gamma$  and TNF- $\alpha$  ( $r = 0.3691/p = 0.0447$  and  $r = 0.5673/p = 0.0009$ , respectively) was found during CVL, and similar situations were observed with respect to IL-4 with TNF- $\alpha$  ( $r = 0.5243/p = 0.0012$ ) and IL-4 with IL-12 ( $r = 0.6643/p < 0.0001$ ) in all infected dogs, independent of clinical status and/or skin parasite burden (Fig. 3).

#### 3.4. The transcription factors GATA-3 and FOXP3 are correlated with less severe clinical forms

In an attempt to determine whether the expression of the transcription factors FOXP3, GATA-3 and T-bet might be reliable biomarkers of clinical status and skin parasite load in CVL, the association between the levels of these variables was investigated. Data analyses revealed significant negative correlations between FOXP3 and GATA-3 with respect to clinical evolution ( $r = -0.6654/p < 0.0001$ ;

$r = -0.3810/p = 0.0239$ , respectively; Fig. 4, left panel), but no correlation between the levels of the transcription factors and skin parasite load (Fig. 4, right panel). The presence of the parasite was associated with an increase in T-bet in all infected groups in comparison with CD ( $p < 0.05$ ; Fig. 4, right panel). In this sense, high levels of T-bet were found in OD and SD compared with CD ( $p < 0.05$ ; Fig. 4, left panel), but no associations could be established between the expression of T-bet and clinical status or dermal parasite burden (Fig. 4).

The data was also evaluated as mean fold-differences relative to the each messenger RNA expression of the GATA-3 and FOXP3 in the clinical groups in relation to the values of the control group. Similar findings were found in comparison to those evaluated during the analysis of the expression of transcription factor genes with statistically significant decrease in target transcript levels of SD to GATA-3 and FOXP3 have been observed as compared to the

transcript levels of the AD ( $p=0.0188$  and  $p<0.05$ ) or OD ( $p=0.0296$  and  $p=0.0256$ ), respectively.

#### 4. Discussion

The skin is an important immune compartment that actively participates in host protection at both the early and later phases of infection. A wide variety of cells, including intra-epithelial T lymphocytes and Langerhans cells, are present in the skin and these provide considerable capacity to generate and maintain local immune reactions. Leishmaniasis is typically transmitted by the bite of sand flies infected with the pathogen and the skin is clearly the first point of contact with the protozoan. Apparently normal skin of dogs naturally infected by *L. chagasi* is intensely parasitised by amastigote forms of *L. chagasi* (Giunchetti et al., 2006) that reflects a compartmentalized profile of cytokine associated with resistance or susceptibility to *Leishmania* infection.

In this context, IL-12 is known to perform a number of key functions including the induction of IFN- $\gamma$  production by PBMC and NK cells, the stimulation of proliferation in pre-activated T-cells and NK cells, production of NO from macrophages and plays in the development of specific type 1 T-cell-mediated immunity (Trinchieri et al., 2003). Additionally, it has been postulated that IL-10 modulates the type 1 immune response in *Leishmania*-infections by inhibiting IFN- $\gamma$  production via the suppression of IL-12 synthesis in antigen presenting cells (Lage et al., 2007; Peruhype-Magalhães et al., 2005). This would imply that the balance between IFN- $\gamma$  and IL-10 during infection is particularly important in the control of VL as suggested in an earlier study involving functional models (Silvestre et al., 2007). In the present study, we have the unique opportunity to perform a compartmentalized characterisation of an immune response in skin from naturally *L. chagasi*-infected dogs. Since skin is important site to transmission of the infection, the study of the immune response in the skin of dogs infected with *L. chagasi* and its association with distinct levels of tissue parasitism and clinical progression of CVL will permit new insights elucidating the progressive or protective mechanisms during the infection (Kemp et al., 1996; Reis et al., 2009).

In the present investigation, dogs showing high skin parasitism exhibited a predominantly immunoregulatory pattern of immune response characterised by increased expression of IL-10 and TGF- $\beta$  in comparison with the CD, LP and MP groups (Fig. 2). These findings are consistent with previous reports relating the ability of IL-10 and TGF- $\beta$  to down-regulate T-cell responses and inhibit the leishmanicidal activity of macrophages thus leading to the progression of leishmaniasis and/or prevention of cure (Vouldoukis et al., 1997; Gantt et al., 2003). Alves et al. (2009) observed that the increased of IL-10 and TGF- $\beta$  in lymph nodes are correlated with high parasite burden and symptomatic diseases in dogs naturally infected with *L. chagasi*. Since the experimental animals typically exhibited active CVL, the present results suggest that an increase in IL-10 and TGF- $\beta$  may lead to the maintenance of parasite multiplication and therefore disease status. Moreover, the IFN- $\gamma$ /IL-10 (LP:  $1845 \pm 6138$ ; MP:  $1780 \pm 4169$ ;

HP:  $40.58 \pm 128.2$ ) and IL-12/IL-10 (LP:  $69.95 \pm 85.06$ ; MP:  $90.80 \pm 97.24$ ; HP:  $16.13 \pm 31.06$ ) ratios were lower in the HP group than in the other groups ( $p<0.05$ ). Accordingly, LP showed a significant increase in IL-12 expression in comparison HP and a negative correlation was observed between IL-12 levels and increase of skin parasite density (Fig. 2). Additionally, the data revealed that increases in IL-12 were negatively correlated with the levels of the immunoregulatory cytokines IL-10 and TGF- $\beta$ 1 (Fig. 3). The results presented here thus re-emphasise the involvement of immunoregulatory cytokines in the suppression of the immune response involved in the control of parasite replication in the skin.

Few studies have been concerned with the involvement of transcription factors in the regulation of cytokine gene transcription in canine models. Biller et al. (2007) have, however, demonstrated increased expression of the mRNAs of FOXP3 and of the cytokines IL-10 and TGF- $\beta$  in regulatory T cells ( $T_{Reg}$ ) of dogs with cancer. In the present study, although specific cell populations such as  $T_{Reg}$  lymphocytes were not assessed, a positive correlation between the expression of FOXP3 and immunoregulatory (IL-10 and TGF- $\beta$ ) cytokines was confirmed ( $r=0.6764/p<0.001$ ;  $r=0.3151/p<0.05$ , respectively). Additionally, low levels of FOXP3 expression were observed in the SD group compared with the AD and OD groups, and this was negatively correlated with clinical progression. Nevertheless, no correlation between the up-regulation of IL-10 and TGF- $\beta$ 1 by FOXP3 and the clinical development of the disease could be observed. An explanation for this finding could be that FOXP3 is involved in regulating the expression of many genes, as described in recent studies involving murine models (Marson et al., 2007; Zheng et al., 2007), or it might indicate that other cells could be involved in the production of these cytokines, how macrophages with the purpose of modulation of the immune response. It is, however, imperative that the role of FOXP3 in the development of regulatory cells in CVL be ascertained in future studies.

Analysis of the cytokine profile with respect to parasite burden revealed increases in the expression of pro-inflammatory cytokines IFN- $\gamma$  and TNF- $\alpha$  and in the transcription factor T-bet in all infected groups (LP, MP and HP) in comparison with the CD group (Figs. 2 and 4). T-bet is a key protein in the immune system and has been described as a  $T_H1$ -specific T-box transcription factor controlling the development of  $T_H1$  cells and the expression of the hallmark type 1 cytokine, IFN- $\gamma$ , in  $T_H1$  and NK cells. A number of studies have established that T-bet plays an essential role in the control of  $T_H1$  cell-dependent protozoan infections (Rosas et al., 2006; Szabo et al., 2002). Recently, Strauss-Ayali et al. (2007) reported an increase in T-bet and IFN- $\gamma$  expression in experimentally and naturally infected dogs presenting parasite load in the spleen. In agreement with the present study, Lage et al. (2007) observed that, independent of the splenic parasitic load, levels of IFN- $\gamma$  were significantly increased in naturally infected dogs compared with their non-infected counterparts. However, in contrast to the present study, no differences in the levels of TNF- $\alpha$  could be established between infected and non-infected dogs as previously described (Lage et al., 2007). Cytokines analysis considering dogs classified according



clinical status revealed in AD group increases in IFN- $\gamma$ , TNF- $\alpha$  (Fig. 1) and IFN- $\gamma$ /IL-4 ratios in comparison with the SD and CD groups (CD:  $0.32 \pm 0.15$ ; AD:  $0.77 \pm 0.50$ ; OD:  $0.80 \pm 0.43$ ,  $p < 0.05$ ). In addition, a negative correlation could be established between high levels of IFN- $\gamma$  and TNF- $\alpha$  and clinical evolution (Fig. 1). These findings are consistent with previous reports implicating the involvement of IFN- $\gamma$  and TNF- $\alpha$  secretion in optimal parasite clearance through activation of macrophages and, consequently, induction of nitric oxide production (Vouldoukis et al., 1997). Furthermore, IL-4 has been proven to have no role in disease progression of the visceralising species (Satoskar et al., 1995). In contrast to the present results, some investigations have found no difference in the expression of IFN- $\gamma$  and TNF- $\alpha$  in bone marrow (Quinnell et al., 2001) or spleen cells (Lage et al., 2007) in naturally infected dogs presenting different clinical forms. Moreover, a recent study by Sanchez-Robert et al. (2008) demonstrated that higher IFN- $\gamma$  expression in PBMCs was associated with an increase of clinical signs in CVL. One possible explanation of this observation is a distinct *in situ* immune response against *L. chagasi* in target organs of naturally infected dogs, as previously described in Sanchez et al. (2004).

Even though IL-12 plays a major role in determining a type 1 immune response, no difference in expression of the mRNA of this cytokine was detected among the CVL clinical groups evaluated in the present study (Fig. 1). In accordance to our results, Lage et al. (2007) and Alves et al. (2009) not observed differences in the frequency and expression of this cytokine in dogs presenting different clinical forms of CVL.

High levels of IL-5 in the skin of asymptomatic *Leishmania*-infected dogs were observed (Fig. 1). Previous authors had suggested that IL-5 and associated IFN- $\gamma$  production could be involved in the control of infection in such animals or humans, possibly by promoting differentiation and activation of eosinophils and enhancing the generation and activation of specific cytotoxic T lymphocytes (Nagasawa et al., 1991; Mary et al., 1999; Peruhype-Magalhães et al., 2005).

In murine leishmaniasis, several researchers have observed that IL-13 synthesis promotes initial IFN- $\gamma$  production and influences the assembly and maturation of tissue granuloma. However, such experiments have not addressed the mechanism(s) by which IL-13 regulates the expression of anti-leishmanial type 1 response (Murray et al., 2006). In the present study it was demonstrated that asymptomatic *Leishmania*-infected animals presented a high expression of IL-13, and a negative correlation of this cytokine with clinical progression in CVL was observed (Fig. 1). In addition, a concomitant high IFN- $\gamma$  expression (Fig. 1) was found in the AD group and this was positively correlated with the expression of IL-13 (Fig. 3). In a recent longitudinal study, Sanchez-Robert et al. (2008) evaluated the cytokine profile in PBMCs of experimentally infected dogs and observed that most of the animals that developed clinical leishmaniasis expressed IL-13 during the first 4 months after infection, a result that was in complete contrast to that presented by the asymptomatic group.

A reduction of GATA-3 in the SD group (Fig. 4B) and a high negative correlation of this transcription factor with

clinical evolution were detected in the present study. The level of GATA-3 also showed a positive correlation with the expression of the type 2 cytokines IL-4, IL-5 and IL-13 (data not shown) in the skin of infected dogs. However, among these cytokines, only IL-13 presented a concomitant expression with GATA-3 in the AD group that was negatively correlated with clinical progression (Figs. 1 and 4) in CVL. This finding is in agreement with that of Kitamura et al. (2005) who evaluated the correlation between the expression of GATA-3 and type 2 cytokines in human helper T-cell clones and demonstrated that only IL-13 was strongly correlated with the mRNA levels of the transcription factor. It has been reported that GATA-3 plays an important role in IL-13 production in both T cells and mast cells, and also facilitates chromatin remodelling of T<sub>H</sub>2 cytokine gene loci, including the IL-13 gene. In addition, a GATA-3 binding site in the proximal IL-13 promoter is necessary for cell type-specific expression of IL-13 (Murray et al., 2006). This interesting correlation found in the dermal compartment may encourage further studies of the role of GATA-3 in the determination of CD4<sup>+</sup> T cell phenotype and in the expression of type 2 cytokines in canine models. Thus, the results presented in this study suggest that high levels of IL-13 and GATA-3 can be considered as good biomarkers of asymptomatic clinical forms in CVL. However, due to high dispersion in the expression of GATA-3 in the groups studied, further investigations should be performed to confirm the importance of this gene as a biomarker in CVL.

Several investigations have demonstrated that a mixed cytokine pattern can be associated with resistance or susceptibility in vaccine models and *Leishmania*-infections (Raziuddin et al., 1994; D'Andrea et al., 1995; Peruhype-Magalhães et al., 2006). The mixed type 1/type 2 immune profile revealed in the present study demonstrated the ability of naturally infected dogs to respond to *L. chagasi* infections independent of clinical status and skin parasite density. The immune profile was characterised by a positive correlation between cytokine levels of type 1 (IFN- $\gamma$ , IL-12 and TNF- $\alpha$ ) and of type 2 (IL-4, IL-5 and IL-13) (Fig. 3). In agreement with these results, Raziuddin et al. (1994) reported enhanced production of IL-4 and TNF- $\alpha$  in both VL and in cutaneous leishmaniasis. Furthermore, a study of the immune response to lipopolysaccharide or *Staphylococcus aureus* in PBMCs pre-treated with IL-4 or IL-13 revealed a significant increase in the production and accumulation of IL-12 and TNF- $\alpha$  in such cells, and this could be inhibited by anti-IL-4 neutralising antibodies (D'Andrea et al., 1995). These findings were confirmed in the present study by the demonstration of positive correlations between IL-4 or IL-13 and IL-12 or TNF- $\alpha$  (Fig. 3). Even though only IL-13 was directly correlated with IFN- $\gamma$ , the concomitant increase in IL-12 and IL-4 suggests an up-regulation of expression of IL-13 cytokine, reflecting a complex regulatory role of the mixed cytokine profile that is conducive to a protective response in *Leishmania*-infected dogs (Fig. 3).

In conclusion, the findings reported in this study are pertinent to understanding the dynamics of the immunological events associated with clinical status and skin parasite density during ongoing CVL. It has been demonstrated that inflammatory cytokine profiles, particularly those driven by IFN- $\gamma$ , TNF- $\alpha$  and IL-13, associated with

enhanced expression of the GATA-3 transcription factor suggest that these genes could be biomarkers for asymptomatic clinical forms in CVL. Moreover, IL-12 could play a protective role against parasite replication. On the other hand, in order to guarantee the survival and persistence of amastigotes in the skin compartment, the establishment of a regulatory profile, triggered by an increase in the immunoregulatory cytokines IL-10 and TGF- $\beta$ , is crucial. The results indicate that a concomitant expression of mixed cytokines, without the necessity for an absolute polarised profile, can tilt the immune system toward either a progressive or protective response in CVL. An advance in our knowledge of the mechanism that determines the protective immune response to *L. chagasi* infection in dogs will permit the establishment of a rational strategy for the development of vaccines and immunological therapies against CVL.

## Acknowledgments

The study was supported by the Fundação de Amparo à Pesquisa do Estado de Minas Gerais, Brazil (PRONEX 2007). RCO, GCO, ABR, ATC and OAMF thank CNPq for fellowships. 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 are also grateful for the use of facilities at CEPIO, Universidade Federal de Minas Gerais and Rede Mineira de Bioterismo (FAPEMIG), and for support with the provision of experimental animals.

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